(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 May 2024 (16.05.2024)

WIPO | PCT

(10) International Publication Number
WO 2024/100040 A1

(51) International Patent Classification:
A61K 47/60 (2017.01) *A61P 35/00* (2006.01)

(21) International Application Number:
PCT/EP2023/080997

(22) International Filing Date:
07 November 2023 (07.11.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PCT/EP2022/080986 07 November 2022 (07.11.2022) EP
23172851.0 11 May 2023 (11.05.2023) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

WO 2024/100040 A1

(54) **Title:** PSMA-TARGETING LINEAR CONJUGATES COMPRISING POLYETHYLENEIMINE AND POLYETHYLENE GLYCOL AND POLYPLEXES COMPRISING THE SAME

(57) **Abstract:** The present invention relates to polyplexes comprising linear conjugates of LPEI and PEG. The LPEI and PEG fragments of the linear conjugates are preferably linked by a [3+2] cycloaddition between an azide and an alkene or an alkyne to produce a 1, 2, 3 triazole or a 4,5- dihydro-1H-[1,2,3]triazole. The linear conjugates are further conjugated to a targeting fragment capable of binding to prostate specific membrane antigen (PSMA) to enable selective interaction with a particular cell type. The conjugates can form polyplexes with therapeutic agents such as nucleic acids to deliver the therapeutic agents to cells.

PSMA-TARGETING LINEAR CONJUGATES COMPRISING POLYETHYLENEIMINE AND POLYETHYLENE GLYCOL AND POLYPLEXES COMPRISING THE SAME

RELATED ART

Cancer remains a leading cause of death world-wide. For most solid tumours after surgical removal, chemotherapy is a key treatment option for managing the remaining cancer cells. A main reason for failure of chemotherapy is inefficient targeting and uptake of the chemotherapeutic agent by the tumour (Vasir & Labhasetwar *Technology in Cancer Research & Treatment* **4**(4), 363-374 (2005)). Poor accessibility to the tumour requires higher doses, and due to the nature of the chemotherapeutic agent this results in non-specific uptake and toxicity of healthy cells. A targeted drug delivery strategy whereby the therapeutic agent is reversibly bound to a targeting ligand and selectively delivered to a cell for treatment is now applied to many chemotherapeutics agents in clinical use. This strategy has shown promise to maximize the safety and efficacy of a given chemotherapeutic agent, as their selective delivery into target cells avoids the nonspecific uptake and associated toxicities to healthy cells (Srinivasarao & Low, *Chem. Rev.*, **117**, 12133-12164, (2017)) that can result in higher maximum tolerated doses.

Cationic polymers are known to form polyplexes with negatively charged nucleic acids in solution. For example, linear polyethyleneimine (LPEI) is protonated at physiological pH and therefore carries a net positive charge. When LPEI is incubated with a nucleic acid, which carries a net negative charge at physiological pH, LPEI and the nucleic acid can form polyplexes that are held together by electrostatic interaction. These polyplexes can be taken up by cells *in vivo* where they can deliver the nucleic acid sequences intracellularly. Accordingly, polyplexes comprising cationic polymers and nucleic acids can be used as vectors for therapy. Despite their promise, technical challenges have arisen related to forming homogeneous and well-characterized cationic polymers. Polyplexes comprising only LPEI can be prone to aggregation and interaction with serum proteins, limiting their potential as nucleic acid delivery agents. To overcome these challenges, polymeric LPEI can be conjugated to polyethylene glycol (PEG). The PEG fragment can help shield the LPEI from the surrounding matrix and improve the biocompatibility and blood circulation of the resulting polyplexes.

However, coupling of PEG to LPEI takes place by formation of covalent bonds between electrophilic PEG fragment(s) and the secondary amines embedded within the LPEI backbone fragment, and thus leads to branched, heterogenous conjugates and vectors with random and

not defined inclusion of PEG fragmentsthat are characterized on the basis of average PEG inclusion density. In such conjugates, typically a multiple number of PEG fragments are bonded orthogonally to the LPEI fragment with no site specificity. Such random synthesis and imprecise characterization of the LPEI-PEG conjugates can make it difficult to establish clear structure-activity relationships (SAR) between the structure of the conjugates and the activity of the resulting polyplex. WO2015/173824 discloses polyplexes of a double stranded RNA such as poly(IC) and a polymeric conjugate which is composed of a LPEI-PEG conjugate with orthogonally bonded PEG fragments, to each of which a targeting moiety capable of binding to a cancer antigen is linked. As an example, a polymeric conjugate and vector is described targeting prostate specific membrane antigen (PSMA).

Prostate specific membrane antigen (PSMA) is a multifunctional transmembrane protein that exhibits a dual enzymatic function as a glutamate carboxypeptidase and folate hydrolase further showing rapid, ligand-induced internalization and recycling (Ghosh A et al., J Cell Biochem 2004, 91: 528-539; Liu H et al., 1998, Cancer Res 58:4055–4060). PSMA is a type II membrane protein originally characterized by the murine monoclonal antibody (mAb) 7E11-C5.3. The PSMA protein has a unique 3-part structure: a 19-amino-acid internal portion, a 24-amino-acid transmembrane portion, and a 707-amino-acid external portion (Chang SS, Rev Urol. 2004, 6(suppl 10):S13-S18). PSMA is also known by additional names, namely glutamate carboxypeptidase II (GCPII), N-acetylated-α-linked acidic dipeptidase, and folate hydrolase (FOLH1) (Jeitner TM et al., Translational Oncology 2022, 22:101450). PSMA is mainly expressed in four tissues of the body, including prostate epithelium, the proximal tubules of the kidney, the jejunal brush border of the small intestine and ganglia of the nervous system (Mhawech-Fauceglia et al., Histopathology 2007, 50:472–483). Since PSMA expression is about 1,000-fold higher in prostate tumors than in healthy tissue, PSMA is particularly considered as a target for diagnosis and therapy in prostate cancer (Kularatne SA et al., Molecular Pharmaceutics 2009, 6(3):780-789; Rowe SP et al., Prostate Cancer Prostatic Dis. 2016, 19(3):223-230; Wang H et al., Small Struct. 2022, 3:220003620;9; Juzeniene A et al, Cancers 2021, 13(4):779). Furthermore, upregulation of PSMA might provide prostate cancer cells with a growth advantage and implicate PSMA in the metabolism of polyglutamated folates and the subsequent uptake of folates (Yao et al., Prostate 2006, 66:867-875; Yao et al., Prostate 2010, 70:305-316). However, PSMA targeting may also be applicable to other PSMA-expressing tumors besides prostate cancer, in particular since PSMA is not expressed on normal vasculature but is expressed on the neovasculature of many solid tumors such as breast cancer,

lung cancer, gastric cancer, colorectal cancer, pancreatic cancer, renal cell carcinoma, and bladder cancer allowing for targeting to occur in the intravascular compartment (Chang SS et al., Cancer Res. 1999, 59(13):3192-3198; Wernicke et al., APMIS 2014, 122(6):482-489; Samplaski MK et al., Mod Pathol. 2011, 24(11):1521-1529; Haffner MC et al., Hum Pathol. 2009, 40(12):1754-1761; Morgenroth A et al., Breast Cancer Research 2019, 21:116; Jian D et al., Clinical and Translational Gastroenterology 2019;10:e-00041; Jeitner TM et al.,Translational Oncology 2022, 22:101450, and references cited therein).

PSMA overexpression in prostate cancer tissue and in the neovasculature of most solid tumors makes it a target for cancer to deliver cancer therapeutics (Barrett JA et al., J Nucl Med 2013, 54: 380-387 and references cited therein). When ligands are recognized by specific receptors on the membrane of cancer cells, an internalization signal is often generated and cellular uptake via receptor-mediated endocytosis follows. Thus, targeting PSMA has been facilitated mostly by PSMA targeted antibodies such as J591 or 7E11 (Viola-Villegas NT et al., Mol Pharm 2014, 11:3965-3973 and references cited therein), PSMA aptamers (Baek SE et al., J Control Release 2014, 196:234-242 and references cited therein), small ligands such as glutamate ureas (Roy J et al, Journal of Medicinal Chemistry 2015, 58(7):3094-3103; Shallal HM et al., Bioconjug Chem 2014, 25:393-405; Lütje S et al., Theranostics 2015, 5:1388; Langut Y et al., PNAS 2017, 114(52):13655-13660; and references cited therein) and, as reported in few studies, by folates (Patil Y et al., Nanomedicine 2018, 14(4):1407-1416; Flores O et al., Theranostics 2017, 7(9):2477-2494; and references cited therein).

SUMMARY OF THE INVENTION

The present invention provides PSMA-targeting conjugates comprising LPEI and specifically defined discrete molecular weight PEG fragments that are connected by discrete linkages formed through defined, chemoselective reactions instead of through random and uncontrolled bonding of an electrophilic PEG fragment to multiple nucleophiles of an LPEI backbone fragment. Thus, the present invention provides more homogeneous PSMA-targeting conjugates with defined chemical structures. The discrete and specifically defined components and linkages not only ensure consistent and predictable ratios of all components of the inventive conjugates including consistent and predictable ratios of LPEI to PEG fragments, but further ensure defined linear instead of randomly branched conjugates. Thus, the LPEI fragment is bonded in a linear end-to-end fashion to a single and specifically defined discrete PEG fragment with a defined and discrete molecular weight which is further connected to a targeting fragment

capable of binding to PSMA. The chemoselective bonding of the LPEI fragments to the specifically defined discrete PEG fragments can take place using any suitable chemical precursors that can form a chemoselective bond. In preferred embodiments, the chemoselective bonding of LPEI fragments to the specifically defined discrete PEG fragments takes place by means of a [3+2] cycloaddition between an azide and an alkyne or alkene leading to a 1,2,3-triazole or a 4,5-dihydro-1H-[1,2,3]triazole.

For the conjugates of the present invention, the PEG fragment is further selectively linked with a targeting fragment capable of binding to prostate specific membrane antigen (PSMA) to target and facilitate the uptake of the inventive compositions, conjugates and/or polyplexes in particular, in PSMA targeted cell types. Thus, preferred embodiments and conjugates comprise one or more, typically and preferably one targeting fragment such as the DUPA residue ($HOOC(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-) or a folate specifically connected to the LPEI-PEG diconjugates forming LPEI-PEG-Targeting fragment triconjugates capable of targeting PSMA on the particular cell types, typically cancer cell types. For the inventive polyplexes, such triconjugates are combined with a polyanion such as a nucleic acid, and hereby preferably with polyinosinic:polycytidylic acid (poly(IC), which polyanion such as poly(IC) can serve as a cytotoxic and/or immunostimulatory payload delivered to and taken up within a cell.

Further surprisingly and advantageously, the inventors have found that the resulting preferred conjugates and polyplexes in accordance with the present invention which have a significantly reduced heterogeneity due to the defined chemoselective bonding of the LPEI fragments to the specifically defined discrete PEG fragments, and thus which have a significantly reduced number of potentially biologically active conjugates and polyplexes, not only form polyplexes of suitable sizes, but also maintain or even increase their overall biological activity such as potency and selectivity for decreasing survival and inducing cell death of targeted cancer cells. In addition, inventive compositions and polyplexes comprising nucleic acids encoding peptides or proteins of interest, in particular encoding pharmaceutically active peptides or proteins such as cytokines, interferons, or toxins, do not only selectively deliver pharmaceutically active nucleic acids encoding pharmaceutically active peptides or proteins to the targeted cells, in particular cancer cells, but furthermore, said delivery results in high expression and efficient protein translation as well as secretion of the encoded pharmaceutically active proteins.

Thus, in one aspect, the present invention provides a composition comprising a conjugate, wherein said conjugate comprises: a linear polyethyleneimine fragment comprising an alpha terminus and an omega terminus; a polyethylene glycol fragment comprising a first terminal end and a second terminal end; wherein said polyethylene glycol fragment comprises, preferably consists of, a discrete number m of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, wherein preferably said discrete number m is a discrete number of contiguous repeating -(O-CH_2-CH_2)- units, and wherein said discrete number of contiguous repeating -(O-CH_2-CH_2)- units) is any discrete number of 25 to 100, preferably of 25 to 60; wherein the alpha terminus of said polyethyleneimine fragment is an initiation residue; wherein the omega terminus of the polyethyleneimine fragment is connected to the first terminal end of the polyethylene glycol fragment by a divalent covalent linking group -Z-X^1-, wherein -Z-X^1- is not a single bond and -Z- is not an amide; wherein the second terminal end of the polyethylene glycol fragment is connected to a targeting fragment by a divalent covalent linking moiety X^2, and wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In another aspect, the present invention provides a composition comprising a conjugate, wherein said conjugate is of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:

R^1-(NR^2-CH_2-CH_2)$_n$-Z-X^1-(O-CH_2-CH_2)$_m$-X^2-L (Formula I*);

wherein

n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H;

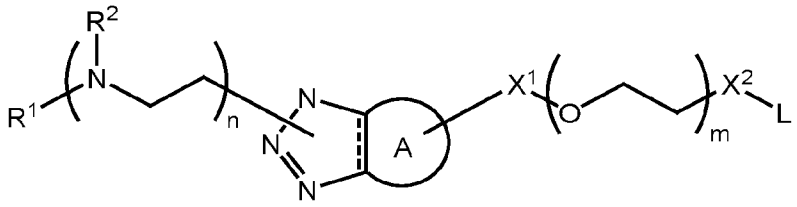
X^1 and X^2 are independently divalent covalent linking moieties;

Z is a divalent covalent linking moiety wherein Z-X^1 is not a single bond and Z is not -NHC(O)-;

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and

wherein preferably said composition consists of said conjugate.

In another aspect, the present invention provides a composition comprising a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

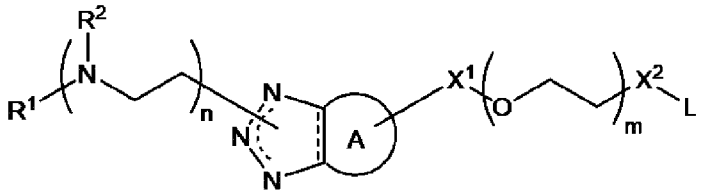
X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

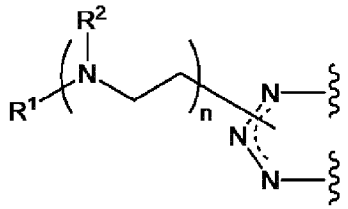
L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting

fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA.

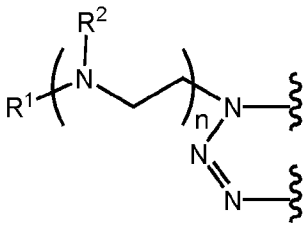
Although the N-N=N fragment of bicyclic ring in Formula I is typically drawn herein using one single bond and one double bond for simplicity, one of skill in the art knows that Formula I and associated conjugate structures as depicted herein can alternatively be drawn as shown below. Such depictions and descriptions of Formula I are interchangeably used herein:



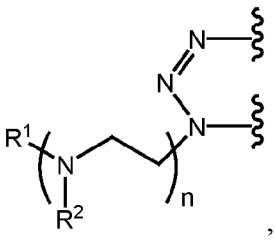
Formula I



wherein the fragment represents two different regioisomeric attachments of the fragment $R^1(NR^2CH_2CH_2)_n$, i.e.,

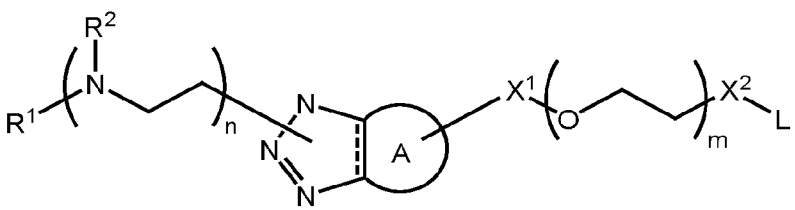


and



, wherein the wavy lines represent chemical bonds to Ring A. Accordingly, Formula I as drawn herein encompasses two regioisomeric embodiments, i.e., wherein the fragment $R^1(NR^2CH_2CH_2)_n$ is bonded at the top nitrogen atom in the structures above or at the bottom nitrogen atom in the structures above, but not at the middle nitrogen atom. One of skill in the art knows that the same applies to other formulae herein, including Formula IA, Formula IB, Formula IC, Formula ID, Formula IE, Formula IH, Formula IJ, Formula IK and the like.

In another aspect, the present invention provides a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H;

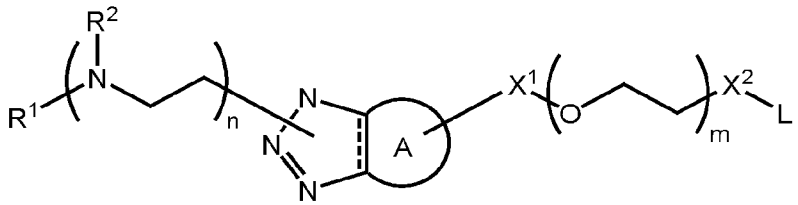
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA.

In another aspect, the present invention provides a composition comprising a conjugate, preferably a plurality of conjugates, of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

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n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$–moieties is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1};

R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2};

R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

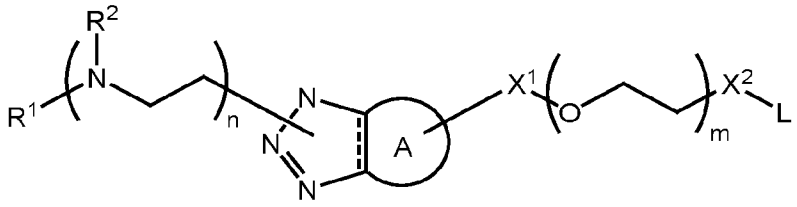
X^1 is a linking moiety of the formula –(Y^1)$_p$–, wherein p is an integer between 1 and 20, and each occurrence of Y^1 is independently selected from a chemical bond, -$CR^{11}R^{12}$-, -C(O)-, -O-, -S-, -NR^{13}-, an amino acid residue, a divalent phenyl moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl or heteroaryl is optionally substituted with one or more R^{13}, and each divalent heterocycle is optionally substituted with one or more R^{14}; wherein R^{11}, R^{12} and R^{13} are independently, at each occurrence, H or C_1-C_6 alkyl; and wherein R^{14} is independently, at each occurrence, H, C_1-C_6 alkyl, or oxo;

X^2 is a linking moiety of the formula –(Y^2)$_q$–, wherein q is an integer between 1 and 50, and each occurrence of Y^2 is independently selected from a chemical bond, -$CR^{21}R^{22}$-, NR^{23}-, -O-, -S-, -C(O)-, an amino acid residue, a divalent phenyl moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl and divalent heteroaryl is optionally substituted with one or more R^{23}, and wherein each divalent heterocycle moiety is optionally substituted with one or more R^{24}; wherein $R^{21,}$ $R^{22,}$ and R^{23} are each independently, at each occurrence, -H, -CO_2H, or C_1-C_6 alkyl, wherein each C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl; and wherein R^{24} is independently, at each occurrence, -H, -CO_2H, C_1-C_6 alkyl, or oxo; and

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is

capable of binding to a cell expressing PSMA, and wherein preferably said composition consists of said conjugate.

In another aspect, the present invention provides a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

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n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$–moieties is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1};

R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2};

R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

X^1 is a linking moiety of the formula –(Y^1)$_p$–, wherein p is an integer between 1 and 20, and each occurrence of Y^1 is independently selected from a chemical bond, -$CR^{11}R^{12}$-, -C(O)-, -O-, -S-, -NR^{13}-, an amino acid residue, a divalent phenyl moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl or heteroaryl is optionally substituted with one or more R^{13}, and each divalent heterocycle is optionally substituted with one or more R^{14}; wherein R^{11}, R^{12} and R^{13} are independently, at each occurrence, H or C_1-C_6 alkyl; and wherein R^{14} is independently, at each occurrence, H, C_1-C_6 alkyl, or oxo;

X^2 is a linking moiety of the formula $–(Y^2)_q–$, wherein q is an integer between 1 and 50, and each occurrence of Y^2 is independently selected from a chemical bond, $-CR^{21}R^{22}-$, $NR^{23}-$, -O-, -S-, -C(O)-, an amino acid residue, a divalent phenyl moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl and divalent heteroaryl is optionally substituted with one or more R^{23}, and wherein each divalent heterocycle moiety is optionally substituted with one or more R^{24}; wherein R^{21}, R^{22}, and R^{23} are each independently, at each occurrence, -H, $-CO_2H$, or C_1-C_6 alkyl, wherein each C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl; and wherein R^{24} is independently, at each occurrence, -H, $-CO_2H$, C_1-C_6 alkyl, or oxo; and

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In a further aspect, the present invention provides a method of synthesizing a composition comprising a conjugate, preferably a plurality of conjugates, of Formula I, comprising reacting an LPEI fragment comprising an azide with a PEG fragment comprising an alkene or alkyne at a pH below about 5, preferably about 4 or below. In some preferred embodiments, the LPEI fragment comprises the azide at the omega terminus, and the PEG fragment comprises the alkene or alkyne at a first terminal end.

In a further aspect, the present invention provides a polyplex comprising a composition as described herein and a polyanion, wherein preferably said polyanion is a nucleic acid, further preferably wherein said nucleic acid is a RNA, and again further preferably wherein said polyanion is polyinosinic:polycytidylic acid (poly(IC).

In a further aspect, the present invention provides a polyplex comprising a composition as described herein and a nucleic acid. In a further aspect, the present invention provides a polyplex comprising a composition as described herein and a nucleic acid, wherein said nucleic acid is a RNA. In a further aspect, the present invention provides a polyplex comprising a composition as described herein and polyinosinic:polycytidylic acid (poly(IC).

In another aspect, the present invention provides a polyplex comprising a triconjugate as described herein, preferably said conjugate of Formula I* or of Formula I, and a polyanion such as a nucleic acid, preferably polyinosinic:polycytidylic acid (poly(IC).

In a further aspect, the present invention provides a polyplex comprising a composition as described herein and a nucleic acid, wherein said nucleic acid is a mRNA. In a further aspect,

the present invention provides a polyplex comprising a composition as described herein and a nucleic acid, wherein said nucleic acid is a DNA, preferably a plasmid DNA.

In one aspect, the present invention provides a pharmaceutical composition comprising a triconjugate, preferably said conjugate of Formula I* or of Formula I, and/or polyplex as described herein, and a pharmaceutically acceptable salt thereof.

In one aspect, the present invention provides a polyplex as described herein, or a pharmaceutical composition comprising a polyplex as described herein for use in the treatment of a disease or disorder, preferably of a cancer, further preferably of a prostate cancer.

In one aspect, the present invention provides the use of a polyplex as described herein for use in the manufacture of a medicament for the treatment of a disease or disorder such as a cancer, further preferably of a prostate cancer.

In another aspect, the present invention provides a method of treating a disease or disorder such as a cancer, preferably of a prostate cancer, in a subject in need thereof, the method comprising administering to the subject an effective amount of a polyplex as described herein.

The linear, nonrandom LPEI-PEG diconjugates described herein, and thus the inventive compositions and polyplexes comprising the triconjugates, not only ensure consistent and predictable ratios of LPEI to PEG fragments, but typically and preferably further ensure structurally defined linear conjugates of LPEI fragment to PEG fragment. Thus, they offer greater batch-to-batch consistency, ease of manufacturing, and more predictable SAR compared with the branched LPEI-PEG diconjugates currently prepared using the random, uncontrolled synthesis strategies described above.

Further advantageously and surprisingly, when the inventive linear, nonrandom conjugates described herein are combined with a polyanion and nucleic acid such as poly(IC) to form a polyplex and administered to cells, the polyplexes surprisingly not only maintain, but may even show superior antitumor activity to polyplexes made using random, branched conjugates. Thus, despite the significant reduction of variability and number in structures of the used conjugates, and thus significant reduction of variability and number in structures of possible (bio)activity including targeting and presenting their targeting fragments to the surface of the targeted cells as well subsequent uptake, there is no loss in efficacy of the linear LPEI-*l*-PEG:nucleic acid polyplexes described herein. To the contrary, the inventive conjugates and compositions are even able to maintain or even increase their overall biological activity. Additional features and advantages of the present technology will be apparent to one of skill in

the art upon reading the Detailed Description of the Invention, below and further aspects and embodiments of the present invention will be become apparent as this description continues.

BRIEF DESCRIPTION OF FIGURES

FIG 1 is a DLS back scatter plot taken in triplicate of a Me-LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(IC) polyplex measuring size distribution and ζ-potential in 20 mM HEPES, 5% glucose at pH 7.2, 0.1875 mg/mL, 1.0 mL volume, N/P ratio of 4. The z-average diameter was 130 nm with a polydispersity index (PDI) of 0.134. The ζ-potential was 26.6 mV.

FIG 2 is a DLS back scatter plot taken in triplicate of a Me-LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) polyplex measuring size distribution and ζ-potential in 20 mM HEPES, 5% glucose at pH 7.2, 0.1875 mg/mL, 1.0 mL volume, N/P ratio of 4. The z-average diameter was 140 nm with a polydispersity index (PDI) of 0.132. The ζ-potential was 28.2 mV.

FIG 3 is a depiction of differential PSMA expression as determined *in vitro* by flow cytometry for an array of human prostate cancer cell lines (LNCaP, VCaP, PC-3, DU145). Staggered histograms of fluorescence intensity are shown and mean fluorescence intensity (MFI) is indicated.

FIG 4A is a flow cytometry analysis of MHC I expression on the cell surface of prostate cancer cells lines with high PSMA expression (LNCaP) as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes at 0.0125 and 0.125 μg/mL of the payload or with no treatment (untreated control). Isotype control and unstained controls indicate background fluorescence. Staggered histograms of fluorescence intensity are shown and mean Fluorecent intensity (MFI) is indicated.

FIG 4B is a flow cytometry analysis of MHC I expression on the cell surface of prostate cancer cells lines with low PSMA expression (DU145) as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes at 0.0125 and 0.125 μg/mL of the payload or with no treatment (untreated control). Isotype control and unstained controls indicate background fluorescence. Staggered histograms of fluorescence intensity are shown and mean Fluorecent intensity (MFI) is indicated.

FIG 5A is a plot of cell survival in LNCaP cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 5B is a plot of cell survival in PC-3 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(Glu). The X

axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 5C is a plot of cell survival in DU145 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 5D is a plot of cell survival in LNCaP cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-folate:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 5E is a plot of cell survival in DU145 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 6A is a plot of cell survival in LNCaP cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 6B is a plot of cell survival in PC-3 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 6C is a plot of cell survival in DU145 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 7 is a plot of cell survival in LNCaP cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC), LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu), Me-LPEI[N3:DBCO]PEG_{36}-[MAL-S]-DUPA:poly(IC), and Me-LPEI[N3:DBCO]PEG_{36}-[MAL-S]-DUPA:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 8 is a plot of cell survival in LNCaP cells as a function of treatment with LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA:poly(IC), LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA:poly(Glu), Me-LPEI[N3:BCN]PEG_{36}-[MAL-S]-DUPA:poly(IC), and Me-LPEI[N3:BCN]PEG_{36}-[MAL-S]-DUPA:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 9 is a plot of cell survival in DU145 prostate cancer cells with low PSMA expression as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC), LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu), Me-LPEI[N3:DBCO]PEG_{36}-[MAL-S]-DUPA:poly(IC), and Me-LPEI[N3:DBCO]PEG_{36}-[MAL-S]-DUPA:poly(Glu). The X axis

indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 10 is a plot of cell survival in DU145 prostate cancer cells with low PSMA expression as a function of treatment with LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(IC), LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(Glu), Me-LPEI[N_3:BCN]PEG_{36}-[MAL-S]-DUPA:poly(IC), and Me-LPEI[N_3:BCN]PEG_{36}-[MAL-S]-DUPA:poly(Glu) The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 11 is a plot of cell survival in LNCaP cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA:poly(IC); LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(IC); LPEI-*l*-[N_3:SCO]-PEG_{36}—[MAL-S]-DUPA:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-[CONH]-DUPA:poly(IC); and LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA:poly(IC) polyplexes. The X axis indicates the log of concentration of poly(IC) delivered.

FIG 12 is a plot of cell survival in VCaP prostate cancer cells with intermediate PSMA cell surface expression as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu). The X axis indicates the concentration of poly(IC) or poly(Glu) delivered.

FIG 13 is a plot of cell survival in DU145 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA:poly(IC); LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(IC); LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-[CONH]-DUPA:poly(IC); and LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA:poly(IC) polyplexes. The X axis indicates the concentration of poly(IC) delivered.

FIG 14A is a plot of IP-10 secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) concentration in LNCaP cells and PC-3 cells.

FIG 14B is a plot of IP-10 secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration in LNCaP cells and PC-3 cells.

FIG 14C is a plot of IP-10 secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration in LNCaP cells and DU145 cells.

FIG 15A is a plot of RANTES secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) concentration in LNCaP cells and PC-3 cells.

FIG 15B is a plot of RANTES secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration in LNCaP cells and PC-3 cells.

FIG 15C is a plot of RANTES secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-

DUPA:poly(IC) concentration in LNCaP cells and DU145 cells.

FIG 16A is a plot of IFNß secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) concentration in LNCaP cells and PC-3 cells.

FIG 16B is a plot of IFNß secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration in LNCaP cells and PC-3 cells.

FIG 16C is a plot of IFNß secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration in LNCaP cells and DU145 cells.

FIG 17 is a Western Blot imaging analysis showing qualitative levels of Caspase 3, cleaved Caspase 3, PARP, cleaved PARP, RIG-1; MDA5, and ISG15 as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes at 0, 0.0625 and 0.625 µg/mL. GAPDH functioned as protein loading control.

FIG 18 is an immunoblot analysis of prostate cancer cells with high PSMA (LNCaP) and low PSMA expression (DU145) as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes at 0.02 and 0.2 µg/mL of the payload (poly(IC) and poly(Glu), respectively) for 5 and 24 hours. The analysis illustrates qualitative levels of IκB, Phospho IκB, IRF3, Phospho IRF3, NFκB, Phospho NFκB, and PD-L1. GAPDH functioned as protein loading control.

FIG 19 is a SEM image of polyplexes particles comprising compounds 31 and 31b and poly(IC), i.e., LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC), formed at an N/P ratio of 4 and a concentration of 0.1875 mg/mL in HEPES 20 mM buffer, 5% glucose (HBG), pH 7.2.

FIG 20 depicts luminescence normalized to survival in human prostate cell lines with differential cell surface expression of PSMA: PSMA high expressing LNCaP cells, and PSMA low expressing DU145 cells following transfection with PSMA targeting polyplexes containing mRNA encoding Luciferase. The X axis indicates the concentration of the mRNA in the polyplexes (0.25, 0.5 and 1.0 µg/mL). The Y axis indicates luminescence normalized to survival in arbitrary units (AU). Selective transfection of PSMA overexpressing cells with Luc mRNA as well as selective expression of Luciferase was demonstrated.

FIG 21 depicts the levels of secreted human IL-2 from two cell lines with differential PSMA expression: PSMA high expressing LNCaP cells, and PSMA low expressing DU145 cells following transfection with PSMA targeting polyplexes containing hIL-2 mRNA. Selective expression of human IL-2 from PSMA overexpressing cells is demonstrated.

FIG 22 depicts the levels of secreted human IFNβ from two cell lines with differential PSMA expression: PSMA high expressing LNCaP cells, and PSMA low expressing DU145 cells following transfection with PSMA targeting polyplexes containing hIFNβ mRNA. Selective expression of human IFNβ from PSMA high expressing cells is demonstrated.

FIG 23 depicts protein biosynthesis inhibition by DT-A protein in two cell lines with differential PSMA expression: high PSMA-expressing LNCaP cells, and low PSMA-expressing DU145 cells following transfection with PSMA targeting polyplexes LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA containing mRNA DT-A. Western blot analysis with an anti-puromycin antibody as probe was utilized to detect inhibition of protein biosynthesis. GAPDH was used as a loading control. Selective inhibition of protein biosynthesis in PSMA overexpressing cells is demonstrated.

FIG 24 depicts luminescence from human prostate cell lines with differential cell surface expression of PSMA: high-PSMA expressing LNCaP cells, and low PSMA-expressing DU145 cells. The cells were treated with PSMA-targeting polyplexes containing plasmid DNA encoding luciferase. The X axis indicates the concentration of the pGreenFire-CMV in the polyplexes (0.25, 0.5 and 1.0 µg/mL). The Y axis indicates luminescence in arbitrary units (AU). Average and standard deviation from triplicate samples are presented. Selective expression of luciferase after transfection of PSMA overexpressing cells with plasmid DNA encoding luciferase (pGreenFire-CMV) is demonstrated.

FIG 25 depicts levels of secreted human IL2 normalized to cell survival, in cell lines with differential PSMA expression: high-expressing LNCaP and C4-2 cells, and low-expressing DU145 cells following transfection with PSMA-targeting polyplexes containing plasmid encoding IL2 protein. The X axis indicates the concentration of the hIL2 plasmid DNA (0.25, 0.5 and 1.0 µg/mL) in the polyplexes. The Y axis indicates the concentration of secreted IL-2 normalized to cell survival in arbitrary units (AU). The selective expression/secretion of human IL2 after transfection of PSMA overexpressing cells with plasmid DNA encoding hIL-2 is demonstrated.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. The herein described and disclosed embodiments, preferred embodiments and very preferred embodiments should apply to all aspects and other embodiments, preferred

embodiments and very preferred embodiments irrespective of whether explicitly or specifically again referred to.

The present invention provides linear conjugates of LPEI and PEG that can form polyplexes with polyanions and nucleic acids such as poly(IC), as outlined herein and below. The conjugates comprise an LPEI fragment, a PEG fragment, and a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein the LPEI fragment and the PEG fragment are coupled in a discrete end-to-end fashion. In some preferred embodiments, the LPEI fragment and the PEG fragment are coupled through the covalent attachment of an azide to an alkene or alkyne to form a 1,2,3-triazole or a 4,5-dihydro-1H-[1,2,3]triazole.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this disclosure belongs.

The articles “a” and “an” are used in this disclosure to refer to one or more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

The term “and/or” is used in this disclosure to mean either “and” or “or” unless indicated otherwise.

The term “about”, as used herein shall have the meaning of +/- 10%. For example about 50% shall mean 45% to 55%. Preferably, the term “about”, as used herein shall have the meaning of +/- 5%. For example about 50% shall mean 47.5% to 52.5%.

The phrase "between number X and number Y", as used herein, shall refer to include the number X and the number Y. For example, the phrase "between 0.01µmol and 50µmol" refers to 0.01µmol and 50µmol and the values in between. The same applies to the phrase "between about number X and about number Y”.

The term “optionally substituted” is understood to mean that a given chemical moiety (e.g. an alkyl group) can (but is not required to) be bonded to other substituents (e.g. heteroatoms). For instance, an alkyl group that is optionally substituted can be a fully saturated alkyl chain (i.e. a pure hydrocarbon). Alternatively, the same optionally substituted alkyl group can have substituents different from hydrogen. For instance, it can, at any point along the chain be bounded to a halogen atom, an alkoxy group, or any other substituent described herein. Thus

the term "optionally substituted" means that a given chemical moiety has the potential to contain other functional groups but does not necessarily have any further functional groups.

The term "optionally replaced" is understood to refer to situations in which the carbon atom of a methylene group (i.e., $-CH_2-$) can be, but is not required to be, replaced by a heteroatom (e.g., -NH-, -O-). For example, a C_3 alkylene (i.e., propylene) group wherein one of the methylene groups is "optionally replaced" can have the structure $-CH_2-O-CH_2-$ or $-O-CH_2-CH_2-$. It will be understood by one of skill in the art that a methylene group cannot be replaced when such replacement would result in an unstable chemical moiety. For example, one of skill in the art will understand that four methylene groups cannot simultaneously be replaced by oxygen atoms. Thus, in some preferred embodiments, when one methylene group of an alkylene fragment is replaced by a heteroatom, one or both of the neighboring carbon atoms are not replaced by a heteroatom.

The term "aryl" refers to cyclic, aromatic hydrocarbon groups that have 1 to 2 aromatic rings, including monocyclic or bicyclic groups such as phenyl, biphenyl or naphthyl. A C_6-C_{10} aryl group contains between 6 and 10 carbon atoms. When containing two aromatic rings (bicyclic, etc.), the aromatic rings of the aryl group may be joined at a single point (e.g., biphenyl), or fused (e.g., naphthyl). The aryl group may be optionally substituted by one or more substituents, e.g., 1 to 5 substituents, at any point of attachment. The substituents can themselves be optionally substituted. Furthermore, when containing two fused rings, the aryl groups herein defined may have an unsaturated or partially saturated ring fused with a fully saturated ring. Exemplary ring systems of these aryl groups include indanyl, indenyl, tetrahydronaphthalenyl, and tetrahydrobenzoannulenyl. In some preferred embodiments, the aryl group is a phenyl group.

Unless otherwise specifically defined, "heteroaryl" means a monovalent monocyclic aromatic ring of 5 to 24 ring atoms or a polycyclic aromatic ring, containing one or more ring heteroatoms selected from N, S, P, or O, the remaining ring atoms being C. A 5-10 membered heteroaryl group contains between 5 and 10 atoms. Heteroaryl as herein defined also means a bicyclic heteroaromatic group wherein the heteroatom is selected from N, S, P, or O. The aromatic radical is optionally substituted independently with one or more substituents described herein. Examples include, but are not limited to, furyl, thienyl, pyrrolyl, pyridyl, pyrazolyl, pyrimidinyl, imidazolyl, isoxazolyl, oxazolyl, oxadiazolyl, pyrazinyl, indolyl, thiophen-2-yl, quinolyl, benzopyranyl, isothiazolyl, thiazolyl, thiadiazole, indazole, benzimidazolyl, thieno[3,2-b]thiophene, triazolyl, triazinyl, imidazo[1,2-b]pyrazolyl, furo[2,3-c]pyridinyl,

imidazo[1,2-a]pyridinyl, indazolyl, pyrrolo[2,3-c]pyridinyl, pyrrolo[3,2-c]pyridinyl, pyrazolo[3,4-c]pyridinyl, thieno[3,2-c]pyridinyl, thieno[2,3-c]pyridinyl, thieno[2,3-b]pyridinyl, benzothiazolyl, indolyl, indolinyl, indolinonyl, dihydrobenzothiophenyl, dihydrobenzofuranyl, benzofuran, chromanyl, thiochromanyl, tetrahydroquinolinyl, dihydrobenzothiazine, dihydrobenzoxanyl, quinolinyl, isoquinolinyl, 1,6-naphthyridinyl, benzo[de]isoquinolinyl, pyrido[4,3-b][1,6]naphthyridinyl, thieno[2,3-b]pyrazinyl, quinazolinyl, tetrazolo[1,5-a]pyridinyl, [1,2,4]triazolo[4,3-a]pyridinyl, isoindolyl, pyrrolo[2,3-b]pyridinyl, pyrrolo[3,4-b]pyridinyl, pyrrolo[3,2-b]pyridinyl, imidazo[5,4-b]pyridinyl, pyrrolo[1,2-a]pyrimidinyl, tetrahydro pyrrolo[1,2-a]pyrimidinyl, 3,4-dihydro-2H-1λ2-pyrrolo[2,1-b]pyrimidine, dibenzo[b,d]thiophene, pyridin-2-one, furo[3,2-c]pyridinyl, furo[2,3-c]pyridinyl, 1H-pyrido[3,4-b][1,4]thiazinyl, benzooxazolyl, benzoisoxazolyl, furo[2,3-b]pyridinyl, benzothiophenyl, 1,5-naphthyridinyl, furo[3,2-b]pyridine, [1,2,4]triazolo[1,5-a]pyridinyl, benzo [1,2,3]triazolyl, imidazo[1,2-a]pyrimidinyl, [1,2,4]triazolo[4,3-b]pyridazinyl, benzo[c][1,2,5]thiadiazolyl, benzo[c][1,2,5]oxadiazole, 1,3-dihydro-2H-benzo[d]imidazol-2-one, 3,4-dihydro-2H-pyrazolo[1,5-b][1,2]oxazinyl, 4,5,6,7-tetrahydropyrazolo[1,5-a]pyridinyl, thiazolo[5,4-d]thiazolyl, imidazo[2,1-b][1,3,4]thiadiazolyl, thieno[2,3-b]pyrrolyl, 3H-indolyl, and derivatives thereof. Furthermore, when containing two fused rings, the heteroaryl groups herein defined may have an unsaturated or partially saturated ring fused with a fully saturated ring. Exemplary ring systems of these heteroaryl groups include indolinyl, indolinonyl, dihydrobenzothiophenyl, dihydrobenzofuran, chromanyl, thiochromanyl, tetrahydroquinolinyl, dihydrobenzothiazine, 3,4-dihydro-1H--isoquinolinyl, 2,3-dihydrobenzofuran, indolinyl, indolyl, and dihydrobenzoxanyl.

The term “alkyl” refers to a straight or branched chain saturated hydrocarbon. C_1-C_6 alkyl groups contain 1 to 6 carbon atoms. Examples of a C_1-C_6 alkyl group include, but are not limited to, methyl, ethyl, propyl, butyl, pentyl, isopropyl, isobutyl, sec-butyl, tert-butyl, isopentyl and neopentyl.

The term “alkylene” refers to a straight or branched chain saturated and bivalent hydrocarbon fragment. C_0-C_6 alkyl groups contain 0 to 6 carbon atoms. Examples of a C_0-C_6 alkylene group include, but are not limited to, methylene, ethylene, propylene, butylene, pentylene, isopropylene, isobutylene, sec-butylene, tert-butylene, isopentylene, and neopentylene.

The term “C_1-C_6-alkoxy”, as used herein, refers to a substituted hydroxyl of the formula (-OR'), wherein R' is an optionally substituted C_1-C_6 alkyl, as defined herein, and the oxygen

moiety is directly attached to the parent molecule, and thus the term "C_1-C_6 alkoxy", as used herein, refers to straight chain or branched C_1-C_6 alkoxy which may be, for example, methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, *sec*-butoxy, *tert*-butoxy, straight or branched pentoxy, straight or branched hexyloxy. Preferred are C_1-C_4 alkoxy and C_1-C_3 alkoxy.

The term "cycloalkyl" means monocyclic or polycyclic saturated carbon rings containing 3-18 carbon atoms. A C_3-C_8 cycloalkyl contains between 3 and 8 carbon atoms. Examples of cycloalkyl groups include, without limitations, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptanyl, cyclooctanyl, norboranyl, norborenyl, bicyclo[2.2.2]octanyl, or bicyclo[2.2.2]octenyl. A C_3-C_8 cycloalkyl is a cycloalkyl group containing between 3 and 8 carbon atoms.

The term "cycloalkenyl" means monocyclic, non-aromatic unsaturated carbon rings containing 5-18 carbon atoms. Examples of cycloalkenyl groups include, without limitation, cyclopentenyl, cyclohexenyl, cycloheptenyl, cyclooctenyl, and norborenyl. A C_5-C_8 cycloalkenyl is a cycloalkenyl group containing between 5 and 8 carbon atoms.

The terms "heterocyclyl" or "heterocycloalkyl" or "heterocycle" refer to monocyclic or polycyclic 3 to 24-membered rings containing carbon and heteroatoms taken from oxygen, nitrogen, or sulfur and wherein there is not delocalized π electrons (aromaticity) shared among the ring carbon or heteroatoms. A 3-10 membered heterocycloalkyl group contains between 3 and 10 atoms. Heterocyclyl rings include, but are not limited to, oxetanyl, azetadinyl, tetrahydrofuranyl, pyrrolidinyl, oxazolinyl, oxazolidinyl, thiazolinyl, thiazolidinyl, pyranyl, thiopyranyl, tetrahydropyranyl, dioxalinyl, piperidinyl, morpholinyl, thiomorpholinyl, thiomorpholinyl S-oxide, thiomorpholinyl S-dioxide, piperazinyl, azepinyl, oxepinyl, diazepinyl, tropanyl, and homotropanyl.

The term "heterocycloalkenyl" refers to monocyclic or polycyclic 3 to 24-membered rings containing carbon and heteroatoms taken from oxygen, nitrogen, or sulfur and wherein there is not delocalized π electrons (aromaticity) shared among the ring carbon or heteroatoms, but there is at least one element of unsaturation within the ring. A 3-10 membered heterocycloalkenyl group contains between 3 and 10 atoms.

As used herein, the term "halo" or "halogen" means fluoro (F), chloro (Cl), bromo (Br), or iodo (I).

The term "carbonyl" refers to a functional group composing a carbon atom double-bonded to an oxygen atom. It can be abbreviated herein as "oxo", as C(O), or as C=O.

The term "overexpression" refers to gene or protein expression within a cell or in a cell

surface that is increased relative to basal or normal expression. In a preferred embodiment, said targeting fragment is capable of binding to a cell overexpressing a cell surface receptor. In one embodiment, said cell overexpressing a cell surface receptor means that the level of said cell surface receptor expressed in said cell of a certain tissue is elevated in comparison to the level of said cell surface receptor as measured in a normal healthy cell of the same type of tissue under analogous conditions. In one embodiment, said cell overexpressing a cell surface receptor refers to an increase in the level of said cell surface receptor in a cell relative to the level in the same cell or closely related non-malignant cell under normal physiological conditions.

The term “polyanion”, as used herein, refers to a polymer, preferably a biopolymer, having more than one site carrying a negative charge. Typically and preferably, the term “polyanion”, as used herein, refers to a polymer, preferably a biopolymer, made up of repeating units comprising residues capable of bearing negative charge. In further embodiments, a polyanion is a polymer, preferably a biopolymer, made up of repeating units comprising negatively charged residues. In another preferred embodiment, said polyanion is a nucleic acid, more preferably a DNA, RNA, polyglutamic acid or hyaluronic acid.

The term “nucleic acid” as used herein, comprises deoxyribonucleic acid (DNA) and/or ribonucleic acid (RNA) or a combination thereof. In a preferred embodiment, the term “nucleic acid” refers to deoxyribonucleic acid (DNA) and/or ribonucleic acid (RNA), and hereby to genomic, viral and recombinantly prepared and chemically synthesized molecules. A nucleic acid may be in the form of a single stranded or double-stranded and linear or covalently closed circular molecule and may comprise a chemical derivatization of a nucleic acid on a nucleotide base, on the sugar or on the phosphate, and may contain non-natural nucleotides and nucleotide analogs.

The term “dispersity” (abbreviated as D), as used herein refers to the distribution of the molar mass in a given polymeric sample such as in polymeric fragments as used herein for the inventive conjugates and polyplexes. It is defined herein as $D = (M_w/M_n)$, wherein D is dispersity; M_w is the weight average molecular weight of the polymeric sample or polymeric fragment; and M_n is the number average molecular weight of the polymeric sample or polymeric fragment.

The term "weight average molecular weight", as used herein refers to the sum of the products of the weight fraction for a given molecule in the mixture times the mass of the molecule for each molecule in the mixture and is typically and preferably represented by the symbol Mw.

The term "number average molecular weight", as used herein refers to the total weight of a mixture divided by the number of molecules in the mixture and is typically and preferably represented by the symbol Mn.

The term “polydispersity index” (abbreviated as PDI) as used herein refers to the polydispersity index in dynamic light scattering measurements of polyplex nanoparticles such as the polyplexes in accordance with the present invention. This index is a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis). The polydispersity index is dimensionless and scaled such that values smaller than 0.05 are rarely seen other than with highly monodisperse standards. Values greater than 0.7 indicate that the sample has a very broad size distribution and is probably not suitable for the dynamic light scattering (DLS) technique. The various size distribution algorithms work with data that falls between these two extremes. The zeta-average diameter (z-average diameter) and polydispersity index of the inventive polyplexes are determined by Dynamic Light Scattering (DLS), based on the assumption that said polyplexes are isotropic and spherically shaped. The calculations for these parameters are defined and determined according to ISO standard document ISO 22412:2017.

The term “amino acid residue” refers to a divalent residue derived from an organic compound containing the functional groups amine (-NH_2) and carboxylic acid (-COOH), typically and preferably, along with a side chain specific to each amino acid. In a preferred embodiment of the present invention, an amino acid residue is a divalent residue derived from an organic compound containing the functional groups amine (-NH_2) and carboxylic acid (-COOH), wherein said divalence is effected with said amine and said carboxylic acid functional group, and thus by –NH- and –CO- moieties. In alternative preferred embodiment of the present invention, an amino acid residue is a divalent residue derived from an organic compound containing the functional groups amine (-NH_2) and carboxylic acid (-COOH), wherein said divalence is effected with said amine or said carboxylic acid functional group, and with a further functional group present in said amino acid residue. By way of a preferred example and embodiment, an amino acid residue in accordance with the present invention derived from cysteine includes the divalent structure –S-(CH2)-CH(COOH)-NH-, wherein said divalence is effected by the amino functionality and the comprised thiol functionality. The term “amino acid residue”, as used herein typically and preferably includes amino acid residues derived from naturally occurring or non-naturally occurring amino acids. Furthermore, the term “amino acid residue”, as used herein, typically and preferably also includes amino acid residues derived from unnatural amino acids that are chemically synthesized including alpha-(α-), beta-(β-),

gamma-(γ-) or delta-(δ-) etc. amino acids as well as mixtures thereof in any ratio. In addition, the term "amino acid residue", as used herein, typically and preferably also includes amino acid residues derived from alpha amino acids including any isomeric form thereof, in particular its D-stereoisomers and L-stereoisomers (alternatively addressed by the (R) and (S) nomenclature), as well as mixtures thereof in any ratio, preferably in a racemic ratio of 1:1. The term "D-stereoisomer", "L-stereoisomer", "D-amino acid" or "L-amino acid" refers to the chiral alpha carbon of the amino acids. Thus, in a preferred embodiment, said amino acid residue is a divalent group of the structure -NH-CHR-C(O)-, wherein R is an amino acid side chain. Two or more consecutive amino acid residues preferably form peptide (i.e., amide) bonds at both the amine portion and the carboxylic acid portion of the amino acid residues respectively. When di, tri or polypeptides are described herein as amino acid residues, typically as $(AA)_a$, the provided sequence is depicted from left to right in the N-C direction. Thus, and by way of example the depiction Trp-Trp-Gly should refer to an amino acid residue, wherein Trp corresponds to the N-terminus of said tripeptide with a –NH- valence, and wherein Gly corresponds to the C-terminus of said tripeptide with a –CO- valence.

The terms "peptide", "polypeptide" and "protein", as used herein refer to substances which comprise about two or more consecutive amino acid residues linked to one another via peptide bonds. The terms "peptide," "polypeptide," and "protein" are used interchangeably herein to refer to polymers of amino acid residues of any length. In one embodiment, the term "protein" refers to large peptides, in particular peptides having at least about 151 amino acids, while in one embodiment, the term "peptide" refers to substances which comprise about two or more, about 3 or more, about 8 or more, or about 20 or more, and up to about 50, about 100 or about 150 amino acids in length,

The term "epitope", as used herein, refers to an antigenic determinant in a molecule such as an antigen. An epitope of a protein preferably comprises a continuous or discontinuous portion of said protein and is preferably between 5 and 100, preferably between 5 and 50, more preferably between 8 and 30, most preferably between 10 and 25 amino acids in length, for example, the epitope may be preferably 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids in length.

The term "antibody" refers to any immunoglobulin, whether natural or wholly or partially synthetically produced and to derivatives thereof and characteristic portions thereof. An antibody may be monoclonal or polyclonal. An antibody may be a member of any immunoglobulin class, including any of the human classes: IgG, IgM, IgA, IgD, and IgE. As

used herein, an antibody fragment (i.e., characteristic portion of an antibody) refers to any derivative of an antibody which is less than full-length. In general, an antibody fragment retains at least a significant portion of the full-length antibody's specific binding ability. Examples of antibody fragments include, but are not limited to, single chain and double strain fragments, Fab, Fab', F(ab')2, scFv, Fv, dsFv diabody, and Fd fragments. An antibody fragment may be produced by any means. For example, an antibody fragment may be enzymatically or chemically produced by fragmentation of an intact antibody and/or it may be recombinantly produced from a gene encoding the partial antibody sequence. Alternatively or additionally, an antibody fragment may be wholly or partially synthetically produced. An antibody fragment may optionally comprise a single chain antibody fragment. Alternatively or additionally, an antibody fragment may comprise multiple chains which are linked together, for example, by disulfide linkages. An antibody fragment may optionally comprise a multimolecular complex. A functional antibody fragment will typically comprise at least about 50 amino acids and more typically will comprise at least about 200 amino acids. In some embodiments, antibodies may include chimeric (e.g. "humanized") and single chain (recombinant) antibodies. In some embodiments, antibodies may have reduced effector functions and/or bispecific molecules. In some embodiments, antibodies may include fragments produced by a Fab expression library. Single-chain Fvs (scFvs) are recombinant antibody fragments consisting of only the variable light chain (VL) and variable heavy chain (VH) covalently connected to one another by a polypeptide linker. Either VL or VH may comprise the NH2-terminal domain. The polypeptide linker may be of variable length and composition so long as the two variable domains are bridged without significant steric interference. Typically, linkers primarily comprise stretches of glycine and serine residues with some glutamic acid or lysine residues interspersed for solubility. Diabodies are dimeric scFvs. Diabodies typically have shorter peptide linkers than most scFvs, and they often show a preference for associating as dimers. An Fv fragment is an antibody fragment which consists of one VH and one VL domain held together by noncovalent interactions. The term "dsFv" as used herein refers to an Fv with an engineered intermolecular disulfide bond to stabilize the VH-VL pair. A F(ab')2 fragment is an antibody fragment essentially equivalent to that obtained from immunoglobulins by digestion with an enzyme pepsin at pH 4.0-4.5. The fragment may be recombinantly produced. A Fab' fragment is an antibody fragment essentially equivalent to that obtained by reduction of the disulfide bridge or bridges joining the two heavy chain pieces in the F(ab')2 fragment. The Fab' fragment may be recombinantly produced. 1. A Fab fragment is an antibody fragment essentially equivalent to

that obtained by digestion of immunoglobulins with an enzyme (e.g. papain). The Fab fragment may be recombinantly produced. The heavy chain segment of the Fab fragment is the Fd sub-fragment.

The term "alpha terminus of the linear polyethyleneimine fragment" (α-terminus of LPEI fragment), as used herein, refers to the terminal end of the LPEI fragment where initiation of polymerization occurs using electrophilic initiators as further described below for the term "initiation residue".

The term "omega terminus of the linear polyethyleneimine fragment" (ω-terminus of LPEI fragment) as used herein, refers to the terminal end of the LPEI fragment where termination of polymerization occurs using nucleophiles such as azides, thiol and other nucleophiles as described herein.

The term "organic residue" refers to any suitable organic group capable of binding to the nitrogen atoms embedded within LPEI fragments. In preferred embodiments the organic residue is connected to the nitrogen atom via a carbonyl group to form an amide linkage. Without wishing to be bound by theory, said organic residue is incorporated on the nitrogen atoms of poly(2-oxazoline) during ring-opening polymerization 2-oxazoline (see, e.g., Glassner et al., (2018), Poly(2-oxazoline)s: A comprehensive overview of polymer structures and their physical properties. *Polym. Int*, **67**: 32-45. https://doi.org/10.1002/pi.5457). Typically and preferably, said organic residue is cleaved (i.e., typically said amide is cleaved) from the poly(2-oxazoline) to yield LPEI and LPEI fragments and thus -(NH-CH_2-CH_2)–moieties embedded within the conjugates of the present invention. However, in case said cleavage reaction is not complete a fraction of said organic residue is not cleaved. Thus, in preferred embodiments of the invention at least 80%, preferably 90% of R^2 in the R^1-(NR^2-CH_2-CH_2)$_n$–moieties of the conjugates of the present invention including the ones of Formula I* and I is H, preferably at least 91%, more preferably 92%, more preferably 93%, more preferably 94%, more preferably 95%, more preferably 96%, more preferably 97%, more preferably 98%, and most preferably 99%, of R^2 in the R^1-(NR^2-CH_2-CH_2)$_n$–moieties of the conjugates of the present invention including the ones of Formula I* or I is H.

The term "initiation residue" refers to the residue present in the LPEI fragment and the R^1-(NR^2-CH_2-CH_2)$_n$–moieties of the conjugates of the present invention, which residue derives from any initiator, typically and preferably any electrophilic initiator, capable of initiating the polymerization of poly(2-oxazoline) from 2-oxazoline. As set forth in Glassner et al., (2018), Poly(2-oxazoline)s: A comprehensive overview of polymer structures and their physical

properties. *Polym. Int*, **67**: 32-45. https://doi.org/10.1002/pi.5457, "different initiator systems can be used including toluenesulfonic acid (TsOH) or alkyl sulfonates such as methyl p-toluenesulfonate (MeOTs), which is most frequently found in literature, p-nitrobenzenesulfonates (nosylates) and trifluoromethanesulfonates (triflates), alkyl, benzyl and acetyl halides, oxazolinium salts and lewis acids." Accordingly, although in preferred embodiments R^1 is -H or $-CH_3$, one of skill in the art will understand that R^1 can also include but is not limited to other suitable residues such as a C_n alkyl group wherein n is greater than 1, typically a $C_{1\text{-}6}$ alkyl group, a benzyl group, or an acetyl group.

Thus, in one aspect, the present invention provides a composition comprising a conjugate, wherein said conjugate comprises: a linear polyethyleneimine fragment comprising an alpha terminus and an omega terminus; a polyethylene glycol fragment comprising a first terminal end and a second terminal end; wherein said polyethylene glycol fragment comprises, preferably consists of, a discrete number m of repeating $-(O\text{-}CH_2\text{-}CH_2)-$ units, wherein said discrete number m of repeating $-(O\text{-}CH_2\text{-}CH_2)-$ units is any discrete number of 25 to 100, preferably of 25 to 60, wherein preferably said discrete number m is a discrete number of contiguous repeating $-(O\text{-}CH_2\text{-}CH_2)-$ units, and wherein said discrete number of contiguous repeating $-(O\text{-}CH_2\text{-}CH_2)-$ units) is any discrete number of 25 to 100, preferably of 25 to 60; wherein the alpha terminus of said polyethyleneimine fragment is an initiation residue; wherein the omega terminus of the polyethyleneimine fragment is connected to the first terminal end of the polyethylene glycol fragment by a divalent covalent linking group $-Z\text{-}X^1-$, wherein $-Z\text{-}X^1-$ is not a single bond and -Z- is not an amide; wherein the second terminal end of the polyethylene glycol fragment is connected to a targeting fragment by a divalent covalent linking moiety X^2, and wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In a further aspect, the present invention provides a composition comprising a conjugate, wherein said conjugate comprises: a linear polyethyleneimine fragment comprising an alpha terminus and an omega terminus; a polyethylene glycol fragment comprising a first terminal end and a second terminal end; wherein said polyethylene glycol fragment comprises, preferably consists of, a discrete number m of repeating $-(O\text{-}CH_2\text{-}CH_2)-$ units, wherein said discrete number m of repeating $-(O\text{-}CH_2\text{-}CH_2)-$ units is any discrete number of 25 to 100, preferably of 25 to 60, wherein preferably said discrete number m is a discrete number of contiguous repeating $-(O\text{-}CH_2\text{-}CH_2)-$ units, and wherein said discrete number of contiguous

repeating -(O-CH_2-CH_2)- units) is any discrete number of 25 to 100, preferably of 25 to 60; wherein the alpha terminus of said polyethyleneimine fragment is an initiation residue; wherein the omega terminus of the polyethyleneimine fragment is connected to the first terminal end of the polyethylene glycol fragment by a divalent covalent linking group -Z-X^1-, wherein -Z- is not a single bond and -Z- is not an amide; and wherein -X^1- is a divalent covalent linking moiety; wherein the second terminal end of the polyethylene glycol fragment is connected to a targeting fragment by a divalent covalent linking moiety X^2, and wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In another aspect, the present invention provides a composition comprising a conjugate, wherein said conjugate is of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:

R^1-(NR^2-CH_2-CH_2)$_n$-Z-X^1-(O-CH_2-CH_2)$_m$-X^2-L (Formula I*);

wherein

n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H;

X^1 and X^2 are independently divalent covalent linking moieties;

Z is a divalent covalent linking moiety wherein Z-X^1 is not a single bond and Z is not -NHC(O)-;

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and

wherein preferably said composition consists of said conjugate.

In still another aspect, the present invention provides a composition comprising a conjugate, wherein said conjugate is of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:

R^1-(NR^2-CH_2-CH_2)$_n$-Z-X^1-(O-CH_2-CH_2)$_m$-X^2-L (Formula I*);

wherein

n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -$(NR^2$-CH_2-$CH_2)_n$– is H;

X^1 and X^2 are independently divalent covalent linking moieties;

Z is a divalent covalent linking moiety wherein Z is not a single bond and Z is not -NHC(O)-;

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and

wherein preferably said composition comprise a plurality of the said conjugates. In a preferred embodiment, the composition of the invention comprises a plurality of said conjugate, further preferably the composition of the invention consists of said conjugate(s).

In another aspect, the present invention provides a composition comprising a conjugate, preferably a plurality of conjugates, of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof: R^1-$(NR^2$-CH_2-$CH_2)_n$-Z-X^1-(O-CH_2-$CH_2)_m$-X^2-L (Formula I*); wherein n is any integer between 1 and 1500; and wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60; R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90%, of said R^2 in said -$(NR^2$-CH_2-$CH_2)_n$–moieties is H; X^1 and X^2 are independently divalent covalent linking moieties; Z is a divalent covalent linking moiety wherein Z is not -NHC(O)-; L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA and wherein further preferably said composition consists of said conjugate.

In another aspect, the present invention provides a conjugate of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:

R^1-$(NR^2$-CH_2-$CH_2)_n$-Z-X^1-(O-CH_2-$CH_2)_m$-X^2-L (Formula I*); wherein n is any integer between 1 and 1500; m is any integer between 1 and 200, preferably m is a discrete number of

repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36; R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$–moieties is H; X^1 and X^2 are independently divalent covalent linking moieties; Z is a divalent covalent linking moiety wherein Z is not -NHC(O)-; L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

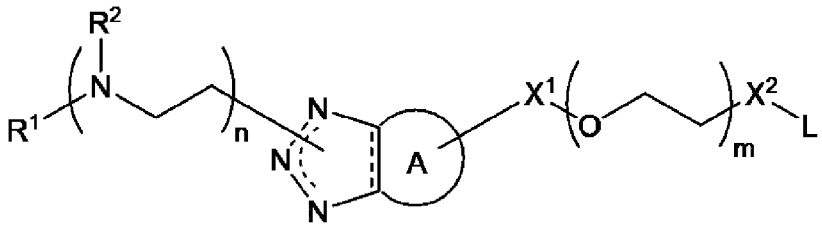
In another aspect, the present invention provides a composition comprising a conjugate preferably a plurality of conjugates, of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof: R^1-(NR^2-CH_2-CH_2)$_n$-Z-X^1-(O-CH_2-CH_2)$_m$-X^2-L (Formula I*); wherein n is any integer between 1 and 1500; m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36; R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H; X^1 and X^2 are independently divalent covalent linking moieties; Z is a divalent covalent linking moiety wherein Z is not a single bond and Z is not -NHC(O)-; L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA, and wherein preferably said composition consists of said conjugate. The term ”wherein said cell surface receptor is PSMA” shall mean that the cell surface receptor is derived from PSMA and is typically and preferably such part and/or portion of the PSMA which is provided on the cell surface and which corresponds further typically and preferably to the extracellular domain of PSMA and/or the cell surface exposed part of PSMA.

In another aspect, the present invention provides a conjugate of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:

R^1-(NR^2-CH_2-CH_2)$_n$-Z-X^1-(O-CH_2-CH_2)$_m$-X^2-L (Formula I*); wherein n is any integer between 1 and 1500; m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100,

preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36; R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H; X^1 and X^2 are independently divalent covalent linking moieties; Z is a divalent covalent linking moiety wherein Z is not a single bond and Z is not -NHC(O)-; L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a a cell surface receptor, wherein said cell surface receptor is PSMA.

In another aspect, the present invention provides a composition comprising a conjugate, preferably a plurality of conjugates, of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:

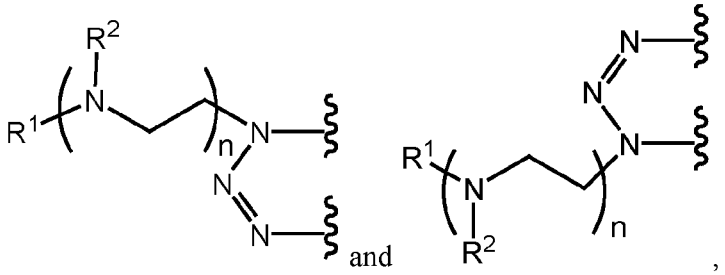


Formula I

wherein: ===== is a single bond or a double bond; n is any integer between 1 and 1500; m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36; R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– moieties is H; Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H; X^1 is a linking moiety of the formula –(Y^1)$_p$–, wherein p is an integer between 1 and 20, and each occurrence of Y^1 is independently selected from a chemical bond, -$CR^{11}R^{12}$-, -C(O)-, -O-, -S-, -NR^{13}-, an amino acid residue, a divalent phenyl moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each

divalent phenyl or heteroaryl is optionally substituted with one or more R^{13}, and each divalent heterocycle is optionally substituted with one or more R^{14}; wherein R^{11}, R^{12} and R^{13} are independently, at each occurrence, H or C_1-C_6 alkyl; and wherein R^{14} is independently, at each occurrence, H, C_1-C_6 alkyl, or oxo; X^2 is a linking moiety of the formula $–(Y^2)_q–$, wherein q is an integer between 1 and 50, and each occurrence of Y^2 is independently selected from a chemical bond, $-CR^{21}R^{22}-$, $NR^{23}-$, -O-, -S-, -C(O)-, an amino acid residue, a divalent phenyl moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl and divalent heteroaryl is optionally substituted with one or more R^{23}, and wherein each divalent heterocycle moiety is optionally substituted with one or more R^{24}; wherein R^{21}, R^{22}, and R^{23} are each independently, at each occurrence, -H, $-CO_2H$, or C_1-C_6 alkyl, wherein each C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl; and wherein R^{24} is independently, at each occurrence, -H, $-CO_2H$, C_1-C_6 alkyl, or oxo; and L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said composition consists of said conjugate.

As noted herein, the depiction of Formual I above represents two different regioisomeric attachments of the fragment $R^1(NR^2CH_2CH_2)_n$, i.e.,



and

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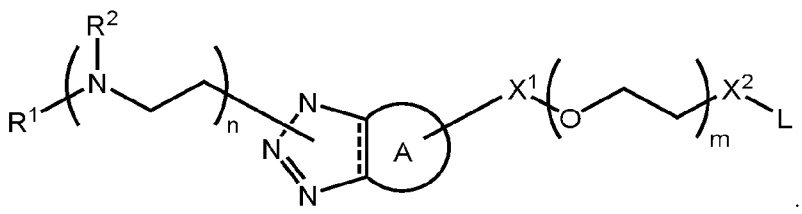
wherein the wavy lines represent chemical bonds to Ring A. Accordingly, Formula I as drawn herein encompasses two regioisomeric embodiments, i.e., wherein the fragment $R^1(NR^2CH_2CH_2)_n$ is bonded at the top nitrogen atom in the structures above or at the bottom nitrogen atom in the structures above, but not at the middle nitrogen atom. Formula I as drawn above is used interchanageably herein with the equivalent depiction of Formula I comprising a fragment N-N=N below, i.e.,



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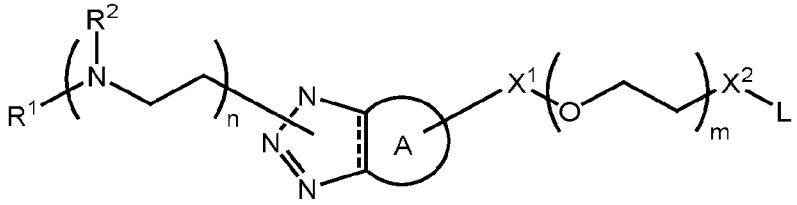
In another aspect, the present invention provides a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:

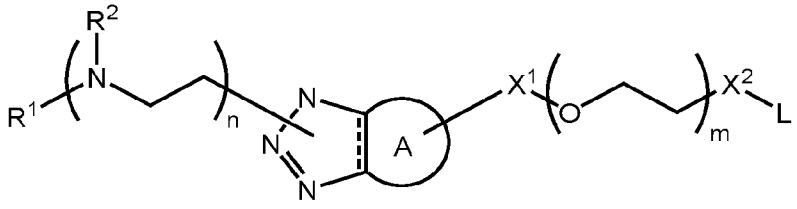


Formula I

wherein: ═══ is a single bond or a double bond; n is any integer between 1 and 1500; m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36; R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– moieties is H; Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H; X^1 is a linking moiety of the formula –$(Y^1)_p$–, wherein p is an integer between 1 and 20, and each occurrence of Y^1 is independently selected from a chemical bond, -$CR^{11}R^{12}$-, -C(O)-, -O-, -S-, -NR^{13}-, an amino acid residue, a divalent phenyl moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl or heteroaryl is optionally substituted with one or more R^{13}, and each divalent heterocycle is optionally substituted with one or more R^{14}; wherein R^{11}, R^{12} and R^{13} are independently, at each occurrence, H or C_1-C_6 alkyl; and wherein R^{14} is independently, at each occurrence, H, C_1-C_6 alkyl, or oxo; X^2 is a linking moiety of the formula –$(Y^2)_q$–, wherein q is an integer between 1 and 50, and each occurrence of Y^2 is independently selected from a chemical bond, -$CR^{21}R^{22}$-, NR^{23}-, -O-, -S-, -C(O)-, an amino acid residue, a divalent phenyl moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl and divalent heteroaryl is optionally substituted with one or more R^{23}, and wherein each divalent heterocycle moiety is optionally substituted with one or more R^{24}; wherein R^{21}, R^{22}, and R^{23} are each independently, at each occurrence, -H, -CO_2H, or C_1-C_6 alkyl, wherein each

C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl; and wherein R^{24} is independently, at each occurrence, -H, -CO_2H, C_1-C_6 alkyl, or oxo; and L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is -CH_3.

In another aspect, the present invention provides a composition comprising a conjugate, preferably a plurality of conjugates, of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H;

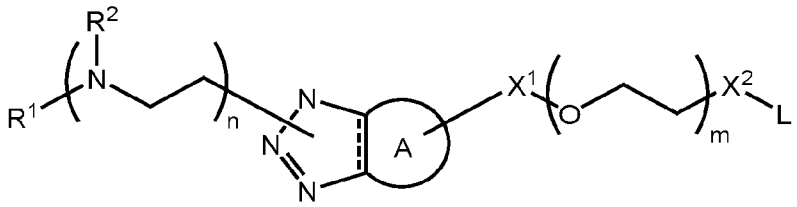
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$.

In another aspect, the present invention provides a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating $-(O-CH_2-CH_2)-$ units, wherein said discrete number m of repeating $-(O-CH_2-CH_2)-$ units is any discrete number of 25 to 100, preferably of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting

fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$.

In some preferred embodiments, the divalent covalent linking moiety Z comprises a triazole.

In some embodiments, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% of the LPEI in the composition is connected to the PEG fragment by a single covalent linking moiety, wherein the covalent linking moiety produces a linear end-to-end linkage between the LPEI fragment and the PEG fragment, as preferably determined by UV spectroscopy or mass spectrometry. In some embodiments, at least 60%, at least 70%, or at least 80%, at least 90%, at least 95% or at least 99% of the LPEI fragments are comprised by said conjugate and are connected to the PEG fragment by a single covalent linking moiety, wherein the covalent linking moiety produces a linear end-to-end linkage between the LPEI fragment and the PEG fragment, as preferably determined by UV spectroscopy or mass spectrometry. In some embodiments, at least 60% at least 70%, or at least 80%, at least 90%, at least 95% or at least 99% of the LPEI comprised in the composition are comprised by said conjugate, as preferably determined by UV spectroscopy or mass spectrometry. In some embodiments, said composition consists essentially of said conjugate. In some embodiments, said composition consists of said conjugate.

In some embodiments, at least 60% of the LPEI in the composition is connected to a single PEG fragment by a single covalent linking moiety Z, preferably wherein the covalent linking moiety Z produces a linear end-to-end linkage between the LPEI fragment and the PEG fragment. In some embodiments, at least 60% of the LPEI fragments comprised in the composition are linked to the PEG fragment by a single triazole linker, as preferably determined by UV spectroscopy or mass spectrometry. In some embodiments, at least 70% of the LPEI in the composition is connected to the PEG fragment by a single covalent linking moiety Z, preferably wherein the covalent linking moiety Z produces a linear end-to-end linkage between the LPEI fragment and the PEG fragment. In some embodiments, at least 70% of the LPEI fragments comprised in the composition are comprised by said conjugate, as preferably determined by UV spectroscopy or mass spectrometry. In some embodiments, at least 80% of the LPEI in the composition is connected to the PEG fragment by a single covalent linking moiety Z, preferably wherein the covalent linking moiety Z produces a linear end-to-end linkage between the LPEI fragment and the PEG fragment. In some embodiments, at least 80% of the LPEI fragments comprised in the composition are comprised by said conjugate, as

preferably determined by UV spectroscopy or mass spectrometry. In some embodiments, at least 90% of the LPEI in the composition is connected to the PEG fragment by a single covalent linking moiety Z, preferably wherein the covalent linking moiety Z produces a linear end-to-end linkage between the LPEI fragment and the PEG fragment. In some embodiments, at least 90% of the LPEI fragments comprised in the composition are comprised by said conjugate, as preferably determined by UV spectroscopy or mass spectrometry. In some embodiments, at least 95% of the LPEI in the composition is connected to the PEG fragment by a single covalent linking moiety Z, preferably wherein the covalent linking moiety Z produces a linear end-to-end linkage between the LPEI fragment and the PEG fragment. In some embodiments, at least 95% of the LPEI fragments comprised in the composition are comprised by said conjugate, as preferably determined by UV spectroscopy or mass spectrometry. In some embodiments, at least 99% of the LPEI in the composition is connected to the PEG fragment by a single covalent linking moiety Z, preferably wherein the covalent linking moiety Z produces a linear end-to-end linkage between the LPEI fragment and the PEG fragment. In some embodiments, at least 99% of the LPEI fragments comprised in the composition are comprised by said conjugate, as preferably determined by UV spectroscopy or mass spectrometry. In some embodiments, said composition consists essentially of said conjugate. In some embodiments, said composition consists of said conjugate. In some embodiments, the LPEI fragment does not comprise substitution beyond its first terminal end and second terminal end.

In some embodiments, the Formula I* does not comprise the structure: R^1-(NH-CH_2-CH_2)$_n$-NHC(O)-(CH_2-CH_2-O)$_m$-X^2-L. In some embodiments, the Formula I* does not comprise the structure R^1-(NR^2-CH_2-CH_2)$_n$-NHC(O)-X^1-(O-CH_2-CH_2)$_m$-X^2-L. In some embodiments, the composition does not comprise a conjugate of the structure R^1-(NH-CH_2-CH_2)$_n$-NHC(O)-X^1-(O-CH_2-CH_2)$_m$-X^2-L. In some embodiments, the composition does not comprise a conjugate of the structure R^1-(NR^2-CH_2-CH_2)$_n$-NHC(O)-(CH_2-CH_2-O)$_m$-X^2-L.

In some embodiments, R^1 is -H.

In some embodiments, at least 80% of the R^2 in the composition is -H. In some embodiments, at least 85%, preferably 90%, preferably 95%, more preferably 99% of the R^2 in the composition is -H. In a preferred embodiment, R^2 is independently -H or an organic residue, wherein at least 85%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$–moieties is H. In another preferred embodiment, R^2 is independently -H or an organic residue, wherein at least 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$–moieties is H. In another preferred embodiment, R^2 is independently -H or an organic residue, wherein at least 90% of said R^2 in said -(NR^2-CH_2-

$CH_2)_n$–moieties is H. In another preferred embodiment, R^2 is independently -H or an organic residue, wherein at least 91%, preferably at least 92%, more preferably 93%, of said R^2 in said -$(NR^2$-CH_2-$CH_2)_n$–moieties is H. In another preferred embodiment, R^2 is independently -H or an organic residue, wherein at least 94%, preferably at least 95%, more preferably 96%, of said R^2 in said -$(NR^2$-CH_2-$CH_2)_n$–moieties is H. In another preferred embodiment, R^2 is independently -H or an organic residue, wherein at least 95%, preferably wherein at least 97%, further preferably at least 98%, more preferably 99%, of said R^2 in said -$(NR^2$-CH_2-$CH_2)_n$–moieties is H.

In some embodiments, Ring A is an 8-membered cycloalkenyl, 5-membered heterocycloalkyl, or 7- to 8-membered heterocycloalkenyl, wherein each cycloalkenyl, heterocycloalkyl or heterocycloalkenyl is optionally substituted at any position with one or more R^{A1}.

In some embodiments, Ring A is cyclooctene, maleimide, or 7- to 8-membered heterocycloalkenyl, wherein the heterocycloalkenyl does not comprise heteroatoms other than N, O and S, and wherein each cyclooctene or heterocycloalkenyl is optionally substituted at any position with one or more R^{A1}.

In some embodiments, Ring A is cyclooctene, maleimide, or 7- to 8-membered heterocycloalkenyl, wherein the heterocycloalkenyl comprises one or more heteroatoms, preferably one or two heteroatoms selected from N, O and S, and wherein each cyclooctene or heterocycloalkenyl is optionally substituted at any position with one or more R^{A1}.

In some embodiments, Ring A is cyclooctene, maleimide, or an 8- membered heterocycloalkene, wherein the heterocycloalkene comprises exactly one heteroatom selected from N, O, and S, wherein each cyclooctene or heterocycloalkene is optionally substituted with one or more R^{A1}.

In some embodiments, R^{A1} is -H, oxo or fluorine, or two R^{A1} combine to form one or more fused phenyl rings, preferably one or two fused phenyl rings, and wherein each phenyl ring is optionally substituted with one or more -OSO_3H or -SO_3H.

In some embodiments, Ring A is cyclooctene, maleimide, or an 8- membered heterocycloalkene, wherein the heterocycloalkene comprises exactly one heteroatom selected from N, O, and S, wherein each cyclooctene or heterocycloalkene is optionally substituted with one or more R^{A1}, wherein R^{A1} is oxo or fluorine, or wherein two R^{A1} combine to form one or more fused phenyl rings, preferably one or two fused phenyl rings.

In some embodiments, Ring A is cyclooctene, maleimide, or an 8- membered heterocycloalkene, wherein the heterocycloalkene comprises exactly one heteroatom selected from N, wherein each cyclooctene or heterocycloalkene is optionally substituted with one or two R^{A1}.

In some embodiments, R^{A1} is -H, oxo or fluorine, or two R^{A1} combine to form one or more fused phenyl rings, preferably one or two fused phenyl rings, and wherein each phenyl ring is optionally substituted with one or more R^{A2}.

In some embodiments, Ring A is cyclooctene, maleimide, or an 8- membered heterocycloalkene, wherein the heterocycloalkene comprises exactly one heteroatom selected from N, wherein each cyclooctene or heterocycloalkene is optionally substituted with one or two R^{A1}, wherein R^{A1} is -H, oxo or fluorine, or wherein two R^{A1} combine to form one or more fused phenyl rings, preferably one or two fused phenyl rings, and wherein each phenyl ring is optionally substituted with one or more $-OSO_3H$ or $-SO_3H$.

In some preferred embodiments, Ring A is cyclooctene, maleimide, or an 8- membered heterocycloalkene, wherein the heterocycloalkene comprises exactly one heteroatom selected from N, wherein each cyclooctene or heterocycloalkene is optionally substituted with one or two R^{A1}, wherein R^{A1} is -H, or wherein two R^{A1} combine to form one or more fused phenyl rings, preferably one or two fused phenyl rings, and wherein each phenyl ring is optionally substituted with one or more $-OSO_3H$ or $-SO_3H$.

Preparation of Linear Conjugates

The conjugates of the invention can be prepared in a number of ways well known to those skilled in the art of polymer synthesis. By way of example, compounds of the present invention can be synthesized using the methods described below, together with synthetic methods known in the art of polymer chemistry, or variations thereon as appreciated by those skilled in the art. The methods include, but are not limited to, those methods described below. The conjugates of the present invention can be synthesized by following the steps outlined in General Schemes 1, 2, 3, 4, 5, 6, 7 and 8, or can be prepared using alternate sequences of assembling intermediates without deviating from the present invention. The conjugates of the present invention can also be synthesized using slight variations on the steps outlined below. For example, where Scheme 3 shows the use of a tetrafluorophenyl ester as an electrophilic functional group for coupling with a PSMA targeting fragment comprising a nucleophilic amine group to form an amide functionality, one of skill in the art will recognize other suitable

electrophilic functional groups besides tetrafluorophenyl ester that can be used for the same purpose.

In some preferred embodiments, the LPEI fragment and the PEG fragment are coupled via a [3+2] cycloaddition between an azide and an alkene or alkyne to form a 1,2,3 triazole or a 4,5-dihydro-1H-[1,2,3]triazole. In some preferred embodiments, the LPEI fragment comprises the azide functional group and the PEG fragment comprises the alkene or alkyne functional group.

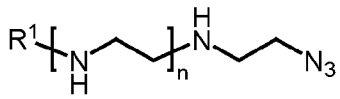
LPEI Fragment

The conjugates of the present invention can comprise LPEI fragments and PEG fragments. Linear polyethyleneimine (LPEI) has the chemical formula –[NH-CH_2-CH_2]–. LPEI can be synthesized according to a number of methods known in the art, including in particular the polymerization of a 2-oxazoline, followed by hydrolysis of the pendant amide bonds (see e.g., Brissault *et al.*, *Bioconjugate Chem.*, **2003**, *14*, 581-587). As noted above, the polymerization of poly(2-oxazolines) (i.e., a suitable precursor for LPEI) from 2-oxazolines can be initiated with any suitable initiator. In some embodiments, the initiator leaves an initiation residue at the alpha terminus of the poly(2-oxazoline). In a preferred embodiment, the initiation residue (i.e., R^1 of Formula I* or Formula I) is a hydrogen atom or a C_1-C_6 alkyl, preferably a hydrogen or C_1-C_4 alkyl, more preferably a hydrogen or methyl group; most preferably a hydrogen atom. In a preferred embodiment, the initiation residue R^1 of Formula I is a hydrogen atom or a C_1-C_6 alkyl, preferably a hydrogen or C_1-C_4 alkyl, more preferably a hydrogen or methyl group; most preferably a hydrogen atom. In preferred embodiments, the initiation residue (i.e., R^1 of Formula I* or Formula I) is -H or -CH_3, most preferably -H. In a preferred embodiment, said initiation residue R^1 of Formula I* is -H. In a preferred embodiment, said initiation residue R^1 of Formula I is -H. In a preferred embodiment, said initiation residue R^1 of Formula I* is -CH_3. In a preferred embodiment, said initiation residue R^1 of Formula I is -CH_3. However, one of skill in the art will understand that the initiation residue can be the residue left from any suitable initiator capable of initiating the polymerization of poly(2-oxazolines) from 2-oxazolines.

In some embodiments, the LPEI fragment can be coupled to the PEG fragment via a [3+2] cycloaddition between an azide and an alkene or alkyne to form a 1, 2, 3 triazole or a 4,5-dihydro-1H-[1,2,3]triazole wherein the LPEI fragment comprises the azide (-N_3) functional group at the omega terminus of the chain. In some preferred embodiments, the LPEI fragment

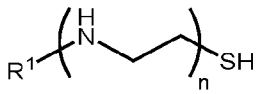
is not further substituted except for a single substitution at the alpha terminus. For example, in some preferred embodiments, the LPEI fragment comprises the repeating formula –[NH-CH_2-CH_2]– and is substituted at the omega terminus with an azide group which can be coupled to an alkyne or alkene substituent on a PEG fragment. In some preferred embodiments, the alpha terminus of the LPEI fragment can be substituted with a hydrogen atom or a C_1-C_6 alkyl, preferably a hydrogen or C_1-C_4 alkyl, more preferably a hydrogen or methyl group; most preferably a hydrogen atom.

For example, in some preferred embodiments, the LPEI fragment can be substituted at the alpha terminus with a hydrogen atom or a C_1-C_6 alkyl, preferably a hydrogen atom or C_1-C_4 alkyl, more preferably a hydrogen atom or methyl group and at the omega terminus with an azide group; in some preferred embodiments, there is no additional substitution present on the LPEI fragment. For example, conjugates of the present invention can be prepared from LPEI fragments of the following formula:



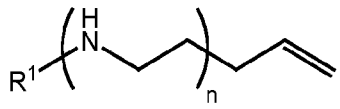
wherein R^1 can be any suitable initiation residue, preferably a hydrogen or C_1-C_6 alkyl, preferably hydrogen or C_1-C_4 alkyl, more preferably hydrogen or methyl, most preferably a hydrogen.

In some embodiments, the LPEI fragment can be terminated with a thiol group, thus, in some embodiments, the omega terminus of said LPEI fragment comprises, preferably is, a thiol group, which can be coupled to a reactive alkene group on the PEG fragment by a thiol-ene reaction. Accordingly, in some embodiments conjugates of the present invention can be prepared from LPEI fragments of the following formula:



wherein R^1 can be any suitable initiation residue, preferably hydrogen or methyl, preferably a hydrogen.

In some embodiments, the LPEI fragment can be terminated with an alkene group, thus, in some embodiments, the omega terminus of said LPEI fragment comprises, preferably is, a alkene group, which can be coupled to a reactive thiol group on the PEG fragment by a thiol-ene reaction. Accordingly, in some embodiments, conjugates of the present invention can be prepared from LPEI fragments of the following formula:



wherein R^1 can be any suitable initiation residue, preferably hydrogen or methyl, preferably a hydrogen.

The LPEI fragment can comprise a range of lengths (i.e., repeating units represented above by the variable "n"). For example, the LPEI fragment can comprise between 1 and 1000 repeating units (i.e., -NH-CH_2-CH_2-). In some embodiments, the LPEI fragment can be present as a disperse polymeric moiety and does not comprise a discrete number of -NH-CH_2-CH_2- repeating units. For example, the LPEI fragment can be present as a disperse polymeric moiety with a molecular weight of between about 5 and 50 KDa, preferably with a dispersity of about 5 or less, preferably of about 4 or less, preferably of about 3 or less, preferably of about 2 or less, preferably of about 1.5 or less. In some embodiments, the LPEI fragment can be present as a disperse polymeric moiety with a molecular weight of between about 10 and 40 KDa with a dispersity of about 4 or less, preferably of about 3 or less, preferably of about 2 or less, preferably of about 1.5 or less. In some embodiments, the LPEI fragment can be present as a disperse polymeric moiety with a molecular weight of between about 12 and 30 KDa with a dispersity of about 3 or less, preferably of about 2 or less, preferably of about 1.5 or less. In some embodiments, the LPEI fragment can be present as a disperse polymeric moiety with a molecular weight of between about 15 and 27 KDa with a dispersity of about 2 or less, preferably of about 1.5 or less. In some embodiments, the LPEI fragment can be present as a disperse polymeric moiety with a molecular weight of between about 17 and 25 KDa, with a dispersity of about 1.2 or less.

For example, the LPEI fragment can be present as a disperse polymeric moiety comprising between about 115 and 1150 repeating units, preferably with a dispersity of about 5 or less, preferably of about 4 or less, preferably of about 3 or less, preferably of about 2 or less, preferably of about 1.5 or less. In some embodiments, the LPEI fragment can be present as a disperse polymeric moiety comprising between about 230 and 930 repeating units with a dispersity of about 4 or less, preferably of about 3 or less, preferably of about 2 or less, preferably of about 1.5 or less. In some embodiments, the LPEI fragment can be present as a disperse polymeric moiety comprising between about 280 and 700 repeating units with a dispersity of about 3 or less, preferably of about 2 or less, preferably of about 1.5 or less. In some embodiments, the LPEI fragment can be present as a disperse polymeric moiety comprising between about 350 and 630 repeating units with a dispersity of about 2 or less,

preferably of about 1.5 or less. In some embodiments, the LPEI fragment can be present as a disperse polymeric moiety comprising between about 400 and 580 repeating units, with a dispersity of about 1.2 or less.

In some embodiments, said R^1-(NR^2-CH_2-CH_2)$_n$-moiety is a disperse polymeric moiety with between 115 and 1150 repeating units n and a dispersity of about 5 or less, wherein preferably said R^1-(NR^2-CH_2-CH_2)$_n$-moiety is a disperse polymeric moiety with between 280 and 700 repeating units n and a dispersity of about 3 or less, and wherein further preferably said R^1-(NR^2-CH_2-CH_2)$_n$-moiety is a disperse polymeric moiety with between 350 and 630 repeating units n and a dispersity of about 2 or less, and again further preferably wherein said R^1-(NR^2-CH_2-CH_2)$_n$-moiety is a disperse polymeric moiety with between 400 and 580 repeating units n and a dispersity of about 1.2 or less.

In a preferred embodiment, said polyethyleneimine fragment is a disperse polymeric moiety with between about 115 and about 1150 repeating units and a dispersity of about 5 or less, preferably between about 230 and about 930 repeating units with a dispersity of about 4 or less; more preferably between about 280 and about 700 repeating units with a dispersity of about 3 or less; again more preferably between about 350 and about 630 repeating units with a dispersity of about 2 or less; yet more preferably between about 400 and about 580 repeating units, with a dispersity of about 1.2 or less.

In a preferred embodiment, said polyethyleneimine fragment is a disperse polymeric moiety with between about 115 and about 1150 repeating units and a dispersity of about 5 or less, preferably of about 4 or less, preferably of about 3 or less, preferably of about 2 or less, preferably of about 1.5 or less. In a preferred embodiment, said polyethyleneimine fragment is a disperse polymeric moiety with between about 230 and about 930 repeating units with a dispersity of about 4 or less, preferably of about 3 or less, preferably of about 2 or less, preferably of about 1.5 or less. In a preferred embodiment, said polyethyleneimine fragment is a disperse polymeric moiety with between about 280 and about 700 repeating units with a dispersity of about 3 or less, preferably of about 2 or less, preferably of about 1.5 or less. In a preferred embodiment, said polyethyleneimine fragment is a disperse polymeric moiety with between about 350 and about 630 repeating units with a dispersity of about 2 or less, preferably of about 1.5 or less. In a preferred embodiment, said polyethyleneimine fragment is a disperse polymeric moiety with between about 400 and about 580 repeating units, with a dispersity of about 1.2 or less.

As noted above, one of skill in the art will understand that in some embodiments, the

LPEI fragment may include organic residues, (i.e., pendant amide groups) connected at the nitrogen atoms embedded within the LPEI chain. One of skill in the art will understand that such organic residues (i.e., amide groups) can be formed during the ring-opening polymerization of 2-oxazolines to form a poly(2-oxazoline). Without wishing to be bound by theory, LPEI can be formed from a poly(2-oxazoline) by cleavage of the amide groups (e.g., using an acid such as HCl). However, in some cases not every amide linkage may be cleaved under these conditions. Accordingly, in some embodiments about 5% or less of the nitrogen atoms in the LPEI fragment may be connected to an organic residue to form an amide. In some embodiments, about 4% or less, about 3% or less, about 2% or less, about 1% or less, about 0.5% or less, about 0.4% or less, about 0.3% or less, about 0.2% or less, or about 0.1% or less of the nitrogen atoms in the LPEI fragment may be connected to an organic residue to form an amide. One of skill in the art will understand that the molecular weight of the LPEI fragment includes the percentage of LPEI fragment that is bonded to an organic residue as an amide. Moreover, one of skill in the art will understand that although chemical structures drawn herein show repeating -NH-CH_2-CH_2- fragments, trace amounts of residual organic residue such as pendant amide groups (e.g., those defined above) may still be present in the resulting triconjugates or polyplexes of the present disclosure. The term "triconguate", as occasionally used herein, shall refer to the inventive conjugate. The prefix "tri-" is caused by the three components comprised by the inventive conjugates, namely the LPEI fragment, the PEG fragment and the targeting fragment.

PEG Fragment

Polyethylene glycol (PEG) has the chemical formula –[O-CH_2-CH_2]–.

The PEG fragment comprised in the inventive conjugates and compositions comprises, preferably consists of, a discrete number m of repeating –(O-CH_2-CH_2)-units and is not defined in terms of an average chain length. Thus, the PEG fragment comprised in the inventive conjugates and compositions comprises, preferably consists of, a discrete number m of repeating –(O-CH_2-CH_2)-units and is not defined in terms of an average chain length but has a specifically defined discrete molecular weight associated with the discrete number m of repeating –(O-CH_2-CH_2)-units. In a preferred embodiment, said PEG fragment comprises, preferably consists of, a discrete number m of repeating units –(O-CH_2-CH_2)-units, wherein typically and preferably said discrete number (m) is a discrete number (m) of and between 25 to 100, further preferably of and between 25 to 60. In a preferred embodiment, said PEG

fragment comprises, preferably consists of, a discrete number m of contiguous repeating units –(O-CH_2-CH_2)-units, wherein typically and preferably said discrete number (m) is a discrete number (m) of and between 25 to 100, further preferably of and between 25 to 60.

The expressions "polyethylene glycol fragment comprising a discrete number (m) of repeating -(O-CH_2-CH_2)- units", or "PEG fragment comprising a discrete number (m) of repeating -(O-CH_2-CH_2)- units" shall refer to a fragment comprising, preferably consisting of, a discrete number – typically herein referred to a discrete number *m* - of repeating -(O-CH_2-CH_2)- units, wherein said discrete number (m) is a discrete, i.e. specific and single defined and integer, number (m) of 25 to 100, preferably of 25 to 60. Thus, the expressions "polyethylene glycol fragment comprising a discrete number (m) of repeating -(O-CH_2-CH_2)- units", or "PEG fragment comprising a discrete number (m) of repeating -(O-CH_2-CH_2)- units" shall refer to a fragment comprising, preferably consisting of, a discrete number *m* - of repeating -(O-CH_2-CH_2)- units, wherein said discrete number (m) is a discrete, i.e. specific and single defined and integer, number (m) of 25 to 100, preferably of 25 to 60, and thus said defined PEG fragments comprise, preferably consist of, a discrete number m of repeating –(O-CH_2-CH_2)- units and are not defined in terms of an average chain length but they each have a specifically defined discrete molecular weight. When herein referring to a discrete number of 25 to 100, it shall refer to any integer of and between 25 to 100, i.e. any integer between 25 and 100 including the integer and discrete numbers mentioned as borders such as here 25 and 100. By way of further example, a PEG fragment comprising a discrete number (m) of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is 36, refers to a PEG fragment comprising a chain of -(O-CH_2-CH_2)- units that contains exactly 36 -(O-CH_2-CH_2)- units. Such chain of exactly 36 -(O-CH_2-CH_2)- units is abbreviated as PEG_{36}. Such PEG fragment is in contrast to a "polymeric PEG fragment", a "polydisperse PEG fragment" or a "disperse PEG fragment", which refers to a heterogenous mixture of sizes and molecular weights as the result of a polymer reaction, typically in a Poisson distribution (J Herzberger et al.; Chem Rev, 2016, 116:2170-2243). The PEG fragments of the present invention comprising a discrete number (m) of repeating -(O-CH_2-CH_2)- units are not synthesized via a polymerization process. The PEG fragments of the present invention comprise a discrete number (m) of repeating -(O-CH_2-CH_2)- units and are single molecule fragments with a discrete, i.e. defined and specified, chain length. Thus, the PEG fragments of the present invention comprising a discrete number (m) of repeating -(O-CH_2-CH_2)- units are single molecule fragments with a discrete, i.e. defined and specified chain length. The PEG fragments of the present invention are not a mixture of molecular entities (such as those resulting from a

random polymerization reaction). The discreteness of the inventive discrete PEG fragments distinguishes them from the polydisperse art.

The PEG fragments of the present invention may comprise, preferably consist of, homogenous discrete PEG fragments or heterogeneous discrete PEG fragments, typically and preferably homogenous discrete PEG fragments. The term "homogenous discrete PEG fragments", as used herein, means a discrete PEG structure whose entire chemical backbone is made up of a continuous and contiguous and specific discrete number of only ethylene oxide units. In other words, no other functionality is present within said homogenous discrete PEG fragments. The termini of the respective reactive precursor molecules comprising homogeneous discrete PEG fragments, however, can and typically do have, for the sake of conjugation with the PEI fragments and the targeting fragments, functional groups. The term "heterogeneous discrete PEG fragments", as used herein, means a discrete PEG structure wherein the basic ethylene oxide backbone comprising a discrete number of ethylene oxide units is broken up by or substituted with other functional groups or units within its structure such as, for example, the inclusion of amide or ester bonds or other functional units. In preferred embodiments of the present invention, the PEG fragment is a homogenous discrete PEG fragment.

In some preferred embodiments, the PEG fragment can be coupled to the LPEI fragment via a [3+2] cycloaddition between an azide and an alkene or alkyne to form a 1,2,3 triazole or a 4,5-dihydro-1H-[1,2,3]triazole, wherein the respective reactive precursor molecule comprising the PEG fragment further comprises the alkene or alkyne functional group. For example, in some preferred embodiments, the reactive precursor molecule comprising the PEG fragment comprises the repeating formula –[O-CH_2-CH_2]– and is substituted at a first end (i.e., terminus) with an alkene or alkyne group (e.g., via a linking moiety "X^1" as discussed herein) which can be coupled to the azide group of a corresponding respective reactive precursor molecule comprising the LPEI fragment. In some preferred embodiments, said alkene or alkyne group is an activated alkene or alkyne group capable of spontaneously reacting with an azide (e.g., without the addition of a catalyst such as a copper catalyst). For example, an activated alkyne group can be incorporated into a 7- or 8-membered ring, resulting in a strained species that reacts spontaneously with the azide group of the LPEI fragment. An activated alkene can include a maleimide moiety, wherein the alkene is activated by conjugation to the neighboring carbonyl groups. In some preferred embodiments, the second end (i.e., terminus) of the PEG fragment can be substituted with a targeting fragment (e.g., DUPA) (e.g., via a linking moiety "X^2" as discussed herein), wherein said targeting fragment is capable of binding to prostate

specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

The PEG fragment comprised in the inventive conjugates and compositions comprises, preferably consists of, a discrete number m of repeating -O-CH_2-CH_2- units and is not defined in terms of an average chain length, as it is the case for polymeric PEG fragments. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$- units comprise, preferably consist of, a discrete number of repeating units m. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$- units comprise, preferably consist of, a discrete number of contiguous repeating units m.

In a preferred embodiment, the PEG fragment comprises, preferably consists of, a discrete number of repeating units m of 25 to 100, preferably of a discrete number of repeating units m of 25 to 60. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 25 to 60, preferably of a discrete number of repeating units m of 30 to 50. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59 or 60. The synthesis of said PEG fragments comprising or consisting of discrete numbers repeating -(O-CH_2-CH_2)$_m$- units and thus discrete PEGs are described in WO2004/073620 and WO2013/033476. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 28, 32, 36, 40, 44, 48, 52, 56, or 60. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 28. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 32. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 36. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 40. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 44. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 48.

In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 25 to 100, preferably of a discrete number of contiguous repeating units m of 25 to 60. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 25 to 60, preferably of a discrete number of contiguous repeating units m of 30 to 50. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of

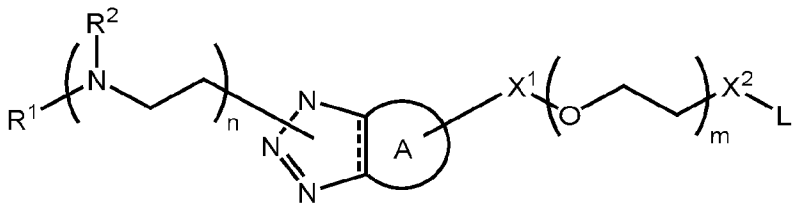
contiguous repeating units m of 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59 or 60. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 28, 32, 36, 40, 44, 48, 52, 56, or 60. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 28. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 32. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 36. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 40. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 44. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 48.

In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety of Formula I* or Formula I consists of a discrete number of repeating units m of 25 to 100, preferably of a discrete number of repeating units m of 25 to 60. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of repeating units m of 25 to 60, preferably of a discrete number of repeating units m of 30 to 50. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of repeating units m of 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59 or 60. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of repeating units m of 28, 32, 36, 40, 44, 48, 52, 56, or 60. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of repeating units m of 28. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of repeating units m of 32. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of repeating units m of 36. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of repeating units m of 40. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of repeating units m of 44. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of repeating units m of 48.

In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety of Formula I* or Formula I consists of a discrete number of contiguous repeating units m of 25 to 100, preferably of a discrete number of contiguous repeating units m of 25 to 60. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of contiguous repeating units m of 25 to

60, preferably of a discrete number of contiguous repeating units m of 30 to 50. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of contiguous repeating units m of 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59 or 60. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of contiguous repeating units m of 28, 32, 36, 40, 44, 48, 52, 56, or 60. In a preferred embodiment said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of contiguous repeating units m of 28. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of contiguous repeating units m of 32. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of contiguous repeating units m of 36. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of contiguous repeating units m of 40. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of contiguous repeating units m of 44. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number of contiguous repeating units m of 48.

In another aspect, the present invention provides a composition comprising a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is

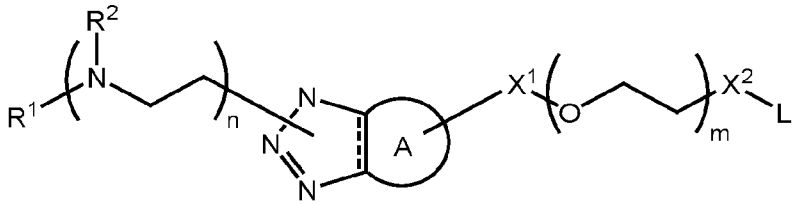
independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$.

In another aspect, the present invention provides a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

═══ is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating $-(O-CH_2-CH_2)-$ units, wherein said discrete number m of repeating $-(O-CH_2-CH_2)-$ units is any discrete number of 25 to 100, preferably of 25 to 60, and wherein further preferably said discrete number m of repeating $-(O-CH_2-CH_2)-$ units is 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl

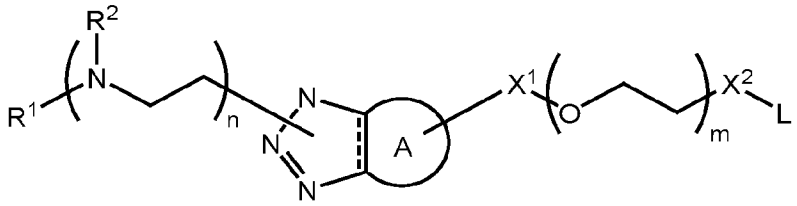
is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, wherein said said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is -CH_3.

In another aspect, the present invention provides a composition comprising a conjugate, preferably a plurality of conjugates, of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

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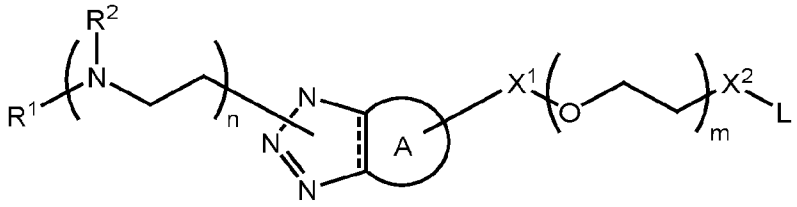

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R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

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Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

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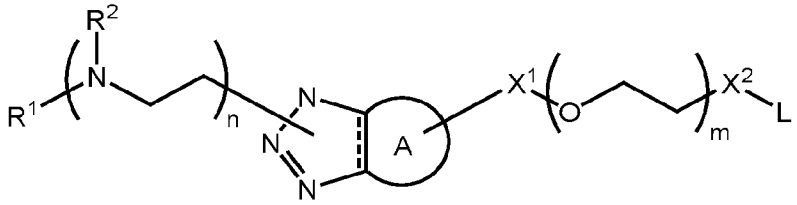
fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$.

In another aspect, the present invention provides a composition comprising a conjugate, preferably a plurality of conjugates, of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



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m is a discrete number of contiguous repeating $-(O-CH_2-CH_2)-$ units m of 25 to 100, preferably of a discrete number of contiguous repeating $-(O-CH_2-CH_2)-$ units m of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

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Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

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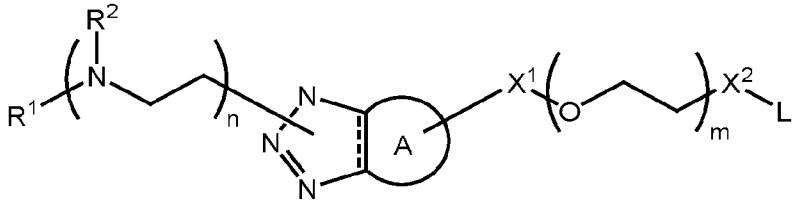
In another aspect, the present invention provides a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



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m is a discrete number of contiguous repeating -(O-CH_2-CH_2)- units m of 25 to 100, preferably of a discrete number of contiguous repeating -(O-CH_2-CH_2)- units m of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

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Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

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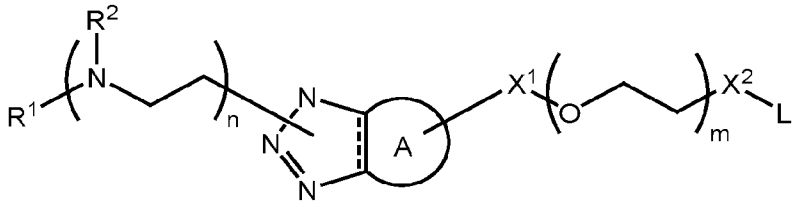
In another aspect, the present invention provides a composition comprising a conjugate, preferably a plurality of conjugates, of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



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m is a discrete number of contiguous repeating -(O-CH_2-CH_2)- units m of 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

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Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

X^1 is a divalent covalent linking moiety;

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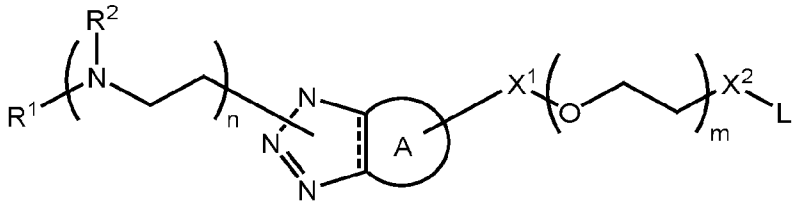
L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is -CH_3.

In another aspect, the present invention provides a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



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X^1 is a divalent covalent linking moiety;

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L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is -CH_3.

In some preferred embodiments, the conjugates of the present invention comprise an LPEI fragment present as a disperse polymeric moiety, wherein n is between about 280 and about 700 with a dispersity of about 3 or less, preferably between about 350 and about 630 with a dispersity of about 2 or less, and more preferably between about 400 and 580 with a dispersity about 1.2 or less, and wherein said conjugates of the present invention further comprise a PEG fragment present as a discrete number of repeating -(O-CH_2-CH_2)- units m, wherein said discrete number of repeating -(O-CH_2-CH_2)- units m is any discrete number of 25 to 100, preferably of 25 to 60, wherein preferably said discrete number m is a discrete number of contiguous repeating -(O-CH_2-CH_2)- units, and wherein said discrete number of contiguous repeating -(O-CH_2-CH_2)- units) is any discrete number of 25 to 100, preferably of 25 to 60 .

In some embodiments, the conjugates of the present invention comprise an LPEI fragment present as a disperse polymeric moiety of about 17 and 25 KDa, with a dispersity of about 1.2

or less and a PEG fragment comprising, preferably consisting of, a discrete number of repeating -(O-CH_2-CH_2)- units m, wherein said discrete number m is any discrete number of 25 to 60. In some preferred embodiments, the conjugates of the present invention can comprise an LPEI fragment present as a disperse polymeric moiety with a molecular weight of between about 17 and 25 KDa, with a dispersity of about 1.2 or less and a PEG fragment comprising, preferably consisting of, a discrete number of repeating -(O-CH_2-CH_2)- units m, wherein said discrete number m is 36.

Targeting Fragment

The inventive conjugates comprise a targeting fragment which allows to direct the inventive conjugate, the inventive composition and the inventive polyplex to a particular target cell type, collection of cells, organ or tissue. Typically and preferably, the targeting fragment is capable of binding to a target cell, preferably to a cell receptor or cell surface receptor thereof.

As used herein, the term "cell surface receptor", refers to a protein, e.g., glycoprotein or lipoprotein, which is present at the surface of the cell, and which is typically and preferably a distinctive marker for the recognition of a cell. Typically and preferably, said cell surface receptor is able to bind to a ligand which include hormones, neurotransmitters, cytokines, growth factors, cell adhesion molecules, or nutrients, in the form of peptides, small molecules, saccharides and oligosaccharides, lipids, amino acids, and such other binding moieties such as antibodies, aptamers, affibodies, antibody fragments and the like.

The inventive conjugate and polyplex comprising the targeting fragment is aiming to mimic such ligand-receptor interaction. Thus, in a preferred embodiment, said targeting fragment is capable of binding to a cell surface receptor.

In a preferred embodiment, said cell surface receptor is a peripheral membrane protein or a transmembrane protein, preferably a transmembrane protein of type II.

In a preferred embodiment, said cell surface receptor is prostate specific membrane antigen (PSMA). The term "wherein said cell surface receptor is PSMA" shall mean that the cell surface receptor is derived from PSMA and is typically and preferably such part and/or portion of the PSMA which is provided on the cell surface, and which corresponds further typically and preferably to the extracellular domain of PSMA and/or the cell surface exposed part of PSMA.

The targeting fragment in accordance with the present invention aims to locate and to deliver, in particular to selectively deliver, the inventive polyplexes and payloads such as the

nucleic acids to the desired target, in particular to the desired target cell. In addition, the inventive conjugate comprising said targeting fragment not only allows to selectively deliver the conjugate and polyplex to a target such as a target cell, but, in addition, allows to enable internalization and to facilitate selective cellular uptake of the polyanion payload by the target, in particular by the target cell. Thus, the targeting fragment in accordance with the present invention represents a portion of the inventive conjugate and polyplex that is capable of specific binding to a selected target, preferably to a selected target cell, further preferably to a cell receptor.

In a preferred embodiment, said targeting fragment is capable of binding to a target cell expressing PSMA. In a preferred embodiment, said targeting fragment is capable of binding to a selected target cell type expressing PSMA. In a preferred embodiment, said targeting fragment is capable of binding to a target cell receptor, wherein said target cell receptor is PSMA. In a preferred embodiment, said targeting fragment is capable of binding to a target cell surface receptor, wherein said target cell surface receptor is PSMA.

In a preferred embodiment, said targeting fragment functions to bind to a target cell expressing PSMA. In a preferred embodiment, said targeting fragment functions to bind to a selected target cell type expressing PSMA. In a preferred embodiment, said targeting fragment functions to bind to a target cell receptor, wherein said target cell receptor is PSMA. In a preferred embodiment, said targeting fragment functions to bind to a target cell surface receptor, wherein said target cell surface receptor is PSMA.

In a preferred embodiment, said targeting fragment is capable of specifically binding to a target cell expressing PSMA. In a preferred embodiment, said targeting fragment is capable of specifically binding to a selected target cell type expressing PSMA. In a preferred embodiment, said targeting fragment is capable of specifically binding to a target cell receptor, wherein said target cell receptor is PSMA. In a preferred embodiment, said targeting fragment is capable of specifically binding to a target cell surface receptor, wherein said target cell surface receptor is PSMA.

In one embodiment, said specifically binding to a target cell, to a target cell or to a target cell surface receptor, means that the targeting fragment and the inventive conjugate and/or inventive polyplex, respectively, binds to said target cell, said target cell receptor, said target cell surface receptor, at least twice, preferably at least three times, further preferably at least four times, again further preferably at least five times as strong as it binds to other non-targeted cells, cell receptors, cell surface receptors, typically and preferably measured by the

dissociation constant (KD). Preferably, a targeting fragment binds to the selected cell surface receptor with a KD of less than 10^{-5} M, preferably less than 10^{-6} M, more preferably less than 10^{-7} M and even more preferably less than 10^{-8} M.

In one embodiment, said specifically binding to a target cell, to a target cell receptor or to a target cell surface receptor means that the targeting fragment and the inventive conjugate and/or inventive polyplex, respectively, binds to said target cell, said target cell receptor or said target cell surface receptor at least twice, preferably at least three times, further preferably at least five times, again further preferably at least ten times, further preferably at least hundred times as strongly as the corresponding conjugate and/or polyplex that is identical to the inventive conjugate and/or the inventive polyplex but comprises instead of the targeting fragment a non-specific fragment such as an hydroxyl group or a -OMe moiety, preferably the -OMe moiety. The binding to the target cell, to the target cell receptor or to the target cell surface receptor is typically and preferably measured by the dissociation constant (KD). Preferably, a targeting fragment binds to the selected target cell surface receptor with a KD of less than 10^{-5} M, preferably less than 10^{-6} M, more preferably less than 10^{-7} M and even more preferably less than 10^{-8} M. In a preferred embodiment, said binding or said specific binding, and thus the level of binding of the inventive conjugate and inventive polyplex, respectively, can be determined by binding assays or displacement assays or by FRET or other measures demonstrating interaction between the targeting fragment and the cell receptor, preferably the cell surface receptor.

The term "binding", as used herein with reference to the binding of the targeting fragment to a cell, a cell receptor or a cell surface receptor refers preferably to interactions via non-covalent binding, such as electrostatic interactions, van der Waals interaction, hydrogen bonds, hydrophobic interactions, ionic bonds, charge interactions, affinity interactions, and/or dipole-dipole interactions.

In another embodiment, said specifically binding to a target cell, to a target cell receptor or to a target cell surface receptor results in a biological effect which is caused by said specific binding of the targeting fragment and inventive conjugate and/or the inventive polyplex, respectively, and/or is caused by the delivered inventive conjugate and/or polyplex and polyanion payload, which biological effect is at least 2-fold, preferably at least 3-fold, further preferably at least 5-fold and again further preferably at least 10-fold, and again further preferably at least 25-fold, at least 50-fold or at least 100-fold greater, as compared to said biological effect of a non-targeted cell, a non-targeted cell receptor or a non-targeted cell

surface receptor.

In another embodiment, said specifically binding to a target cell, to a target cell receptor, or to a target cell surface receptor results in a biological effect which is caused by said specific binding of the targeting fragment and inventive conjugate and/or the inventive polyplex, respectively, and/or is caused by the delivered inventive conjugate and/or polyplex and polyanion payload, which biological effect is at least 2-fold, preferably at least 3-fold, further preferably at least 5-fold and again further preferably at least 10-fold, and again further preferably at least 25-fold, at least 50-fold or at least 100-fold greater, as compared to said biological effect caused by the corresponding conjugate and/or polyplex that is identical to the inventive conjugate and/or the inventive polyplex but comprises instead of the targeting fragment a non-specific fragment such as an hydroxyl group or a -OMe moiety, preferably the -OMe moiety.

The binding and specific binding can be determined as well by measures of activation of protein signalling and therefore can be measured by protein phosphorylation or protein expression, mRNA expression in cells or tissues (using western blot analysis, real time PCR, RNAseq IHC etc). The level of delivery of an inventive polyplex to a particular tissue may be measured by comparing the amount of protein produced in a cell with overexpression as compared to a cell with normal and low expression by means of western blot analysis or luminescence/fluorescent assay, flow cytometry assays or measuring the secretion of the protein by measures of such as ELISA, ECLIA: By comparing the amount of expression or secretion of a downstream protein (from the nucleic acid delivered such as poly(IC) in cells/tissues with overexpression of the target receptor as compared to normal cells/tissues or cells/tissues with low expression by means of western blot analysis or luminescence/fluorescent assay, flow cytometry assays or measuring the secretion of the protein by measures of such as ELISA, ECLIA. The level of delivery can also be measured by means of cytotoxicity using cell survival assays or cell death assays including (MTT, Methylene Blue assays, CellTiter-Glo assays, propidium iodide assay): By comparing the amount of protein produced in a tissue to the weight of said tissue, comparing the amount of therapeutic and/or prophylactic in a tissue to the weight of said tissue, comparing the amount of protein produced in a tissue to the amount of total protein in said tissue, or comparing the amount of therapeutic and/or prophylactic in a tissue to the amount of total therapeutic and/or prophylactic in said tissue. It will be understood that the delivery of an inventive polyplex to a target cell or target tissue need not be determined in a subject being treated, it may be determined in a surrogate such as an animal model or a

cellular model.

Thus, in a preferred embodiment, said biological effect is selected from (i) activation of protein signalling, (ii) protein expression, (iii) mRNA expression in cells or tissues, (iv) expression or secretion of a downstream protein from a nucleic acid delivered such as the delivered poly(IC) in cells/tissues with overexpression of the target cell surface receptor as compared to normal cells/tissues or cells/tissues with low expression, (v) cytotoxicity.

In one embodiment, said target cells include, but are not limited to, hepatocytes, epithelial cells, hematopoietic cells, epithelial cells, endothelial cells, lung cells, bone cells, stem cells, mesenchymal cells, neural cells, cardiac cells, adipocytes, vascular smooth muscle cells. Thus, in one embodiment, the target cell is a cell in the liver. In one embodiment, the target cell is an epithelial cell. In one embodiment, the target cell is a hepatocyte. In one embodiment, the target cell is a hematopoietic cell. In one embodiment, the target cell is a muscle cell. In one embodiment, the target cell is an endothelial cell. In one embodiment the target cell is a tumor cell or a cell in the tumor microenvironment. In one embodiment, the target cell is a blood cell. In one embodiment, the target cell is a cell in the lymph nodes. In one embodiment, the target cell is a cell in the lung. In one embodiment, the target cell is a cell in the skin. In one embodiment, the target cell is a spleen cell. In one embodiment, the target cell is an antigen presenting cell such as a professional antigen presenting cell in the spleen. In one embodiment, the target cell is a dendritic cell in the spleen. In one embodiment, the target cell is a T cell. In one embodiment, the target cell is a B cell. In one embodiment, the target cell is a NK cell. In one embodiment, the target cell is a monocyte.

In some embodiments, said targeting fragment selectively or preferentially interacts with a particular cell type. The targeting fragment not only serves to selectively target the conjugates and polyplexes of present invention to a certain cell, but further typically facilitates selective uptake of the conjugates and corresponding polyplexes of the present invention within a certain cell type. In some embodiments, said targeting fragment selectively or preferentially interacts with a particular cell surface receptor. When the targeting fragment of a conjugate and/or polyplex selectively or preferentially interacts with a cell surface receptor, the conjugate and/or polyplex can be selectively or preferentially taken up into the cell that comprises said cell surface receptor.

In a preferred embodiment, said targeting fragment is a peptide, a protein, a small molecule ligand, a saccharide, an oligosaccharide, a lipid, an amino acid, wherein said peptide, said protein, said small molecule ligand, said saccharide, said oligosaccharide, said lipid, said

amino acid is selected from a hormone, a neurotransmitter, a cytokine, a growth factor, a cell adhesion molecule, or a nutrient, and wherein said targeting fragment is an antibody, an antibody fragment, an aptamer or an affibody.

The term "small molecule ligand" as used herein, and in particular with reference to the inventive targeting fragment relates to a chemical moiety that has a molecular weight of at least 75 g/mol, preferably of at least 100 g/mol, and further preferably of at least 200 g/mol and has, preferably, a molecular weight of less than about 2000 g/mol. In some embodiments, the small molecule has a molecular weight of less than about 1500 g/mol, more preferably less than about 1000 g/mol. In a further preferred embodiment, the small molecule has a molecular weight of less than about 800 g/mol, again more preferably less than about 500 g/mol. The term "small molecule ligand" as used herein, and in particular with reference to the inventive targeting fragment shall further preferably relates to such ligand capable of binding, preferably specifically binding, to a target cell, to a target cell receptor, or preferably to a target cell surface receptor. In a preferred embodiment, said small molecule ligand has a molecular weight of at least 75 g/mol, preferably of at least 100 g/mol, and further preferably of at least 200 g/mol and has, preferably, a molecular weight of less than about 2000 g/mol, preferably of less than about 1500 g/mol. In a preferred embodiment, said small molecule ligand has a molecular weight of at least 75 g/mol, preferably of at least 100 g/mol, and further preferably of at least 200 g/mol and has, preferably, a molecular weight of less than about 2000 g/mol, preferably of less than about 1500 g/mol, and wherein said small molecule ligand is capable of binding, preferably specifically binding, to a target cell surface receptor.

In some embodiments, the targeting fragment is a native, natural or modified ligand or a paralog thereof, or a non-native ligand such as an antibody, a single-chain variable fragment (scFv), or an antibody mimetic such as an affibody. In a preferred embodiment, the targeting fragment is a native, natural or modified cell surface antigen ligand or a paralog thereof, or a non-native cell surface antigen ligand such as an antibody, a single-chain variable fragment (scFv), or an antibody mimetic such as an affibody. In a preferred embodiment, the targeting fragment is a native, natural or modified cell surface receptor ligand or a paralog thereof, or a non-native cell surface receptor ligand such as an antibody, a single-chain variable fragment (scFv), or an antibody mimetic such as an affibody. In a preferred embodiment, the targeting fragment is a small molecule ligand, a peptide, a protein, an aptamer, a native, natural or modified ligand and/or a paralog thereof. In a preferred embodiment, the targeting fragment is a small molecule ligand, a peptide, a protein, an aptamer, a native, natural or modified cell

surface antigen ligand and/or a paralog thereof, wherein said small molecule ligand has a molecular weight of at least 75 g/mol, preferably of at least 100 g/mol, and further preferably of at least 200 g/mol and has, preferably, a molecular weight of less than about 2000 g/mol, preferably of less than about 1500 g/mol. In a preferred embodiment, the targeting fragment is a small molecule ligand, a peptide, a protein, an aptamer, a native, natural or modified cell surface receptor ligand and/or a paralog thereof, wherein said small molecule ligand has a molecular weight of at least 75 g/mol, preferably of at least 100 g/mol, and further preferably of at least 200 g/mol and has, preferably, a molecular weight of less than about 2000 g/mol, preferably of less than about 1500 g/mol. In a preferred embodiment, the targeting fragment is a small molecule ligand, a peptide, a protein, an aptamer, a native, natural or modified ligand and/or a paralog thereof, an antibody, a single-chain variable fragment (scFv), or an antibody mimetic such as an affibody.

In a preferred embodiment, the targeting fragment is a small molecule ligand, a peptide, a protein, an aptamer, a native, natural or modified cell surface receptor ligand and/or a paralog thereof. In a preferred embodiment, the targeting fragment is a small molecule ligand, a peptide, a protein, an aptamer, a native, natural or modified ligand and/or a paralog thereof, and wherein said small molecule ligand, said peptide, said protein, said aptamer, said native, natural or modified ligand and/or said paralog thereof is capable of binding, preferably selectively binding, to a cell surface receptor. In a preferred embodiment, said targeting fragment is a small molecule ligand. In a preferred embodiment, said targeting fragment is a small molecule ligand, wherein said small molecule ligand is capable of binding, preferably selectively binding, to a cell surface receptor. In a preferred embodiment, said targeting fragment is a peptide. In a preferred embodiment, said targeting fragment is a peptide, wherein said peptide is capable of binding, preferably selectively binding, to a cell surface receptor. In a preferred embodiment, said targeting fragment is a protein. In a preferred embodiment, said targeting fragment is a protein, wherein said protein is capable of binding, preferably selectively binding, to a cell surface receptor. In a preferred embodiment, said targeting fragment is an aptamer. In a preferred embodiment, said targeting fragment is an aptamer, wherein said aptamer is capable of binding, preferably selectively binding, to a cell surface receptor. In a preferred embodiment, said targeting fragment is a native, natural or modified ligand and/or a paralog thereof, preferably a native, natural or modified cell surface receptor ligand and/or a paralog thereof. In a preferred embodiment, said targeting fragment is a native, natural or modified ligand and/or a paralog thereof, wherein said native, natural or modified ligand and/or said paralog thereof is

capable of binding, preferably selectively binding, to a cell surface receptor. In a preferred embodiment, said targeting fragment is an antibody, a single-chain variable fragment (scFv), or an antibody mimetic such as an affibody. In a preferred embodiment, said targeting fragment is an antibody, a single-chain variable fragment (scFv), or an antibody mimetic such as an affibody, wherein said antibody, a single-chain variable fragment (scFv), or an antibody mimetic such as an affibody is capable of binding, preferably selectively binding, to a cell surface receptor.

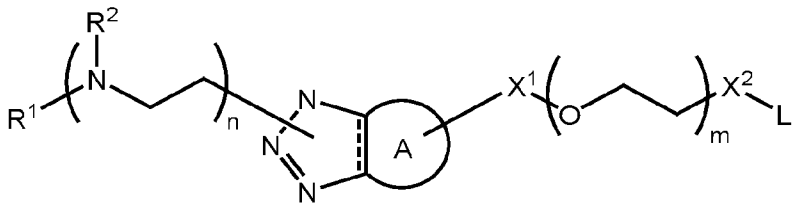
In a preferred embodiment, the targeting fragment is a small molecule ligand, a peptide, a protein, an aptamer, an antibody, an antibody fragment, preferably a single-chain variable fragment (scFv), an antibody mimetic, preferably selected from an affibody, nanobody, diabody, designed ankyrin repeat protein (DARPin), a cytokine or a functional fragment thereof, an integrin, an interleukin or a functional fragment thereof, an enzyme, a nucleic acid, a fatty acid, a carbohydrate, mono-, oligo- or polysaccharides, a peptidoglycan, a glycopeptide, asialoorosomucoid, mannose-6-phospate, mannose, Sialyl-Lewis^x, N-acetyllactosamine, galactose, lysosomotropic agents, and/or a nucleus localizing agents, preferably T-antigen, a tumor low pH insertion peptide (PHLIP), a p32 targeting peptide, preferably LyP-1 tumor homing peptide, insulin-like growth factor 1, vascular endothelial growth factor, platelet-derived growth factor, and/or a fibroblast growth factor.

In some embodiments the targeting fragment is a non-native ligand such as an antibody or an antibody fragment (e.g., a single-chain variable fragment (scFv), an antibody mimetic such as an affibody, nanobody, diabody, designed ankyrin repeat protein (DARPin), or other antibody variant). In some embodiment, the targeting fragment is a hormone or a fragment preferably a functional fragment, thereof (e.g., insulin), asialoorosomucoid, mannose-6-phospate, mannose, Sialyl-Lewis^x, N-acetyllactosamine, galactose, lysosomotropic agents, and/or a nucleus localizing agents (e.g., T-antigen), a tumor low pH insertion peptide (PHLIP), a p32 targeting peptide such as LyP-1 tumor homing peptide, insulin-like growth factor 1, vascular endothelial growth factor, platelet-derived growth factor, and/or a fibroblast growth factor. Further non-limiting examples of targeting fragments include an enzyme, a nucleic acid, a fatty acid, a carbohydrate, mono-, oligo- or polysaccharides, a peptidoglycan, a glycopeptide.

In a preferred embodiment, said targeting fragment is a small molecule ligand, a peptide, a protein, an aptamer, an antibody, an antibody fragment, preferably a Fab, Fab', F(ab')2 or a scFv fragment, an antibody mimetic, preferably selected from an affibody, nanobody, diabody, designed ankyrin repeat protein (DARPin), a growth factor or a functional fragment thereof, a

hormone or a functional fragment thereof, preferably insulin, a cytokine or a functional fragment thereof, an interleukin or a functional fragment thereof, an enzyme, a nucleic acid, a fatty acid, a carbohydrate, mono-, oligo- or polysaccharides, a peptidoglycan, a glycopeptide, asialoorosomucoid, mannose-6-phospate, mannose, Sialyl-Lewis^x, N-acetyllactosamine, galactose, lysosomotropic agents, and/or a nucleus localizing agents, preferably T-antigen, a tumor low pH insertion peptide (PHLIP), a p32 targeting peptide, preferably LyP-1 tumor homing peptide, insulin-like growth factor 1, vascular endothelial growth factor, platelet-derived growth factor, and/or a fibroblast growth factor.

In another aspect, the present invention provides a composition comprising a conjugate, preferably a plurality of conjugates, of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating units m of 25 to 100, preferably of a discrete number of repeating units m of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

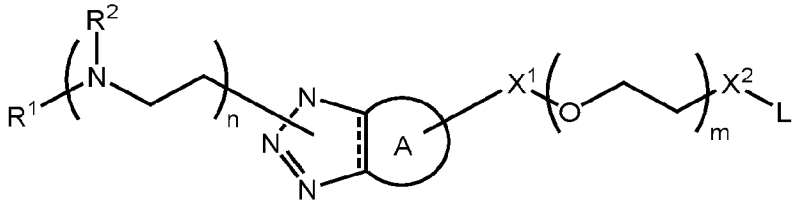
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In another aspect, the present invention provides a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

═══ is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of contiguous repeating units m of 25 to 100, preferably of a discrete number of contiguous repeating units m of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

Said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), which is also named herein as PSMA targeting fragment.

PSMA is mainly expressed in four tissues of the body, including prostate epithelium, the

proximal tubules of the kidney, the jejunal brush border of the small intestine and ganglia of the nervous system (Mhawech-Fauceglia et al., Histopathology 2007, 50:472–483). PSMA is overexpressed in neoplastic tissue and in malignant prostate, especially in prostatic adenocarcinoma relative to normal tissue, and the level of PSMA expression is further up-regulated as the disease progresses into metastatic phases (Silver et al., 1997, Clin. Cancer Res., 3:81). Since PSMA expression is about 1,000-fold higher in prostate tumors, PSMA is particularly considered as target for diagnosis and therapy in prostate cancer (Kularatne SA et al., Molecular Pharmaceutics 2009, 6(3):780-789; Rowe SP et al., Prostate Cancer Prostatic Dis. 2016, 19(3):223-230; Wang H et al., Small Struct. 2022, 3:220003620;9). Furthermore, upregulation of PSMA might provide prostate cancer cells with a growth advantage and implicate PSMA in the metabolism of polyglutamated folates and the subsequent uptake of folates (Yao et al., Prostate 2006, 66:867-875; Yao et al., Prostate 2010, 70:305-316). However, PSMA targeting may also be applicable to other PSMA-expressing tumors besides prostate cancer, in particular since PSMA is not expressed on normal vasculature but it is expressed on the neovasculature of many solid tumors such as breast cancer, lung cancer, gastric cancer, colorectal cancer, pancreatic cancer, renal cell carcinoma, and bladder cancer allowing for targeting to occur in the intravascular compartment (Chang SS et al., Cancer Res. 1999, 59(13):3192-3198; Wernicke et al., APMIS 2014, 122(6):482-489; Samplaski MK et al., Mod Pathol. 2011, 24(11):1521-1529; Haffner MC et al., Hum Pathol. 2009, 40(12):1754-1761; Morgenroth A et al., Breast Cancer Research 2019, 21:116; Jian D et al., Clinical and Translational Gastroenterology 2019;10:e-00041; Jeitner TM et al.,Translational Oncology 2022, 22:101450, and references cited therein).

In a preferred embodiment, said targeting fragment is capable of binding to a cell expressing PSMA. In a preferred embodiment, said targeting fragment is capable of binding to a cell overexpressing PSMA. In one embodiment, said overexpressing PSMA means that the level of PSMA expressed in said cell of a certain tissue is elevated in comparison to the level of PSMA as measured in a normal healthy cell of the same type of tissue under analogous conditions. In one embodiment, said overexpressing PSMA refers to an increase in the level of PSMA in a cell relative to the level in the same cell or closely related non-malignant cell under normal physiological conditions. In one embodiment, said cell overexpressing PSMA relates to expression of PSMA that is at least 10-fold higher as compared to a normal cell or a normal tissue. In one embodiment, said cell overexpressing PSMA relates to expression of PSMA with a cut-off of 5% or more PSMA positive cells, as e.g. described in Mhawech-Fauceglia et al.,

2007, which can be used to define PSMA expression in different types of tissues or cells. Thus, cells or tissue with < 5% positive cells was considered to be negative, or where the PSMA expression is categorized according to its intensity and scored as 0 (no expression), 1 (low expression), 2 (medium expression), and 3 (high expression), as described in Hupe et al., 2018 2018 (Hupe MC et al, Frontiers in Oncology 2018, 8 (623): 1-7).

In a preferred embodiment, said targeting fragment is capable of binding to a cell expressing or overexpressing PSMA. Cells expressing PSMA typically include tumor cells, such as prostate, bladder, pancreas, lung, breast, kidney, colon tumor cells, melanomas, and sarcomas. In a preferred embodiment said targeting fragment is capable of binding to a cell expressing or overexpressing PSMA, wherein said cell is a tumor cell, preferably selected from a prostate, a bladder, a pancreas, a lung, a breast cancer, a kidney and a colon tumor cell, a melanoma, and a sarcoma. In a preferred embodiment said targeting fragment is capable of binding to a cell expressing or overexpressing PSMA, wherein said cell is a tumor cell, wherein said tumor cell is a prostate tumor cell.

In a preferred embodiment, said targeting fragment is capable of specifically binding to PSMA, wherein typically and preferably said affinity or specific binding is measured by the dissociation constant (K_D) and said affinity or specific binding refers to a K_D of less than 10^{-3} M, preferably of less than 10^{-4} M, further preferably of less than 10^{-5} M, further preferably of less than 10^{-6} M, more preferably of less than 10^{-7} M and even more preferably of less than 10^{-8} M, and again further preferably of less than 10^{-9} M, and again further preferably of less than 10^{-10} M. In a preferred embodiment, said targeting fragment is capable of specifically binding to PSMA, wherein typically and preferably said affinity or specific binding is measured by the dissociation constant (K_D) and said affinity or specific binding refers to a K_D of less than 10^{-3} M, of less than 10^{-4} M, of less than 10^{-5} M, of less than 10^{-6} M, of less than 10^{-7} M, of less than 10^{-8} M, and of less than 10^{-9} M. Preferably, binding results in formation of a complex between the targeting fragment and PSMA, wherein the binding or complex can be detected, typically and preferably using a Biacore 3000 instrument (Biacore Inc., Piscataway NJ) or cell based binding assays or Flow Induced Dispersion Analysis (FIDA), typically and preferably as described in Kularatne et al, Mol Pharm. 2009; 6(3): 790–800.

In a preferred embodiment, said targeting fragment is capable of binding to the extracellular domain of PSMA or parts thereof. In a preferred embodiment, said targeting fragment is capable of binding to epitopes on the extracellular domain of PSMA.

In a preferred embodiment, said targeting fragment is a PSMA antibody, a PSMA

aptamer, or a small-molecule PSMA targeting fragment.

In a preferred embodiment, said targeting fragment is a PSMA antibody, a PSMA aptamer or a small-molecule PSMA targeting fragment. In a preferred embodiment, said PSMA targeting fragment is a PSMA antibody, a PSMA aptamer or a small-molecule PSMA targeting fragment. The term "small molecule PSMA targeting fragment" as used herein relates to a chemical moiety that has a molecular weight of less than about 2000 g/mol, and that is typically and preferably capable of binding to PSMA. In some embodiments, the small molecule PSMA targeting fragment has a molecular weight of less than about 1800 g/mol. In some embodiments, the small molecule PSMA targeting fragment has a molecular weight of less than about 1500 g/mol, more preferably less than about 1000 g/mol. In a further preferred embodiment, the small molecule has a molecular weight of less than about 800 g/mol, again more preferably less than about 500 g/mol.

In some embodiments, said PSMA targeting fragment is a PSMA antibody that is an antibody capable of binding to PSMA, thus also referred to and known by the skilled person in the art as anti-PSMA antibodies. In some embodiments, said antibody is a monoclonal antibody, a polyclonal antibody, and/or an antibody fragment, preferably a functional fragment thereof, a chimeric antibody, a recombinant antibody, and/or a bi- or multispecific antibody. Such PSMA antibodies include, but are not limited to, scFv antibodies A5, G0, G1, G2, and G4 and mAbs 3/E7, 3/F11, 3/A12, K7, K12, and D20 (Elsasser-Beile et al., 2006, Prostate, 66:1359); mAbs E99, J591, J533, and J415 (Liu et al., 1997, Cancer Res., 57:3629; Liu et al., 1998, Cancer Res., 58:4055; Fracasso et al., 2002, Prostate, 53:9; McDevitt et al., 2000, Cancer Res., 60:6095; McDevitt et al., 2001, Science, 294:1537; Smith-Jones et al., 2000, Cancer Res., 60:5237; Vallabhajosula et al., 2004, Prostate, 58:145; Bander et al., 2003, J. Urol., 170:1717; Patri et al., 2004, Bioconj. Chem., 15:1174; Viola-Villegas NT et al., Mol Pharm 2014, 11:3965-3973; and U.S. Patent 7,163,680); mAb 7E11-C5.3 (Horoszewicz et al., 1987, Anticancer Res., 7:927); antibody 7E11 (Horoszewicz et al., 1987, Anticancer Res., 7:927; and U.S. Patent 5,162,504); and antibodies described in Chang et al., 1999, Cancer Res., 59:3192; Murphy et al., 1998, J. Urol., 160:2396; Grauer et al., 1998, Cancer Res., 58:4787; and Wang et al., 2001, Int. J. Cancer, 92:871. One of ordinary skill in the art will appreciate that any antibody that recognizes and/or specifically binds to PSMA may be used in accordance with the present invention. All foregoing documents and disclosures are incorporated herein by reference in their entirety.

In some embodiments, said targeting fragment capable of binding to PSMA is an aptamer. PSMA targeting aptamers include, but are not limited to, the A10 aptamer or A9 aptamer, derivatives thereof, and/or functional fragments thereof (Lupold et al., 2002, Cancer Res., 62:4029; and Chu et al., Nucleic Acids Res 2006, 34(10):e73; Baek SE, et al., J Control Release 2014, 196:234-242). In some embodiments, in the aptamer derivatives fewer than 30, 25, 20, 15, 10, 5, 4, 3, 2, or 1 nucleic acid is substituted relative to the aptamer. In some embodiments, the sequences of the aptamer derivatives are at least 80%, preferably 85%, more preferably 90%, again more preferably 95%, most preferably 99% identical.

In a preferred embodiment, said targeting fragment is a small molecule PSMA targeting fragment. In a preferred embodiment, said PSMA targeting fragment is a small molecule PSMA targeting fragment, preferably a small molecule PSMA targeting peptidase inhibitor. In a preferred embodiment, said small molecule PSMA peptidase inhibitors include 2-PMPA, GPI5232, VA-033, phenylalkylphosphonamidates (Jackson et al., 2001, Curr. Med. Chem., 8:949; Bennett et al., 1998, J. Am. Chem. Soc., 120:12139; Jackson et al., 2001, J Med. Chem., 44:4170; Tsukamoto et al., 2002, Bioorg. Med. Chem. Lett., 12 :2189; Tang et al., 2003, Biochem. Biophys. Res. Commun., 307: 8; Oliver et al., 2003, Bioorg. Med. Chem., 11:4455; and Maung et al., 2004, Bioorg. Med. Chem., 12:4969), and/or analogs and derivatives thereof. All of the foregoing documents (scientific and other publications, patents and patent applications) are incorporated herein by reference in their entirety. In some embodiments, said small molecule PSMA targeting fragment is a protein, a peptide, an amino acid or a derivative thereof. In a preferred embodiment, said small molecule PSMA targeting fragment includes thiol and indole thiol derivatives, such as 2-MPPA and 3-(2-mercaptoethyl)-1H-indole-2-carboxylic acid derivatives (Majer et al., 2003, J Med. Chem., 4611989; and U.S. Patent Publication 2005/0080128). In some embodiments, said small molecule PSMA targeting fragments comprise hydroxamate derivatives (Stoermer et al., 2003, Bioorg. Med. Chem. Lett., 1312097). In a preferred embodiment, said small molecule PSMA peptidase inhibitors include androgen receptor targeting agents (ARTAs), such as those described in U.S. Patents 7,026,500; 7,022,870; 6,998,500; 6,995,284; 6,838,484; 6,569,896; 6,492,554; and in U.S. Patent Publications 2006/0287547; 2006/0276540; 2006/0258628; 2006/0241180; 2006/0183931; 2006/0035966; 2006/0009529; 2006/0004042; 2005/0033074; 2004/0260108; 2004/0260092; 2004/0167103; 2004/0147550; 2004/0147489; 2004/0087810; 2004/0067979; 2004/0052727; 2004/0029913; 2004/0014975; 2003/0232792; 2003/0232013; 2003/0225040; 2003/0162761; 2004/0087810; 2003/0022868; 2002/0173495; 2002/0099096; 2002/0099036. In some

embodiments, said small molecule PSMA targeting fragments include polyamines, such as putrescine, spermine, and spermidine (U.S. Patent Publications 2005/0233948 and 2003/0035804). All foregoing documents and disclosures are incorporated herein by reference in their entirety.

In a preferred embodiment, said small molecule PSMA peptidase inhibitors include PBDA- and urea-based inhibitors, such as ZJ 43, ZJ , ZJ 17, ZJ 38 (Nan et al., 2000, J. Med. Chem., 43:772; and Kozikowski et al., 2004, J. Med. Chem., 47, 7, 1729-1738), and/or and analogs and derivatives thereof. Other agents which bind PSMA can also be used as PSMA targeting fragment including, for example those found in Clin. Cancer Res., 2008 14:3036-43, or PSMA targeting fragments prepared by sequentially adding components to a preformed urea, such as the lysine-urea-glutamate compounds described in Banerjee et al. (J. Med. Chem. vol. 51, pp. 4504-4517, 2008). In a preferred embodiment, said one or more targeting fragments capable of binding to prostate specific membrane antigen (PSMA) are small-molecule PSMA targeting fragments, more preferably small urea-based inhibitors.

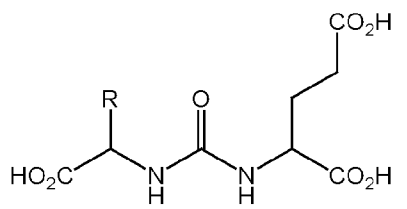
In preferred embodiments, said small molecule PSMA targeting fragments are urea-based inhibitors (herein also called urea-based peptidase inhibitors or urea-based PSMA peptidase inhibitors), more preferably small urea-based inhibitors, such as disclosed in Kularatne et al., Mol Pharmaceutics 2009, 6, 780; Kularatne et al., Mol. Pharmaceutics 2009, 6, 790; Kopka et al., J Nucl Med 2017, 58:17S-26S, Kozikowski et al., J Med Chem. 2001, 44:298–301, Kozikowski et al., J Med Chem. 2004, 47:1729-1738, WO2017/044936, WO2011/084518, WO2011/084521, WO2011/084513, WO2012/166923, WO2008/105773, WO2008/121949, WO2012/135592, WO2010/005740, WO2015/168379, WO03/045436, WO03/045436, WO2016/183447, US2015/258102, WO2011/084513, WO 2017/089942, US2010/278927, WO2012/016188, WO2008/124634, WO2009/131435, US 2007/225213, WO2017/086467, WO2009/026177, WO2012005572, WO2014/072357, and WO2011/108930. All foregoing documents and disclosures are incorporated herein by reference in their entirety.

In a preferred embodiment, said targeting fragment is a dipeptide urea based PSMA peptidase inhibitor, preferably a small molecule dipeptide urea-based PSMA peptidase inhibitor. In a preferred embodiment, said PSMA targeting fragment is a dipeptide urea based PSMA peptidase inhibitor, preferably a small molecule dipeptide urea-based PSMA peptidase inhibitor.

The term “urea-based PSMA peptidase inhibitor” relates to a PSMA peptidase inhibitor

comprising a urea group. The term "dipeptide urea based PSMA peptidase inhibitor" relate to PSMA peptidase inhibitor comprising a urea group and two peptides or amino acids each independently attached to the -NH_2 groups of the urea group, while the term "small molecule dipeptide urea-based PSMA peptidase inhibitor" further refers that the dipeptide urea based PSMA peptidase inhibitor has a molecular weight of less than about 2000 g/mol, and that is typically and preferably capable of binding to PSMA. In some embodiments, the small molecule dipeptide urea-based PSMA peptidase inhibitor has a molecular weight of less than about 1800 g/mol, less than about 1500 g/mol, preferably less than about 1000 g/mol. In a further preferred embodiment, the small molecule dipeptide urea-based PSMA peptidase inhibitor has a molecular weight of less than about 800 g/mol, again more preferably less than about 500 g/mol. PSMA peptidase inhibitors are able to reduce the activity of the PSMA transmembrane zinc(II) metalloenzyme that catalyzes the cleavage of terminal glutamates. More preferably, said small molecule urea-based PSMA peptidase inhibitor has a molecular weight of less than about 500 g/mol. Again more preferably, said small molecule urea-based PSMA peptidase inhibitor is a Glutamate-urea based PSMA peptidase inhibitor, preferably such as mentioned in Kopka et al., J Nuc Med, 58(9), suppl. 2, 2017; Wirtz et al., EJNMMI Research (2018) 8:84 and references cited therein, all incorporated herein by reference in their entirety.

In a preferred embodiment, said targeting fragment, preferably said urea based PSMA peptidase inhibitor is a glutamate-urea moiety of formula **1**, preferably of formula **1***:



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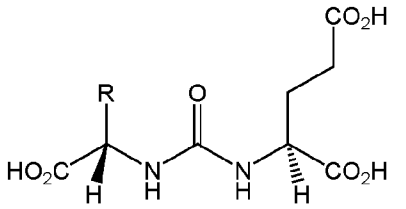
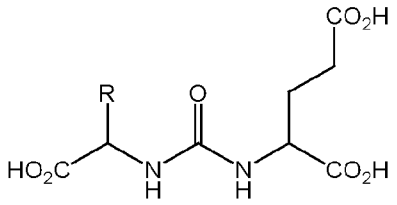


1*

and enantiomers, stereoisomers, rotamers, tautomers, diastereomers, or racemates thereof; wherein R is preferably substituted or unsubstituted alkyl, substituted or unsubstituted aryl, and any combination thereof; more preferably R is C_{1-6}-alkyl, preferably C_2-C_4-alkyl, substituted one or more times, preferably one time with OH, SH, NH_2, or COOH, wherein one of said NH_2, OH or SH or COOH groups serves as the point of covalent attachment to the X^2 linking moiety and the PEG fragment respectively, wherein the alkyl group can optionally be interrupted by N(H), S or O. In another preferred embodiment, R is C_{1-6}-alkyl, preferably C_2-C_4-alkyl, substituted one time with OH, SH, NH_2, or COOH, wherein said NH_2, OH, or SH or COOH group serves as the point of covalent attachment to the X^2 linking moiety and the PEG fragment respectively. In a very preferred embodiment, R is C_2-alkyl substituted one time with

COOH, wherein said COOH group serves as the point of covalent attachment to the X^2 linking moiety and the PEG fragment respectively. In preferred embodiments, when said COOH group serves as said point of covalent attachment to the X^2 linking moiety, said COOH group is condensed with an amine group of the X^2 linking moiety to form an amide.

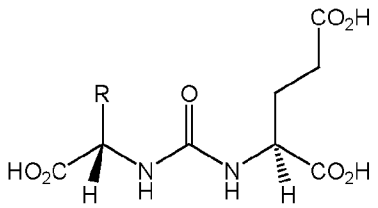
In a preferred embodiment, said targeting fragment is a glutamate-urea moiety of formula **1**:



1,

wherein R is C_{1-6}-alkyl, preferably C_2-C_4-alkyl, substituted one or more times, preferably one time with OH, SH, NH_2, or COOH, wherein one of said NH_2, OH or SH or COOH group serves as the point for covalent attachment to the X^2 linking moiety and the PEG fragment respectively, and wherein the alkyl group is optionally be interrupted by N(H), S or O. In another preferred embodiment, R is C_{1-6}-alkyl, preferably C_2-C_4-alkyl, substituted one time with OH, SH, NH_2, or COOH, wherein said NH_2, OH, or SH or COOH group serves as the point for covalent attachment to the X^2 linking moiety and the PEG fragment respectively. In a very preferred embodiment, R is C_2-alkyl substituted one time with COOH, wherein said COOH group serve as the point for covalent attachment to the X^2 linking moiety and the PEG fragment respectively. In preferred embodiments, when said COOH group serves as said point of covalent attachment to the X^2 linking moiety, said COOH group is condensed with an amine group of the X^2 linking moiety to form an amide.

In another preferred embodiment, said targeting fragment is a glutamate-urea moiety of formula **1***



1*

wherein R is C_{1-6}-alkyl, preferably C_2-C_4-alkyl, substituted one or more times, preferably one time with OH, SH, NH_2, or COOH, wherein one of said NH_2, OH or SH or COOH group serve as the point for covalent attachment to the X^2 linking moiety and the PEG fragment respectively, and wherein the alkyl group is optionally be interrupted by N(H), S or O. In another preferred embodiment, R is C_{1-6}-alkyl, preferably C_2-C_4-alkyl, substituted one time

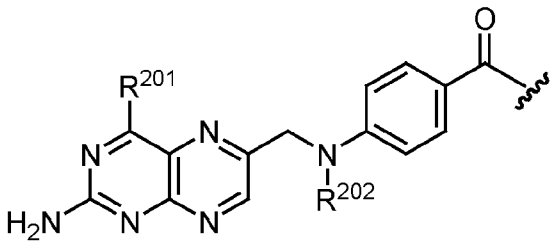
with OH, SH, NH_2, or COOH, wherein said NH_2, OH, or SH or COOH group serve as the point for covalent attachment to the X^2 linking moiety and the PEG fragment respectively. In a very preferred embodiment, R is C_2-alkyl substituted one time with COOH, wherein said COOH group serve as the point for covalent attachment to the X^2 linking moiety and the PEG fragment respectively. In preferred embodiments, when said COOH group serves as said point of covalent attachment to the X^2 linking moiety, said COOH group is condensed with an amine group of the X^2 linking moiety to form an amide.

In a further preferred embodiment, said targeting fragment comprises or preferably consists of the DUPA residue (HOOC-$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-). In a further very preferred embodiment, said targeting fragment consists of the DUPA residue (HOOC$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-), wherein both chiral C-atoms having (*S*)-configuration, as depicted in formula **1***.

In a further preferred embodiment, said PSMA targeting fragment is a folate ligand. In a further preferred embodiment, said PSMA targeting fragment is a small molecule PSMA targeting fragment, wherein said small molecule PSMA targeting fragment is a folate ligand.

In preferred embodiments, said folate ligand binds to a cell surface receptor, wherein said cell surface receptor is PSMA. As recently reported, targeting of cells expressing PSMA has been achieved by amides of folic acid (Flores O et al., Theranostics 2017, 7(9):2477-2494).

As used herein, the term "folate ligand" is understood as folic acid or methotrexate or a derivative or analogue thereof. Preferably said folic acid or methotrexate derivative or analogue thereof comprises a glutamate functionality R-NH-[CH(COOH)-CH_2-CH_2-C(O)NH]$_\eta$-CH(COOH)-CH_2-CH_2-COOH, wherein η is an integer from 0 to 100, and wherein R is a group of Formula 2:



(Formula 2), wherein

R^{201} is -OH or -NH_2;

R^{202} is -H or -CH_3; and

the wavy line indicates the point of attachment to said glutamate functionality. In preferred embodiments, η is an integer from 0 to 10, preferably η is an integer from 0 to 5, and further preferably η is 0.

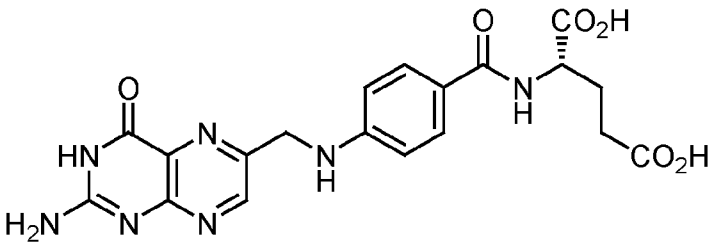
One of skill in the art will understand that when R^{201} is -OH, in preferred embodiments said OH will tautomerize to a carbonyl group (=O), and the neighboring nitrogen atom of said R^{201} will be protonated.

One of skill in the art will futher understand that said glutamate functionality R-NH-[CH(COOH)-CH_2-CH_2-C(O)NH]$_\eta$-CH(COOH)-CH_2-CH_2-COOH comprises at least one *alpha* carboxylate group and a *gamma* carboxylate group. Specifically, the one or more -COOH groups bonded to the same carbon as the -NH- group or groups are understood herein as *alpha* carboxylate groups. When $\eta = 0$, the -COOH group bonded to the same carbon as the R-NH group is understood herein as the *alpha* carboxylate group. The -COOH group bonded to the –$(CH_2)_2$- group is understood herein as the *gamma* carboxylate group. Moreover, one of skill in the art will understand that the carboxylate groups discussed herein, e.g., the *alpha* and the *gamma* carboxylate groups, can be protonated or deprotonated depending on the pH of the surrounding solution. Accordingly, one of skill in the art will understand that although the carboxylate groups are drawn as neutral species (-COOH) for simplicity and clarity, these can exist (e.g., can primarily exist) as deprotonated, i.e., negatively charged species ($-COO^-$) at physiological pH.

In some embodiments, an *alpha* carboxylate group of said glutamate functionality serves as the point of covalent attachment to the X^2 linking moiety. In preferred embodiments, when said *alpha* carboxylate group of said glutamate functionality serves as said point of attachment to the X^2 linking moiety, said *alpha* carboxylate group is condensed with an amine group of the X^2 linking moiety to form an amide. In some embodiments, when said *alpha* carboxylate group of said glutamate functionality serves as said point of attachment to the X^2 linking moiety, said *alpha* carboxylate group is condensed with a hydroxy group of the X^2 linking moiety to form an ester.

In preferred embodiments, the *gamma* carboxylate group of said glutamate functionality serves as the point of covalent attachment to the X^2 linking moiety. In preferred embodiments, when said *gamma* carboxylate group of said glutamate functionality serves as said point of attachment to the X^2 linking moiety, said *gamma* carboxylate group is condensed with an amine group of the X^2 linking moiety to form an amide. In some embodiments, when said *gamma* carboxylate group of said glutamate functionality serves as said point of attachment to the X^2 linking moiety, said *gamma* carboxylate group is condensed with a hydroxy group of the X^2 linking moiety to form an ester.

In a preferred embodiment, said folate ligand is folic acid:

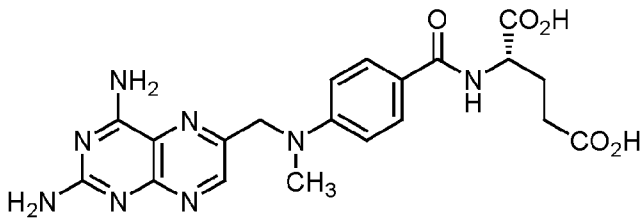


wherein either the *alpha* carboxylate group or the *gamma* carboxylate group of said folic acid serves as the point of covalent attachment to the X^2 linking moiety.

In some embodiments, the *alpha* carboxylate group of said folic acid serves as the point of covalent attachment to the X^2 linking moiety. In preferred embodiments, when said *alpha* carboxylate group of said folic acid serves as said point of attachment to the X^2 linking moiety, said *alpha* carboxylate group is condensed with an amine group of the X^2 linking moiety to form an amide. In some embodiments, when said *alpha* carboxylate group of said folic acid serves as said point of attachment to the X^2 linking moiety, said *alpha* carboxylate group is condensed with a hydroxy group of the X^2 linking moiety to form an ester.

In preferred embodiments, the *gamma* carboxylate group of said folic acid serves as the point of covalent attachment to the X^2 linking moiety. In preferred embodiments, when said *gamma* carboxylate group of said folic acid serves as said point of attachment to the X^2 linking moiety, said *gamma* carboxylate group is condensed with an amine group of the X^2 linking moiety to form an amide. In some embodiments, when said *gamma* carboxylate group of said folic acid serves as said point of attachment to the X^2 linking moiety, said *gamma* carboxylate group is condensed with a hydroxy group of the X^2 linking moiety to form an ester.

In a preferred embodiment, said folate ligand is methotrexate:



wherein either the *alpha* carboxylate group or the *gamma* carboxylate group of said methotrexate serves as the point of covalent attachment to the X^2 linking moiety.

In some embodiments, the *alpha* carboxylate group of said methotrexate serves as the point of covalent attachment to the X^2 linking moiety. In preferred embodiments, when said *alpha* carboxylate group of said methotrexate serves as said point of attachment to the X^2 linking moiety, said *alpha* carboxylate group is condensed with an amine group of the X^2

linking moiety to form an amide. In some embodiments, when said *alpha* carboxylate group of said methotrexate serves as said point of attachment to the X^2 linking moiety, said *alpha* carboxylate group is condensed with a hydroxy group of the X^2 linking moiety to form an ester.

In preferred embodiments, the *gamma* carboxylate group of said methotrexate serves as the point of covalent attachment to the X^2 linking moiety. In preferred embodiments, when said *gamma* carboxylate group of said methotrexate serves as said point of attachment to the X^2 linking moiety, said *gamma* carboxylate group is condensed with an amine group of the X^2 linking moiety to form an amide. In some embodiments, when said *gamma* carboxylate group of said methotrexate serves as said point of attachment to the X^2 linking moiety, said *gamma* carboxylate group is condensed with a hydroxy group of the X^2 linking moiety to form an ester.

In a further aspect, the present invention provides a composition comprising a conjugate of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof: R^1-(NR^2-CH_2-CH_2)$_n$-Z-X^1-(O-CH_2-CH_2)$_m$-X^2-L (Formula I*); wherein n is any integer between 1 and 1500; m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60; R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$–moieties is H; X^1 and X^2 are independently divalent covalent linking moieties; Z is a divalent covalent linking moiety wherein Z is not -NHC(O)-, wherein preferably Z is a divalent covalent linking moiety wherein Z-X^1 is not a single bond and Z is not -NHC(O)-; L is a targeting fragment capable of binding to a cell overexpressing prostate specific membrane antigen (PSMA), wherein preferably said L is the DUPA residue (HOOC(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-(CH_2)$_2$-CO-), and wherein preferably said composition consists of said conjugate.

In a further aspect, the present invention provides a composition comprising a conjugate of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof: R^1-(NR^2-CH_2-CH_2)$_n$-Z-X^1-(O-CH_2-CH_2)$_m$-X^2-L (Formula I*); wherein n is any integer between 1 and 1500; m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60; R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$–moieties is H; X^1 and X^2 are independently divalent covalent linking moieties; Z is a divalent covalent linking moiety wherein Z is not -NHC(O)-, wherein preferably

Z is a divalent covalent linking moiety wherein Z-X^1 is not a single bond and Z is not -NHC(O)-; L is a targeting fragment capable of binding to prostate specific membrane antigen (PSMA), wherein preferably said L is the DUPA residue ($HOOC(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-), and wherein preferably said composition consists of said conjugate.

In a further aspect, the present invention provides a composition comprising a conjugate of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof: R^1-$(NR^2$-CH_2-$CH_2)_n$-Z-X^1-$(O$-CH_2-$CH_2)_m$-X^2-L (Formula I*); wherein n is any integer between 1 and 1500; m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60; R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -$(NR^2$-CH_2-$CH_2)_n$–moieties is H; X^1 and X^2 are independently divalent covalent linking moieties; Z is a divalent covalent linking moiety wherein Z is not -NHC(O)-, wherein preferably Z is a divalent covalent linking moiety wherein Z is not a single bond and Z is not -NHC(O)-; L is a targeting fragment, wherein said targeting fragment L is the DUPA residue ($HOOC(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-), and wherein preferably said composition consists of said conjugate.

In another aspect, the present invention provides a conjugate of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:

R^1-$(NR^2$-CH_2-$CH_2)_n$-Z-X^1-$(O$-CH_2-$CH_2)_m$-X^2-L (Formula I*); wherein n is any integer between 1 and 1500; m is any integer between 1 and 200, preferably m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60; R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -$(NR^2$-CH_2-$CH_2)_n$–moieties is H; X^1 and X^2 are independently divalent covalent linking moieties; Z is a divalent covalent linking moiety wherein Z is not -NHC(O)-, wherein preferably Z is a divalent covalent linking moiety wherein Z is not a single bond and Z is not -NHC(O)-; L is a targeting fragment capable of binding to a cell overexpressing prostate specific membrane antigen (PSMA), wherein preferably said L is the DUPA residue ($HOOC(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-).

In another aspect, the present invention provides a conjugate of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:

R^1-$(NR^2$-CH_2-$CH_2)_n$-Z-X^1-$(O$-CH_2-$CH_2)_m$-X^2-L (Formula I*);

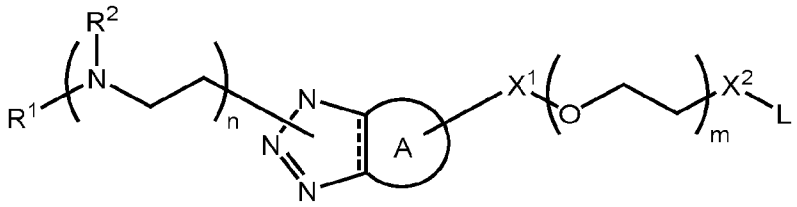
wherein n is any integer between 1 and 1500; m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60; R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$–moieties is H; X^1 and X^2 are independently divalent covalent linking moieties; Z is a divalent covalent linking moiety wherein Z is not -NHC(O)-, wherein preferably Z is a divalent covalent linking moiety wherein Z is not a single bond and Z is not -NHC(O)-; L is a targeting fragment capable of binding to prostate specific membrane antigen (PSMA), wherein preferably L is the DUPA residue (HOOC(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-(CH_2)$_2$-CO-).

In another aspect, the present invention provides a conjugate of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:

R^1-(NR^2-CH_2-CH_2)$_n$-Z-X^1-(O-CH_2-CH_2)$_m$-X^2-L (Formula I*);

wherein n is any integer between 1 and 1500; m is any integer between 1 and 200, preferably m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60; R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$–moieties is H; X^1 and X^2 are independently divalent covalent linking moieties; Z is a divalent covalent linking moiety wherein Z is not -NHC(O)-, wherein preferably Z is a divalent covalent linking moiety wherein Z is not a single bond and Z is not -NHC(O)-; L is a targeting fragment, wherein said targeting fragment L is the DUPA residue (HOOC(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-(CH_2)$_2$-CO-).

In some embodiments, said conjugate is of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$–moieties is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1};

R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings , wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2};

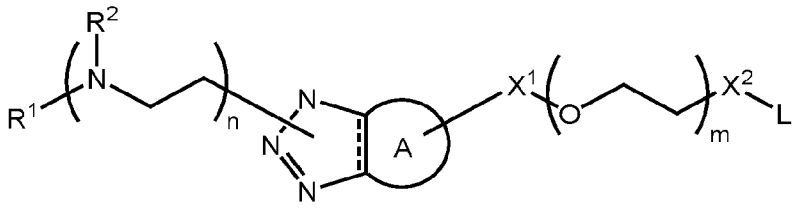
R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

X^1 is a linking moiety of the formula –(Y^1)$_p$–, wherein p is an integer between 1 and 20, and each occurrence of Y^1 is independently selected from a chemical bond, -$CR^{11}R^{12}$-, -C(O)-, -O-, -S-, -NR^{13}-, an amino acid residue, a divalent phenyl moiety, a divalent carbocycle moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl or heteroaryl is optionally substituted with one or more R^{13}, and each divalent heterocycle is optionally substituted with one or more R^{14}; wherein R^{11}, R^{12} and R^{13} are independently, at each occurrence, H, -SO_3H, -NH_2, -CO_2H, or C_1-C_6 alkyl, wherein each alkyl is optionally substituted with -CO_2H or -NH_2; and wherein R^{14} is independently, at each occurrence, H, C_1-C_6 alkyl, or oxo, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl;

X^2 is a linking moiety of the formula –(Y^2)$_q$–, wherein q is an integer between 1 and 50, and each occurrence of Y^2 is independently selected from a chemical bond, -$CR^{21}R^{22}$-, NR^{23}-, -O-, -S-, -C(O)-, an amino acid residue, a divalent phenyl moiety, a divalent carbocycle moiety a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl and divalent heteroaryl is optionally substituted with one or more R^{23}, and wherein each divalent heterocycle moiety is optionally substituted with one or more R^{24}; wherein $R^{21,}$ $R^{22,}$ and R^{23} are each independently, at each occurrence, -H, -SO_3H, -NH_2, -CO_2H, or C_1-C_6 alkyl, wherein each C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, -CO_2H, -NH_2, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl; and wherein R^{24} is independently, at each occurrence, -H, -CO_2H, C_1-C_6 alkyl, or oxo; and

L is a targeting fragment, wherein preferably said targeting fragment L is the DUPA residue (HOOC(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-(CH_2)$_2$-CO-).

In another aspect, the present invention provides a composition comprising a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating units m of 25 to 100, preferably of a discrete number of repeating units m of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

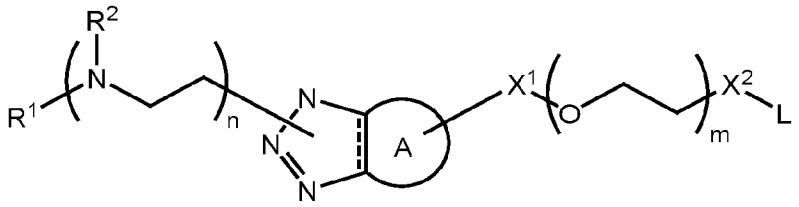
X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein said targeting fragment is an PSMA targeting fragment, wherein preferably said PSMA targeting fragment is capable of specifically binding to a cell expressing, preferably overexpressing, PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is -CH_3. In a further preferred embodiment, said targeting fragment comprises or preferably consists of the DUPA residue (HOOC-(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-(CH_2)$_2$-CO-). In a further very preferred embodiment, said targeting

fragment consists of the DUPA residue (HOOC(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-(CH_2)$_2$-CO-), wherein both chiral C-atoms having (*S*)-configuration, as depicted in formula **1***.

In another aspect, the present invention provides a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating units m of 25 to 100, preferably of a discrete number of repeating units m of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

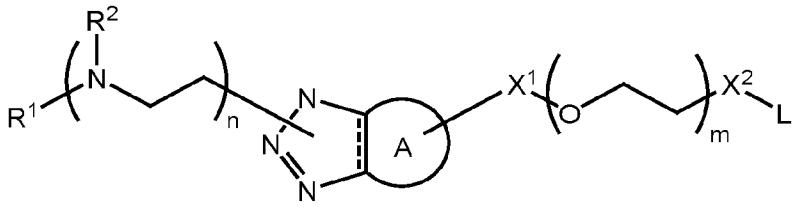
X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein said targeting fragment is an PSMA targeting fragment, wherein preferably said PSMA targeting fragment is capable of specifically binding to a cell expressing, preferably overexpressing, PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is -CH_3. In a further preferred embodiment, said targeting fragment comprises or preferably consists of the DUPA residue (HOOC-(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-(CH_2)$_2$-CO-). In a further very preferred embodiment, said targeting fragment consists of the DUPA residue (HOOC(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-

$(CH_2)_2$-CO-), wherein both chiral C-atoms having (*S*)-configuration, as depicted in formula **1***.

In another aspect, the present invention provides a composition comprising a conjugate of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating units m of 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

X^1 is a divalent covalent linking moiety;

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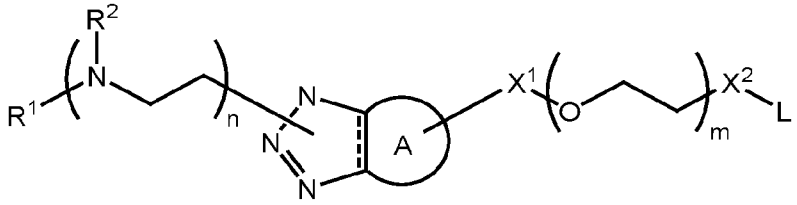

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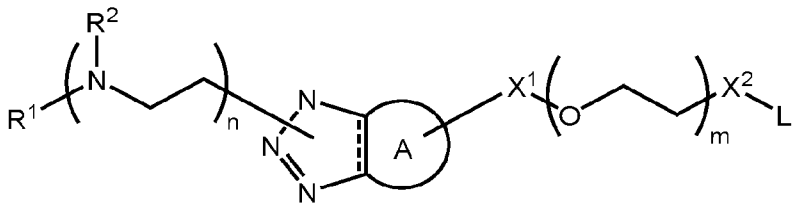

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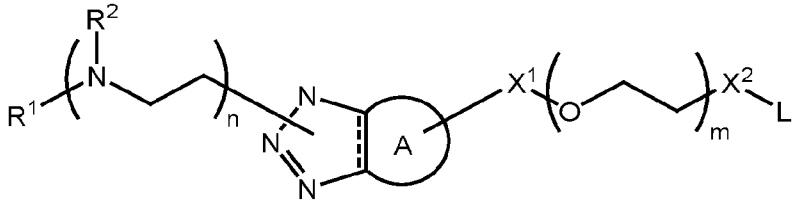

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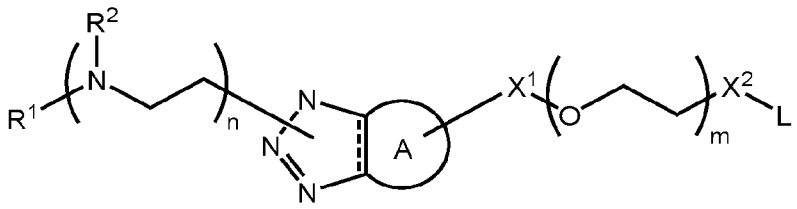

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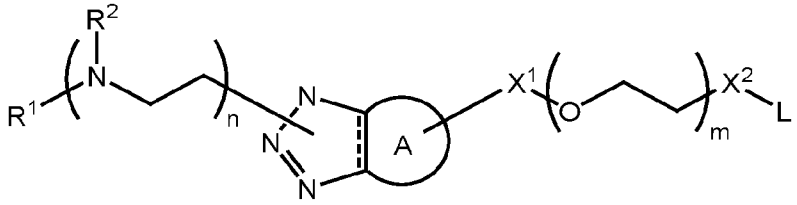

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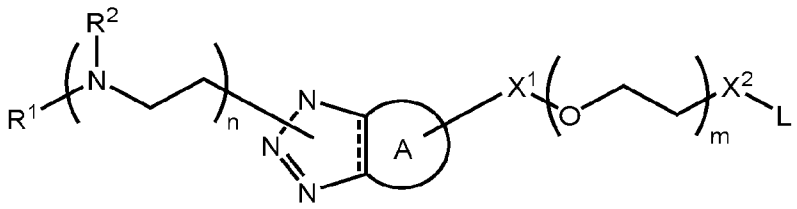

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X^1 is a linking moiety of the formula $-(Y^1)_p-$, wherein p is an integer between 1 and 20, and each occurrence of Y^1 is independently selected from a chemical bond, $-CR^{11}R^{12}-$, -C(O)-, -O-, -S-, $-NR^{13}-$, an amino acid residue, a divalent phenyl moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl or heteroaryl is optionally substituted with one or more R^{13}, and each divalent heterocycle is optionally substituted with one or more R^{14}; wherein R^{11}, R^{12} and R^{13} are independently, at each occurrence, H or C_1-C_6 alkyl; and wherein R^{14} is independently, at each occurrence, H, C_1-C_6 alkyl, or oxo;

X^2 is a linking moiety of the formula $-(Y^2)_q-$, wherein q is an integer between 1 and 50, and each occurrence of Y^2 is independently selected from a chemical bond, $-CR^{21}R^{22}-$, $NR^{23}-$, -O-, -S-, -C(O)-, an amino acid residue, a divalent phenyl moiety, a divalent heterocycle moiety,

and a divalent heteroaryl moiety, wherein each divalent phenyl and divalent heteroaryl is optionally substituted with one or more R^{23}, and wherein each divalent heterocycle moiety is optionally substituted with one or more R^{24}; wherein R^{21}, R^{22}, and R^{23} are each independently, at each occurrence, -H, $-CO_2H$, or C_1-C_6 alkyl, wherein each C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl; and wherein R^{24} is independently, at each occurrence, -H, $-CO_2H$, C_1-C_6 alkyl, or oxo; and

L is a targeting fragment, wherein said targeting fragment is an PSMA targeting fragment, wherein preferably said PSMA targeting fragment is capable of specifically binding to a cell expressing, preferably overexpressing, PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$. In a further preferred embodiment, said targeting fragment comprises or preferably consists of the DUPA residue (HOOC-$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-). In a further very preferred embodiment, said targeting fragment consists of the DUPA residue (HOOC$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-), wherein both chiral C-atoms having (*S*)-configuration, as depicted in formula **1***.

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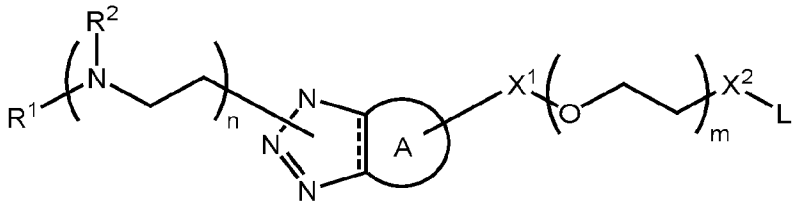

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C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

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In a preferred embodiment, said DUPA residue is linked to said PEG targeting fragment by way of the linking moiety X^2.

Such linking moieties are known to the skilled person and are disclosed in US2020/0188523A1, US2011/0288152A1, US2010/324008A1, the disclosures of said patent applications incorporated herein by way reference in its entirety.

In a preferred embodiment, said linking moiety X^2 is a peptide linker or a C_1-C_{10} alkylene linker or a combination of both. In a preferred embodiment, said linking moiety X^2 is a peptide linker.

In a preferred embodiment, said linking moiety X^2 is a peptide linker, wherein said peptide linker comprises, preferably consists of, the sequence of SEQ ID NO: 3 (-(NH-$(CH_2)_7$-CO)-Phe-Phe-(NH-CH_2-CH(NH_2)-CO)-Asp-Cys-) or SEQ ID NO: 1 (-(NH-$(CH_2)_7$-CO)-Phe-Gly-Trp-Trp-Gly-Cys-). In a preferred embodiment, said linking moiety X^2 is a peptide linker, wherein said peptide linker comprises, preferably consists of, the sequence of SEQ ID NO: 1 (-(NH-$(CH_2)_7$-CO)-Phe-Gly-Trp-Trp-Gly-Cys-). In a further preferred embodiment, said linking moiety X^2 comprises, preferably consists of, SEQ ID NO: 1 or 3 and the targeting fragment is HOOC$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO- (DUPA residue). In a very preferred embodiment, said linking moiety X^2 comprises, preferably consists of, SEQ ID NO: 1 and the targeting fragment L is HOOC$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO- (DUPA residue). In a preferred embodiment, said targeting fragment L is HOOC-$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO- capable of binding to a cell overexpressing

PSMA, wherein said linking moiety X^2 comprises, preferably consists of SEQ ID NO: 1.

In another preferred embodiment, the targeting fragment is 2-[3-(1,3-dicarboxypropyl) ureido]pentanedioic acid (DUPA), wherein typically and preferably said coupling to the rest of said conjugate is effected via a terminal carboxyl group of said DUPA. Thus, in a further preferred embodiment, said targeting fragment L is the DUPA residue ($HOOC(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-). The DUPA can be selectively taken up in cells that have increased expression (e.g., overexpression) of prostate-specific membrane antigen (PSMA).

Coupling of PEG Fragment to Targeting fragment

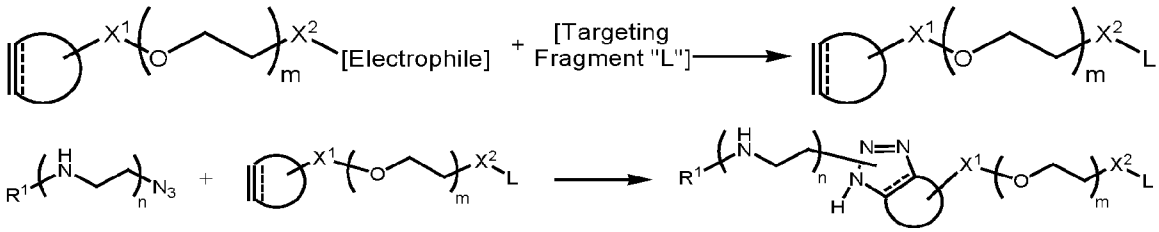
In some embodiments, the second terminal end of the PEG fragment is functionalized with a linking group (i.e., X^2) that links the PEG fragment to a targeting fragment. Typically, the linking moiety X^2 comprises a reactive group for coupling to an appropriate, i.e. complementary reactive group on the targeting fragment. One of skill in the art will understand the various complementary reactive groups of such coupling reaction between said X^2 reactive groups and said reactive groups of the targeting fragments. In some embodiments, the targeting fragment L can be unmodified and used directly as a reactive partner for covalent coupling to a PEG fragment and linking moiety X^2 respectively. For example, Scheme 3 shows the nucleophilic addition of hEGF to an electrophilic tetrafluorophenyl ester bonded to a PEG fragment. As shown in Scheme 3, a nucleophilic amine of the hEGF displaces the tetrafluorophenol of the tetrafluorophenyl ester to form a covalent bond with the PEG fragment and linking moiety X^2 respectively. In some embodiments, the targeting fragment L can be coupled to a PEG fragment by the linking moiety X^2 using a suitable chemical linkage such as an amide or ester bond. For example, Schemes 4 and 5 show DUPA and folate groups, respectively, that are bonded to a PEG fragment by an X^2 linker comprising an amide linkage. The amide groups are formed by a dehydration synthesis reaction between an appropriate carboxylic acid group on DUPA and folate and an appropriate amine on the PEG-X^2 fragment.

In some preferred embodiments, a first end (i.e., terminus) of the PEG fragment is functionalized with an alkene or alkyne group which can in some embodiments be used to react with an azide-functionalized LPEI; and a second end (i.e., terminus) of the PEG fragment is functionalized with a targeting fragment, which in some embodiments can be used to facilitate uptake of the conjugates and corresponding polyplexes in specific cell types. Accordingly, in some preferred embodiments, the resulting conjugates of the present invention can have the

general structure LPEI-PEG-Targeting fragment, arranged in a linear end-to-end fashion.

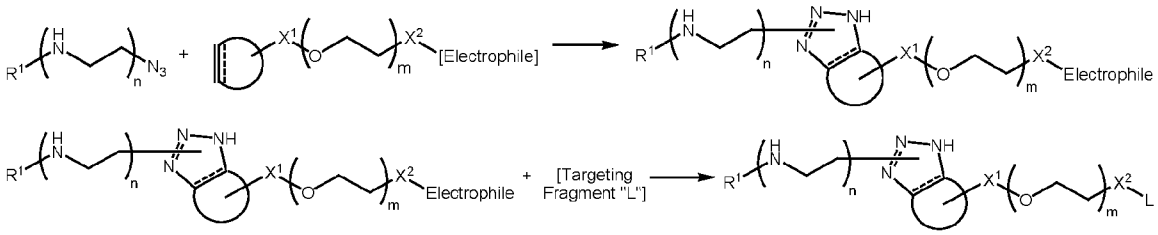
The conjugates of the present invention can be prepared using a variety of different methods and steps. Schemes 1 and 2 below show different strategies for arranging the conjugates of the present invention. As shown below in Scheme 1, conjugates of the present invention can be prepared by first coupling a PEG fragment to a targeting fragment, followed by coupling targeting fragment-modified PEG fragment to the LPEI fragment. As shown below in Scheme 2, conjugates of the present invention can be prepared by first coupling a PEG fragment to the LPEI fragment, followed by coupling the LPEI-modified PEG fragment to a targeting fragment.

Scheme 1. Exemplary coupling difunctional PEG to targeting fragment followed by LPEI



As shown in Scheme 1, a difunctional PEG (e.g, a PEG containing an alkene or alkyne and an electrophile) can be reacted first with a targeting fragment (e.g., hEGF, DUPA, or folate) to produce a PEG fragment covalently bonded to the targeting fragment. The alkene or alkyne group of the targeting fragment-modified PEG can then be reacted with the azide group of an LPEI fragment via a [3+2] cycloaddition to produce a linear conjugate of the general structure LPEI-PEG-targeting fragment.

Scheme 2. Exemplary coupling difunctional PEG to LPEI followed by targeting fragment.

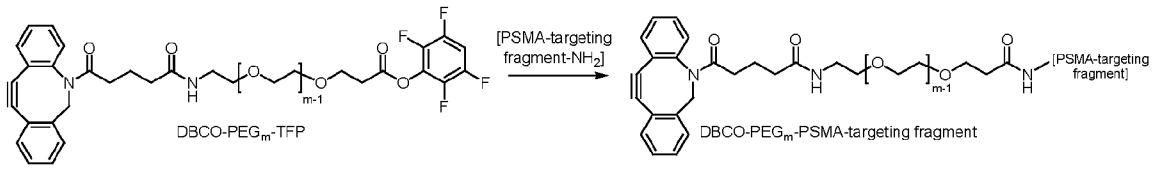


As shown in Scheme 2, a bifunctional PEG (e.g., a PEG containing an alkene or alkyne and an electrophile) can be reacted first with the azide group of an LPEI fragment via a [3+2] cycloaddition to produce a linear conjugate of LPEI and PEG covalently attached by a 1, 2, 3 triazole or A 4,5-dihydro-1H-[1,2,3]triazole. The linear LPEI-PEG fragment can then be reacted with a targeting fragment (e.g., hEGF, DUPA, or folate) to produce a linear conjugate

of the general structure LPEI-PEG-targeting fragment.

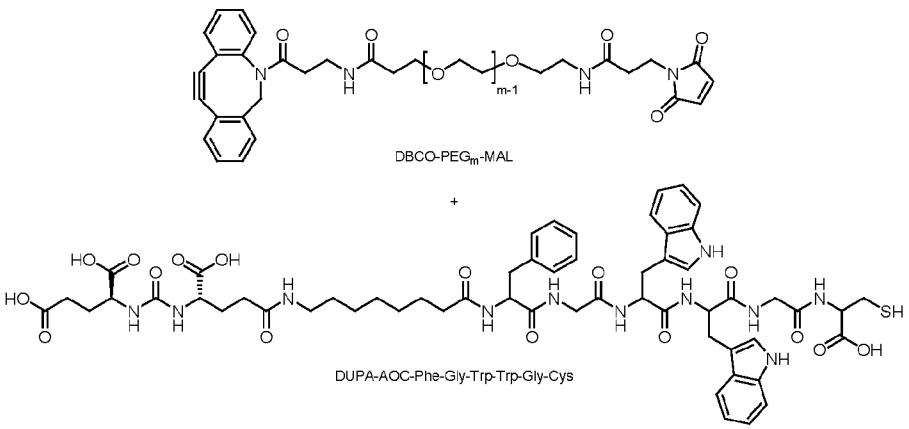
Schemes 3-5 below show general methods for coupling a PEG fragment to various targeting fragments. One of skill in the art will appreciate that the PEG fragment can be coupled to various targeting fragments using any suitable chemistries (e.g., nucleophilic substitution, peptide coupling and the like). For example, one of skill in the art will appreciate that it is not necessary to use a tetrafluorophenyl ester as an electrophile to couple a PEG fragment to hEGF as shown in Scheme 3, but that other electrophilic groups such as a maleate (as shown in Scheme 4) can also be used. Moreover, one of skill in the art will appreciate that the reactive group of the bi-functionalized PEG fragment does not necessarily need to be an electrophilic group, but instead can be a nucleophilic group that reacts, e.g., with an electrophilic portion of a targeting fragment.

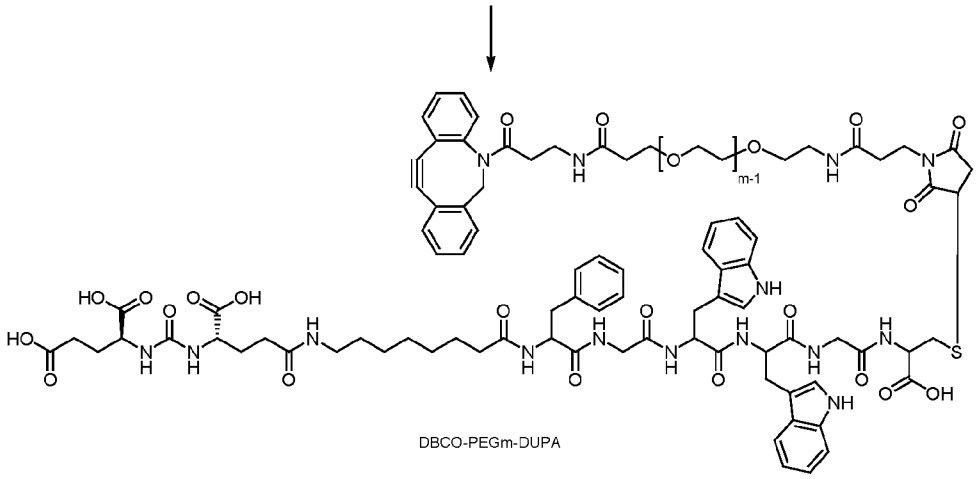
Scheme 3. Exemplary coupling of bifunctional PEG to hEGF.



As shown above in Scheme 3, in some embodiments PEG can be modified to include an electrophilic group such as a tetrafluorophenyl ester and/or an activated alkyne group such as DBCO. Treatment of the tetrafluorophenyl ester-modified PEG a PSMA-targeting fragment comprising a nucleophilic group such as an $-NH_2$ group in solution results in a nucleophilic substitution to produce a PEG fragment conjugated to a PSMA-targeting fragment. The DBCO group can be used in subsequent reactions for coupling to an LPEI fragment. The variable m represents a discrete number of repeating $-(O-CH_2-CH_2)-$units, wherein said discrete number m of repeating $-(O-CH_2-CH_2)-$units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

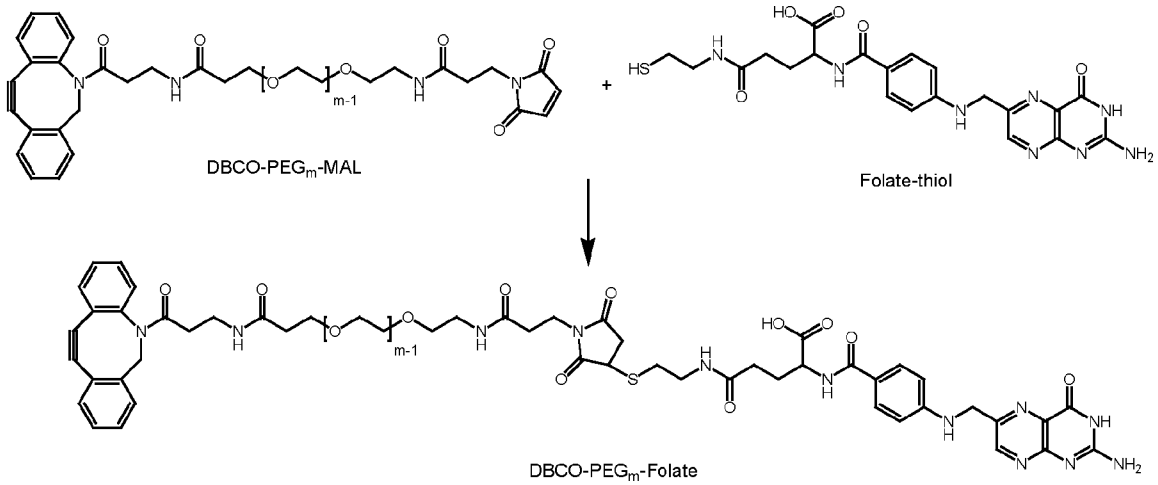
Scheme 4. Exemplary coupling of bifunctional PEG to DUPA.





As shown above in Scheme 4, PEG can be modified to include an electrophilic maleimide (MAL) group and/or an activated alkyne group such as DBCO. The maleimide-substituted PEG can be coupled to a nucleophilic partner such as the depicted DUPA derived moiety (as depicted in the scheme above comprising a peptidic spacer Aoc-Phe-Gly-Trp-Trp-Gly-Cys (SEQ ID NO:1), N-terminally derivatized with 2-[3-(1,3-dicarboxypropyl)ureido]pentanedioic acid (DUPA) which due to the amino acid residue derived from cysteine contains a nucleophilic group, namely a thiol. Treatment of the MAL-modified PEG in solution with the thiol-modified DUPA derived moiety in solution results in a nucleophilic 1,4-addition via the nucleophilic thiol of the DUPA derived moiety to produce a DUPA-modified PEG. The variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

Scheme 5. Exemplary coupling of bifunctional PEG to folate.



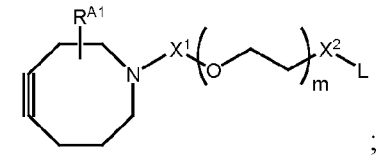
As shown above in Scheme 5, PEG can be modified to include an electrophilic

maleimide (MAL) group. The maleimide-substituted PEG can be coupled to nucleophilic partner such as a folate residue which itself is modified to contain a nucleophilic group (e.g., thiol). Treatment of the MAL-modified PEG in solution with folate thiol in solution results in a nucleophilic 1,4-addition via the nucleophilic thiol of folate to produce a folate-modified PEG. The variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

Coupling of PEG Fragment to LPEI Fragment

Before or after coupling the bi-functionalized PEG fragment to a targeting fragment L, the bi-functionalized PEG fragment can be coupled to an LPEI fragment. In preferred embodiments, the bi-functionalized PEG fragment is coupled to LPEI using cycloaddition chemistry, e.g., a 1,3-dipolar cycloaddition or [3+2] cycloaddition between an azide and an alkene or alkyne to form a 1, 2, 3 triazole or a 4,5-dihydro-1H-[1,2,3]triazole. In other preferred embodiments, the bi-functionalized PEG fragment is coupled to LPEI using thiol-ene chemistry, between a thiol and an alkene to form a thioether.

One of skill in the art will appreciate that any suitable alkene or alkyne groups can be used to react with an azide group to couple the LPEI fragment to the PEG fragment. In some preferred embodiments, incorporation of alkene or alkyne groups into ring systems introduces strain into the ring systems. The strain of the ring systems can be released upon reaction of the alkene or alkyne group to produce a 1, 2, 3 triazole or a 4,5-dihydro-1H-[1,2,3]triazole, preferably without the use of an added catalyst such as copper. Thus, in some preferred embodiments, suitable ring systems include seven-, eight-, or nine-membered rings that include an alkyne group, or eight-membered rings that include a *trans* alkene group. For example, suitable alkyne groups such as cyclooctyne (OCT), monofluorinated cyclooctyne (MOFO), difluorocycloalkyne (DIFO), dibenzocyclooctynol (DIBO), dibenzoazacyclooctyne (DIBAC), bicyclononyne (BCN), biarylazacyclooctynone (BARAC) and tetramethylthiepinium (TMTI) can be used. Additionally, suitable alkene groups such as *trans* cyclooctene, *trans* cycloheptene, and maleimide can be used. For example, conjugates of the present invention can be prepared from moieties comprising a PEG fragment and an alkene or alkyne group according to one of the following formulae: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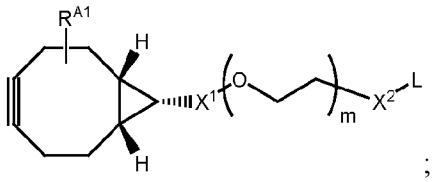
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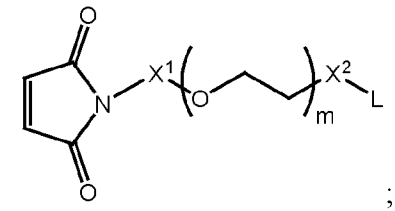
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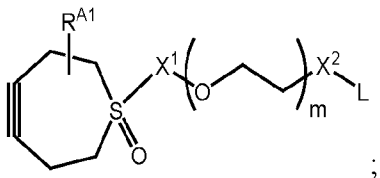
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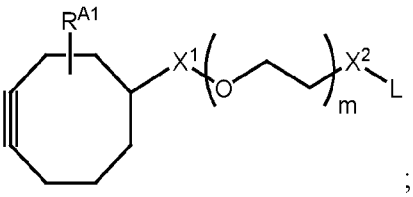
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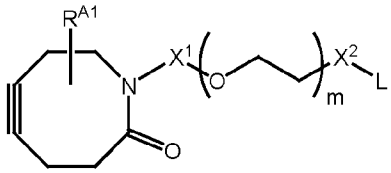
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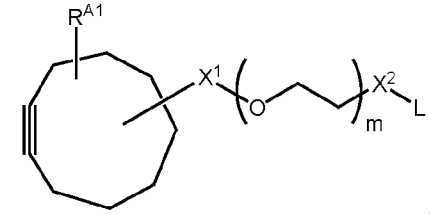
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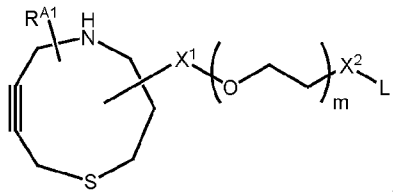
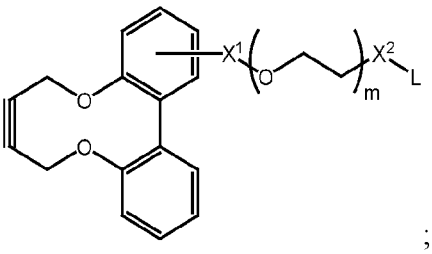

; or


;

wherein the variables X^1, X^2, R^{A1}, L and m are defined above.

Without wishing to be bound by theory, the azide and the alkene or alkyne groups can spontaneously (i.e., without the addition of a catalyst) react to form a 1, 2, 3 triazole or a 4,5-dihydro-1H-[1,2,3]triazole. In some embodiments, the azide group reacts with an alkyne to form a 1, 2, 3 triazole. In some embodiments, the azide group reacts with an alkene to form a 4,5-dihydro-1H-[1,2,3]triazole.

One of skill in the art will appreciate that both the LPEI fragment and the PEG fragment can be functionalized to include an azide group, and both the LPEI fragment and the PEG fragment can be functionalized to include an alkene or alkyne fragment (e.g., a strained alkene or alkyne). Thus, in some embodiments, the LPEI fragment comprises the alkene or alkyne group (e.g., a strained alkene or alkyne) and the bi-functionalized PEG fragment comprises an azide group. In some preferred embodiments, the bi-functionalized PEG fragment comprises

the alkene or alkyne group (e.g., a strained alkene or alkyne) and the LPEI fragment comprises an azide group.

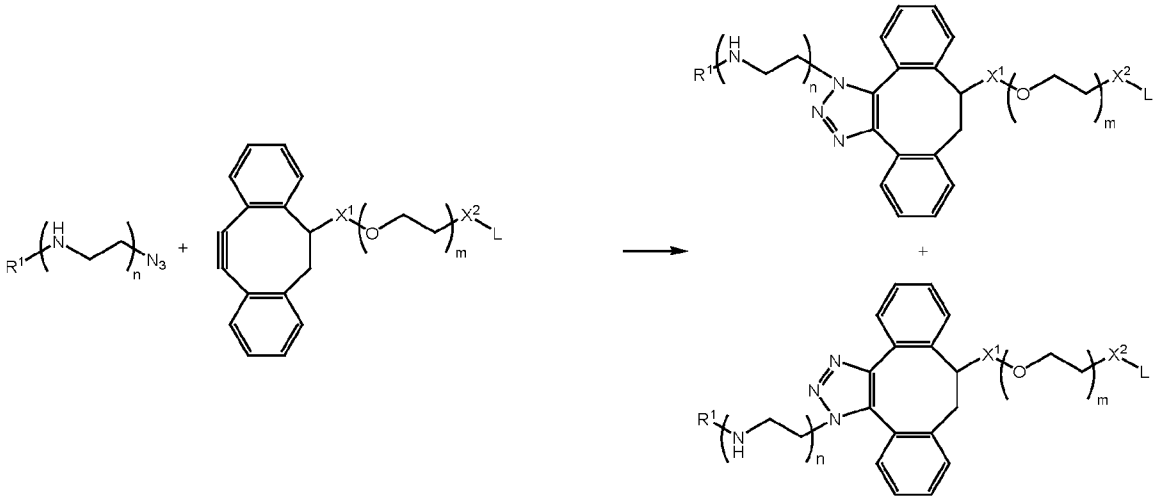
One of skill in the art will also appreciate that a [3+2] cycloaddition between an azide and an alkene or alkyne group can give adducts with different regiochemistries as shown in Schemes 6-8, below. One of skill in the art will understand that all possible regiochemistries of [3+2] cycloaddition are contemplated by this invention.

In some preferred embodiments, the [3+2] azide-alkyne cycloaddition reaction takes place at a pH of 5 or below, preferably 4 or below. As set forth below in the Comparative Example, no reaction occurred when a PEG fragment modified with an activated alkyne was treated with a non-azide containing LPEI fragment at a pH of 4. Without wishing to be bound by theory, these results suggest that the azide group of the LPEI fragment chemoselectively reacts with the alkyne or alkene (preferably a strained alkyne or alkene) group of the PEG fragment. However, at higher pH, the Comparative Example teaches that a side product was formed, characterized as a hydroamination reaction between the nitrogen atoms of the LPEI fragment and the alkene or alkyne. Without wishing to be bound by theory, the present invention teaches that an LPEI fragment (e.g., comprising a terminal azide) can be chemoselectively bonded to a PEG fragment (e.g., comprising an activated, preferably strained alkene or alkyne), at a pH below about 5, preferably about 4 or below.

In another aspect, the present invention provides a method of synthesizing a conjugate of Formula I, comprising reacting an LPEI fragment comprising a thiol with a PEG fragment comprising an alkene, as shown below in Scheme 9.

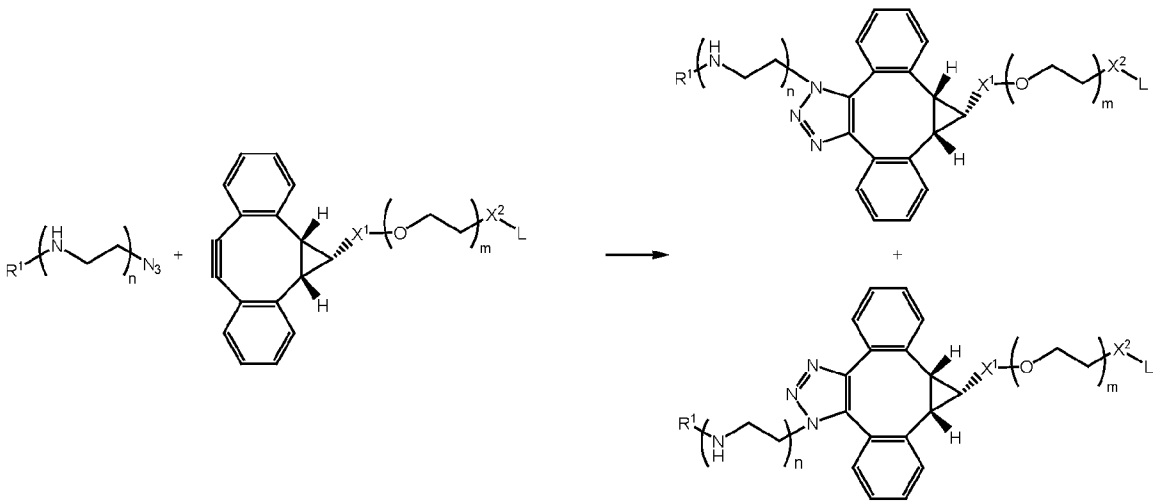
In another aspect, the present invention provides a method of synthesizing a conjugate as described and defined herein, and preferably a method of synthesizing a conjugate of Formula I, wherein the method comprises reacting the omega terminus of a linear polyethyleneimine fragment with a first terminal end of a polyethylene glycol fragment, wherein said reaction occurs at a pH below about 5, preferably 4 or below, and wherein preferably said omega terminus of said linear polyethyleneimine fragment comprises an azide, and wherein said first terminal end of said polyethylene glycol fragment comprises an alkene or an alkyne, and wherein said reaction is between said azide and said alkene or an alkyne.

Scheme 6. Coupling of LPEI to Dibenzocyclooctyne (DBCO)-modified PEG



As shown above in Scheme 6, in some embodiments PEG can be modified to include a strained alkyne group such DBCO. Treatment of the DBCO-modified PEG in solution with an azide-modified LPEI results in a [3+2] cycloaddition of the azide to the alkyne of DBCO to produce a 1, 2, 3 triazole. One of skill in the art will appreciate that the reaction shown above in Scheme 6 can produce triazole adducts with different regiochemistries as shown above. The variables m and n represent the number of repeating PEG and LPEI units as described herein, wherein m is any discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

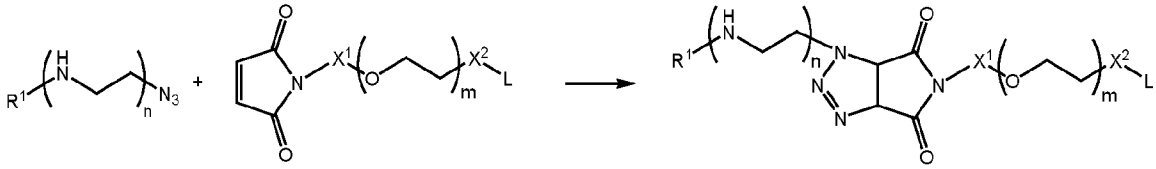
Scheme 7. Coupling of LPEI to Bicyclononyne (BCN)-modified PEG



As shown above in Scheme 7, in some embodiments PEG can be modified to include a strained alkyne group such bicyclononyne (BCN). Treatment of the BCN-modified PEG in solution with an azide-modified LPEI results in a [3+2] cycloaddition of the azide to the alkyne

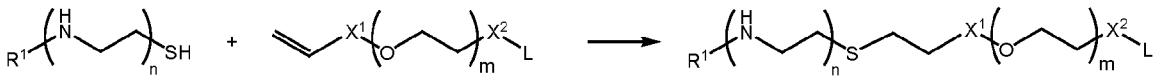
of BCN to produce a 1, 2, 3 triazole. One of skill in the art will appreciate that the reaction shown above in Scheme 7 can produce triazole adducts with different regiochemistries as shown above. The variables m and n represent the number of repeating PEG and LPEI units as described herein, wherein m is any discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

Scheme 8. Coupling of LPEI to Maleimide (MAL)-Modified PEG



As shown above in Scheme 8, in some embodiments PEG will be modified to include an alkene group such as maleimide (MAL). Treatment of the MAL-modified PEG in solution with an azide-modified LPEI will result in a [3+2] cycloaddition of the azide to the alkene of MAL to produce a 4,5-dihydro-1H-[1,2,3]triazole. The variables m and n will represent the number of repeating PEG and LPEI units as described herein, wherein m is any discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

Scheme 9. Coupling LPEI to Alkene-Modified PEG



As shown above in Scheme 9, in some embodiments PEG can be modified to include a terminal alkene group and LPEI can be modified to include a terminal thiol group. Treatment of the thiol-modified LPEI in solution with an alkene-modified PEG can result in a thiol-ene reaction to produce a thioether. The variables m and n will represent the number of repeating PEG and LPEI units as described herein, wherein m is any discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

X^1 and X^2 Linking Moieties

In some embodiments, the PEG fragments of the conjugates of the present invention can be connected to alkene or alkyne groups and/or targeting fragments by covalent linking

moieties.

X^1 Linking Moieties

In some embodiments, PEG fragments of the conjugates of the present invention are connected to an activated (e.g., cyclic) alkene or alkyne group on a terminal end by a linking moiety. For instance, the X^1 linking moiety can be formed as the result of selecting a PEG fragment and an alkene or alkyne group that each contain reactive functional groups that can be combined by well-known chemical reactions. For example, a PEG fragment can be coupled to an activated (e.g., cyclic) alkene or alkyne group by standard means such as peptide coupling (e.g., to form an amide), nucleophilic addition, or other means known to one of skill in the art.

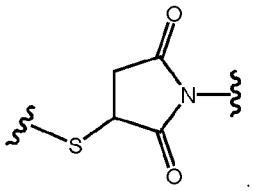
In one aspect, X^1 is a linking moiety of the formula $–(Y^1)_p–$, wherein p is an integer between 1 and 20, and each occurrence of Y^1 is independently selected from a chemical bond, $-CR^{11}R^{12}-$, -C(O)-, -O-, -S-, $-NR^{13}-$, an amino acid residue, a divalent phenyl moiety, a divalent carbocyle moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl or heteroaryl is optionally substituted with one or more R^{11}, and each divalent heterocycle is optionally substituted with one or more R^{14}; R^{11}, R^{12} and R^{13} are independently, at each occurrence, H, $-SO_3H$, $-NH_2$, or C_1-C_6 alkyl, wherein each alkyl is optionally substituted with $-CO_2H$ or NH_2; and R^{14} is independently, at each occurrence, H, C_1-C_6 alkyl, or oxo, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl.

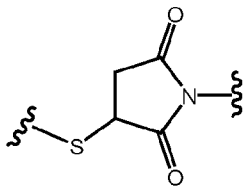
In some embodiments, when Y^1 is an amino acid residue, it can be oriented in any direction, i.e., -C(O)-CHR-NH- or -NH-CHR-C(O)-, wherein "R" represents the side-chain of a naturally occurring amino acid.

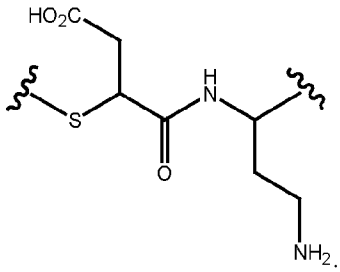
In some embodiments, the divalent heteroaryl moiety is a divalent heteroaryl group comprising one or more heteroatoms selected from O, N, S, and P, preferably one or two atoms selected from O and N. In some embodiments, the divalent heteroaryl moiety is a divalent furan, pyrrole, imidazole, pyrazole, triazole, pyridine, pyrimidine, pyridazine, pyrazine, thiophene, oxazole, or isoxazole; wherein the divalent heteroaryl is optionally substituted with one or more, preferably one or zero R^{14}.

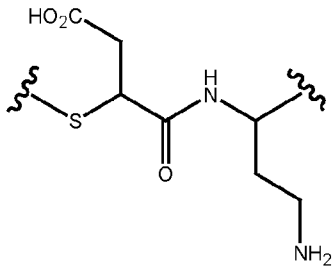
In the embodiments below for X^1, unless otherwise specified, a wavy line indicates a bond in any direction, i.e., to a PEG fragment or to the divalent covalent linking moiety (e.g., "Z" or Ring A).

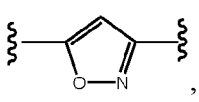
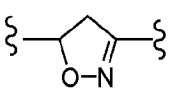
In some embodiments, the divalent heterocycle moiety is a divalent heterocycle group comprising one or more heteroatoms selected from O, N, S, and P, preferably one or two atoms

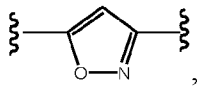
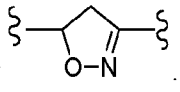
selected from O and N. In some embodiments, the divalent heterocycle moiety is a divalent tetrahydrofuran, pyrrolidine, piperidine, or 4,5-Dihydro-isoxazole, each optionally substituted with one or more R^{14}. In some preferred embodiments, the divalent heterocycle moiety is a succinimide. In some preferred embodiments, two Y^1 can combine to form a linking moiety or partial linking moiety of the formula  .

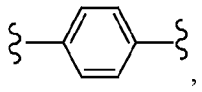
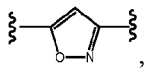
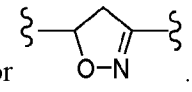
In a further preferred embodiment, two Y^1 can combine to form a linking moiety or partial linking moiety of the formula  , wherein the wavy line next to the sulfur represents the direction of connectivity towards the targeting fragment.

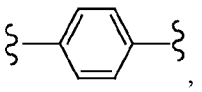
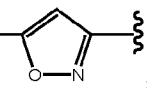
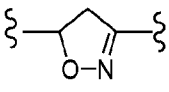
In a further preferred embodiment, Y^1 can comprise a linking moiety or partial linking moiety of the formula:  .

In a further preferred embodiment, Y^1 can comprise a linking moiety or partial linking moiety of the formula:  , wherein the wavy line next to the sulfur represents the direction of connectivity towards the targeting fragment.

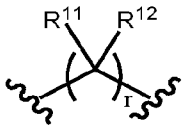
In some embodiments, X^1 is a linking moiety of the formula $–(Y^1)_p–$, wherein p is an integer between 1 and 8, and each occurrence of Y^1 is independently selected from a chemical bond, $-CHR^{11}-$, -C(O)-, -O-, -S-, -NH-, $-C_6H_4-$,  , or  .

In some embodiments, X^1 is a linking moiety of the formula $–(Y^1)_p–$, wherein p is an integer between 1 and 8, and each occurrence of Y^1 is independently selected from a chemical bond, $-CH_2-$, -C(O)-, -O-, -S-, -NH-, $-C_6H_4-$,  , or  .

In some embodiments, X^1 is a linking moiety of the formula $–(Y^1)_p–$, wherein p is an integer between 1 and 8, and each occurrence of Y^1 is independently selected from a chemical bond, $-CH_2-$, -C(O)-, -O-, -S-, -NH-,  ,  , or  .

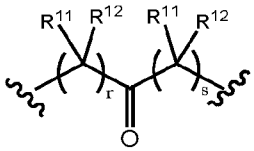
In some embodiments, X^1 is a linking moiety of the formula $–(Y^1)_p–$, wherein p is an integer between 1 and 8, and each occurrence of Y^1 is independently selected from a chemical bond, $-CH_2-$, -C(O)-, -O-, -NH-,  ,  , or  , wherein Y^1 is only -NH- when it is adjacent to a -C(O)- group to form a carbamate or amide.

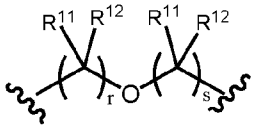
In some embodiments, X^1 is



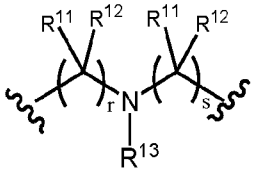
, wherein r is an integer between 1 and 8, preferably between 1 and 4, more preferably between 1 and 2; and wherein R^{11} and R^{12} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H.

In some embodiments, X^1 is



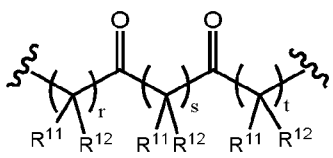
, or  , wherein r and s are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 7; and wherein R^{11} and R^{12} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "s" is a bond to the PEG fragment $–[OCH_2-CH_2]_m–$, wherein m is a discrete number of repeating $-(O-CH_2-CH_2)-$ units, wherein said discrete number m of repeating $-(O-CH_2-CH_2)-$ units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, X^1 is



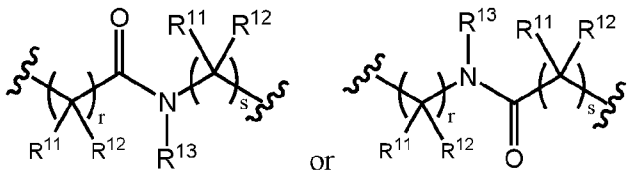
, wherein s and t are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 7; and wherein R^{11}, R^{12}, and R^{13} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "s" is a bond to the PEG fragment $–[OCH_2\text{-}CH_2]_m–$, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, X^1 is



, wherein r is an integer between 0 and 3, preferably between 1 and 3, more preferably between 1 and 2; s and t are each independently an integer between 0 and 2, preferably 0 and 1; wherein the sum of r, s, and t is less than or equal to 6; and wherein R^{11} and R^{12} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "t" is a bond to the PEG fragment $–[OCH_2\text{-}CH_2]_m–$, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

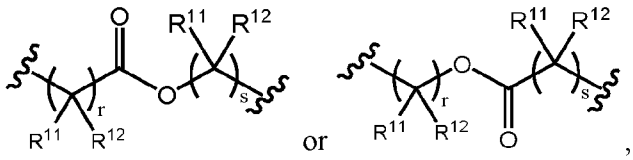
In some embodiments, X^1 is



, wherein r and s are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 6; and wherein R^{11}, R^{12} and R^{13} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "s" is a bond to the PEG fragment –

$[OCH_2\text{-}CH_2]_m$–, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

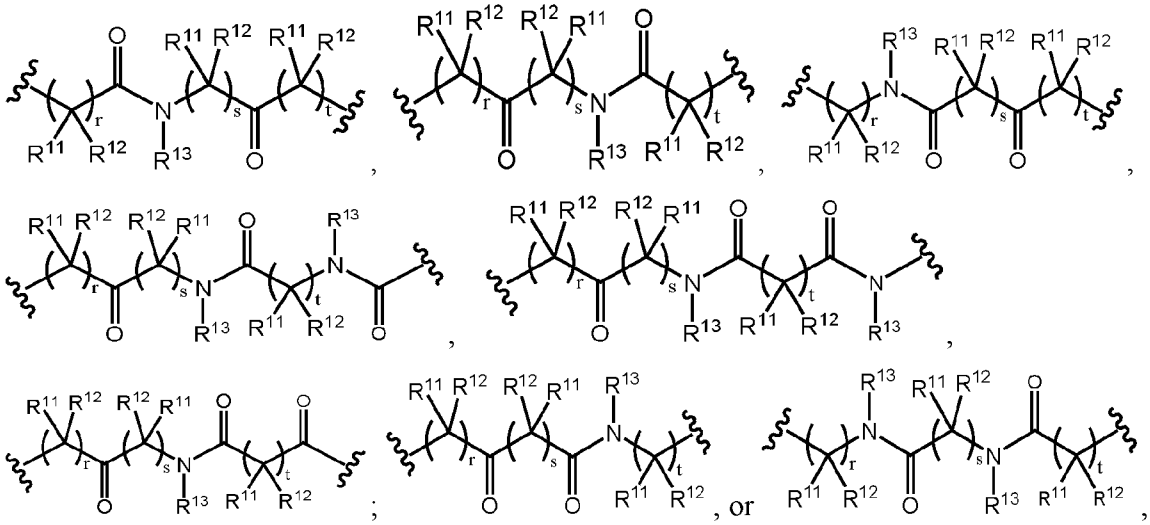
In some embodiments, X^1 is



or

, wherein r and s are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 6; and wherein R^{11}, R^{12} and R^{13} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "s" is a bond to the PEG fragment –$[OCH_2\text{-}CH_2]_m$–, wherein m is a discrete number of repeating –(O-CH_2-CH_2)- units, wherein said discrete number m of repeating –(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

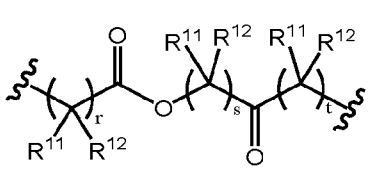
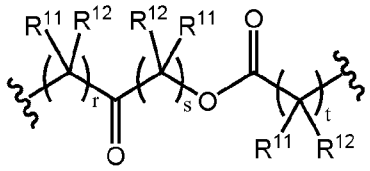
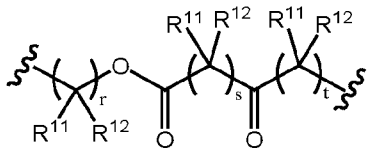
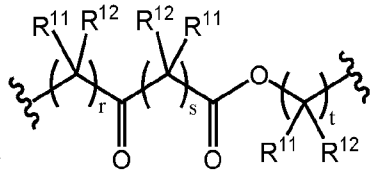
In some embodiments, X^1 is



wherein r and t are each an integer between 0 and 3 and s is an integer between 0 and 3; preferably wherein r is 0, s is 2 or 3, and t is 2; wherein the sum of r, s and t is less than or equal to 5; and wherein R^{11}, R^{12} and R^{13} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "t" is a bond to the PEG fragment –$[OCH_2\text{-}CH_2]_m$–, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-

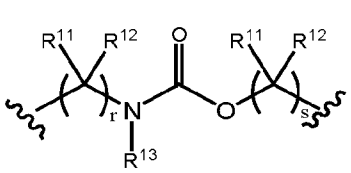
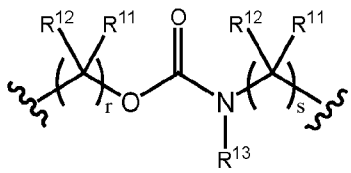
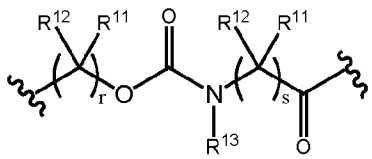
CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, X^1 is

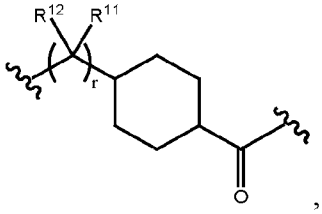
or ,wherein r and t are each an integer between 0 and 3; s is an integer between 0 and 3; wherein the sum or r, s and t is less than or equal to 5; and wherein R^{11} and R^{12} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "t" is a bond to the PEG fragment –$[OCH_2$-$CH_2]_m$–, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, X^1 is



, or  ,

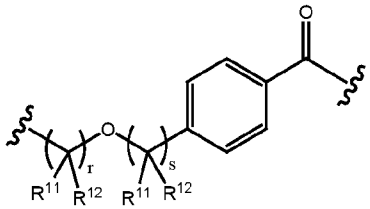
wherein r and s are each independently an integer between 0 and 3, preferably between 0 and 2; wherein the sum of r and s is less than or equal to 5; and wherein R^{11}, R^{12} and R^{13} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "s" is a bond to the PEG fragment –$[OCH_2$-$CH_2]_m$–, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, X^1 is



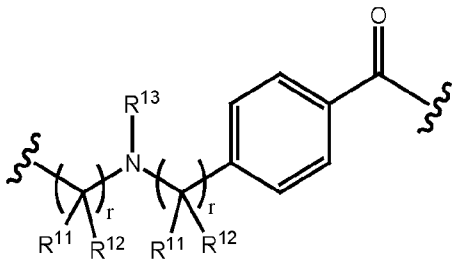
, wherein r is independently an integer between 0 and 4, preferably between 0 and 2, more preferably between 1 and 2; and wherein R^{11}, and R^{12} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the carbonyl group is a bond to the PEG fragment –$[OCH_2$-$CH_2]_m$–, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, X^1 is



, wherein r and s are each independently an integer between 0 and 4, preferably between 0 and 2, more preferably between 1 and 2; wherein the sum of r and s is less than or equal to 5; and wherein R^{11}, and R^{12} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the carbonyl group is a bond to the PEG fragment –$[OCH_2$-$CH_2]_m$–, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

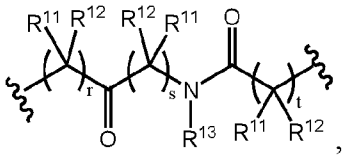
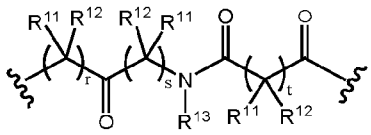
In some embodiments, X^1 is



, wherein r and s are each independently an integer between 0 and 4, preferably between 0 and 2; wherein the sum of r and s is less than or equal to 5; and wherein R^{11}, R^{12} and R^{13} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2

alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the carbonyl group is a bond to the PEG fragment $–[OCH_2\text{-}CH_2]_m–$, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some preferred embodiments, X^1 is selected from:


,

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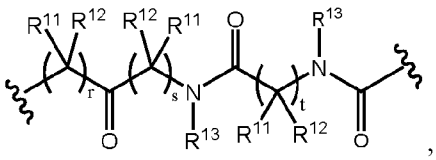
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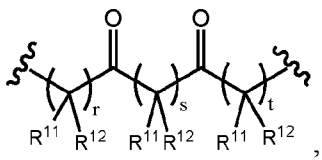
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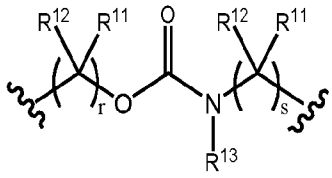
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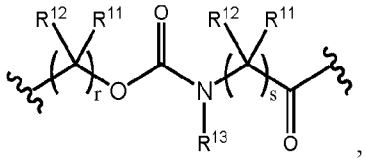
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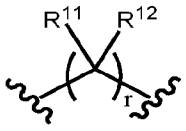
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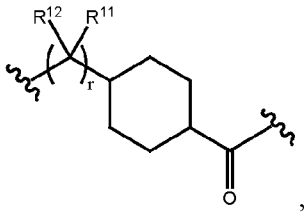
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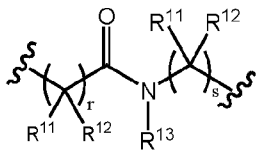
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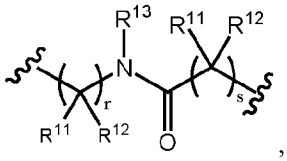
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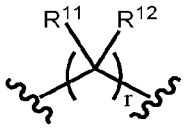
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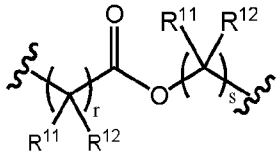
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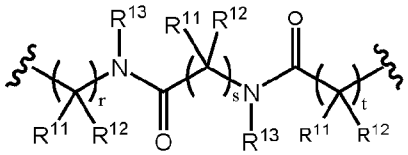
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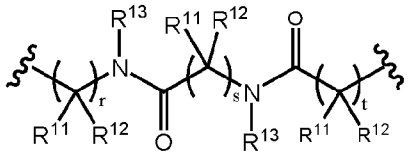

; wherein:

r is independently, at each occurrence, 0-6, preferably 0, 1, 2, or 5;

s is independently, at each occurrence, 0-6, preferably 0, 2, 4;

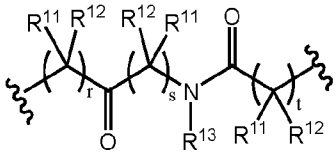
t is independently, at each occurrence, 0-6, preferably 0, 1, 2, 4;

R^{11} and R^{12} are independently, at each occurrence, selected from -H, -C_1-C_2 alkyl, -SO_3H, and -NH_2; more preferably -H, -SO_3H, and -NH_2; yet more preferably -H; and

R^{13} is -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "s" or "t" or carbonyl group is a bond to the PEG fragment $–[OCH_2\text{-}CH_2]_m–$, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-

CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some preferred embodiments, X^1 is selected from: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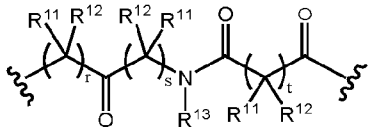
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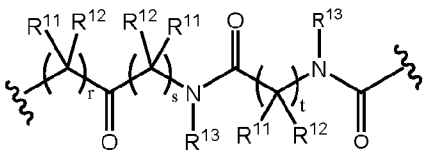
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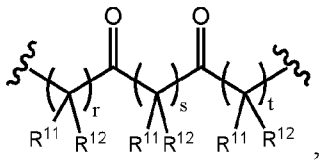
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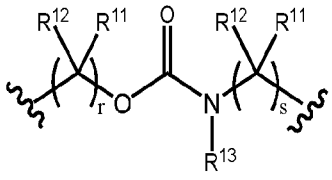
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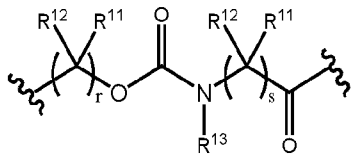
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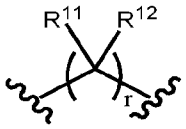
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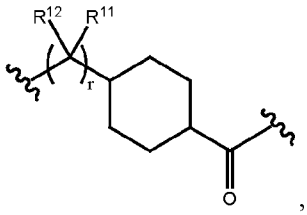
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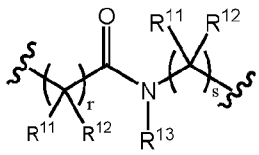
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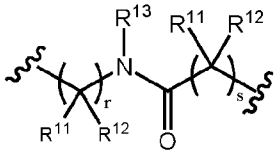
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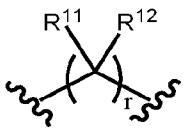
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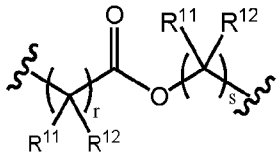
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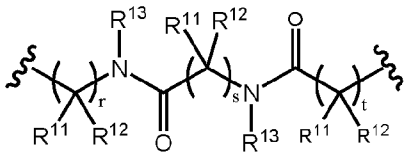
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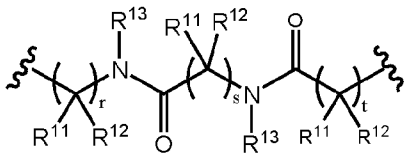

; wherein:

r is independently, at each occurrence, 0-6, preferably 0, 1, 2, or 5;

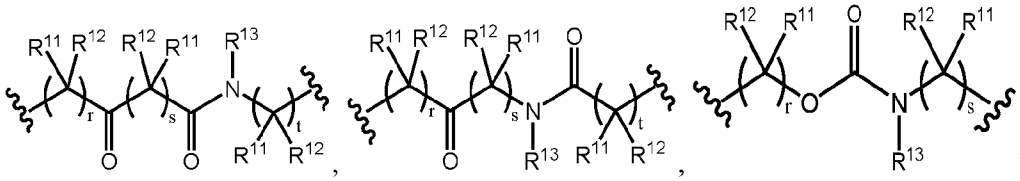
s is independently, at each occurrence, 0-6, preferably 0, 2, 4;

t is independently, at each occurrence, 0-6, preferably 0, 1, 2, 4;

R^{11} and R^{12} are independently, at each occurrence, selected from -H and -C_1-C_2 alkyl, preferably -H; and

R^{13} is -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "s" or "t" or carbonyl group is a bond to the PEG fragment –[OCH_2-CH_2]$_m$–, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some preferred embodiments, X^1 is a group selected from:


wherein:

r is independently, at each occurrence, 0-6, preferably 0, 1, 2, or 5; more preferably 0;

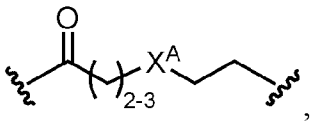
s is independently, at each occurrence, 0-6, preferably 0, 2, 3, or 4; more preferably 2 or 3;

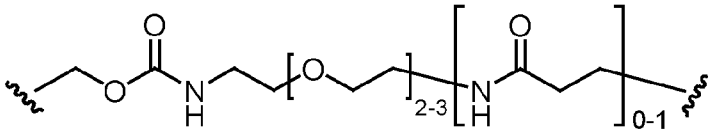
t is independently, at each occurrence, 0-6, preferably 0, 1, 2, 4; more preferably 2;

R^{11} and R^{12} are independently, at each occurrence, selected from -H and -C_1-C_2 alkyl, preferably -H; and

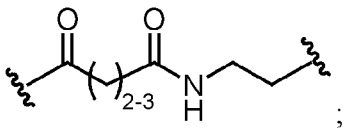
R^{13} is -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "s" or "t" group is a bond to the PEG fragment –[OCH_2-CH_2]$_m$–, wherein m is a discrete number of repeating –(O-CH_2-CH_2)- units, wherein said discrete number m of repeating –(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some preferred embodiments, X^1 is selected from:


, wherein X^A is -NHC(O)- or -C(O)NH-; and


. Preferably the wavy line on the left side is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line on the right side is a bond to the PEG fragment –[OCH_2-CH_2]$_m$–, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some preferred embodiments, X^1 is selected from: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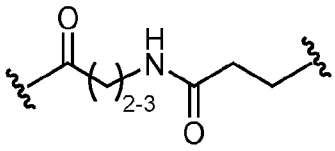
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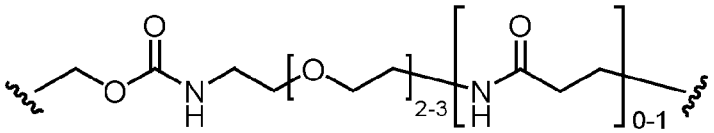
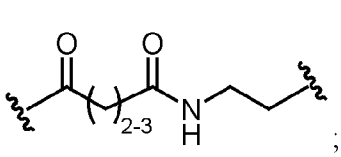

. Preferably the wavy line on the left side is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line on the right side is a bond to the PEG fragment $–[OCH_2\text{-}CH_2]_m–$, wherein m is a discrete number of repeating $–(O\text{-}CH_2\text{-}CH_2)-$ units, wherein said discrete number m of repeating $–(O\text{-}CH_2\text{-}CH_2)-$ units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some preferred embodiments, X^1 is selected from: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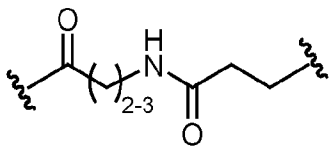
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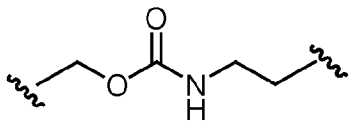
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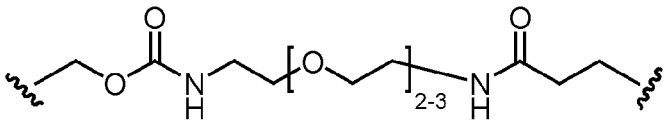
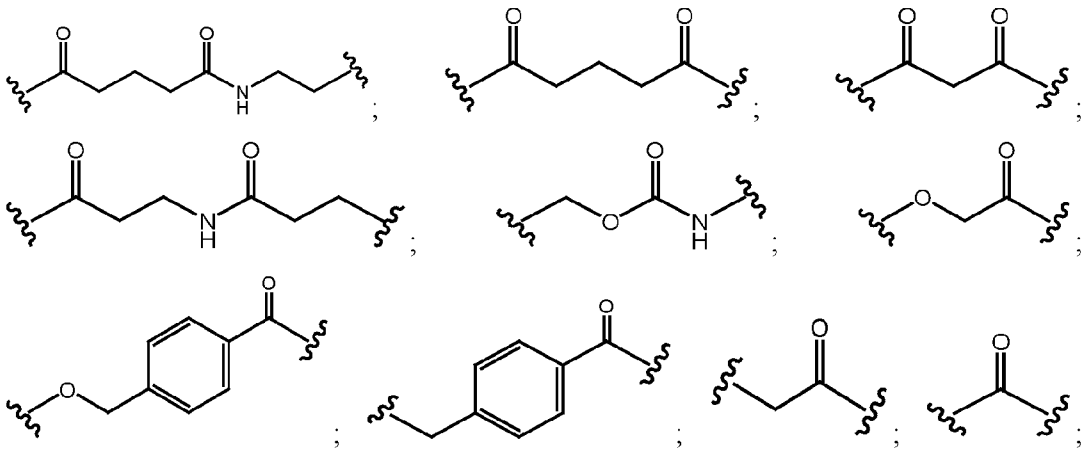
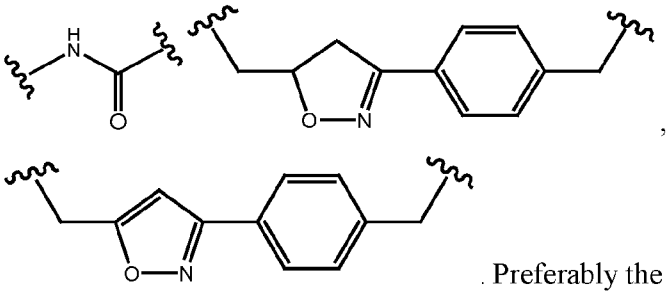

. Preferably the wavy line on the left side is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line on the right side is a bond to the PEG fragment $–[OCH_2\text{-}CH_2]_m–$, wherein m is a discrete number of repeating $–(O\text{-}CH_2\text{-}CH_2)-$ units, wherein said discrete number m of repeating $–(O\text{-}CH_2\text{-}CH_2)-$ units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

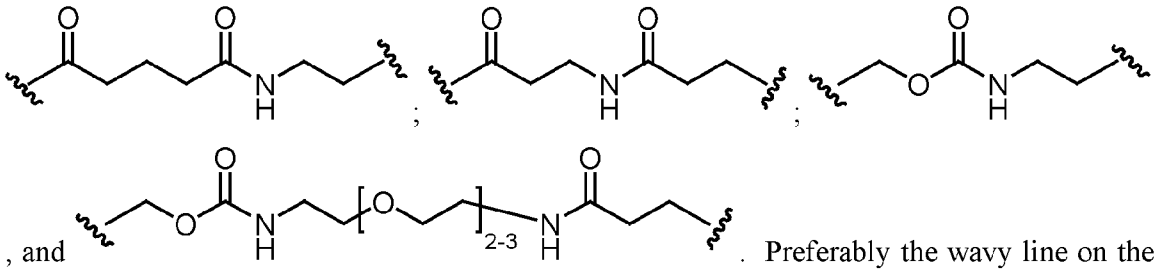
In some embodiments, X^1 is selected from:




,and

. Preferably the wavy line on the left side is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line on the right side is a bond to the PEG fragment $-[OCH_2-CH_2]_m-$, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some preferred embodiments, X^1 is selected from:


;
;
, and
. Preferably the wavy line on the left side is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line on the right side is a bond to the PEG fragment $-[OCH_2-CH_2]_m-$, wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some preferred embodiments, X^1 is $-(CH_2)_{1-6}-$; preferably X^1 is $-(CH_2)_{2-4}-$; more preferably X^1 is $-(CH_2)_2-$.

X^2 Linking Moieties

In some embodiments, PEG fragments of the conjugates of the present invention are connected to a targeting fragment on a terminal end by a linking moiety. For instance, the X^2 linking moiety can be formed as the result of selecting a PEG fragment and a targeting fragment that each contain reactive functional groups that can be combined by well-known chemical reactions. For example, a PEG fragment can be coupled to a targeting group by standard means such as peptide coupling (e.g., to form an amide), nucleophilic addition, or other means known to one of skill in the art.

In one aspect, X^2 is a linking moiety of the formula $–(Y^2)_q–$, wherein q is an integer between 1 and 50, and each occurrence of Y^2 is independently selected from a chemical bond, $-CR^{21}R^{22}-$, $NR^{23}-$, -O-, -S-, -C(O)-, an amino acid residue, a divalent phenyl moiety, a divalent carbocyle moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl and divalent heteroaryl is optionally substituted with one or more R^{23}, and wherein each divalent heterocycle moiety is optionally substituted with one or more R^{24};

R^{21}, R^{22}, and R^{23} are each independently, at each occurrence, -H, $-SO_3H$, $-NH_2$, $-CO_2H$, or C_1-C_6 alkyl, wherein each C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, $-CO_2H$, $-NH_2$, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl;

R^{24} is independently, at each occurrence, -H, $-CO_2H$, C_1-C_6 alkyl, or oxo.

In some embodiments, R^{21}, R^{22} and R^{23} are each independently, at each occurrence, -H, $-CO_2H$, or C_1-C_6 alkyl. In some embodiments, R^{21}, R^{22} and R^{23} are each, independently -H or C_1-C_4 alkyl, preferably C_1-C_2 alkyl.

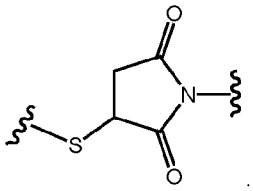
In some embodiments, R^{21}, R^{22}, R^{23}, and R^{24} are -H.

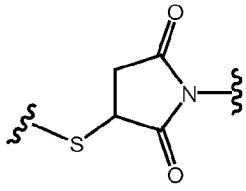
In some embodiments, R^{24} is independently -H, C_1-C_6 alkyl, or oxo.

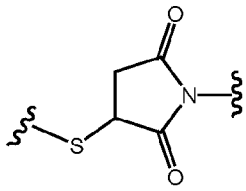
In some embodiments, the divalent heteroaryl moiety is a divalent heteroaryl group comprising one or more heteroatoms selected from O, N, S, and P, preferably one or two atoms selected from O and N. In some embodiments, the divalent heteroaryl moiety is a divalent furan, pyrrole, imidazole, pyrazole, triazole, pyridine, pyrimidine, pyridazine, pyrazine, thiophene, oxazole, or isoxazole; wherein the divalent heteroaryl is optionally substituted with one or more, preferably one or zero R^{21}.

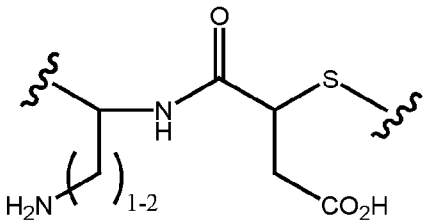
In the embodiments below for X^2, unless otherwise specified, a wavy line indicates a bond in any direction, i.e., to a PEG fragment ($-[OCH_2CH_2]_m-$), wherein the variable m represents a discrete number of repeating $-(O-CH_2-CH_2)-$units, wherein said discrete number m of repeating $-(O-CH_2-CH_2)-$units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; or to a targeting fragment (i.e., "L"), wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

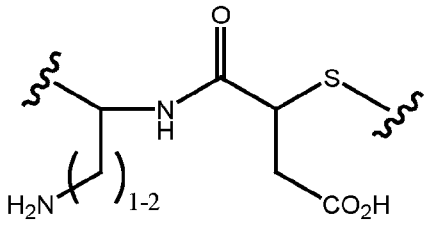
In some embodiments, the divalent heterocycle moiety is a divalent heterocycle group comprising one or more heteroatoms selected from O, N, S, and P, preferably one or two atoms selected from O and N. In some embodiments, the divalent heterocycle moiety is a divalent tetrahydrofuran, pyrrolidine, piperidine, or 4,5-dihydro-isoxazole, each optionally substituted with one or more R^{24}. In some preferred embodiments, the divalent heterocycle moiety is a

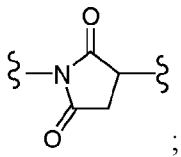
succinimide. In some preferred embodiments, two Y^2 can combine to form a linking moiety or partial linking moiety of the formula  .

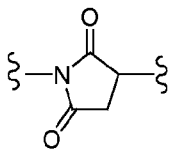
In a further preferred embodiment, two Y^2 can combine to form a linking moiety or partial linking moiety of the formula  , wherein the wavy line next to the sulfur represents a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA; and the wavy line next to the nitrogen represents a bond to the the PEG fragment ($-[OCH_2-CH_2]_m-$), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

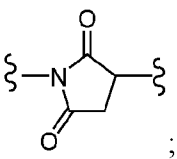
In a further preferred embodiment, two Y^2 can combine to form a linking moiety or partial linking moiety of the formula  , wherein the wavy line next to the sulfur represents a bond to the PEG fragment ($-[OCH_2-CH_2]_m-$) wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line next to nitrogen represents a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In a further preferred embodiment, Y^2 can comprise a linking moiety or partial linking moiety of the formula:  .

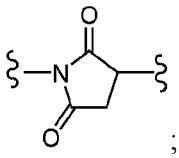
In a further preferred embodiment, Y^2 can comprise a linking moiety or partial linking moiety of the formula:  , wherein the wavy line next to the sulfur represents the direction of connectivity towards the targeting fragment.

In some embodiments, X^2 is a linking moiety of the formula $-(Y^2)_q-$, wherein q is an integer between 1 and 40, and each occurrence of Y^2 is independently selected from a chemical bond, $-CR^{21}R^{22}-$, NH-, -O-, -S-, -C(O)-, an amino acid residue, and  ; and R^{21} and R^{22} are independently, at each occurrence, -H, $-CO_2H$, or C_1-C_6 alkyl, wherein each C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl.

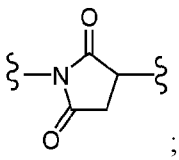
In some embodiments, X^2 is a linking moiety of the formula $-(Y^2)_q-$, wherein q is an integer between 1 and 40, and each occurrence of Y^2 is independently selected from a chemical bond, $-CHR^{21}-$, NH-, -O-, -S-, -C(O)-, an amino acid residue, and  ; and R^{21} is independently, at each occurrence, -H, $-CO_2H$, or C_1-C_4 alkyl (preferably C_1 alkyl), wherein each C_1-C_4 alkyl is optionally substituted with one or more C_6-C_{10} aryl or 5 to 8-membered heteroaryl.

In some embodiments, X^2 is a linking moiety of the formula $-(Y^2)_q-$, wherein q is an integer between 1 and 40, and each occurrence of Y^2 is independently selected from a chemical bond, $-CHR^{21}-$, -NH-, -O-, -S-, -C(O)-, an amino acid residue, and  ; and

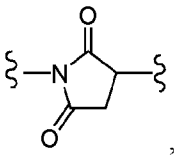
R^{21} is independently, at each occurrence, -H, $-CO_2H$, or C_1-C_4 alkyl (preferably C_1 alkyl), wherein each C_1-C_4 alkyl is optionally substituted with one or more C_6-C_{10} aryl or 5 to 8-membered heteroaryl.

In some embodiments, X^2 is a linking moiety of the formula $-(Y^2)_q-$, wherein q is an integer between 1 and 40, and each occurrence of Y^2 is independently selected from a chemical bond, $-CHR^{21}-$, -NH-, -O-, -S-, -C(O)-, an amino acid residue, and  ; and

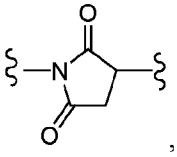
R^{21} is independently, at each occurrence, -H, $-CO_2H$, or C_1-C_3 alkyl (preferably C_1 alkyl), wherein each C_1-C_3 alkyl is optionally substituted with one or more phenyl or indole.

In some embodiments, X^2 is a linking moiety of the formula $-(Y^2)_q-$, wherein q is an integer between 1 and 40, and each occurrence of Y^2 is independently selected from a chemical bond, $-CHR^{21}-$, -NH-, -O-, -S-, -C(O)-, an amino acid residue, and  ; and

R^{21} is independently, at each occurrence, -H, $-CO_2H$, or C_1-C_3 alkyl (preferably C_1 alkyl), wherein each C_1-C_3 alkyl is optionally substituted with one or more phenyl or 3-indole.

In some embodiments, X^2 is a linking moiety of the formula $-(Y^2)_q-$, wherein q is an integer between 1 and 40, and each occurrence of Y^2 is independently selected from a chemical bond, $-CHR^{21}-$, -NH-, -O-, -S-, -C(O)-, an amino acid residue, and  , wherein Y^2 is only -NH- when it is adjacent to a -C(O)- group to form a carbamate or amide; and

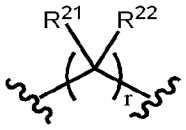
R^{21} is independently, at each occurrence, -H, $-CO_2H$, or C_1-C_3 alkyl (preferably C_1 alkyl), wherein each C_1-C_3 alkyl is optionally substituted with one or more phenyl or 3-indole.

In some embodiments, X^2 is a linking moiety of the formula $-(Y^2)_q-$, wherein q is an integer between 1 and 40, and each occurrence of Y^2 is independently selected from a chemical bond, $-CHR^{21}-$, -NH-, -O-, -S-, -C(O)-, an amino acid residue, and  , wherein Y^2 is only -NH- when it is adjacent to a -C(O)- group to form an amide; and

R^{21} is independently, at each occurrence, -H, $-CO_2H$, or C_1-C_3 alkyl (preferably C_1 alkyl), wherein each C_1-C_3 alkyl is optionally substituted with one or more phenyl or 3-indole.

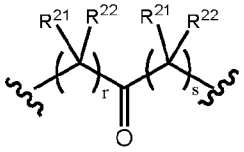
In some embodiments, when Y^2 is an amino acid residue, Y^2 represents a naturally occurring, L- amino acid residue. When Y^2 is an amino acid residue, it can be oriented in any direction, i.e., -C(O)-CHR-NH- or -NH-CHR-C(O)-, wherein "R" represents the side-chain of a naturally occurring amino acid.

In some embodiments, X^2 is

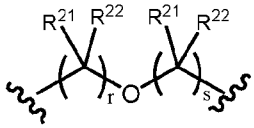


, wherein r is an integer between 1 and 8, preferably between 1 and 4, more preferably between 1 and 2; and wherein R^{21} and R^{22} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H.

In some embodiments, X^2 is

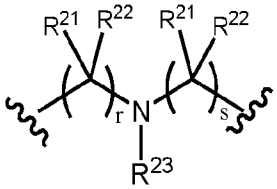


, or



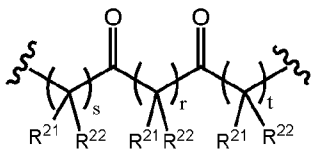
, wherein r and s are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 7; and wherein R^{21} and R^{22} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H.

In some embodiments, X^2 is



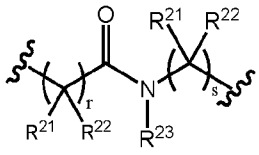
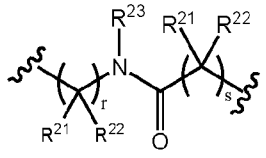
, wherein s and t are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 7; and wherein R^{21}, R^{22}, and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H.

In some embodiments, X^2 is

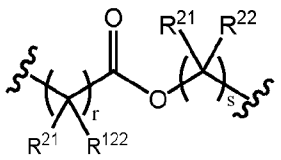
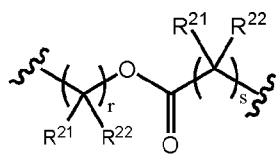


, wherein r is an integer between 0 and 3, preferably between 1 and 3, more preferably between 1 and 2; s and t are each independently an integer between 0 and 2, preferably 0 and 1; wherein the sum of r, s, and t is less than or equal to 6; and wherein R^{21} and R^{22} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H.

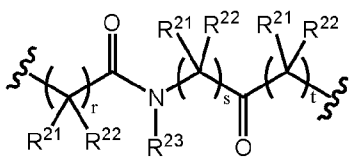
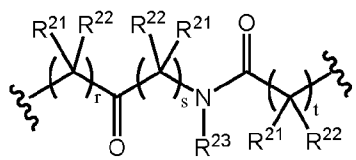
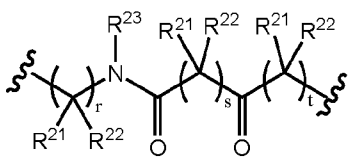
In some embodiments, X^2 is

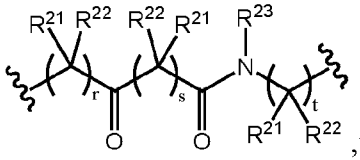
 or  , wherein r and s are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 6; and wherein R^{21}, R^{22} and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the PEG fragment (–$[OCH_2$-$CH_2]_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line nearest to the integer "s" is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some embodiments, X^2 is

 or  , wherein r and s are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 6; and wherein R^{21}, R^{22} and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the PEG fragment (–$[OCH_2$-$CH_2]_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line nearest to the integer "s" is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

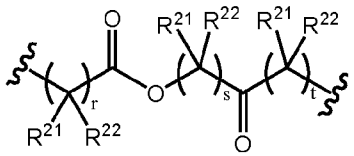
In some embodiments, X^2 is

 ,  ,  , or



,wherein r and t are each an integer between 0 and 3 and s is an integer between 0 and 3; preferably wherein r is 0, s is 2 or 3, and t is 2; wherein the sum of r, s and t is less than or equal to 5; and wherein R^{21}, R^{22} and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line nearest to the integer "t" is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some embodiments, X^2 is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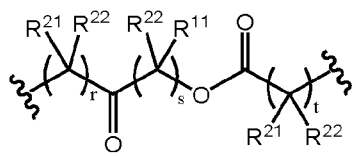
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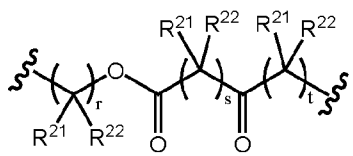
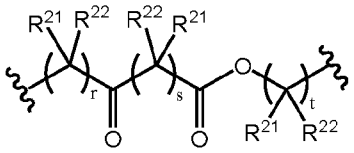
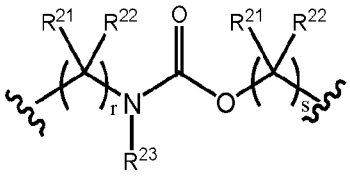
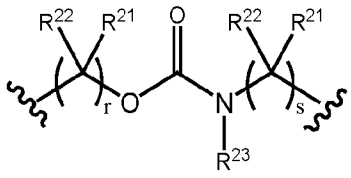

, or


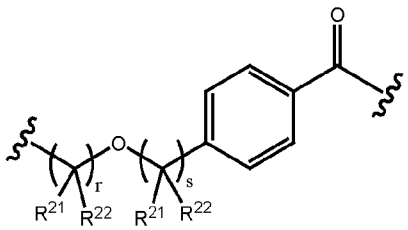
, wherein r and t are each an integer between 0 and 3; s is an integer between 0 and 3; wherein the sum or r, s and t is less than or equal to 5; and wherein R^{21} and R^{22} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line nearest to the integer "t" is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some embodiments, X^2 is

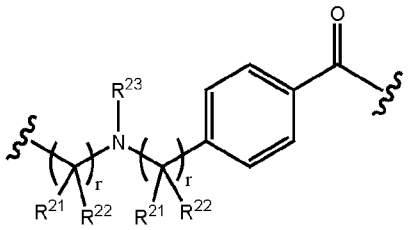
, or ,wherein r and s are each independently an integer between 0 and 3, preferably between 0 and 2; wherein the sum of r and s is less than or equal to 5; and wherein R^{21}, R^{22} and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line nearest to the integer "s" is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some embodiments, X^2 is



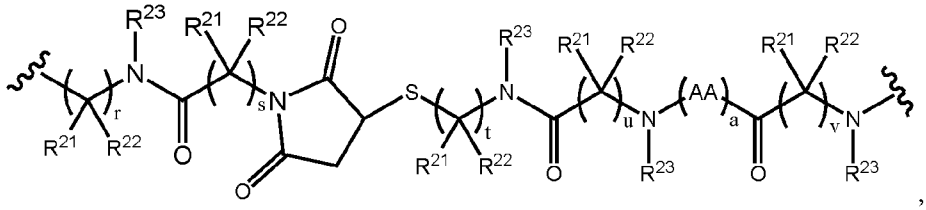
, wherein r and s are each independently an integer between 0 and 4, preferably between 0 and 2, more preferably between 1 and 2; wherein the sum of r and s is less than or equal to 5; and wherein R^{21}, and R^{22} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line nearest to the carbonyl group is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some embodiments, X^2 is



, wherein r and s are each independently an integer between 0 and 4, preferably between 0 and 2; wherein the sum of r and s is less than or equal to 5; and wherein R^{21}, R^{22} and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line nearest to the carbonyl group is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some embodiments, X^2 is selected from:



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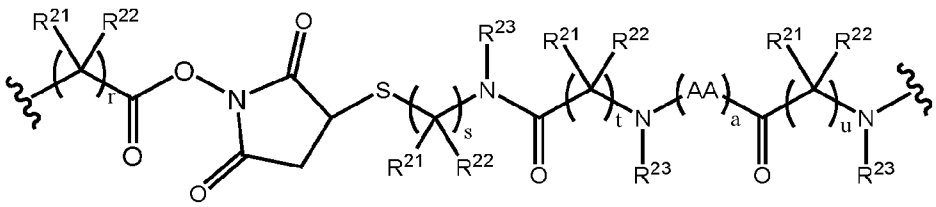

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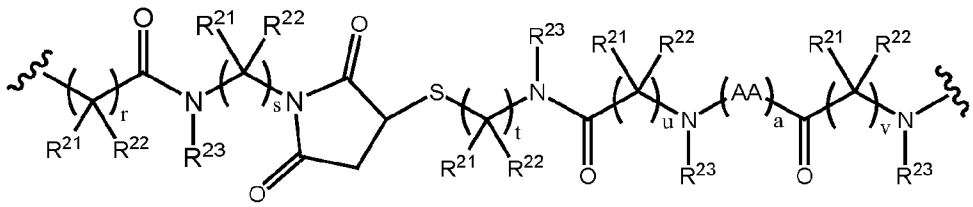

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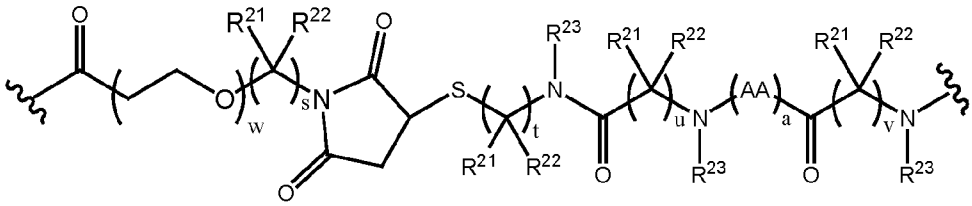

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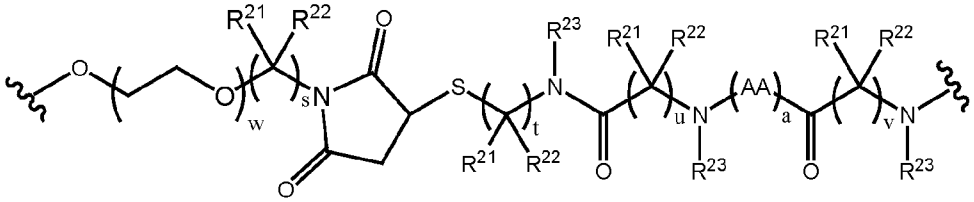

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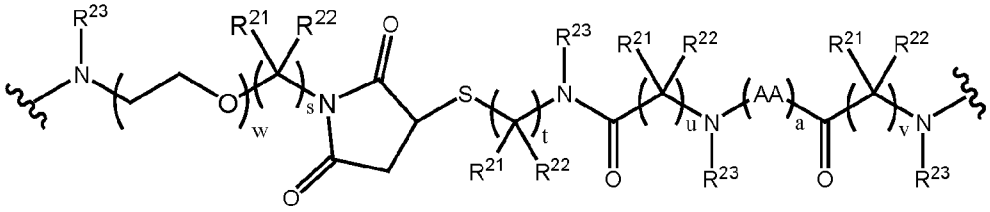

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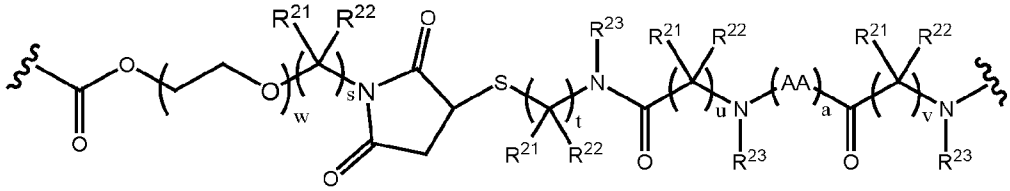

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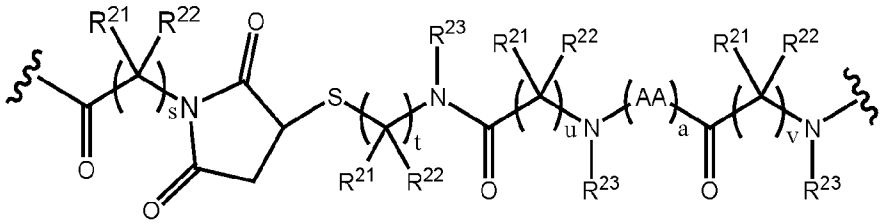

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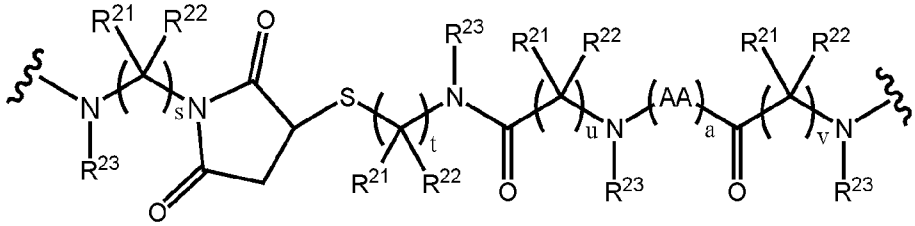

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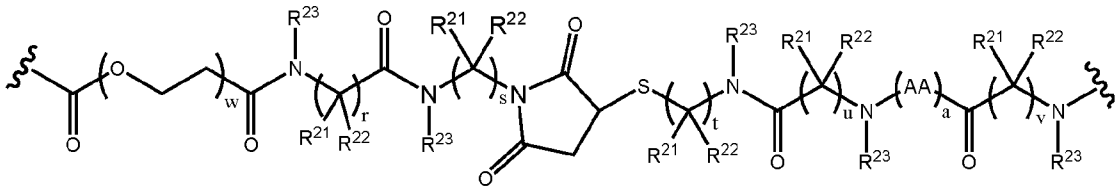

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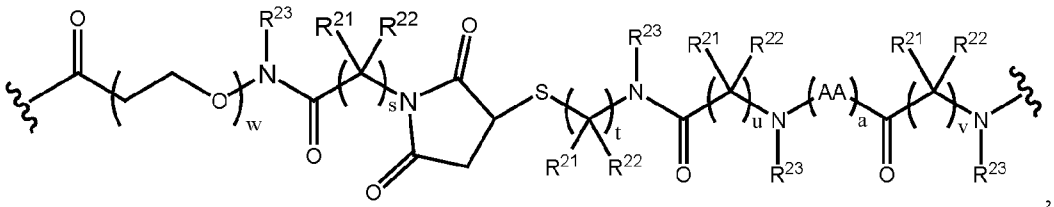

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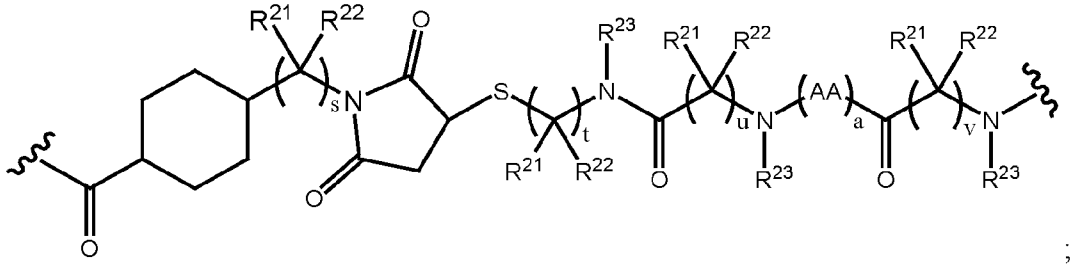


;

wherein r, s, t and u are each independently an integer between 0 and 6, preferably between 0 and 4; v is an integer between 0 and 10; w is an integer between 0 and 10;

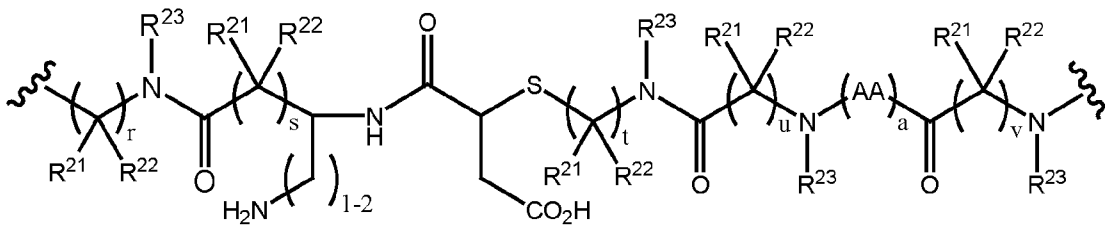
AA is an amino acid residue, preferably a naturally occurring amino acid residue; yet more preferably wherein AA is an an amino acid selected from Arg, His, Lys, Asp, Glu, Ser, Thr, Asn, Gln, Cys, Sec, Gly, Pro, Ala, Val, Ile, Leu, Met, Phe, Tyr, and Trp;

a is an integer between 0 and 10, preferably between 0 and 6; more preferably between 0 and 4;

and wherein R^{21}, R^{22} and R^{23} are independently –H, C_1-C_6 alkyl or (-COOH), preferably –H, C_1-C_2 alkyl or (-COOH), more preferably –H or (-COOH). Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some preferred embodiments, $(AA)_a$ comprises a tri-peptide selected from Trp-Trp-Gly or Trp-Gly-Phe. In some preferred embodiments, $(AA)_a$ is Trp-Trp-Gly-Phe (SEQ ID NO:2).

In some embodiments, X^2 is selected from:



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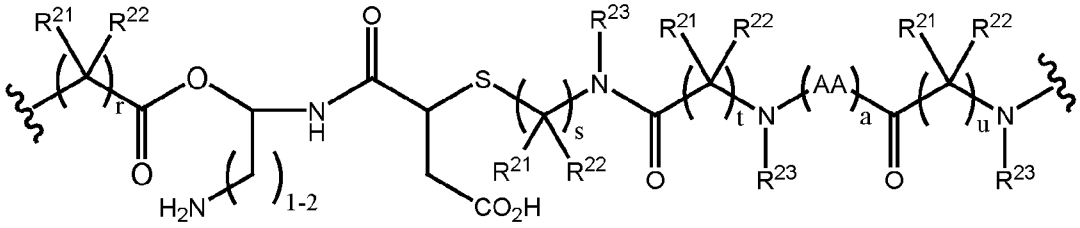
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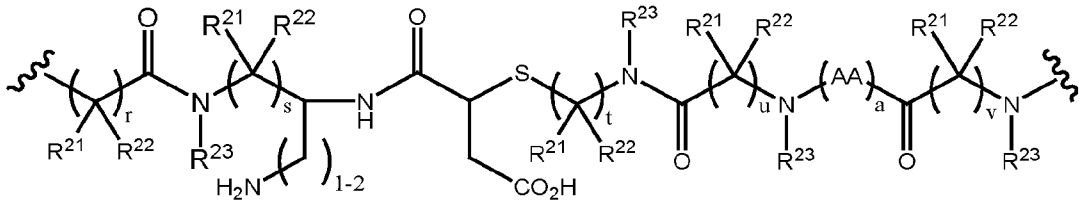
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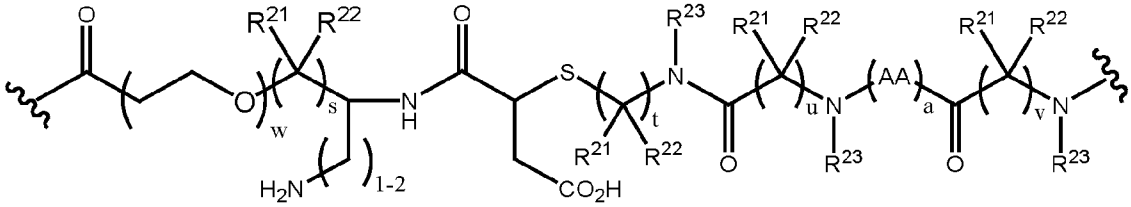
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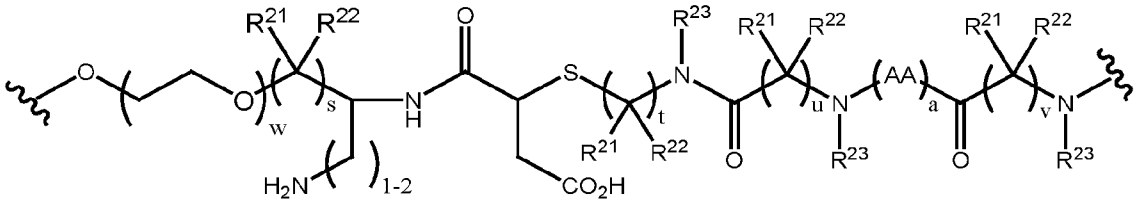
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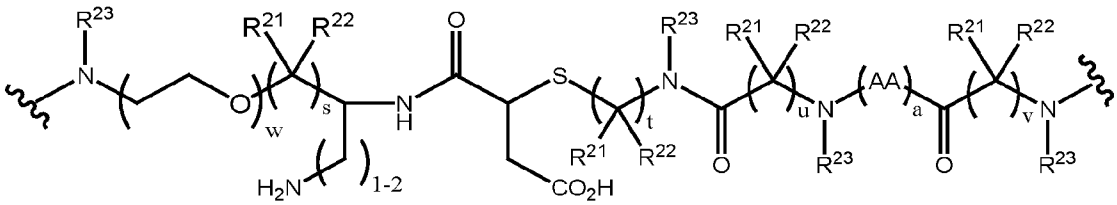
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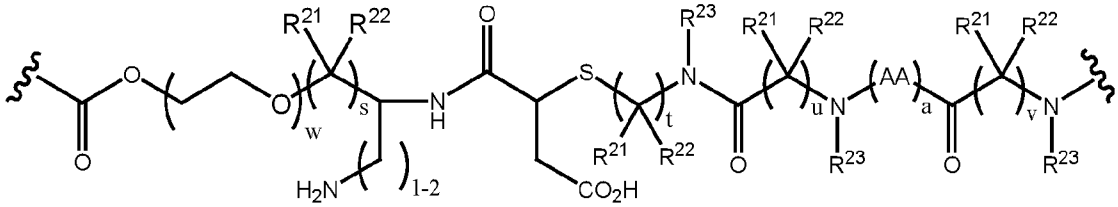
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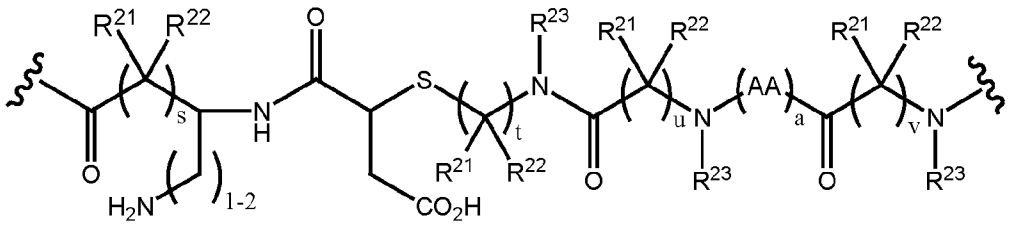
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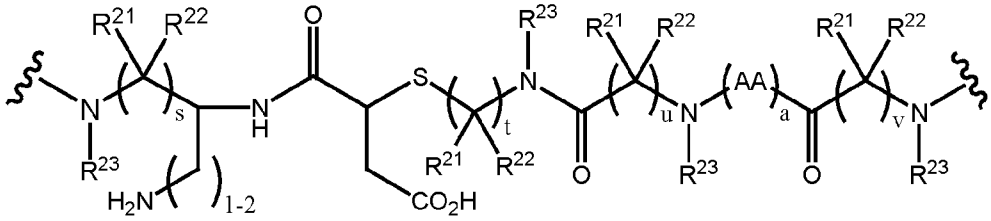
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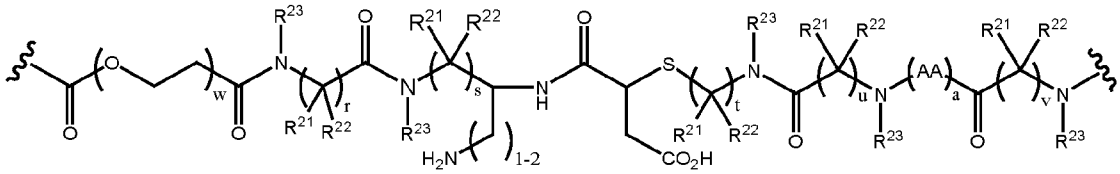
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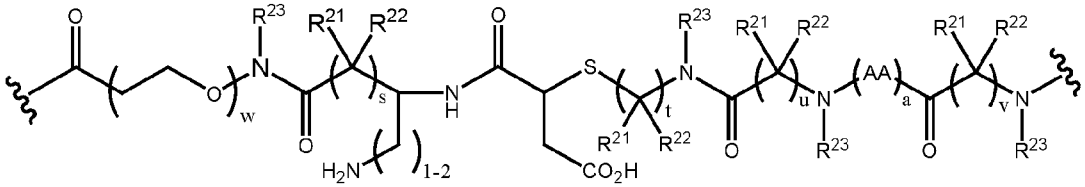
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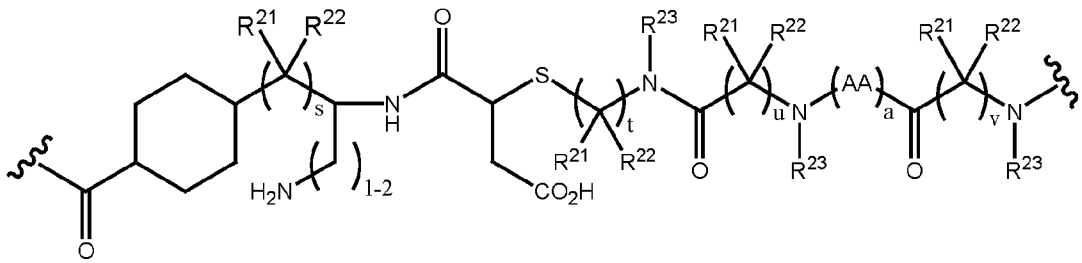
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wherein r, s, t and u are each independently an integer between 0 and 6, preferably between 0 and 4; v is an integer between 0 and 10; w is an integer between 0 and 10;

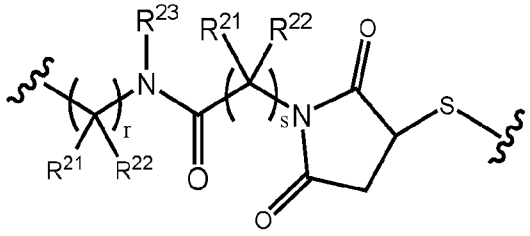
AA is an amino acid residue, preferably a naturally occurring amino acid residue; yet more preferably wherein AA is an amino acid selected from Arg, His, Lys, Asp, Glu, Ser, Thr, Asn, Gln, Cys, Sec, Gly, Pro, Ala, Val, Ile, Leu, Met, Phe, Tyr, and Trp;

a is an integer between 0 and 10, preferably between 0 and 6; more preferably between 0 and 4;

and wherein R^{21}, R^{22} and R^{23} are independently –H, C_1-C_6 alkyl or (-COOH), preferably –H, C_1-C_2 alkyl or (-COOH), more preferably –H or (-COOH). Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some preferred embodiments, $(AA)_a$ is Trp-Trp-Gly-Phe (SEQ ID NO:2).

In some embodiments, X^2 is selected from: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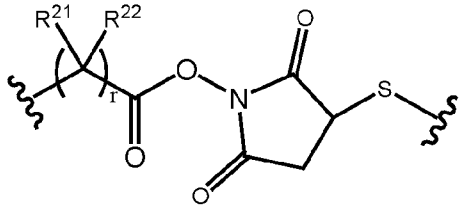
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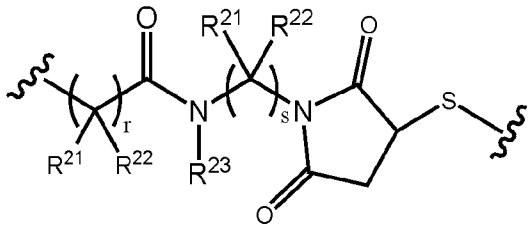
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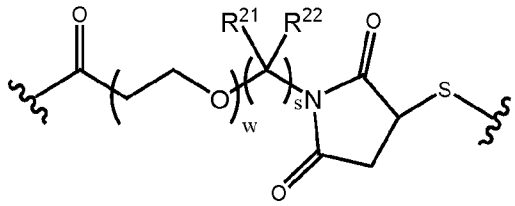
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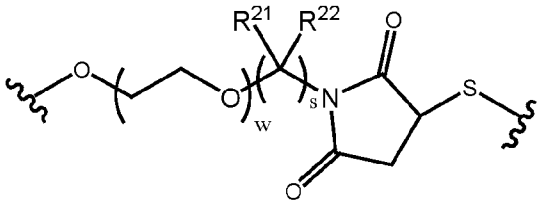
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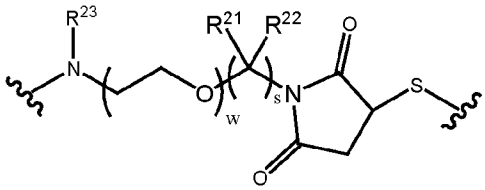
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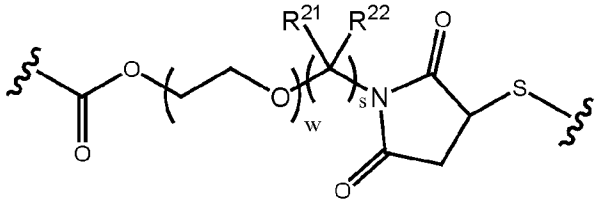
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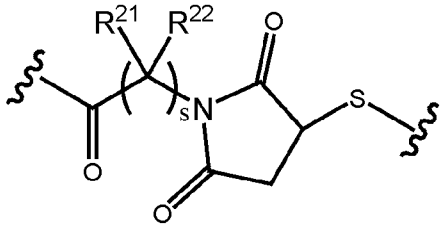
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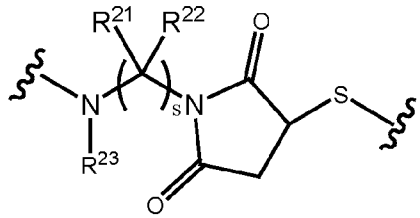
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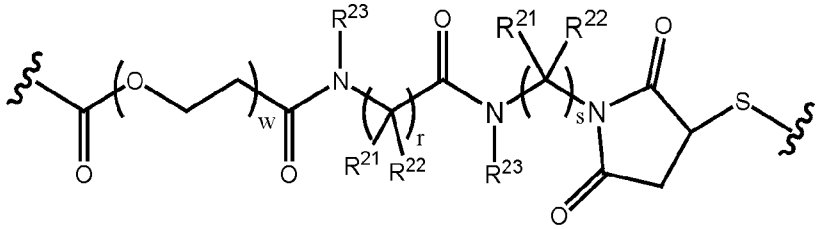
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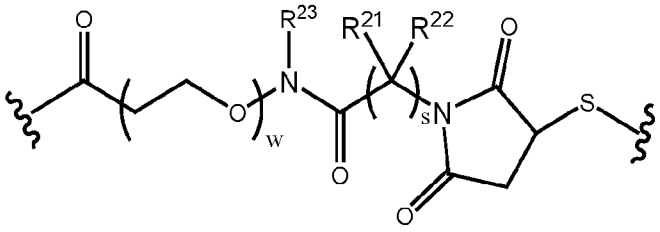

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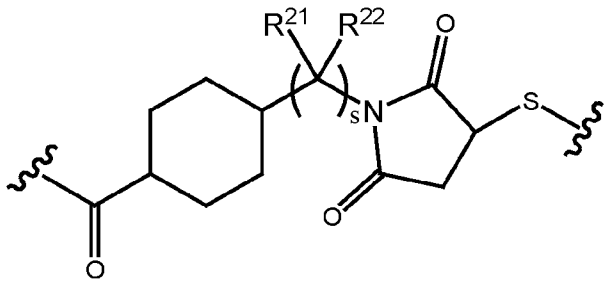
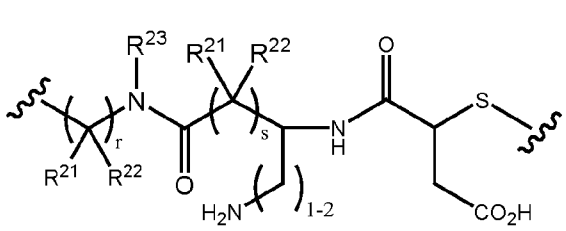


;

wherein r and s are each independently an integer between 0 and 4, preferably between 0 and 2; w is an integer between 0 and 10;

and wherein R^{21}, R^{22} and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line on the left side is a bond to the PEG fragment (–$[OCH_2$-$CH_2]_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some embodiments, X^2 is selected from:



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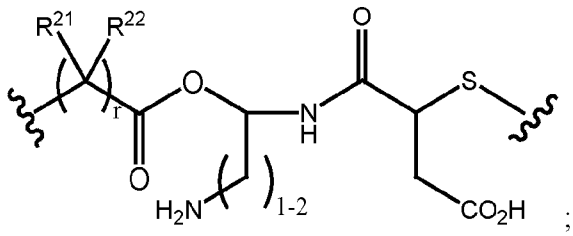

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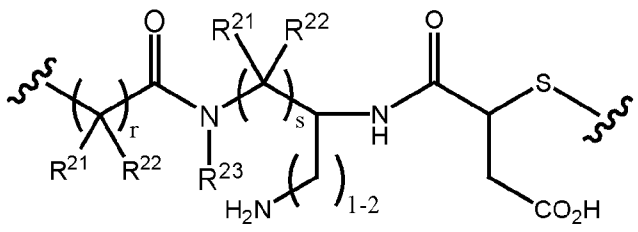

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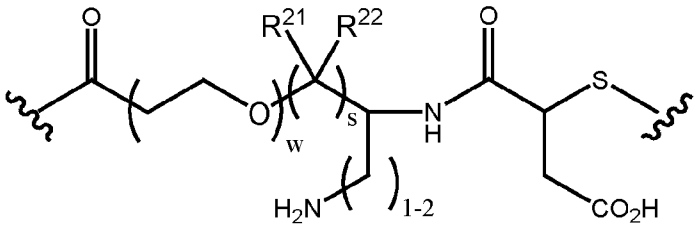
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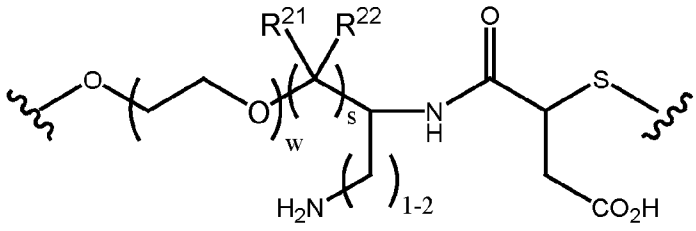
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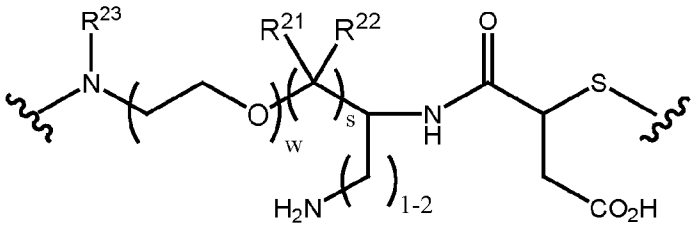
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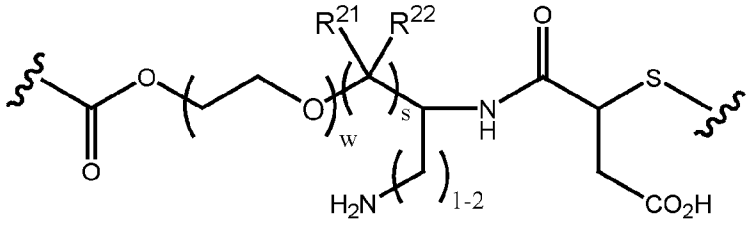
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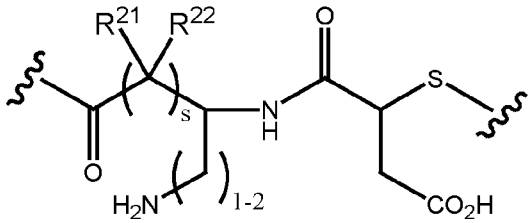
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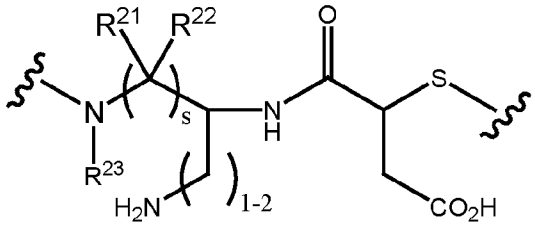
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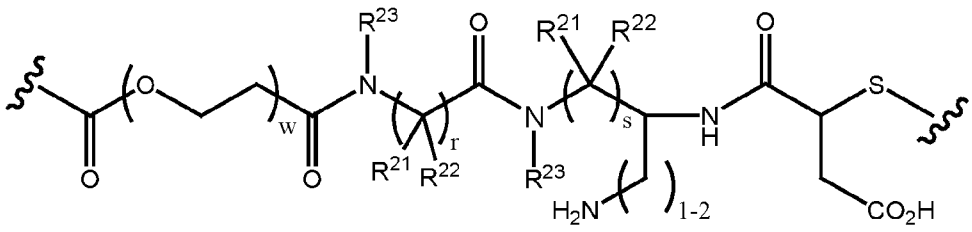
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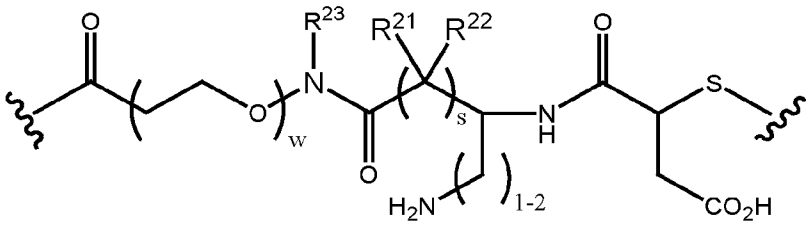
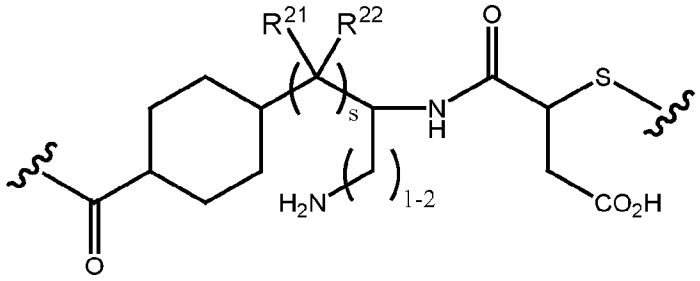


; or

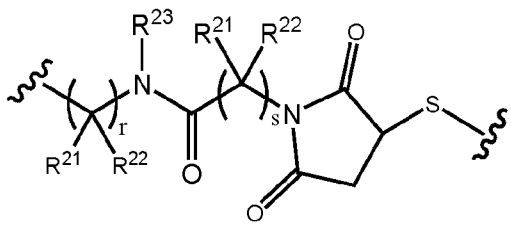


;

wherein r and s are each independently an integer between 0 and 4, preferably between 0 and 2; w is an integer between 0 and 10;

and wherein R^{21}, R^{22} and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line on the left side is a bond to the PEG fragment (–$[OCH_2$-$CH_2]_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some preferred embodiments, X^2 is selected from:



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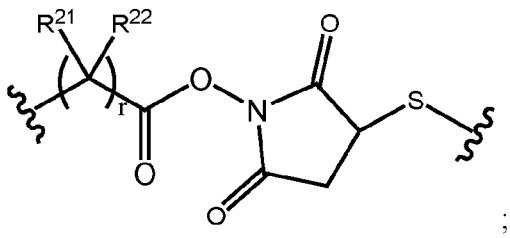

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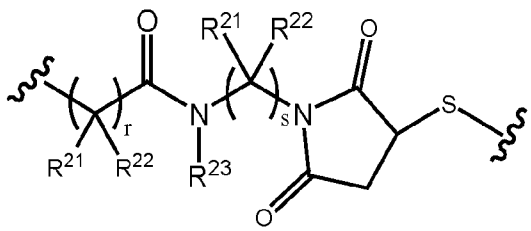

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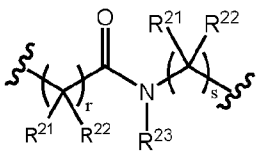

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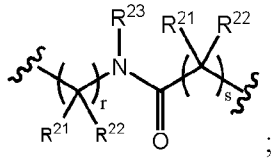

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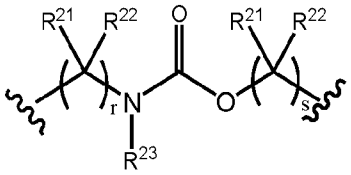
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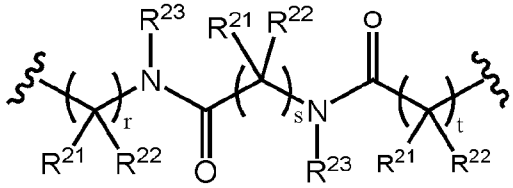
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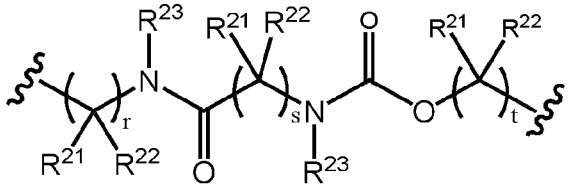
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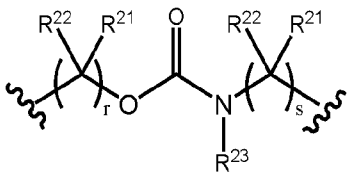
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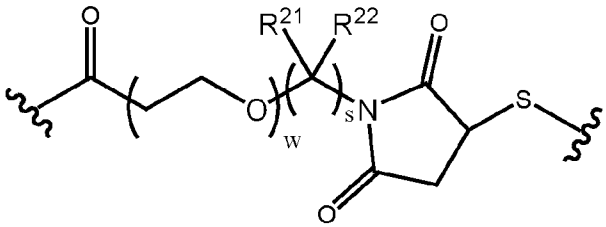
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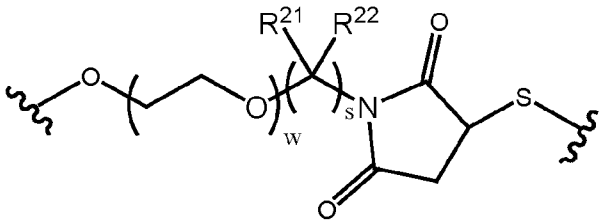
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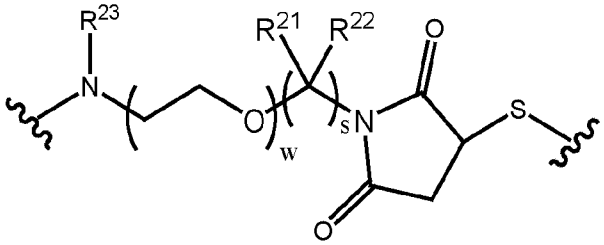
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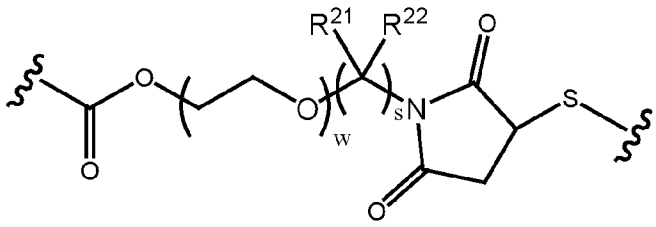
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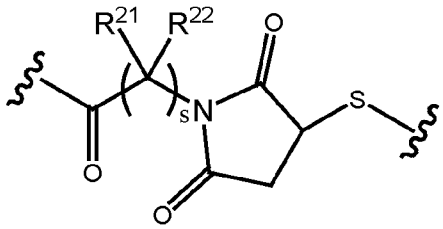
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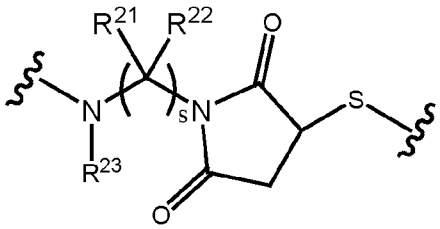
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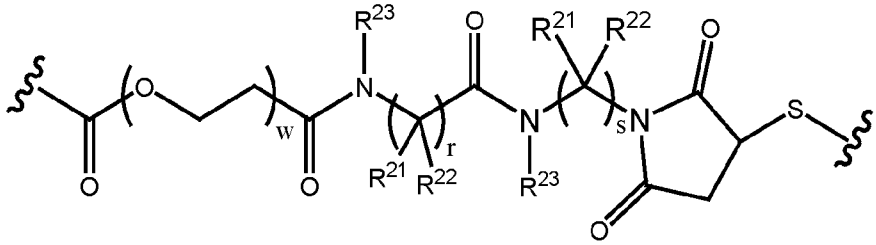

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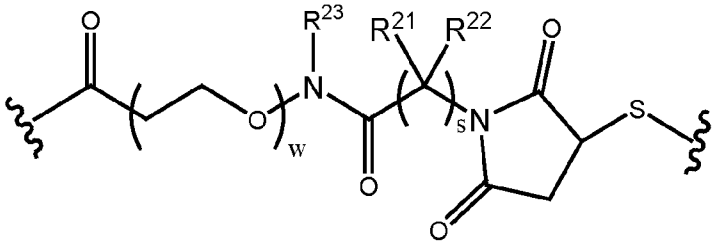

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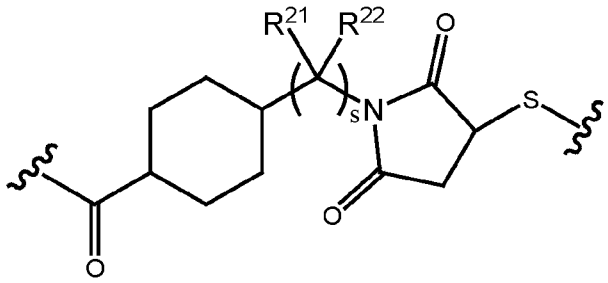


;

wherein;

r, s, and t, are each independently an integer between 0 and 4, preferably between 0 and 2; w is an integer between 0 and 10;

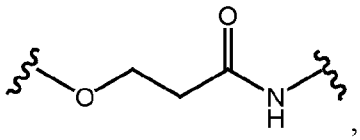
AA is an amino acid selected from Arg, His, Lys, Asp, Glu, Ser, Thr, Asn, Gln, Cys, Sec, Gly, Pro, Ala, Val, Ile, Leu, Met, Phe, Tyr, and Trp;

a is an integer between 0 and 10, preferably between 0 and 6; more preferably between 0 and 4;

and wherein R^{21}, R^{22} and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line on the left side is a bond to the PEG fragment (–$[OCH_2$-$CH_2]_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In yet more preferred embodiments, $(AA)_a$ is Trp-Trp-Gly-Phe (SEQ ID NO:2).

In some embodiments, X^2 comprises or alternatively is a urea, a carbamate, a carbonate, or an ester. In preferred embodiments, X^2 is selected from:



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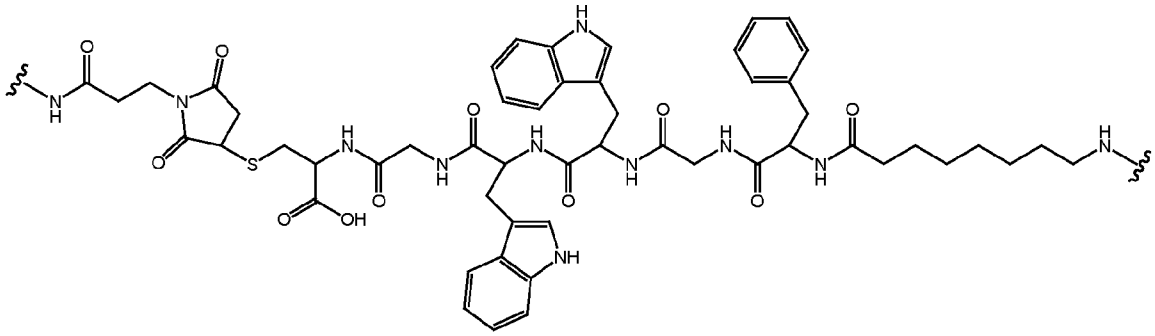
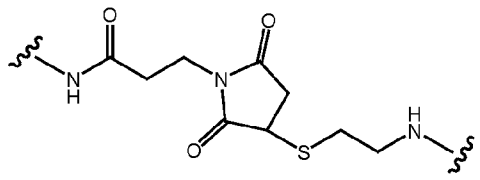
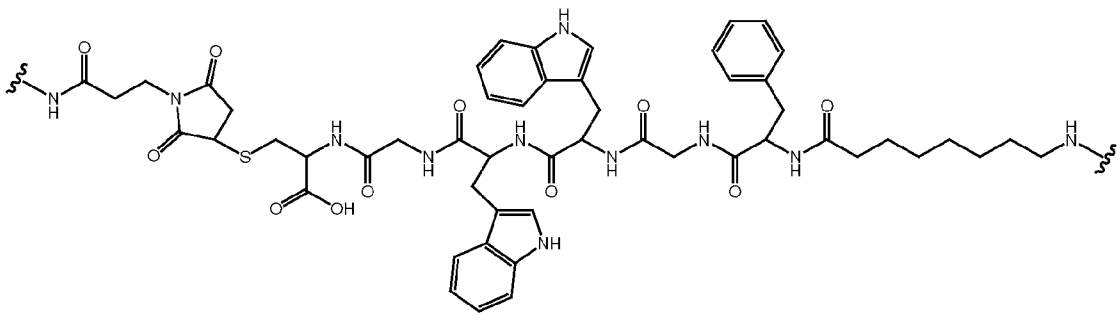




and . Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

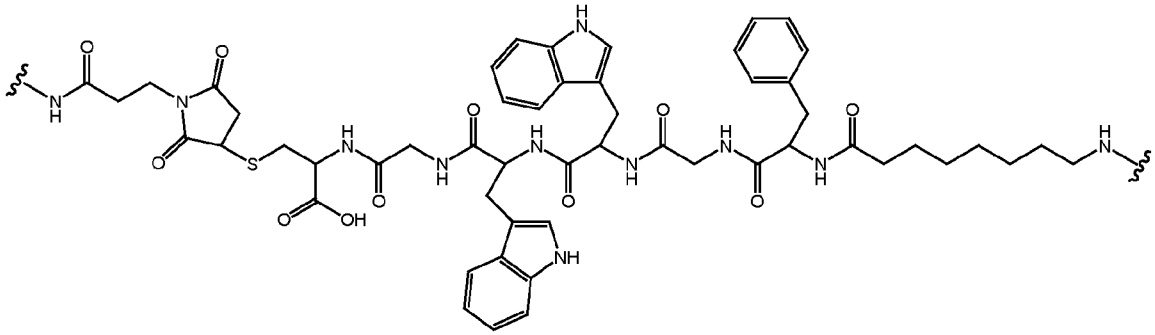
In a preferred embodiment said X^2 is



.

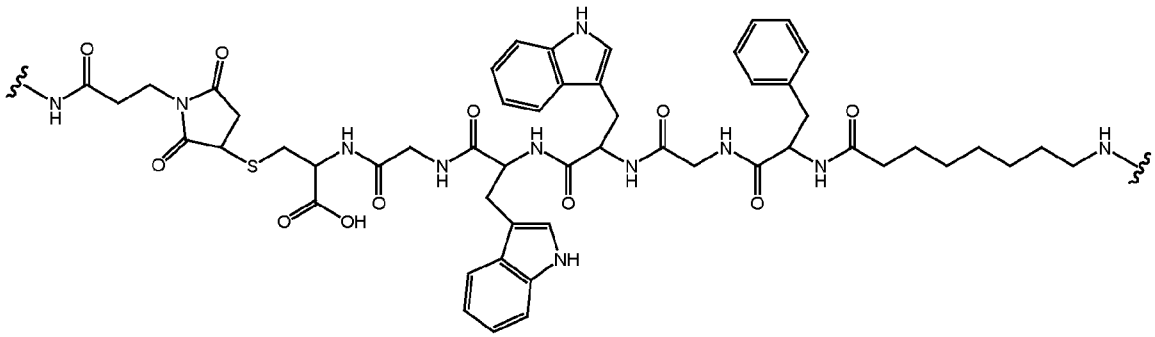
Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In a further preferred embodiment said X^2 is



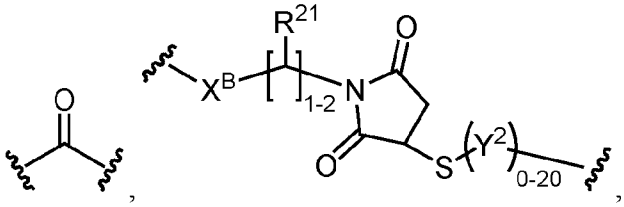
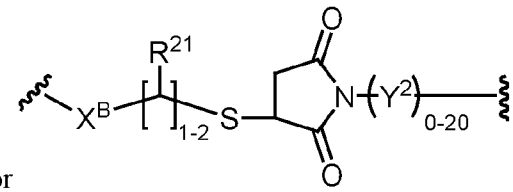
and said L of said triconjugate is the DUPA residue (HOOC(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-(CH_2)$_2$-CO-). Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the DUPA residue.

In a further preferred embodiment said X^2 is



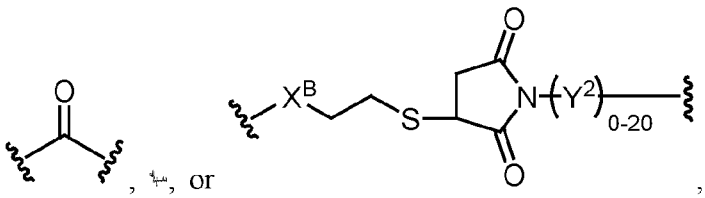
and said L of said triconjugate is the DUPA residue (HOOC(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-(CH_2)$_2$-CO-), wherein the terminus with the amide group of said X^2 is bonded to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and wherein the terminus with the amine functionality is bonded to the DUPA residue (HOOC(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-(CH_2)$_2$-CO-).

In some embodiments, X^2 is selected from:

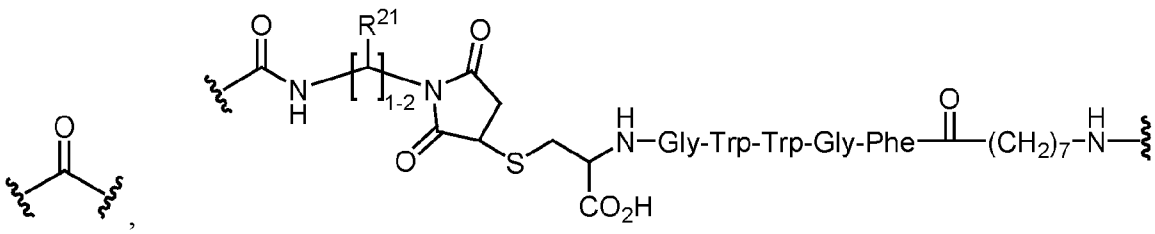
, wherein X^B is -C(O)NH- or -NH-C(O)-, and wherein Y^2 and R^{21} are as defined above. Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some embodiments, X^2 is selected from:

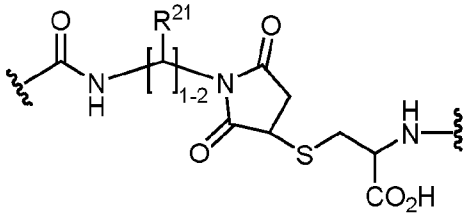


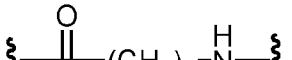
, wherein X^B is -C(O)NH- or -NH-C(O)-, and wherein Y^2 and R^{21} are as defined above. Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

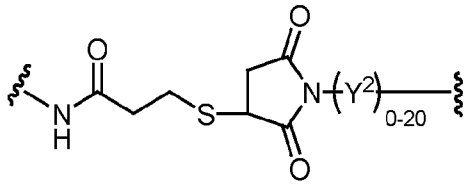
In some embodiments, X^2 is selected from:



(SEQ ID NO. 10, wherein SEQ ID NO:10 is defined as W1-Gly-Trp-Trp-Gly-Phe-W2,

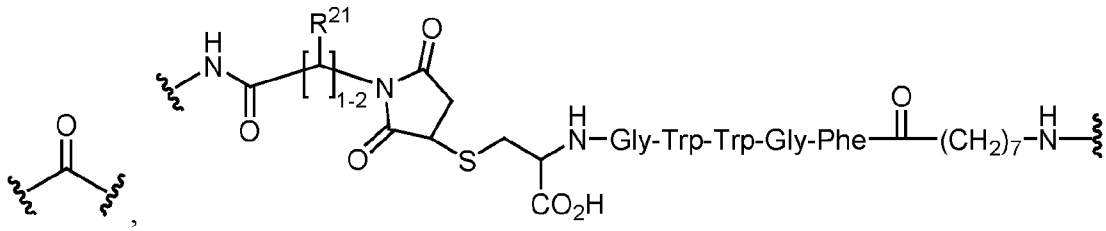


wherein W1 is and W2 is ), or

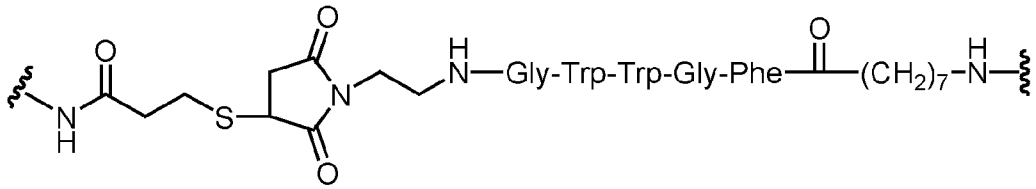


; wherein Y^2 and R^{21} are as defined above. Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

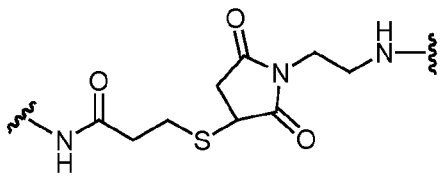
In some embodiments, X^2 is selected from:



, , or



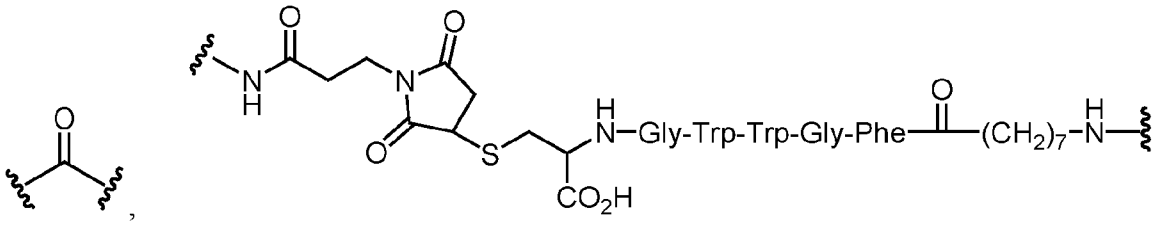
(SEQ ID NO. 14, wherein SEQ ID NO:14 is defined as W9-Gly-Trp-Trp-Gly-Phe-W10, wherein W9 is

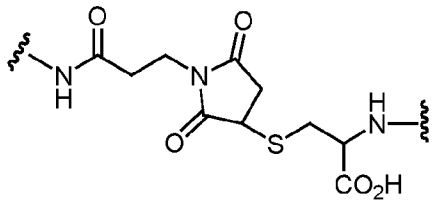
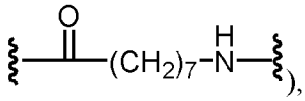


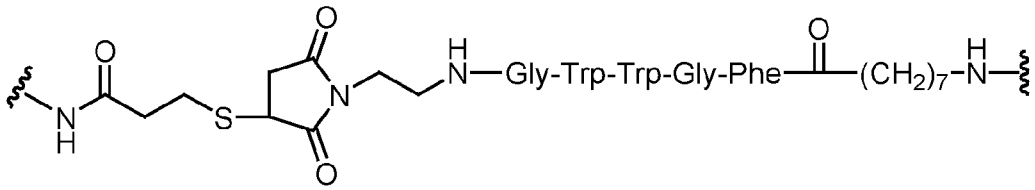
and W10 is ); wherein R^{21} is as defiend above; preferably R^{21} is -H or -CH_2-NH_2; more preferably -H. Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60,

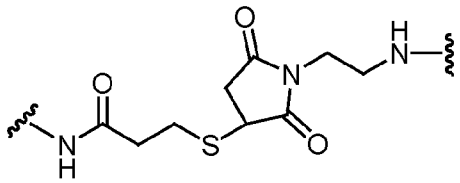
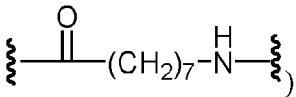
and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some embodiments, X^2 is selected from:

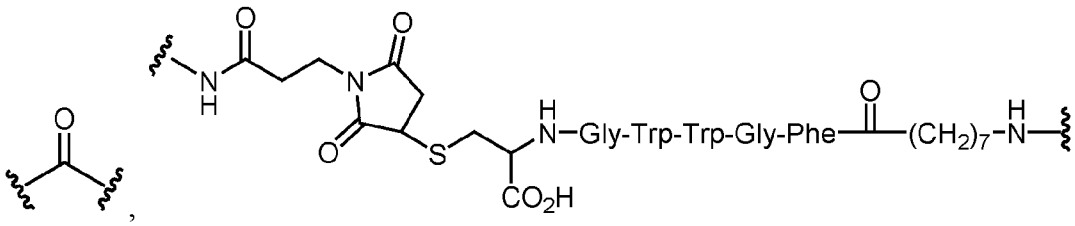


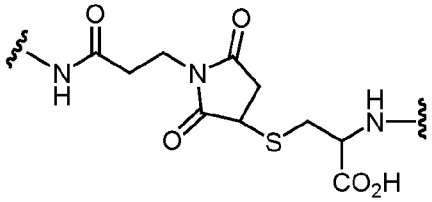
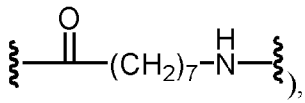
(SEQ ID No. 11, wherein SEQ ID NO:11 is defined as W3-Gly-Trp-Trp-Gly-Phe-W4, wherein W3 is  and W4 is ),

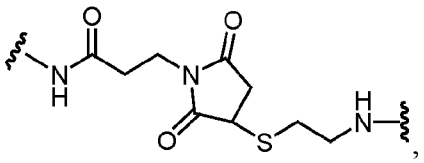


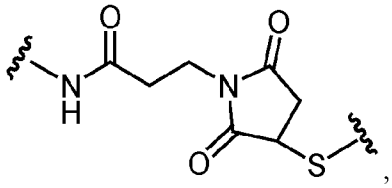
or (SEQ ID NO. 14, wherein SEQ ID NO:14 is defined as W9-Gly-Trp-Trp-Gly-Phe-W10, wherein W9 is  and W10 is ). Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

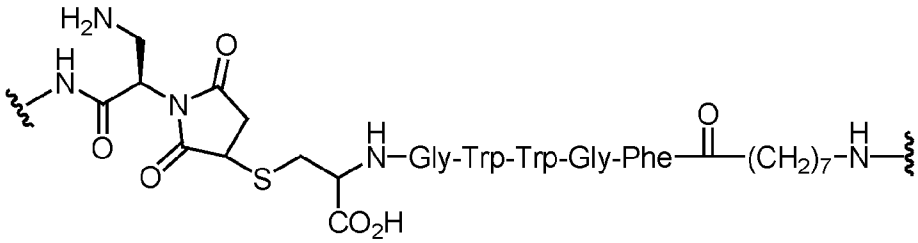
In some embodiments, X^2 is selected from:

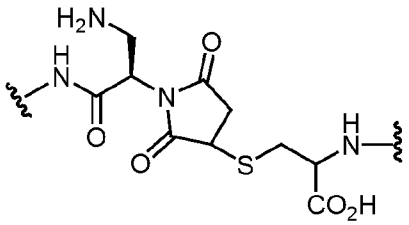
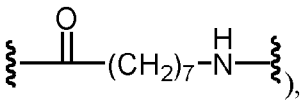


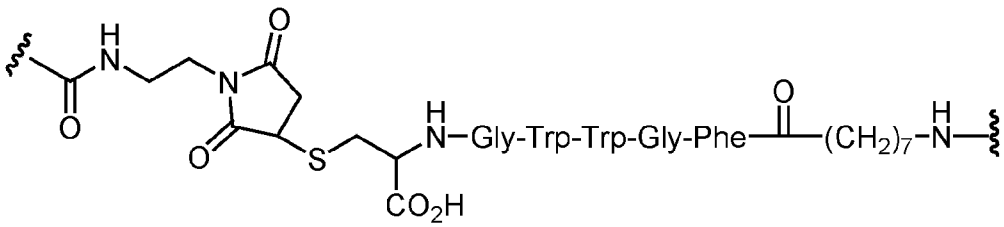
(SEQ ID No. 11, wherein SEQ ID NO:11 is defined as W3-Gly-Trp-Trp-Gly-Phe-W4, wherein W3 is  and W4 is ),

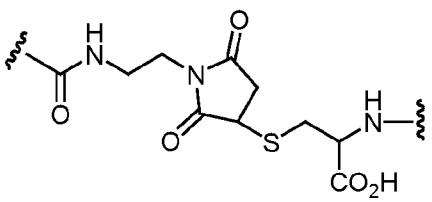
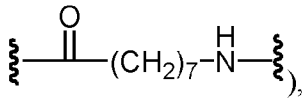


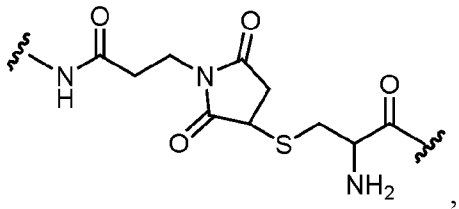




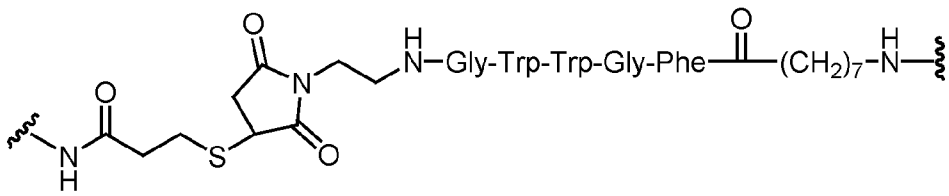
(SEQ ID No. 12, wherein SEQ ID NO:12 is defined as W5-Gly-Trp-Trp-Gly-Phe-W6, wherein W5 is  and W6 is ),



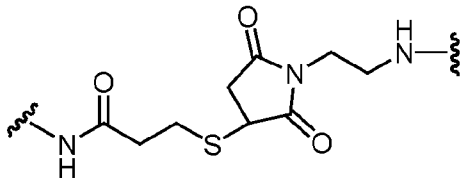
(SEQ ID No. 13, wherein SEQ ID NO:13 is defined as W7-Gly-Trp-Trp-Gly-Phe-W8, wherein W7 is  and W8 is ),



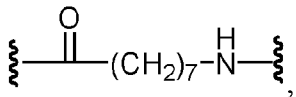
,



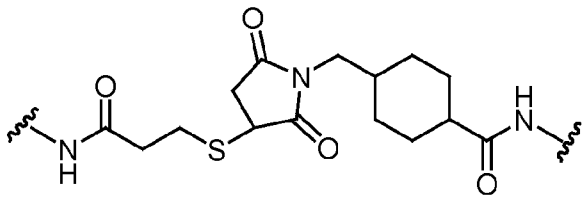
(SEQ ID NO. 14, wherein SEQ ID NO:14 is defined as W9-Gly-Trp-Trp-Gly-Phe-W10, wherein W9 is



and W10 is

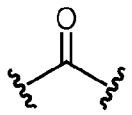


, or



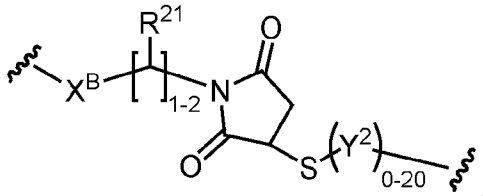
. Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some embodiments, X^2 is:



.

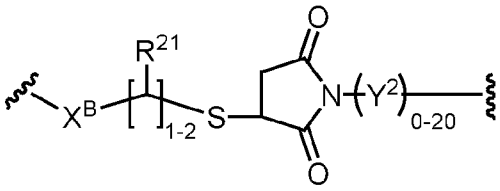
In some embodiments, X^2 is:



, wherein X^B is -C(O)NH- or -NH-C(O)-. Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable

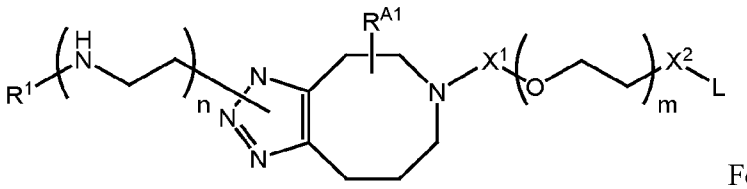
m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

In some embodiments, X^2 is:



, wherein X^B is -C(O)NH- or -NH-C(O)-. Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–), wherein the variable m represents a discrete number of repeating -(O-CH_2-CH_2)-units, wherein said discrete number m of repeating -(O-CH_2-CH_2)-units is any discrete number of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36; and the wavy line on the right side is a bond to the targeting fragment (L), wherein said targeting fragment L is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

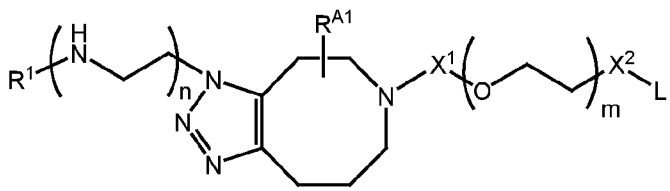
In some embodiments, the composition comprises a conjugate of the Formula IA:



Formula IA,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

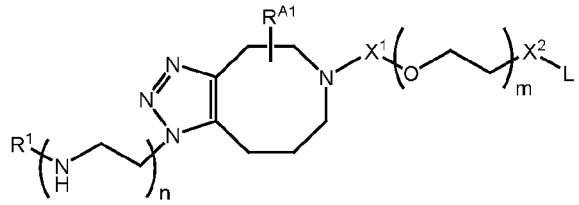
In some embodiments, the composition comprises a conjugate of the Formula IA-1:



Formula IA-1,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

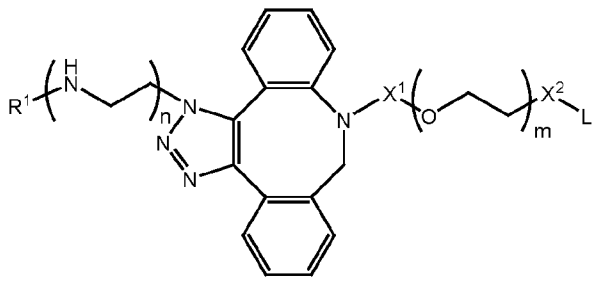
In some embodiments, the composition comprises a conjugate of the Formula IA-2:



Formula IA-2,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

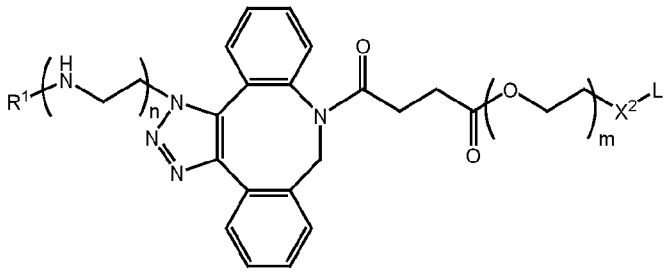
In some embodiments, the composition comprises a conjugate of the Formula IA-3:



Formula IA-3,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

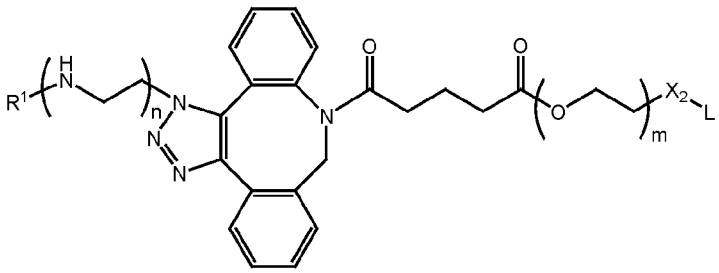
In some embodiments, the composition comprises a conjugate of the Formula IA-3a:



Formula IA-3a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

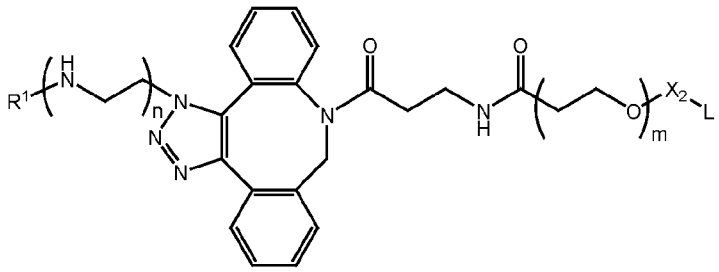
In some embodiments, the composition comprises a conjugate of the Formula IA-3b:



Formula IA-3b,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IA-3c:

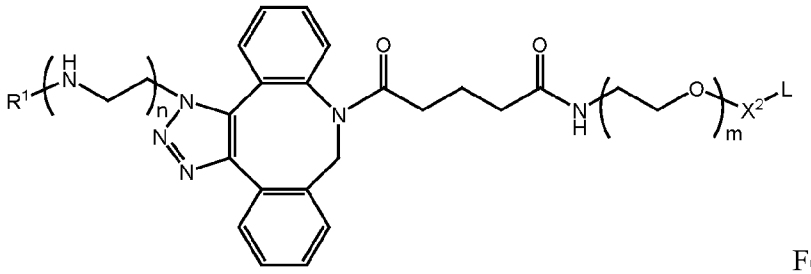


Formula IA-3c,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and

preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

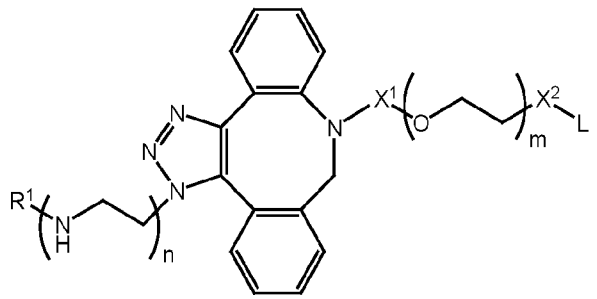
In some embodiments, the composition comprises a conjugate of the Formula IA-3d:



Formula IA-3d,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

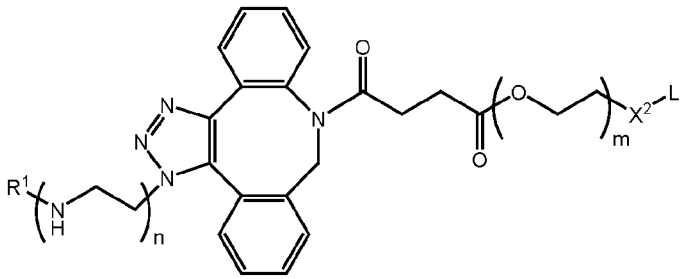
In some embodiments, the composition comprises a conjugate of the Formula IA-4:



Formula IA-4,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

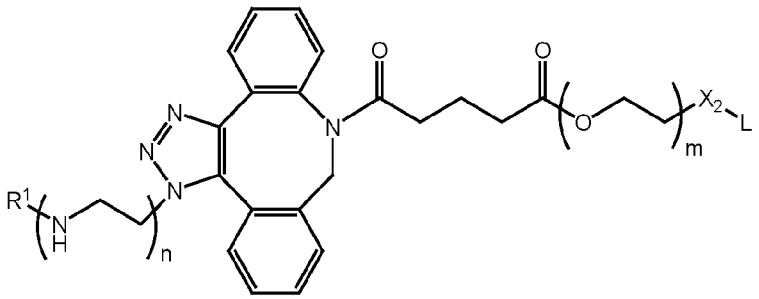
In some embodiments, the composition comprises a conjugate of the Formula IA-4a:



Formula IA-4a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

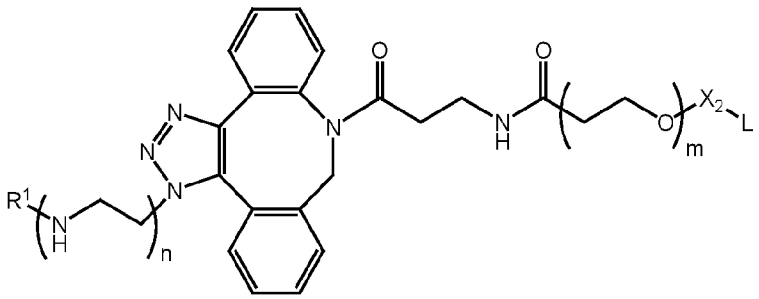
In some embodiments, the composition comprises a conjugate of the Formula IA-4b:



Formula IA-4b,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IA-4c:

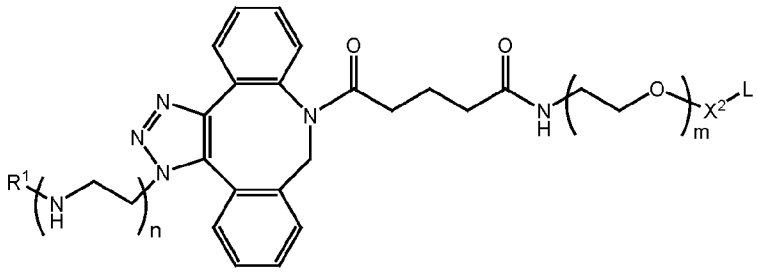


Formula IA-4c,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less,

and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

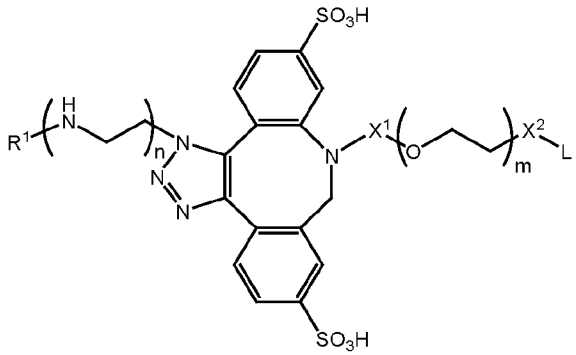
In some embodiments, the composition comprises a conjugate of the Formula IA-4d:



Formula IA-4d,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

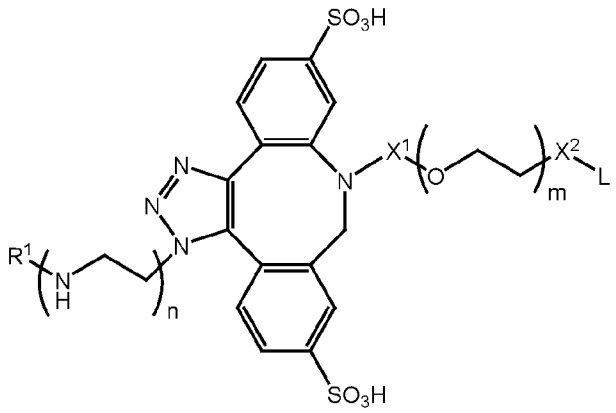
In some embodiments, the composition comprises a conjugate of the Formula IA-5:



Formula IA-5,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

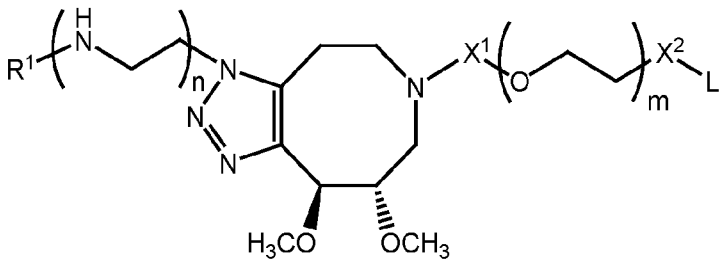
In some embodiments, the composition comprises a conjugate of the Formula IA-6:



Formula IA-6,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

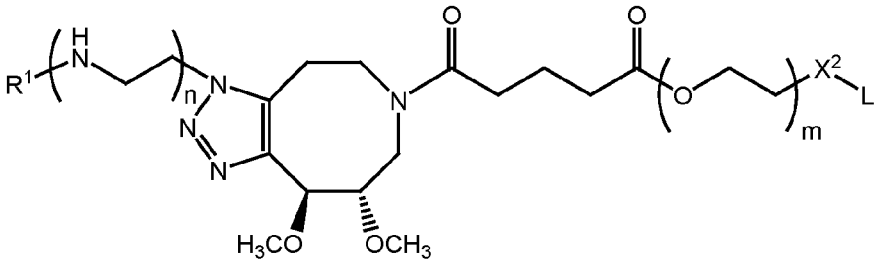
In some embodiments, the composition comprises a conjugate of the Formula IA-7:



Formula IA-7,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

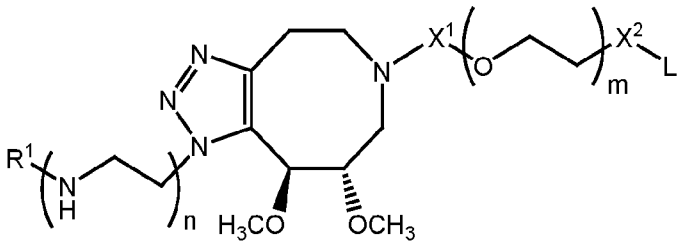
In some embodiments, the composition comprises a conjugate of the Formula IA-7a:



Formula IA-7a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

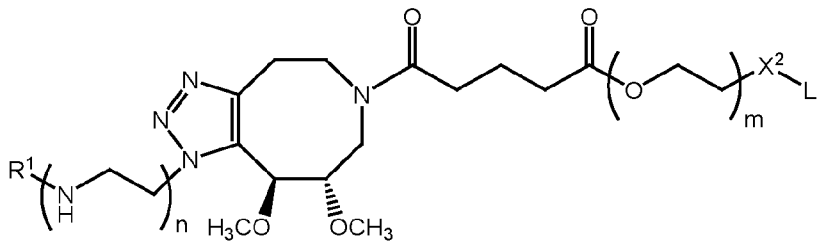
In some embodiments, the composition comprises a conjugate of the Formula IA-8:



Formula IA-8,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

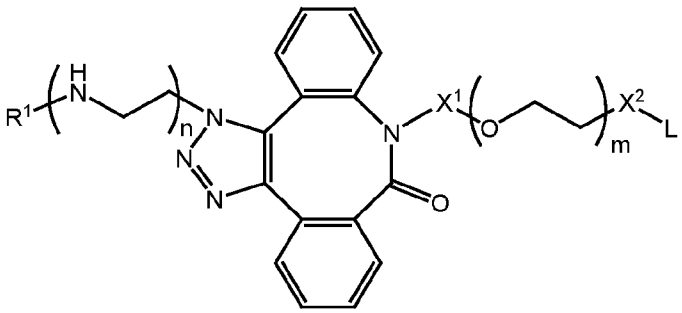
In some embodiments, the composition comprises a conjugate of the Formula IA-8a:



Formula IA-8a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

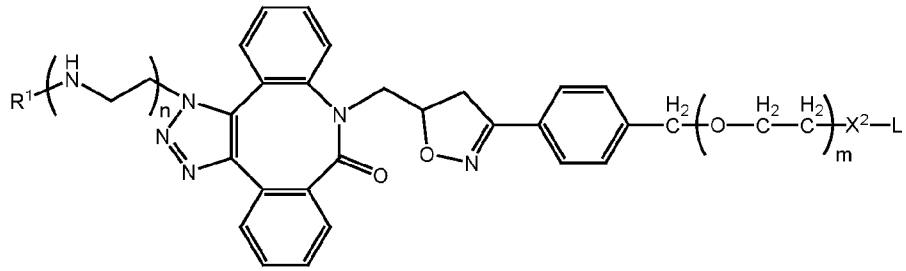
In some embodiments, the composition comprises a conjugate of the Formula IA-9:



Formula IA-9,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

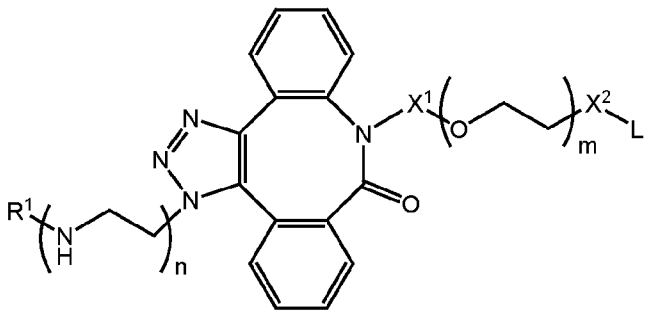
In some embodiments, the composition comprises a conjugate of the Formula IA-9a:



Formula IA-9a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

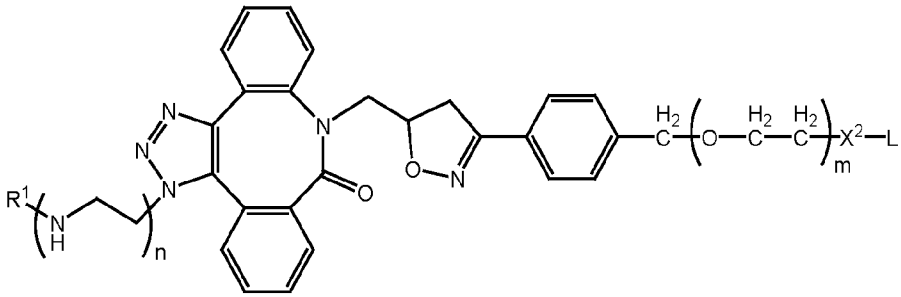
In some embodiments, the composition comprises a conjugate of the Formula IA-10:



Formula IA-10,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

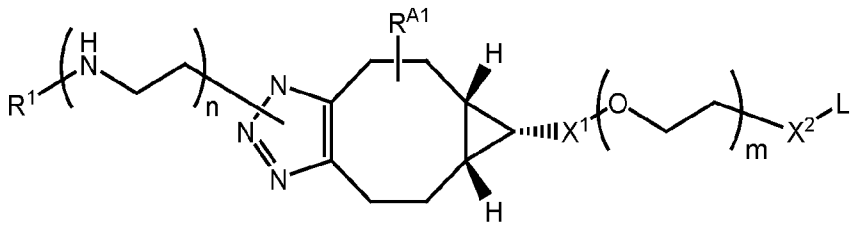
In some embodiments, the composition comprises a conjugate of the Formula IA-10a:



Formula IA-10a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

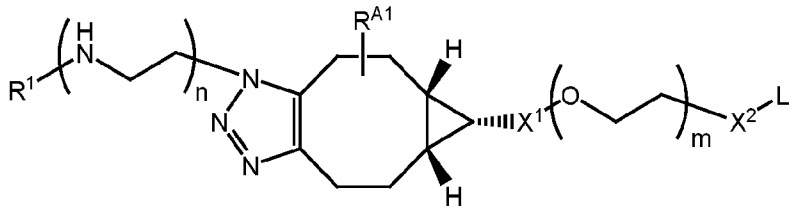
In some embodiments, the composition comprises a conjugate of the Formula IB:



Formula IB,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

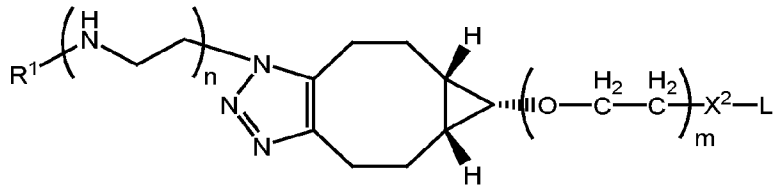
In some embodiments, the composition comprises a conjugate of the Formula IB-1:



Formula IB-1,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

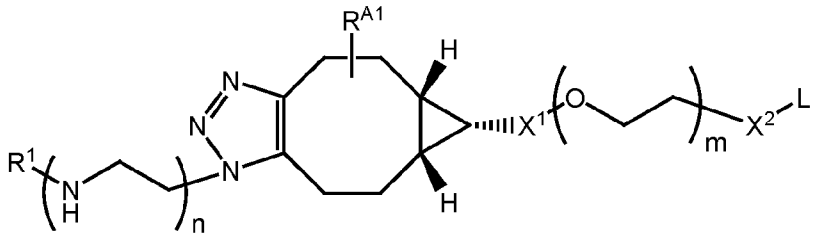
In some embodiments, the composition comprises a conjugate of the Formula IB-1a:



Formula IB-1a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

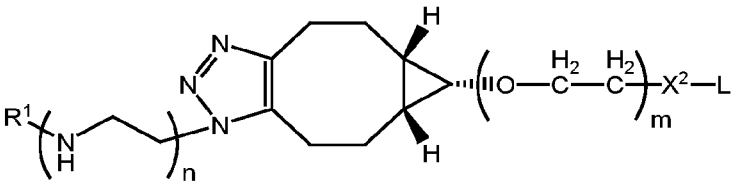
In some embodiments, the composition comprises a conjugate of the Formula IB-2:



Formula IB-2,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

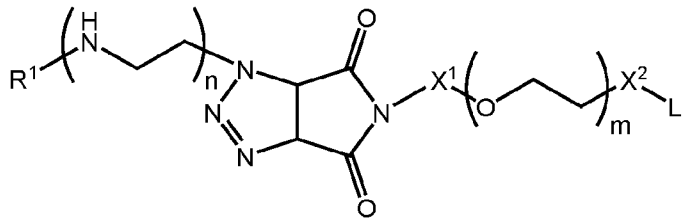
In some embodiments, the composition comprises a conjugate of the Formula IB-2a:



Formula IB-2a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

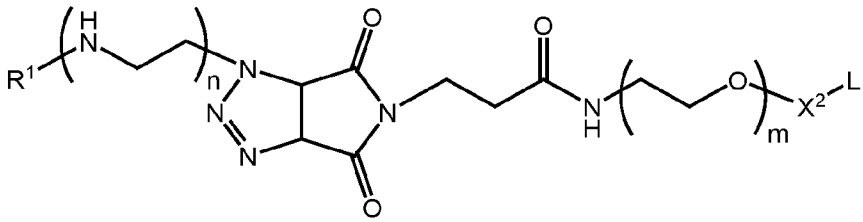
In some embodiments, the composition comprises a conjugate of the Formula IC:



Formula IC,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IC-1:

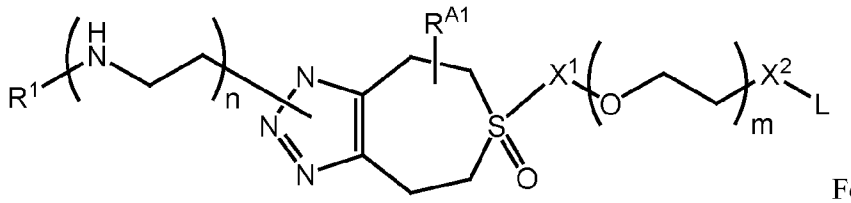


Formula IC-1,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said

discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

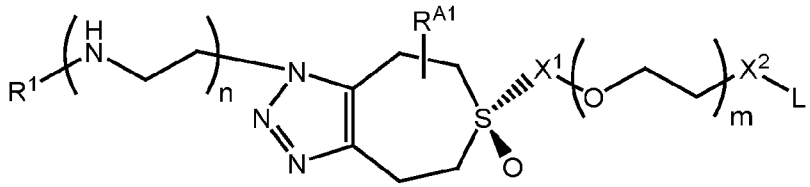
In some embodiments, the composition comprises a conjugate of the Formula ID:



Formula ID,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

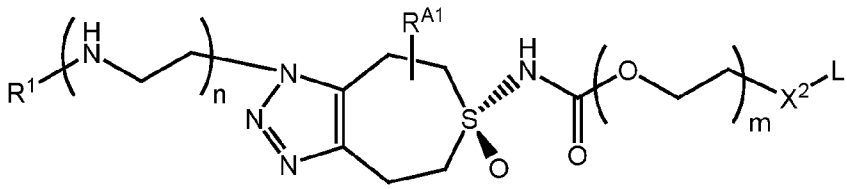
In some embodiments, the composition comprises a conjugate of the Formula ID-1:



Formula ID-1,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula ID-1a:

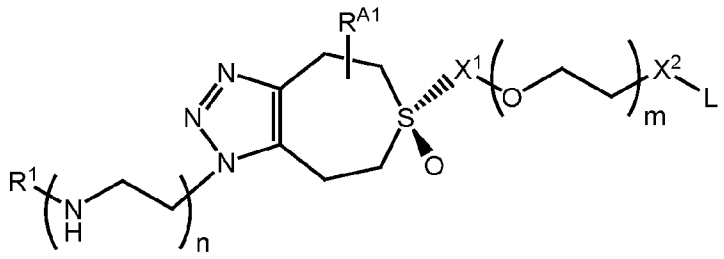


Formula ID-1a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said

discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

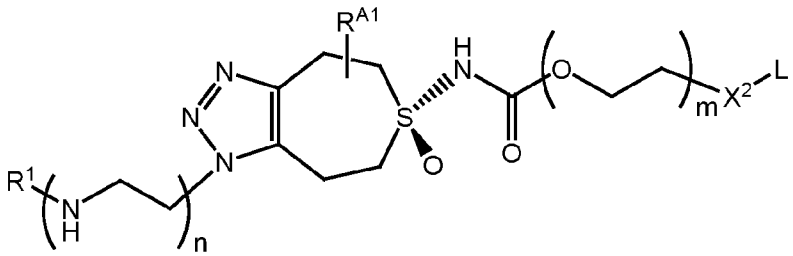
In some embodiments, the composition comprises a conjugate of the Formula ID-2:



Formula ID-2,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

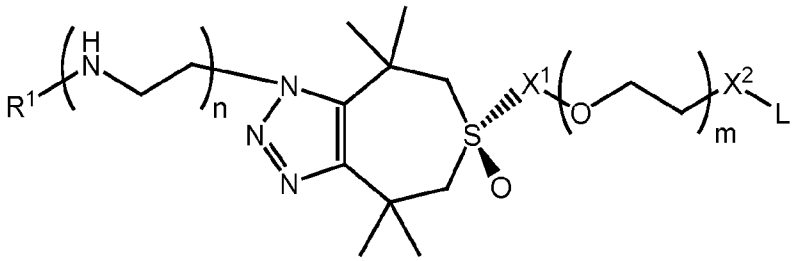
In some embodiments, the composition comprises a conjugate of the Formula ID-2a:



Formula ID-2a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

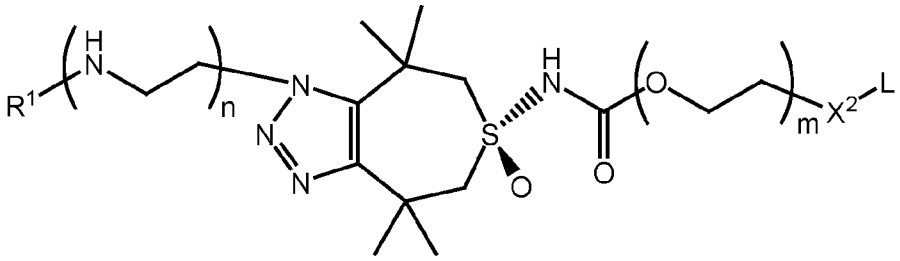
In some embodiments, the composition comprises a conjugate of the Formula ID-3:



Formula ID-3,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

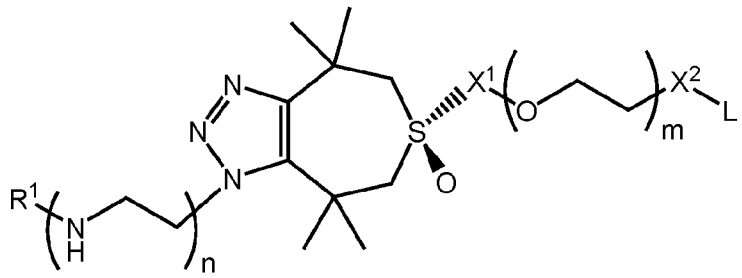
In some embodiments, the composition comprises a conjugate of the Formula ID-3a:



Formula ID-3a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

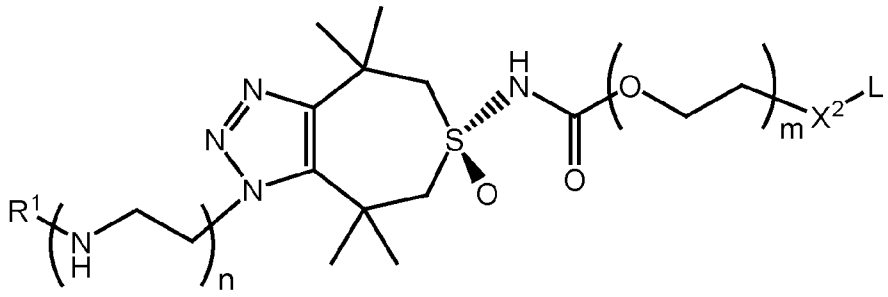
In some embodiments, the composition comprises a conjugate of the Formula ID-4:



Formula ID-4,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

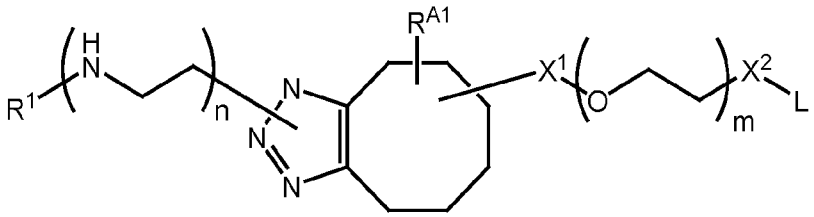
In some embodiments, the composition comprises a conjugate of the Formula ID-4a:



Formula ID-4a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

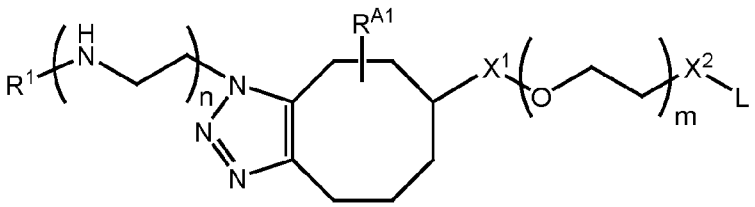
In some embodiments, the composition comprises a conjugate of the Formula IE:



Formula IE,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IE-1:

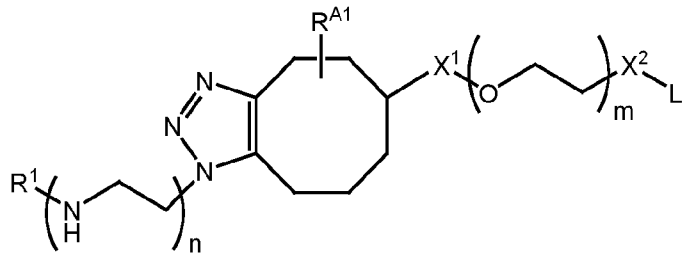


Formula IE-1,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said

discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

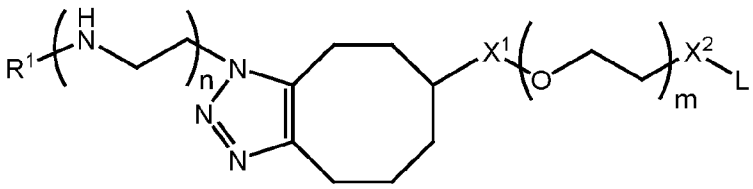
In some embodiments, the composition comprises a conjugate of the Formula IE-2:



Formula IE-2,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

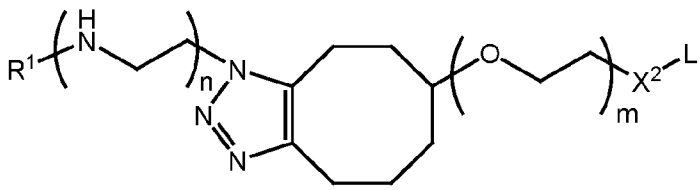
In some embodiments, the composition comprises a conjugate of the Formula IE-3:



Formula IE-3,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IE-3a:

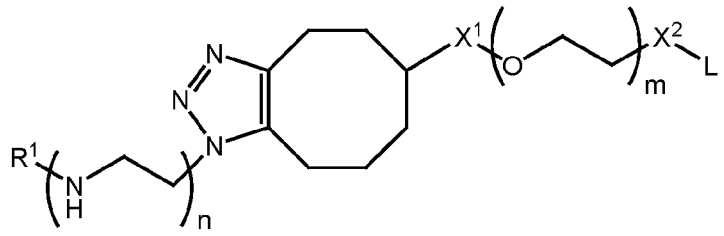


Formula IE-3a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and

preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

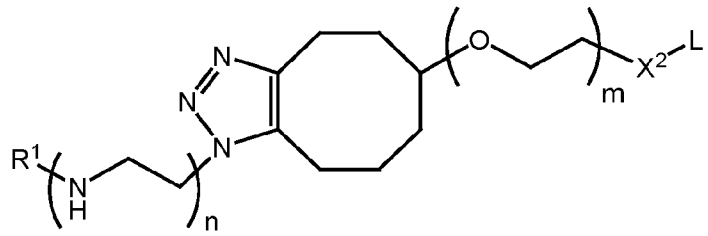
In some embodiments, the composition comprises a conjugate of the Formula IE-4:



Formula IE-4,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

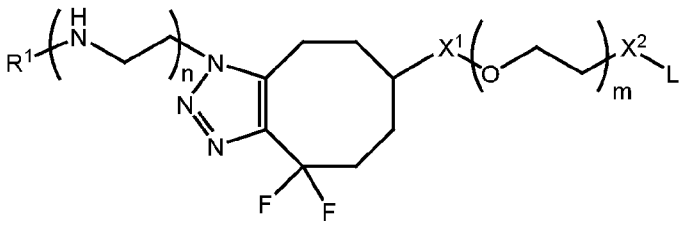
In some embodiments, the composition comprises a conjugate of the Formula IE-4a:



Formula IE-4a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

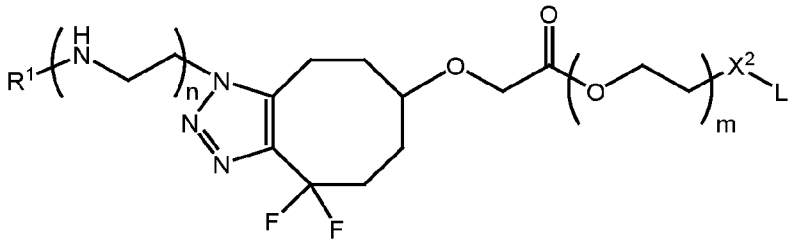
In some embodiments, the composition comprises a conjugate of the Formula IE-5:



Formula IE-5,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 3624.

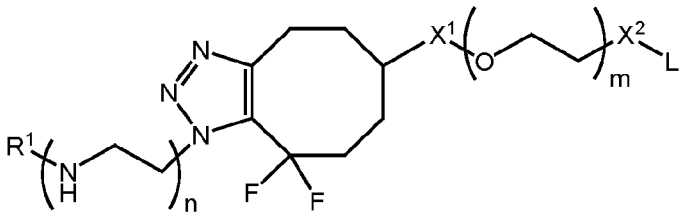
In some embodiments, the composition comprises a conjugate of the Formula IE-5a:



Formula IE-5a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

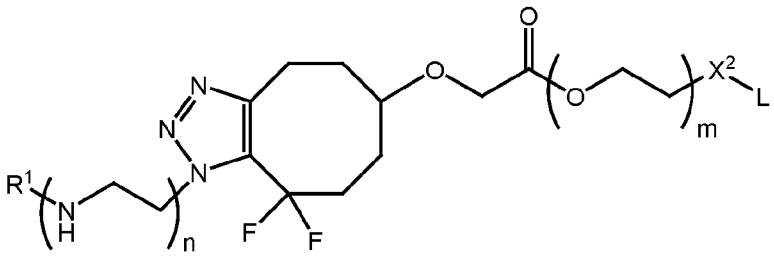
In some embodiments, the composition comprises a conjugate of the Formula IE-6:



Formula IE-6,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

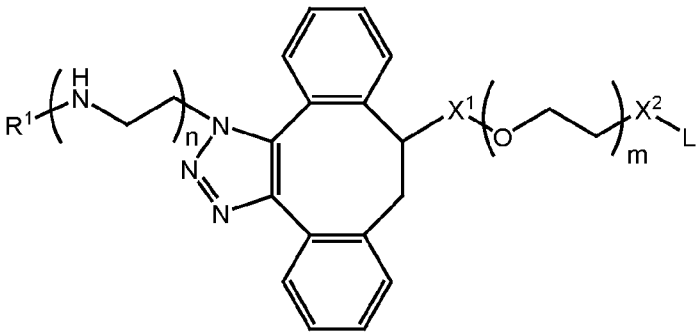
In some embodiments, the composition comprises a conjugate of the Formula IE-6a:



Formula IE-6a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

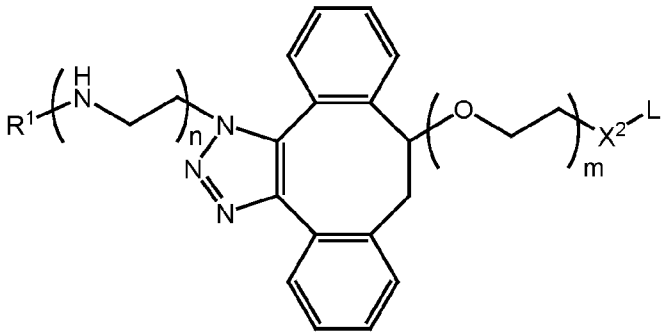
In some embodiments, the composition comprises a conjugate of the Formula IE-7:



Formula IE-7,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

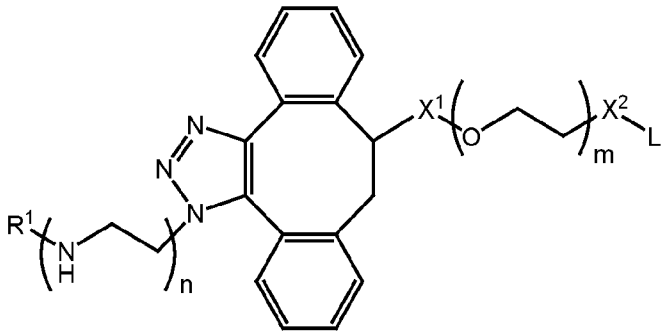
In some embodiments, the composition comprises a conjugate of the Formula IE-7a:



Formula IE-7a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

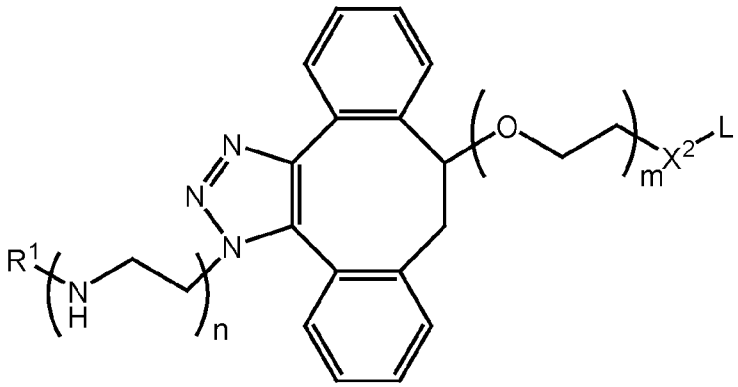
In some embodiments, the composition comprises a conjugate of the Formula IE-8:



Formula IE-8,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IE-8a:

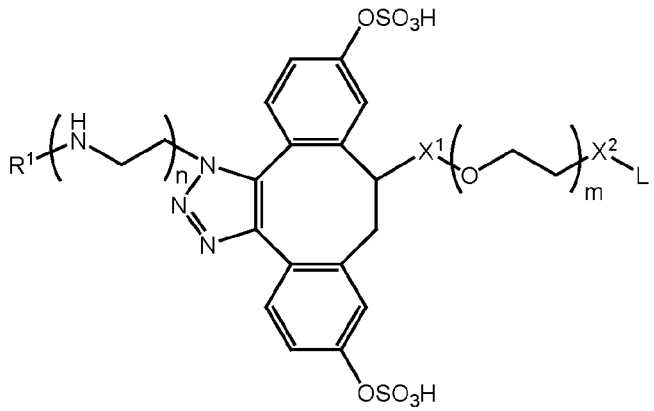


Formula IE-8a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said

discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

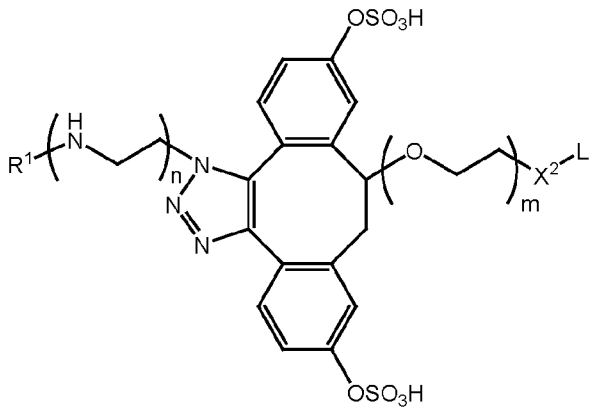
In some embodiments, the composition comprises a conjugate of the Formula IE-9:



Formula IE-9,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

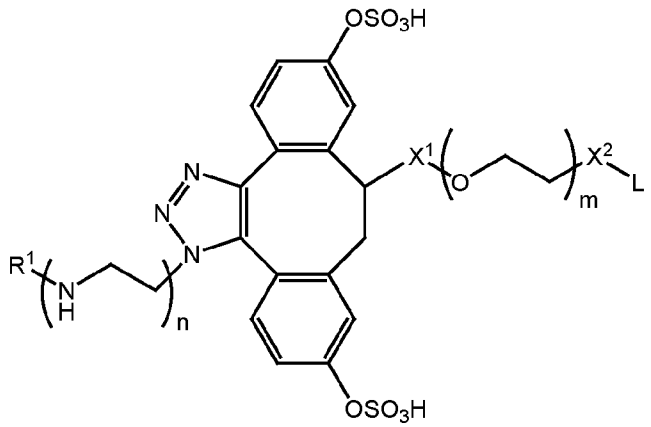
In some embodiments, the composition comprises a conjugate of the Formula IE-9a:



Formula IE-9a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

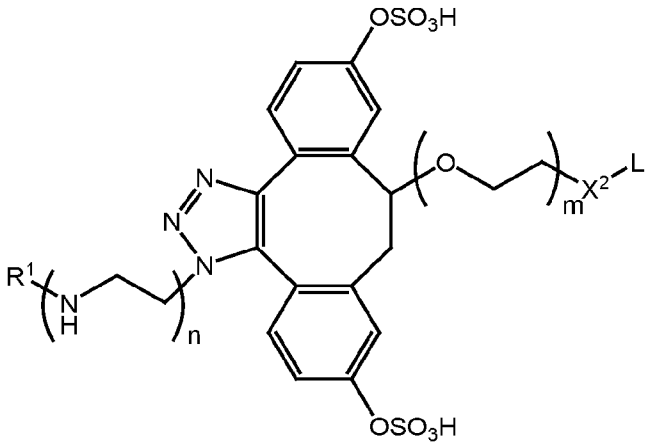
In some embodiments, the composition comprises a conjugate of the Formula IE-10:



Formula IE-10,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

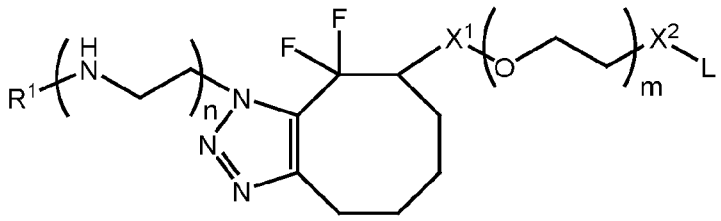
In some embodiments, the composition comprises a conjugate of the Formula IE-10a:



Formula IE-10a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

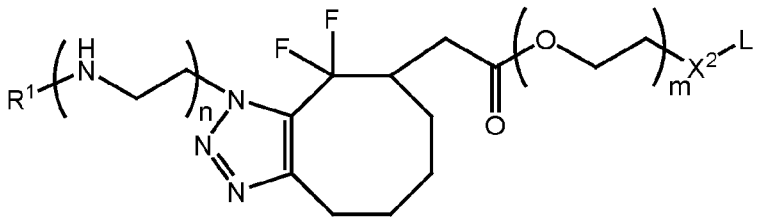
In some embodiments, the composition comprises a conjugate of the Formula IE-11:



Formula IE-11,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

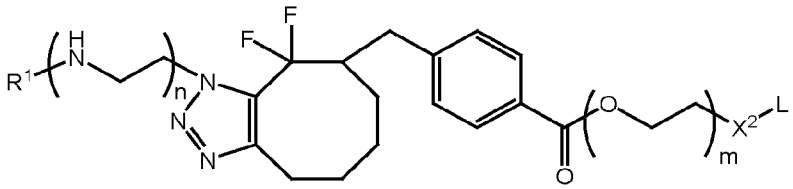
In some embodiments, the composition comprises a conjugate of the Formula IE-11a:



Formula IE-11a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

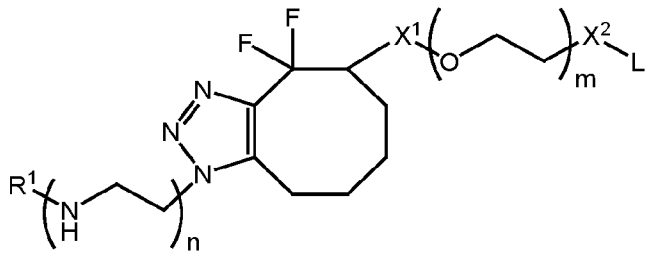
In some embodiments, the composition comprises a conjugate of the Formula IE-11b:



Formula IE-11b,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

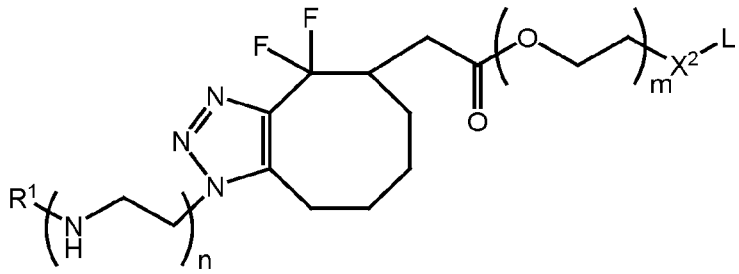
In some embodiments, the composition comprises a conjugate of the Formula IE-12:



Formula IE-12,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

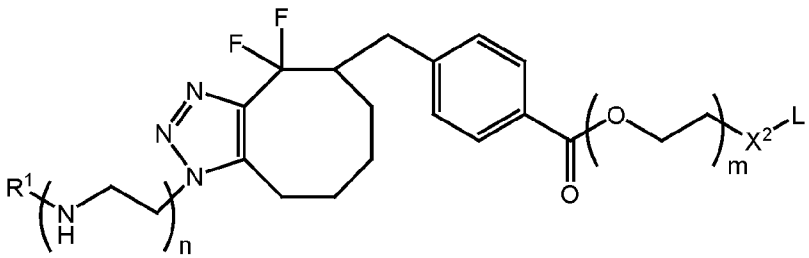
In some embodiments, the composition comprises a conjugate of the Formula IE-12a:



Formula IE-12a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IE-12b:

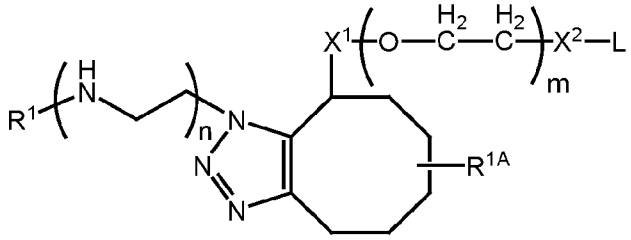


Formula IE-12b,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less,

and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

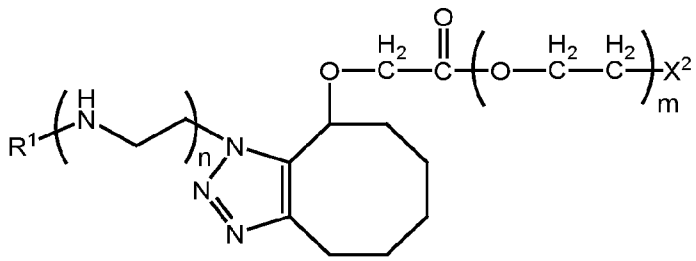
In some embodiments, the composition comprises a conjugate of the Formula IE-13:



Formula IE-13,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

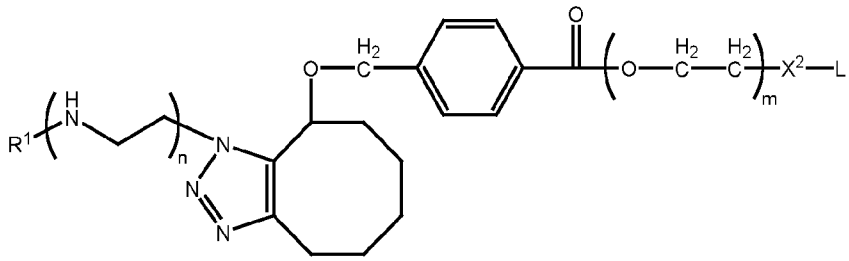
In some embodiments, the composition comprises a conjugate of the Formula IE-13a:



Formula IE-13a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

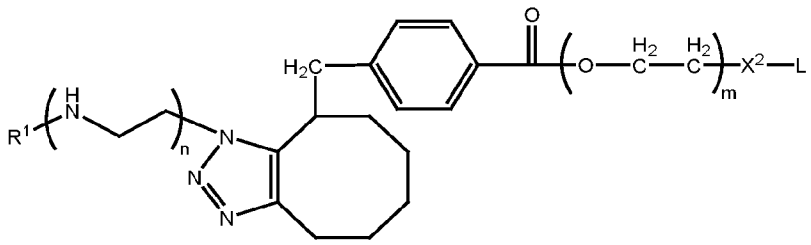
In some embodiments, the composition comprises a conjugate of the Formula IE-13b:



Formula IE-13b,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

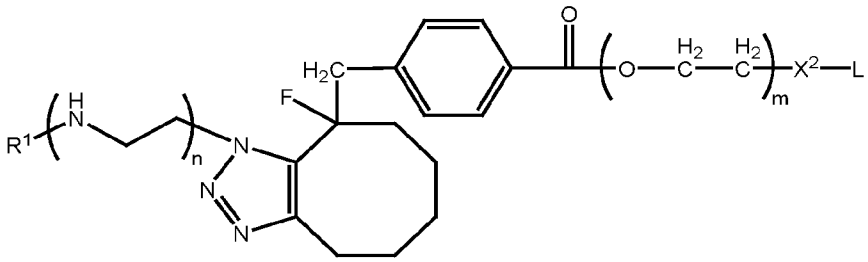
In some embodiments, the composition comprises a conjugate of the Formula IE-13c:



Formula IE-13c,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IE-13d:

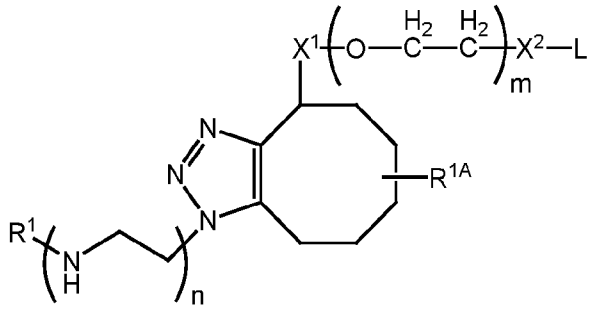


Formula IE-13d,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said

discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

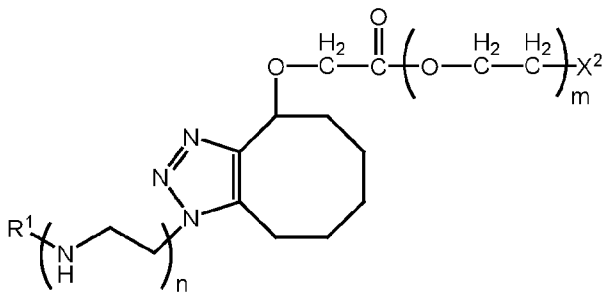
In some embodiments, the composition comprises a conjugate of the Formula IE-14:



Formula IE-14,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

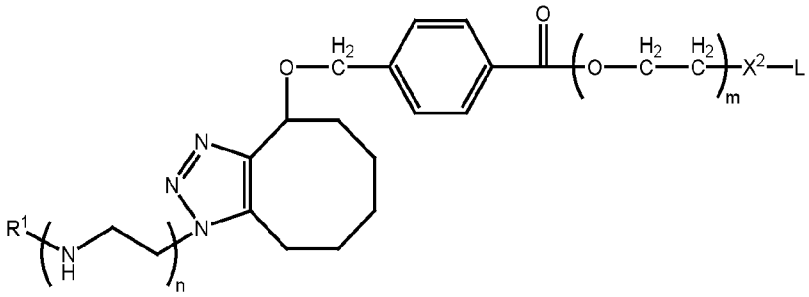
In some embodiments, the composition comprises a conjugate of the Formula IE-14a:



Formula IE-14a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

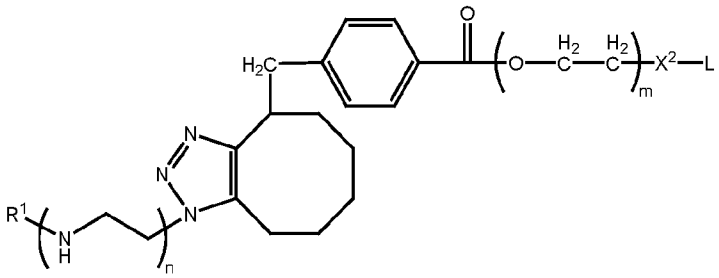
In some embodiments, the composition comprises a conjugate of the Formula IE-14b:



Formula IE-14b,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

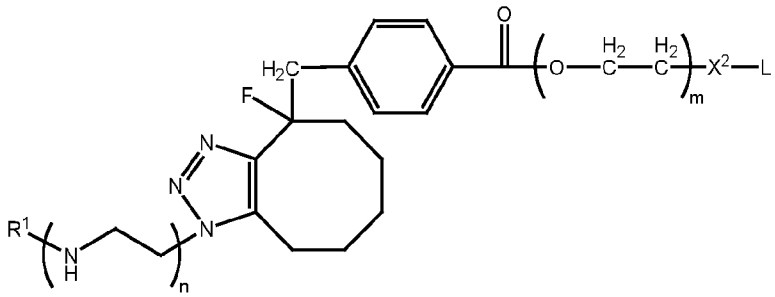
In some embodiments, the composition comprises a conjugate of the Formula IE-14c:



Formula IE-14c,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IE-14d:

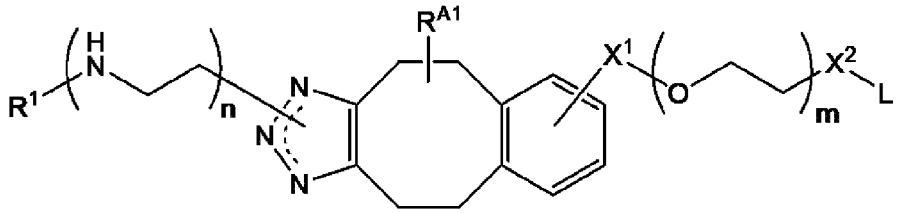


Formula IE-14d,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less,

and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

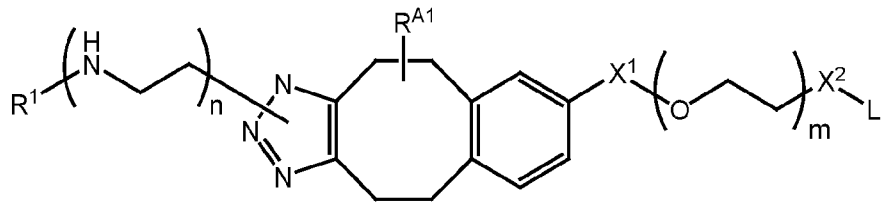
In some embodiments, the composition comprises a conjugate of the Formula IH:



Formula IH,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

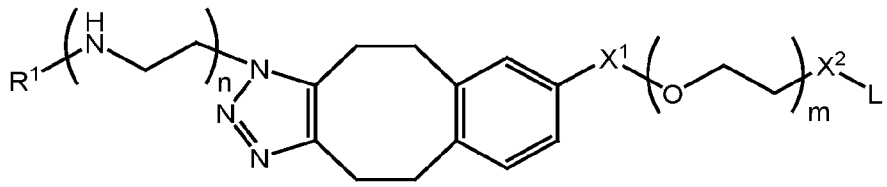
In some embodiments, the composition comprises a conjugate of the Formula IH':



Formula IH',

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IH-1:

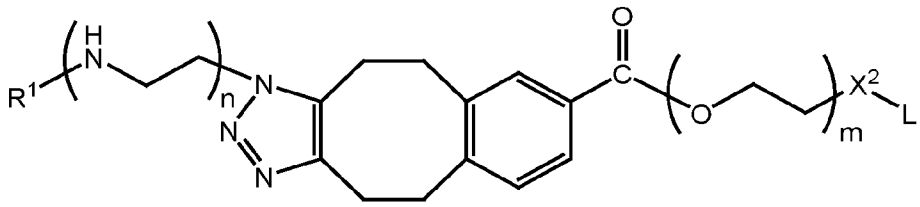


Formula IH-1,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less,

and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

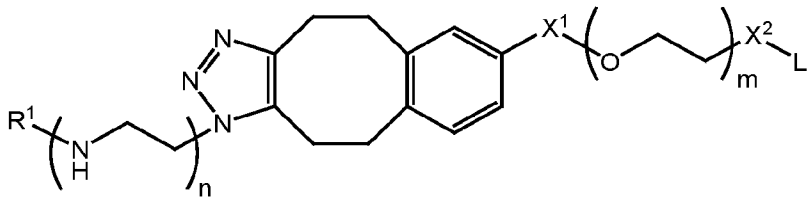
In some embodiments, the composition comprises a conjugate of the Formula IH-1a:



Formula IH-1a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

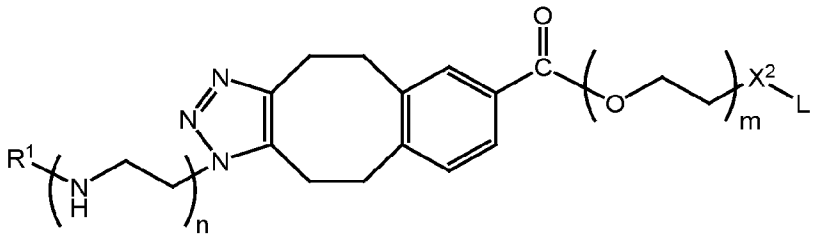
In some embodiments, the composition comprises a conjugate of the Formula IH-2:



Formula IH-2,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

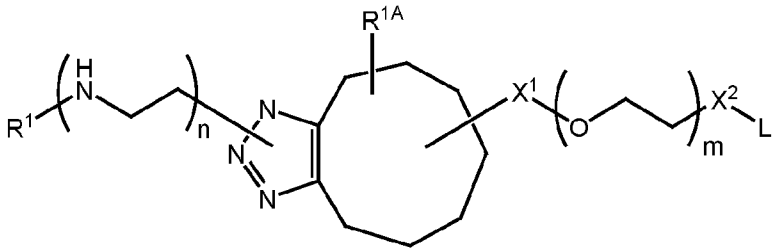
In some embodiments, the composition comprises a conjugate of the Formula IH-2a:



Formula IH-2a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

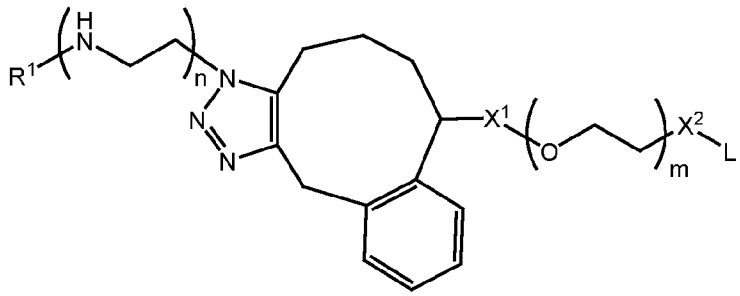
In some embodiments, the composition comprises a conjugate of the Formula IJ:



Formula IJ

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

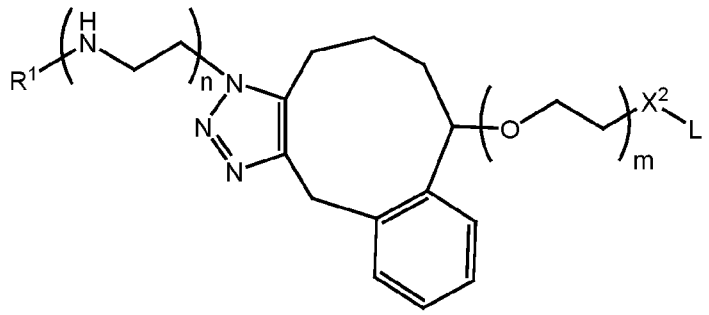
In some embodiments, the composition comprises a conjugate of the Formula IJ-1:



Formula IJ-1,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

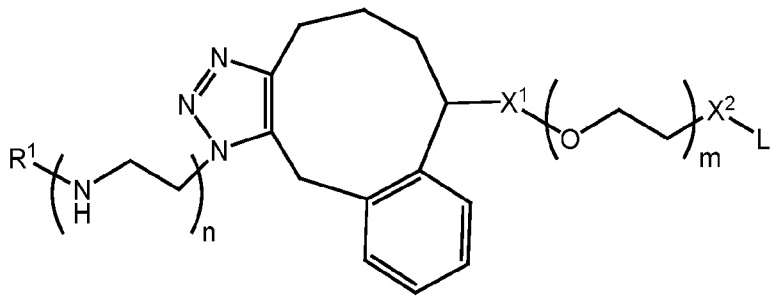
In some embodiments, the composition comprises a conjugate of the Formula IJ-1a:



Formula IJ-1a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

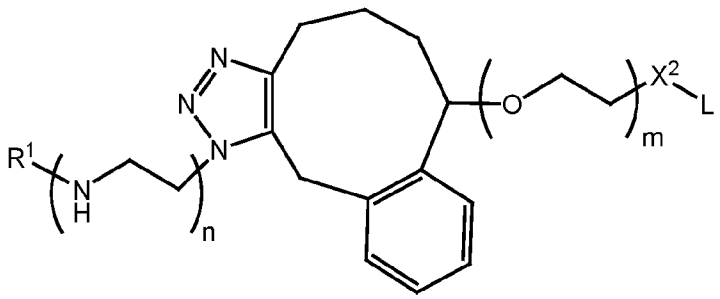
In some embodiments, the composition comprises a conjugate of the Formula IJ-2:



Formula IJ-2,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

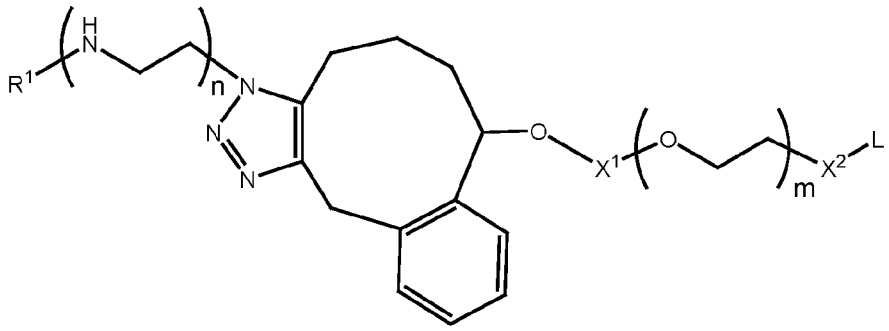
In some embodiments, the composition comprises a conjugate of the Formula IJ-2a:



Formula IJ-2a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

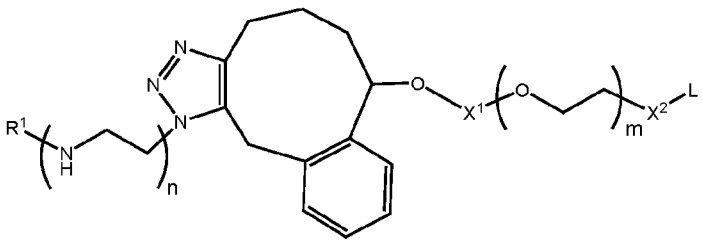
In some embodiments, the composition comprises a conjugate of the Formula IJ-3:



Formula IJ-3,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

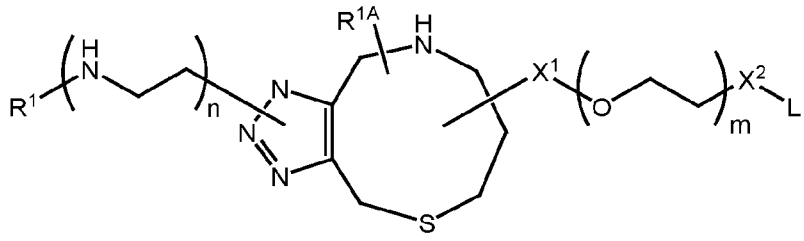
In some embodiments, the composition comprises a conjugate of the Formula IJ-4:



Formula IJ-4,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

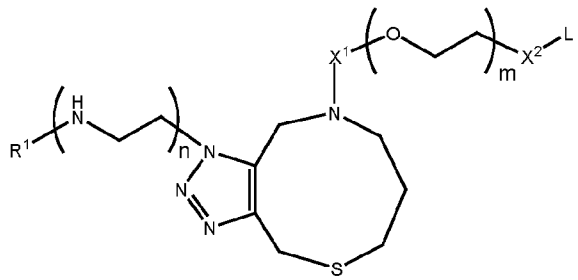
In some embodiments, the composition comprises a conjugate of the Formula IK:



Formula IK,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

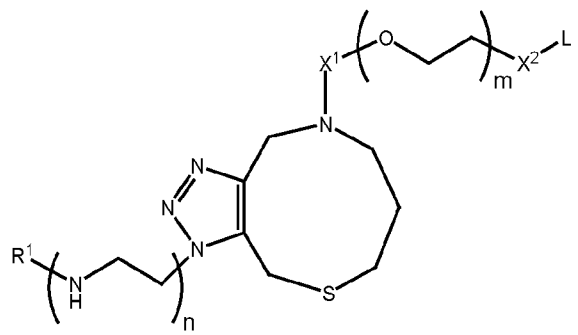
In some embodiments, the composition comprises a conjugate of the Formula IK-1:



Formula IK-1,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IK-2:

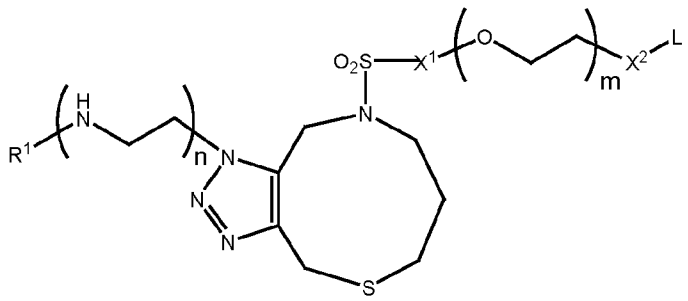


Formula IK-2,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less,

and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

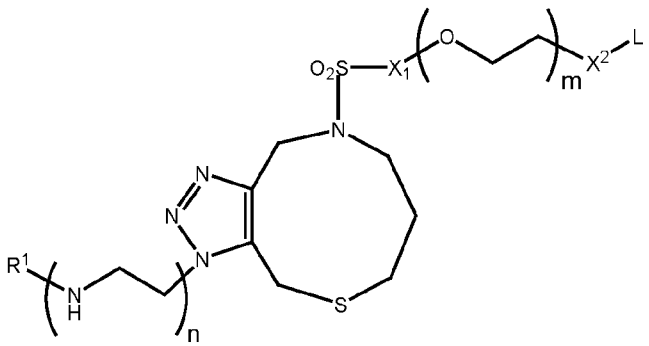
In some embodiments, the composition comprises a conjugate of the Formula IK-3:



Formula IK-3,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

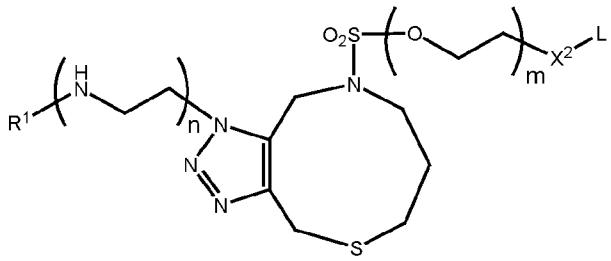
In some embodiments, the composition comprises a conjugate of the Formula IK-4:



Formula IK-4,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

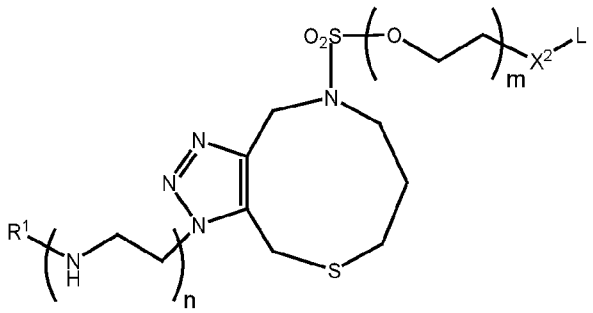
In some embodiments, the composition comprises a conjugate of the Formula IK-3a:



Formula IK-3a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

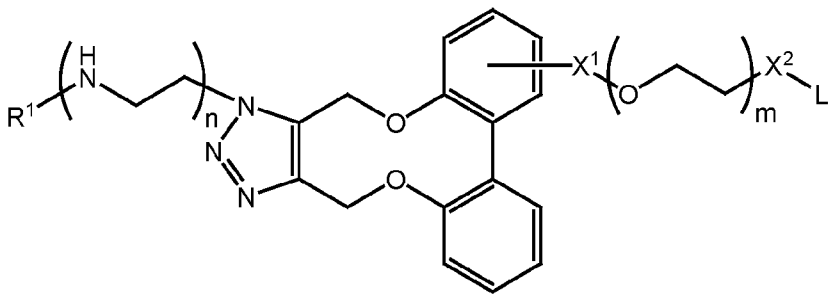
In some embodiments, the composition comprises a conjugate of the Formula IK-4a:



Formula IK-4a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IL:

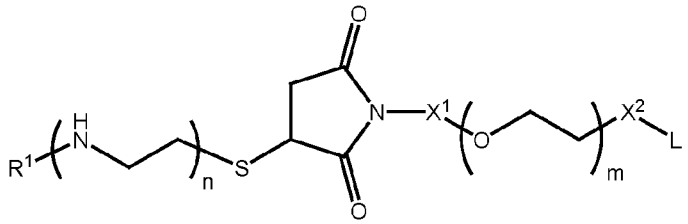


Formula IL,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less,

and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

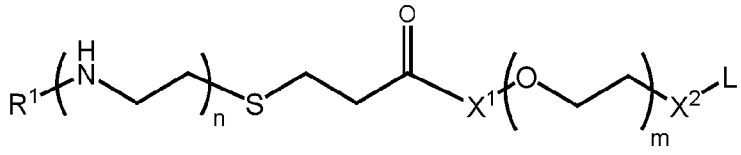
In some embodiments, the composition comprises a conjugate of the Formula IM:



Formula IM,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

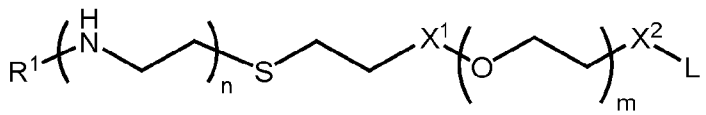
In some embodiments, the composition comprises a conjugate of the Formula IN:



Formula IN,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, the composition comprises a conjugate of the Formula IO:

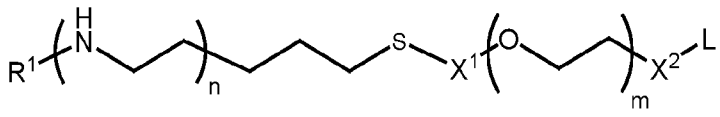


Formula IO,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said

discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

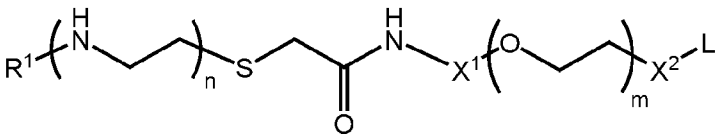
In some embodiments, the composition comprises a conjugate of the Formula IP:



Formula IP,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

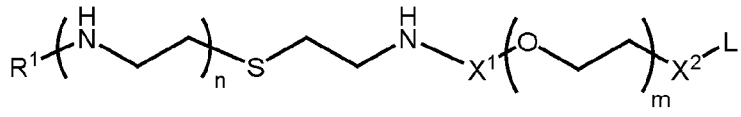
In some embodiments, the composition comprises a conjugate of the Formula IQ:



Formula IQ,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

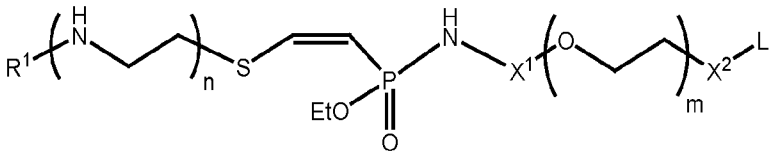
In some embodiments, the composition comprises a conjugate of the Formula IR:



Formula IR,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

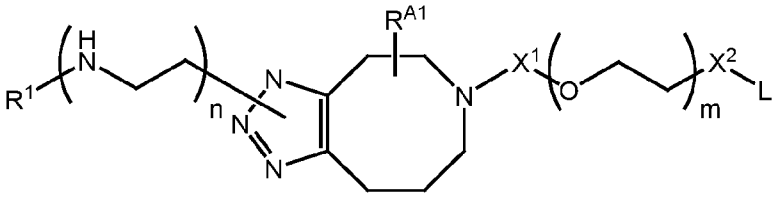
In some embodiments, the composition comprises a conjugate of the Formula IS:



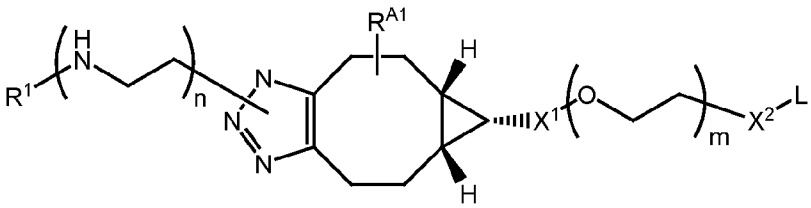
Formula IS,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

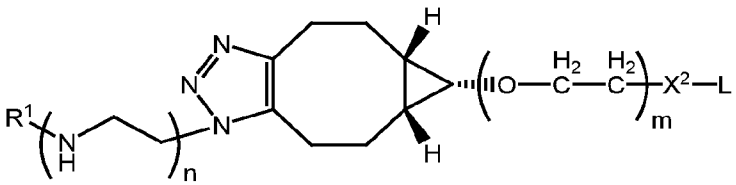
In a further preferred embodiment, said conjugate of Formula I is selected from:



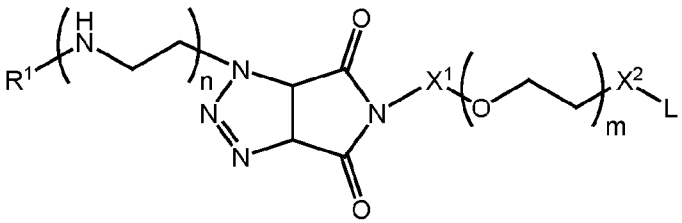
Formula IA,



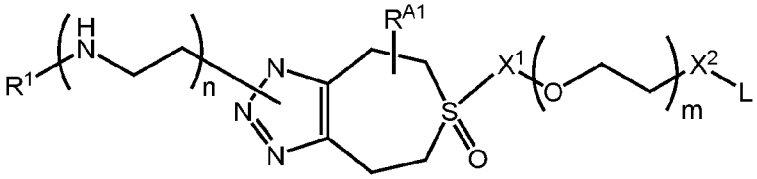
Formula IB,



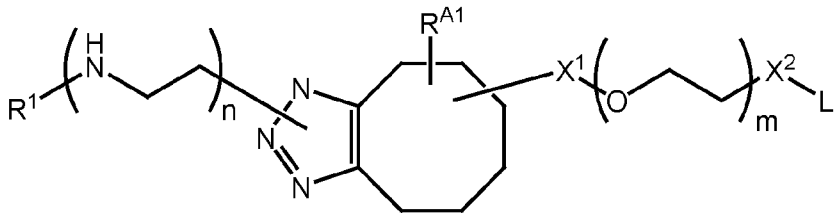
Formula IB-2a,



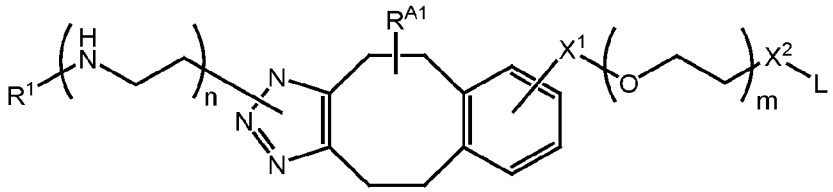
Formula IC,



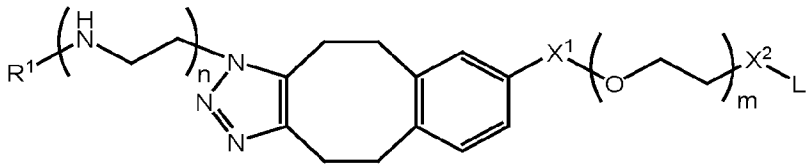
Formula ID,



Formula IE,



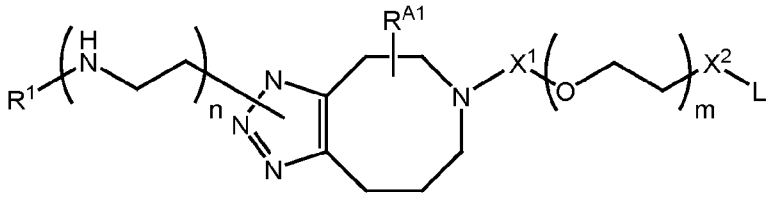
Formula IH,



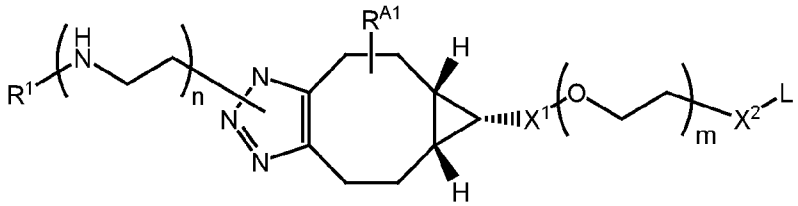
Formula IH-1,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

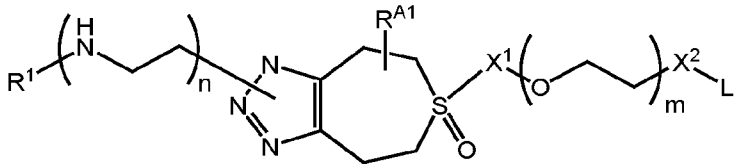
In some embodiments, said conjugate of Formula I is selected from:



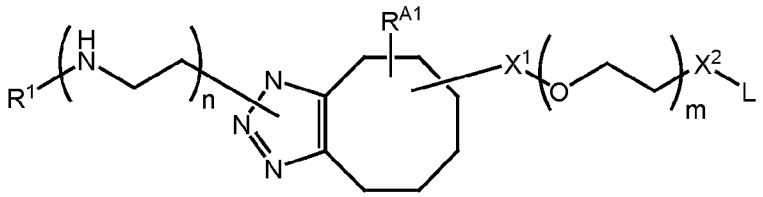
Formula IA,



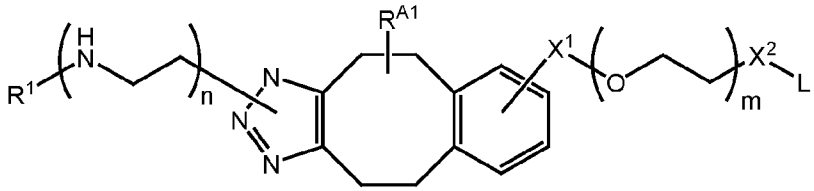
Formula IB,



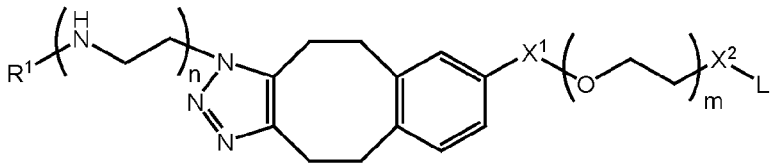
Formula ID,



Formula IE,



Formula IH, and

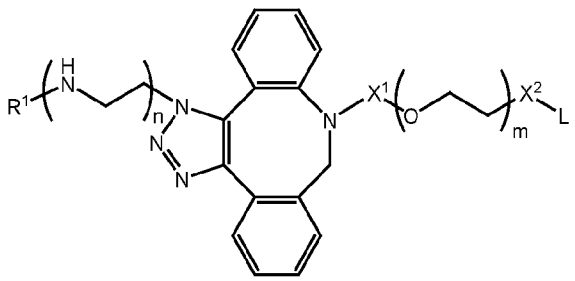


Formula IH-1,

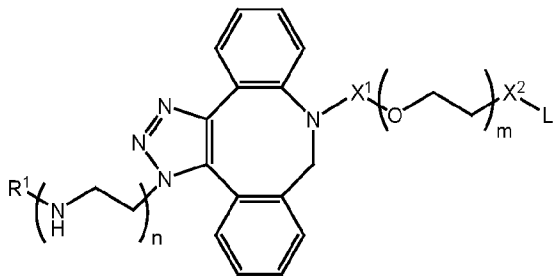
preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In preferred embodiments, of any of Formulae IA, IB, IC, ID, IE, and/or IH, R^{A1} is -H.

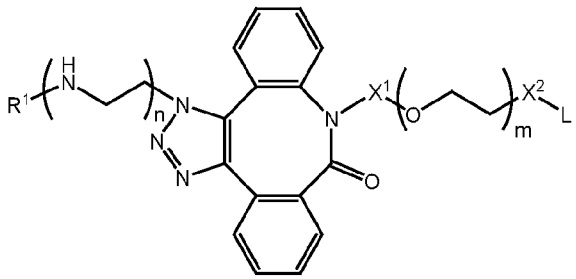
In another preferred embodiment, said conjugate of Formula I is selected from:



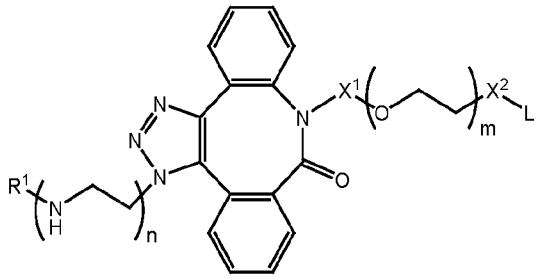
Formula IA-3,



Formula IA-4,



Formula IA-9,



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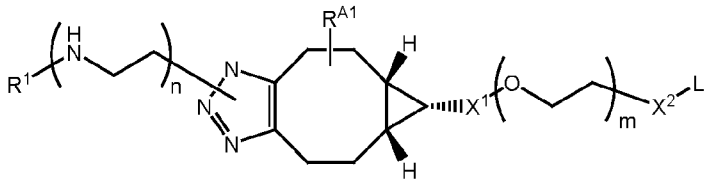
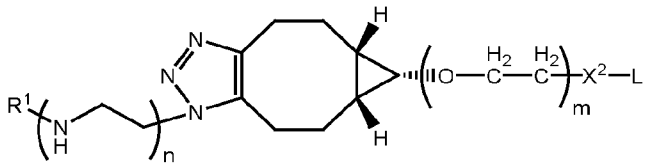


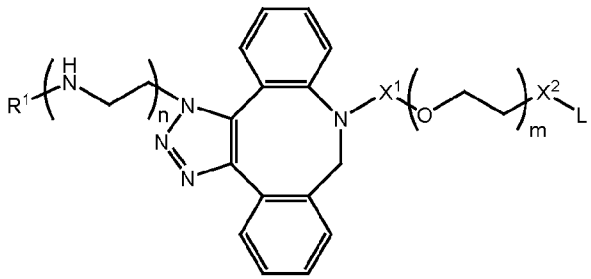
Formula IB,



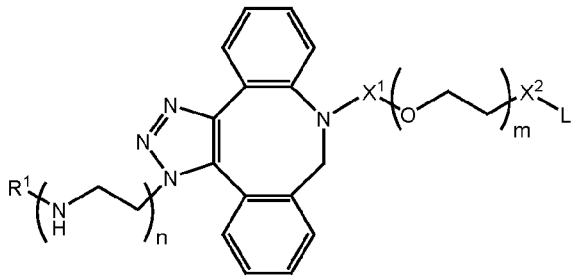
Formula IB-2a,

preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

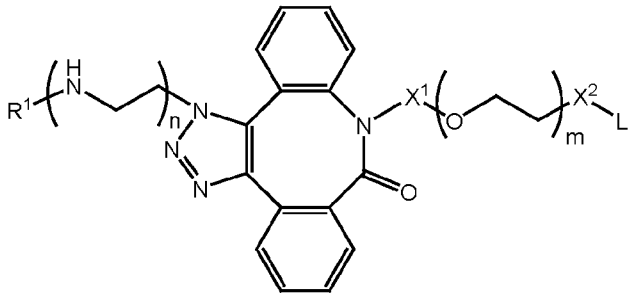
In some embodiments, said conjugate of Formula I is selected from:



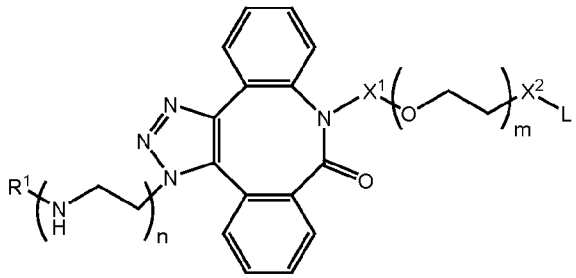
Formula IA-3,



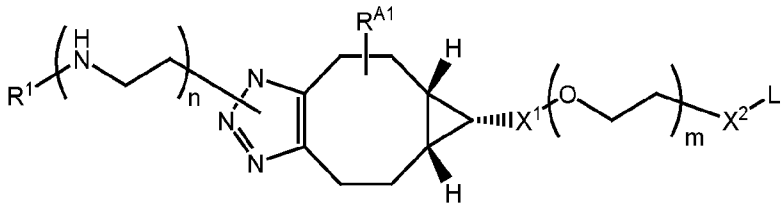
Formula IA-4,



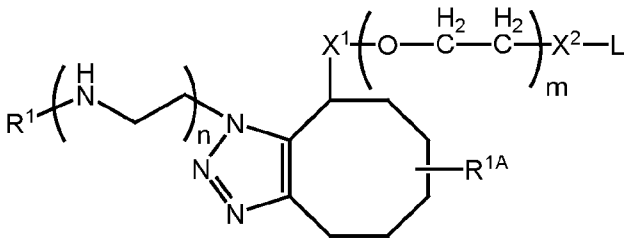
Formula IA-9,



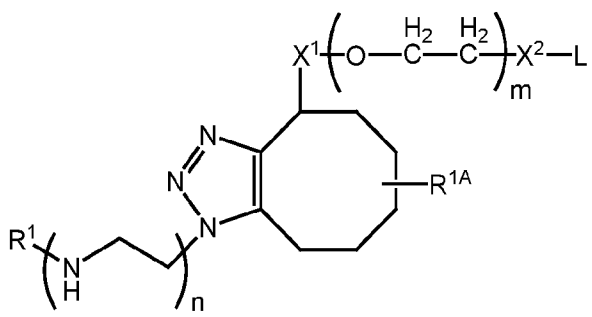
Formula IA-10,



Formula IB,



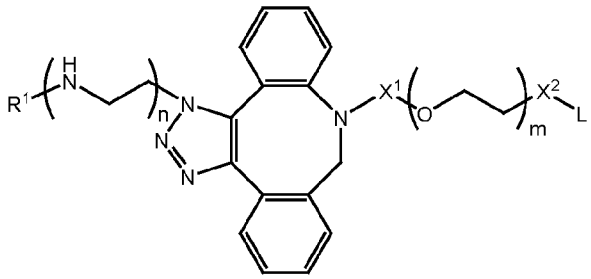
Formula IE-13, and



Formula IE-14,

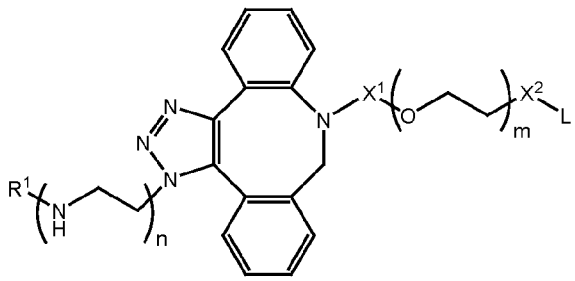
preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m is any integer of 25 to 100, preferably of 25 to 60, and further preferably wherein m is 36.

In some embodiments, said conjugate of Formula I is selected from:



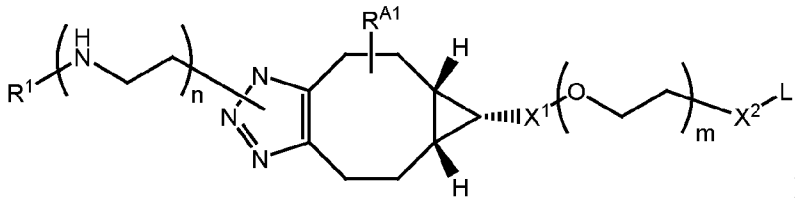
Formula IA-3,

and



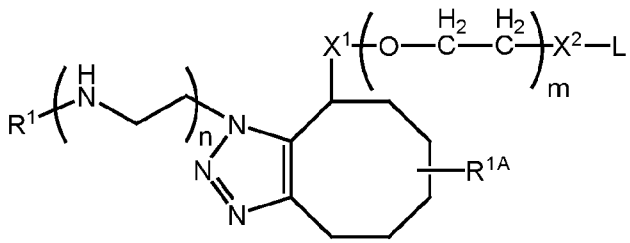
Formula IA-4.

In some embodiments, said conjugate of Formula I is selected from:

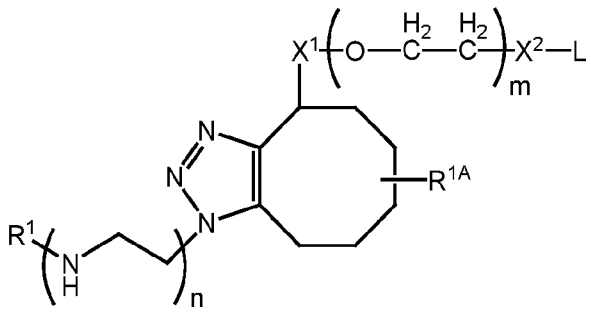


Formula IB.

In some embodiments, said conjugate of Formula I is selected from:



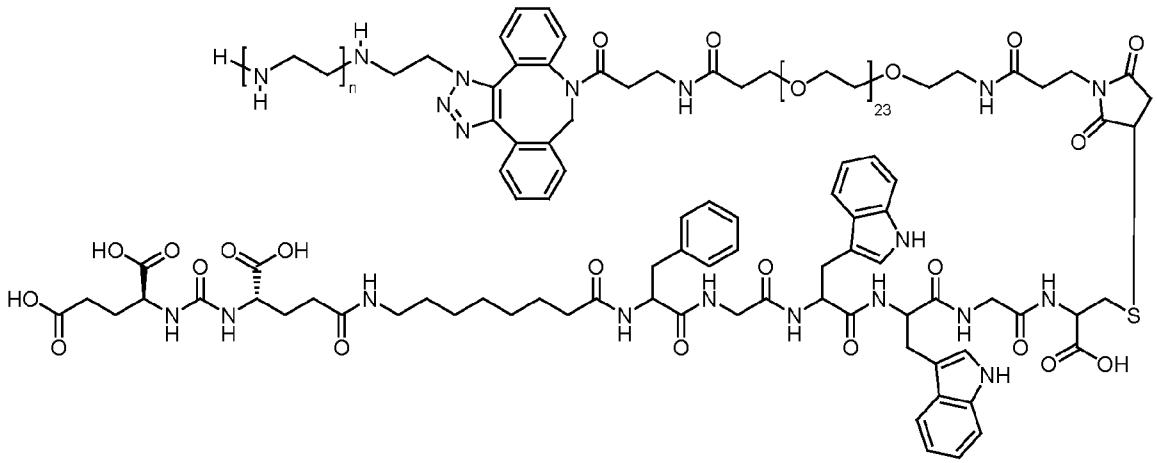
Formula IE-13, and



Formula IE-14.

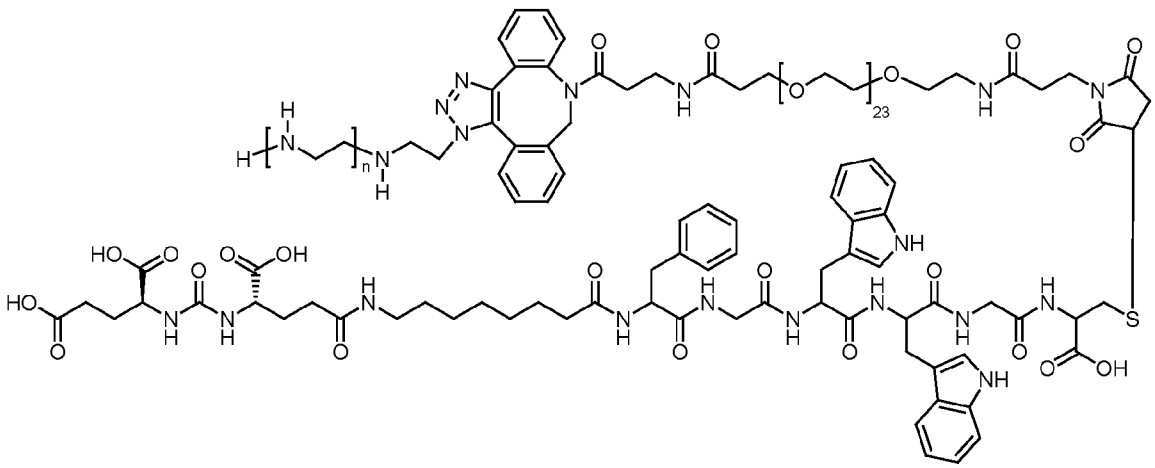
:

In some embodiments, the composition comprises a conjugate of the formula:



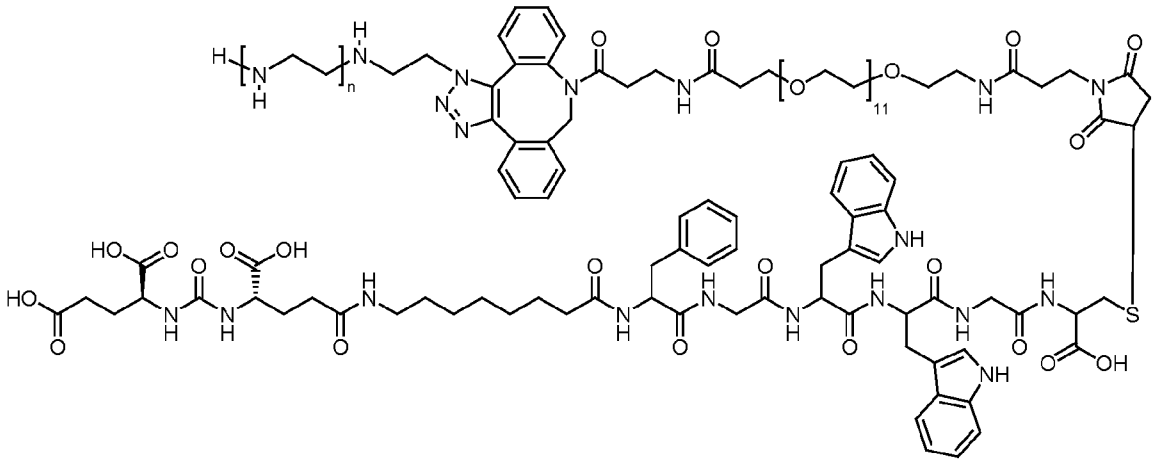
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



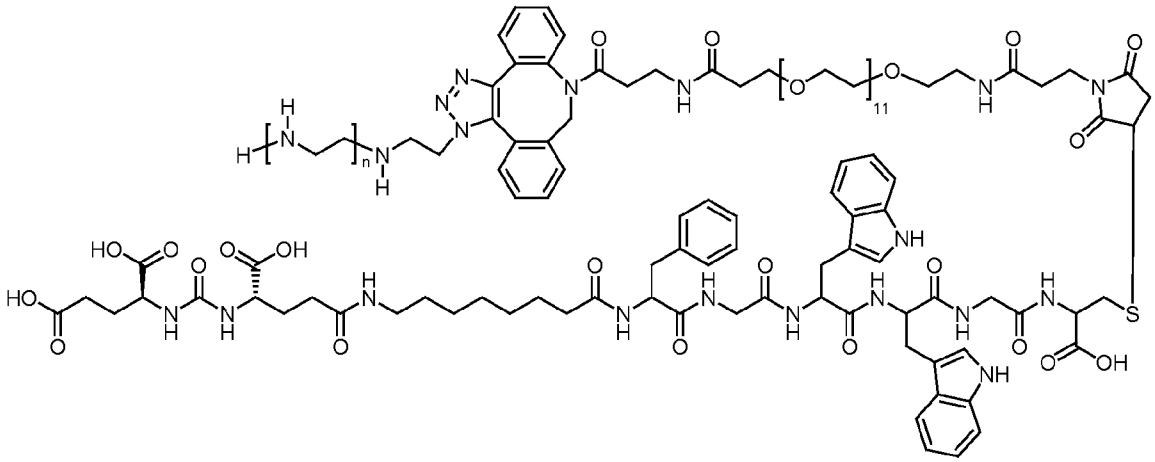
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



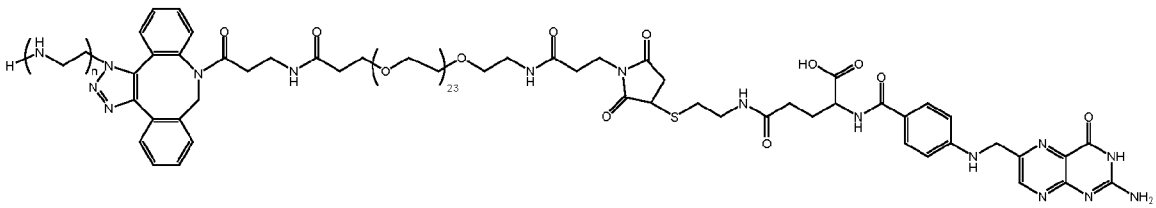
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



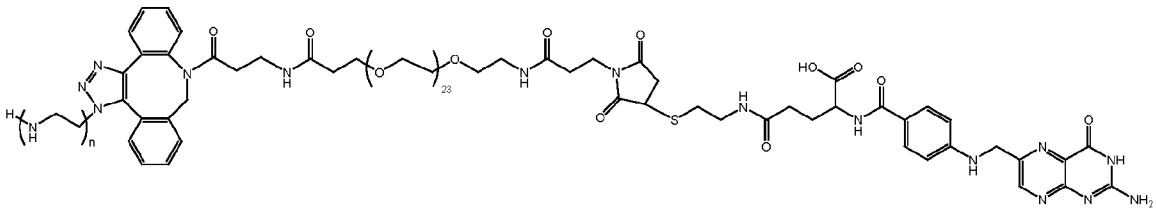
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



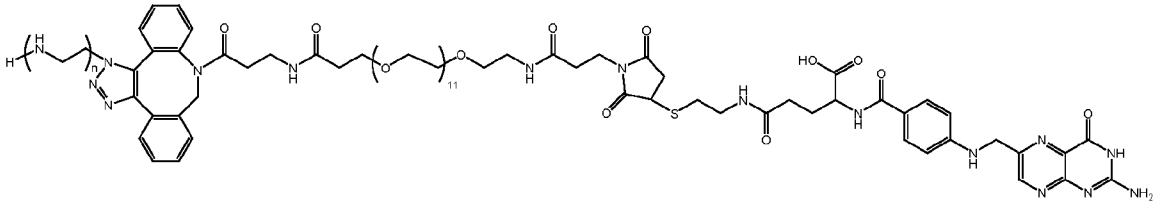
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



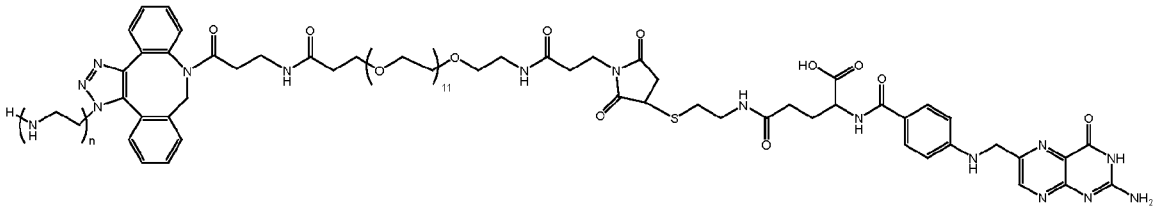
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less

In some embodiments, the composition comprises a conjugate of the formula:



preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In a preferred embodiment, the composition comprises a conjugate comprising Compound 12a, Compound 12b, Compound 19a, Compound 19b, Compound 24, Compound 28a, Compound 28b, Compound 32a, Compound 32b, Compound 37a, Compound 37b, Compound 43, Compound 44a, Compound 44b, Compound 45, Compound 49a, and/or Compound 49b.

In a preferred embodiment, the composition comprises a conjugate selected from Compound 12a, Compound 12b, Compound 19a, Compound 19b, Compound 24, Compound 28a, Compound 28b, Compound 32a, Compound 32b, Compound 37a, Compound 37b, Compound 43, Compound 44a, Compound 44b, Compound 45, Compound 49a, and/or Compound 49b.

In a preferred embodiment, the composition comprises a conjugate comprising Compound 12a and/or Compound 12b. In a preferred embodiment, the composition comprises a conjugate comprising Compound 19a and/or Compound 19b. In a preferred embodiment, the composition comprises a conjugate comprising Compound 24. In a preferred embodiment, the composition comprises a conjugate comprising Compound 28a and/or Compound 28b. In a preferred embodiment, the composition comprises a conjugate comprising Compound 32a and/or Compound 32b. In a preferred embodiment, the composition comprises a conjugate comprising Compound 37a and/or Compound 37b. In a preferred embodiment, the composition comprises a conjugate comprising Compound 43. In a preferred embodiment, the composition comprises a conjugate comprising Compound 44a and/or Compound 44b. In a preferred embodiment, the composition comprises a conjugate comprising Compound 45. In a preferred

embodiment, the composition comprises a conjugate comprising Compound 49a, and/or Compound 49b.

In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 12a and/or Compound 12b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 19a and/or Compound 19b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 24. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 28a and/or Compound 28b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 32a and/or Compound 32b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 37a and/or Compound 37b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 43. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 44a and/or Compound 44b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 45. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 49a, and/or Compound 49b.

Polyplexes

The inventive compositions further comprise polyanions, preferably wherein said polyanions are a nucleic acids, and wherein said polyanions and said conjugates preferably form polyplexes. In a preferred embodiment, said polyanion is non-covalently bound to said conjugate. This facilitates the dissociation of the polyanion and, preferably the nucleic acid, from the targeting fragment following arrival to the targeted cell or tissue and its internalization in the targeted cell or tissue, preferably tumor cell or tumort issue causing the production of, for example, chemokines, as shown herein. The production of chemokines will attract immune cells to the tumor site.

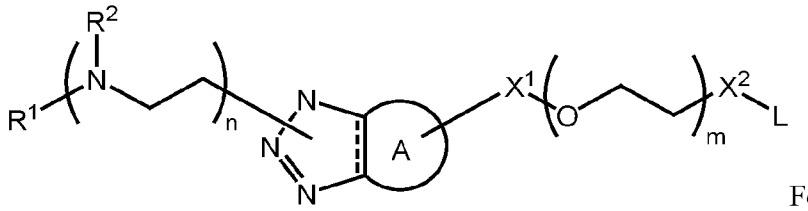
The inventive polyplex provides efficient delivery of the polyanion, preferably the nucleic acid, into cells harboring the target cell surface receptor. As described herein, the targeting fragment comprised by the inventive polyplex is capable of binding to the target cell surface receptor.

In a preferred embodiment, said polyanion is a nucleic acid. In a preferred embodiment, said nucleic acid is a dsRNA. In a very preferred embodiment, said dsRNA is polyinosinic:polycytidylic acid (poly(IC)). In a preferred embodiment, said nucleic acid is a

ssRNA. In a very preferred embodiment, said ssRNA is a mRNA. In another preferred embodiment said polyanion is a nucleic acid, wherein said nucleic acid is a DNA, wherein preferably said DNA is a plasmid DNA.

Thus, in another aspect, the present invention provides a polyplex comprising a conjugate as described herein and a polyanion, wherein said polyanion is preferably non-covalently bound to said conjugate. In a preferred embodiment, said conjugate is a conjugate of Formula I* or is a conjugate of Formula I. In a preferred embodiment, said polyanion is a nucleic acid. In a preferred embodiment, said polyanion is a nucleic acid, wherein said nucleic acid is a RNA. In a preferred embodiment, said RNA is a ssRNA or dsRNA. In a preferred embodiment, said RNA is a ssRNA. In another preferred embodiment, said RNA is a dsRNA. In a preferred embodiment, said ssRNA is a mRNA. In a preferred embodiment, said dsRNA is polyinosinic:polycytidylic acid poly(IC). In a preferred embodiment, said RNA is a mRNA or poly(IC). In a preferred embodiment, said RNA is a mRNA. In a preferred embodiment, said RNA is polyinosinic:polycytidylic acid (poly(IC).

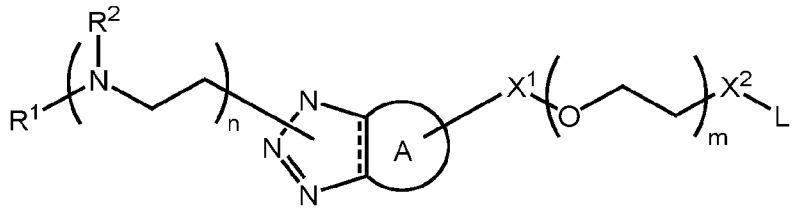
In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof, and a nucleic acid, wherein said nucleic acid is preferably non-covalently bound to said conjugate



Formula I,

wherein A, R^1, R^2, X^1, X^2. L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter A, R^1, R^2, X^1, X^2, L, m and n or collectively to some or all of A, R^1, R^2, X^1, X^2, L m and n.

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof, and a nucleic acid, wherein said nucleic acid is preferably non-covalently bound to said conjugate:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500, preferably any integer between 2 and 1500;

m is a discrete number of repeating units m of 25 to 100, preferably of a discrete number of repeating units m of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

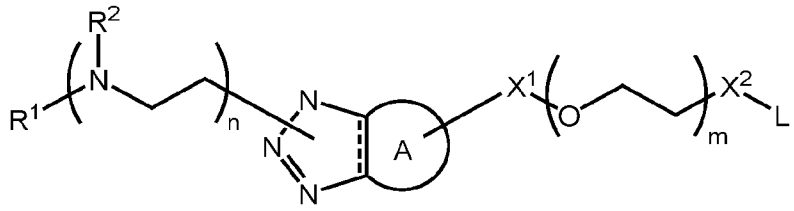
X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment capable of binding to a cell expressing PSMA, preferably overexpressing PSMA, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, wherein said target cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$. In a preferred embodiment, said nucleic acid is a RNA. In a preferred embodiment, said RNA is a ssRNA or dsRNA. In a preferred embodiment, said RNA is a ssRNA. In another preferred embodiment, said RNA is a dsRNA. In a preferred embodiment, said RNA is a mRNA or poly(IC). In a preferred embodiment, said RNA is a mRNA. In a preferred embodiment, said RNA is polyinosinic:polycytidylic acid (poly(IC). In a preferred embodiment, said ssRNA is a mRNA. In a preferred embodiment, said dsRNA is polyinosinic:polycytidylic acid poly(IC). In another preferred embodiment said nucleic acid is a DNA, wherein preferably said DNA is a plasmid DNA.

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer

thereof, and a nucleic acid, wherein said nucleic acid is preferably non-covalently bound to said conjugate:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500, preferably any integer between 2 and 1500;

m is a discrete number of repeating units m of 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, preferably overexpressing PSMA, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$. In a preferred embodiment, said nucleic acid is a RNA. In a preferred embodiment, said RNA is a ssRNA or dsRNA. In a preferred embodiment, said RNA is a ssRNA. In another preferred embodiment, said RNA is a dsRNA. In a preferred embodiment, said RNA is a mRNA or poly(IC). In a preferred embodiment, said RNA is a mRNA. In a preferred embodiment, said RNA is polyinosinic:polycytidylic acid (poly(IC). In a preferred embodiment, said ssRNA is a mRNA. In a preferred embodiment, said dsRNA is polyinosinic:polycytidylic acid poly(IC). In

another preferred embodiment said nucleic acid is a DNA, wherein preferably said DNA is a plasmid DNA.

The term "RNA" as used herein relates to a nucleic acid which comprises ribonucleotide residues and preferably being entirely or substantially composed of ribonucleotide residues. "Ribonucleotide" relates to a nucleotide with a hydroxyl group at the 2'-position of a β-D-ribofuranosyl group. The term "RNA" as used herein comprises double stranded RNA (dsRNA) and single stranded RNA (ssRNA). The term "RNA" further includes isolated RNA such as partially or completely purified RNA, essentially pure RNA, synthetic RNA, recombinantly generated RNA, *in vitro* transcribed RNA, *in vivo* transcribed RNA from a template such as a DNA template, and replicon RNA, in particular self-replicating RNA, and includes modified RNA which differs from naturally occurring RNA by addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of an RNA or internally. The RNA may have modified naturally occurring or synthetic ribonucleotides. Nucleotides in RNA can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides.

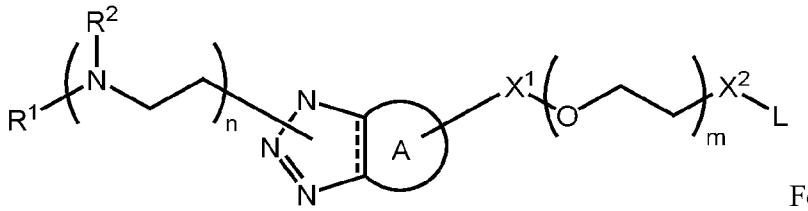
The term "single stranded RNA (ssRNA)" generally refers to an RNA molecule to which no complementary nucleic acid molecule (typically no complementary RNA molecule) is associated. ssRNA may contain self-complementary sequences that allow parts of the RNA to fold back and pair with itself to form double helices and secondary structure motifs including without limitation base pairs, stems, stem loops and bulges. The size of the ssRNA strand may vary from 8 nucleotides up to 20000 nucleotides.

The term "double stranded RNA (dsRNA)" is RNA with two partially or completely complementary strands. The dsRNA is preferably a fully or partially (interrupted) pair of RNA hybridized together. It can be prepared for example by mixing partially or completely complementary strands ssRNA molecules. It also can be made by mixing defined fully or partially pairing non- homopolymeric or homopolymeric RNA strands. The size of the dsRNA strands may vary from 8 nucleotides up to 20000 nucleotides independently for each strand.

In a preferred embodiment, the RNA is a ssRNA. In a preferred embodiment, the RNA is a ssRNA consisting of one single strand of RNA. Single stranded RNA can exist as minus strand [(-) strand] or as plus strand [(+) strand]. The (+) strand is the strand that comprises or encodes genetic information. The genetic information may be for example a nucleic acid sequence encoding a protein or polypeptide. When the (+) strand RNA encodes a protein, the

(+) strand may serve directly as template for translation (protein synthesis). The (-) strand is the complement of the (+) strand. In the case of ssRNA, (+) strand and (-) strand are two separate RNA molecules. (+) strand and (-) strand RNA molecules may associate with each other to form a double-stranded RNA ("duplex RNA").

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, and a RNA, wherein said RNA is preferably non-covalently bound to said conjugate



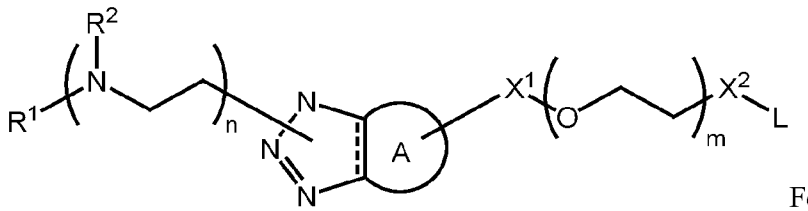
Formula I,

wherein A, R^1, R^2, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter A, R^1, R^2, X^1, X^2, L,m and n, or collectively to some or all of A, R^1, R^2, X^1, X^2 L, m and n.

In a preferred embodiment, size of the RNA strand may vary from 8 nucleotides up to 20000 nucleotides.

In a preferred embodiment, said RNA is a ssRNA or a dsRNA. In a preferred embodiment, said ssRNA is a mRNA. In a preferred embodiment, said dsRNA is polyinosinic:polycytidylic acid (poly(IC)). In a preferred embodiment, said RNA is a mRNA or poly(IC). In a preferred embodiment, said RNA is a mRNA. In a preferred embodiment, said RNA is polyinosinic:polycytidylic acid (poly(IC)). In another preferred embodiment said nucleic acid is a DNA, wherein preferably said DNA is a plasmid DNA.

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, and a mRNA, wherein said mRNA is preferably non-covalently bound to said conjugate



Formula I

wherein A, R^1, R^2, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter A, R^1, R^2, X^1, X^2, L,m and n, or collectively to some or all of A, R^1, R^2, X^1, X^2 L, m and n.

In a preferred embodiment, said RNA is a "messenger-RNA" (mRNA). In preferred

embodiments, the term mRNA relates to a RNA transcript which encodes a peptide or protein. mRNA may be modified by stabilizing modifications and capping. Typically, a mRNA comprises a 5' untranslated region (5'-UTR), a protein coding region, and a 3' untranslated region (3'-UTR). Preferably, mRNA, in particular synthetic mRNA, contains a 5′ cap, UTRs embracing the coding region and a 3′ poly(A) tail. In one embodiment, the mRNA is produced by *in vitro* transcription using a DNA template where DNA refers to a nucleic acid that contains deoxyribonucleotides. The term "untranslated region" or "UTR" relates to a region in a DNA molecule which is transcribed but is not translated into an amino acid sequence, or to the corresponding region in an RNA molecule, such as an mRNA molecule. An untranslated region (UTR) can be present 5' (upstream) of an open reading frame (5'-UTR) and/or 3' (downstream) of an open reading frame (3'-UTR). A 3'-UTR, if present, is preferably located at the 3' end of a gene, downstream of the termination codon of a protein-encoding region, but the term "3'-UTR" does preferably not include the poly(A) tail. Thus, the 3'-UTR is preferably upstream of the poly(A) tail (if present), e.g. directly adjacent to the poly(A) tail. A 5'-UTR, if present, is preferably located at the 5' end of a gene, upstream of the start codon of a protein-encoding region. A 5'-UTR is preferably downstream of the 5'-cap (if present), e.g. directly adjacent to the 5'-cap. 5'- and/or 3'-untranslated regions may, according to the invention, be functionally linked to an open reading frame, so as for these regions to be associated with the open reading frame in such a way that the stability and/or translation efficiency of the RNA comprising said open reading frame are increased. The terms "poly(A) sequence" or "poly(A) tail" refer to an uninterrupted or interrupted sequence of adenylate residues which is typically located at the 3' end of a mRNA molecule. An uninterrupted sequence is characterized by consecutive adenylate residues. While a poly(A) sequence is normally not encoded in eukaryotic DNA, but is attached during eukaryotic transcription in the cell nucleus to the free 3' end of the RNA by a template-independent RNA polymerase after transcription, the present invention also encompasses poly(A) sequences encoded by DNA. Terms such as "5'-cap", "cap", "5'-cap structure", or "cap structure" are used synonymously and refer preferably to a nucleotide modification at the 5' end of the mRNA, more preferably to a dinucleotide that is found on the mRNA 5' end. A 5'-cap can be a structure wherein a (optionally modified) guanosine is bonded to the first nucleotide of an mRNA molecule via a 5' to 5' triphosphate linkage (or modified triphosphate linkage in the case of certain cap analogs). The term cap can refer to a naturally occurring cap or modified cap. RNA molecules may be characterized by a 5'-cap, a 5'- UTR, a 3'-UTR, a poly(A) sequence, and/or adaptation of the codon usage. The mRNA may be generated by

chemical synthesis, *in vivo* or *in vitro* transcription, e.g. from a DNA or other nucleic acid template, or it may be recombinantly prepared or viral RNA. The mRNA includes non-self-amplifying mRNAs, such as endogenous mRNAs of mammalian cells, and self-amplifying mRNAs. Endogenous mRNA includes pre-mature and mature mRNA. The mRNA is preferably exogenous mRNA that has to enter the cell from outside the cell, e.g. by directly passing through the cytoplasmic membrane or by endocytosis followed by endosomal escape. mRNA preferably does not enter the nucleus, nor integrates into the genome. In a preferred embodiment, said mRNAs have a size of about and more than 100 nucleotides up to 20000 nucleotides.

The formation of the inventive polyplex is typically caused by electrostatic interactions between positive charges on side of the inventive conjugate and negative charges on side of the polyanion, nucleic acid and RNA respectively. This results in complexation and spontaneous formation of polyplexes. In one embodiment, a an inventive polyplex refers to a particle having a z-average diameter suitable for parenteral administration.

In a preferred embodiment, said nucleic acid is a single stranded RNA (ssRNA). In a preferred embodiment, said ssRNA is a messenger RNA (mRNA). In a further preferred embodiment, said mRNA encodes a peptide or protein of interest. In a further preferred embodiment, said mRNA encodes a peptide or protein of interest, wherein said peptide or protein of interest is selected from reporter proteins and pharmaceutically active peptides or proteins. In a further preferred embodiment, said mRNA encodes a peptide or protein of interest, wherein said peptide or protein of interest is a reporter protein. In a further preferred embodiment, said mRNA encodes a peptide or protein of interest, wherein said peptide or protein of interest is a pharmaceutically active peptide or protein. In a further preferred embodiment, said mRNA is a pharmaceutically active nucleic acid. In a further preferred embodiment, said mRNA is a pharmaceutically active nucleic acid, wherein said pharmaceutically active nucleic acid is a nucleic acid that encodes a pharmaceutically active peptide or protein.

In another preferred embodiment said polyanion is a nucleic acid, wherein said nucleic acid is a DNA, wherein preferably said DNA is a plasmid DNA. In another preferred embodiment, said nucleic acid is a DNA. In a further preferred embodiment, said DNA is a plasmid DNA (pDNA). In a further preferred embodiment, said pDNA encodes a peptide or protein of interest. In a further preferred embodiment, said pDNA encodes a peptide or protein of interest, wherein said peptide or protein of interest is selected from reporter proteins and

pharmaceutically active peptides or proteins. In a further preferred embodiment, said pDNA encodes a peptide or protein of interest, wherein said peptide or protein of interest is a reporter protein. In a further preferred embodiment, said pDNA encodes a peptide or protein of interest, wherein said peptide or protein of interest is a pharmaceutically active peptide or protein. In a further preferred embodiment, said pDNA is a pharmaceutically active nucleic acid. In a further preferred embodiment, said pDNA is a pharmaceutically active nucleic acid, wherein said pharmaceutically active nucleic acid is a nucleic acid that encodes a pharmaceutically active peptide or protein.

In a preferred embodiment, said nucleic acid is a nucleic acid that encodes a peptide or protein of interest. In a further preferred embodiment, said nucleic acid encodes a peptide or protein of interest, wherein said peptide or protein of interest is selected from reporter proteins and pharmaceutically active peptides or proteins. In a further preferred embodiment, said nucleic acid encodes a peptide or protein of interest, wherein said peptide or protein of interest is a reporter protein. In a further preferred embodiment, said nucleic acid encodes a peptide or protein of interest, wherein said peptide or protein of interest is a pharmaceutically active peptide or protein. In a further preferred embodiment, said nucleic acid is a pharmaceutically active nucleic acid. In a further preferred embodiment, said nucleic acid is a pharmaceutically active nucleic acid, wherein said pharmaceutically active nucleic acid is a nucleic acid that encodes a pharmaceutically active peptide or protein. In a further preferred embodiment, said pharmaceutically active nucleic acid is a mRNA. In a further preferred embodiment, said pharmaceutically active nucleic acid is a pDNA.

In a further preferred embodiment, said nucleic acid is a pharmaceutically active nucleic acid, wherein said pharmaceutically active nucleic acid is pharmaceutically active in its own. In a further preferred embodiment, said nucleic acid is a pharmaceutically active nucleic acid, wherein said pharmaceutically active nucleic acid is a nucleic acid that encodes a pharmaceutically active peptide or protein.

In a preferred embodiment, said nucleic acid encodes a peptide or protein of interest, wherein said peptide or protein of interest is a reporter protein. In these embodiments, the nucleic acid comprises a reporter gene. Certain genes may be chosen as reporters because the characteristics they confer on cells or organisms expressing them may be readily identified and measured, or because they are selectable markers. Reporter genes are often used as an indication of whether a certain gene has been taken up by or expressed in the cell or organism population. Preferably, the expression product of the reporter gene is visually detectable. Common visually

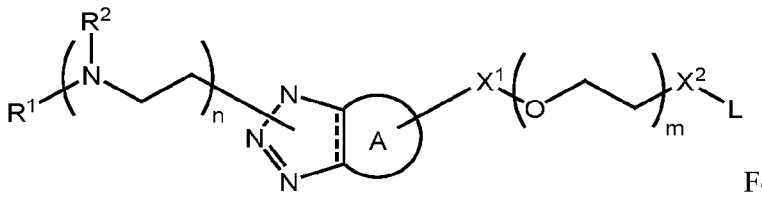
detectable reporter proteins typically possess fluorescent or luminescent proteins. Examples of specific reporter genes include the gene that encodes jellyfish green fluorescent protein (GFP), which causes cells that express it to glow green under blue light, the enzyme luciferase, which catalyzes a reaction with luciferin to produce light, and the red fluorescent protein (RFP) as well as the ones known by the skilled person as described in Concilio SC et al., Molecular Therapy: Oncolytics, 2021, 21:98-109, incorporated herein by way of reference in its entirety. Variants of any of these specific reporter genes are possible, as long as the variants possess visually detectable properties. For example, eGFP is a point mutant variant of GFP.In a preferred embodiment, said RNA is coding RNA, i.e. RNA encoding a peptide or protein. Said RNA may express the encoded peptide or protein. In a very preferred embodiment, said RNA, ssRNA or encoding RNA is a "messenger-RNA" (mRNA).

In a preferred embodiment, said RNA is a pharmaceutically active RNA. A "pharmaceutically active RNA" is an RNA that encodes a pharmaceutically active peptide or protein or is pharmaceutically active in its own, e.g., it has one or more pharmaceutical activities such as those described for pharmaceutically active proteins, e.g., immunostimulatory activity.

The term "encoding" refers to the inherent property of specific sequences of nucleotides in a RNA, such as an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. The terms "RNA encodes" or “RNA encoding”, as interchangeably used, means that the RNA, preferably the mRNA, if present in the appropriate environment, such as within cells of a target tissue, can direct the assembly of amino acids to produce the peptide or protein it encodes during the process of translation. In one embodiment, RNA is able to interact with the cellular translation machinery allowing translation of the peptide or protein. A cell may produce the encoded peptide or protein intracellularly (e.g. in the cytoplasm), may secrete the encoded peptide or protein, or may produce it on the surface.

With respect to RNA, and in particular with respect to mRNA, the term "expression" or "translation" relates to the process, typically in the ribosomes of a cell, by which a strand of mRNA directs the assembly of a sequence of amino acids to make a peptide or protein. The term "expression" is used in its most general meaning and comprises production of RNA and/or protein.

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, and a pDNA, wherein said pDNA is preferably non-covalently bound to said conjugate



Formula I

wherein A, R^1, R^2, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter A, R^1, R^2, X^1, X^2, L,m and n, or collectively to some or all of A, R^1, R^2, X^1, X^2 L, m and n.

In a preferred embodiment, said pDNA is coding DNA, i.e. DNA encoding a peptide or protein. Said pDNA may express the encoded peptide or protein.

In a preferred embodiment, said pDNA is a pharmaceutically active pDNA. A "pharmaceutically active pDNA " is a pDNA that encodes a pharmaceutically active peptide or protein or is pharmaceutically active in its own, e.g., it has one or more pharmaceutical activities such as those described for pharmaceutically active proteins, e.g., immunostimulatory activity.

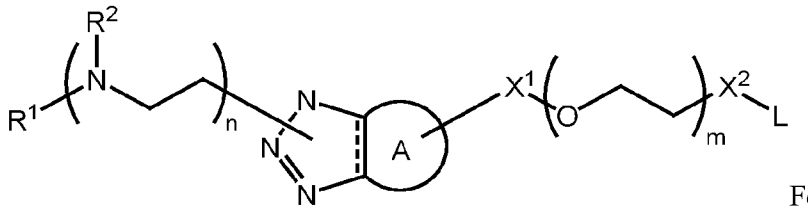
As known by the skilled person, for said preferred embodiments, such a double-stranded (ds) circular plasmid, i.e. a plasmid DNA, encoding peptide or protein of interest, preferably a pharmaceutically active peptide or protein, consists of, at minimum, a promoter and a gene of interest encoding said peptide or protein of interest, preferably said pharmaceutically active peptide or protein, and typically and preferably comprises further control elements such as appropriate promoters and terminators operably linked to said gene of interest encoding said pharmaceutically active peptide or protein.

A "pharmaceutically active peptide or protein" or "therapeutic peptide or protein" is a peptide or a protein that has a positive or advantageous effect on a condition or disease state of a subject when provided to the subject in a therapeutically effective amount. In one embodiment, a pharmaceutically active peptide or protein has curative or palliative properties and may be administered to ameliorate, relieve, alleviate, reverse, delay onset of or lessen the severity of one or more symptoms of a disease or disorder. A pharmaceutically active peptide or protein may have prophylactic properties and may be used to delay the onset of a disease or to lessen the severity of such disease or pathological condition. The term "pharmaceutically active peptide or protein" includes entire proteins or polypeptides, and can also refer to pharmaceutically active fragments thereof. It can also include pharmaceutically active analogs

of a peptide or protein. The term "pharmaceutically active peptide or protein" includes peptides and proteins that are antigens, i.e., the peptide or protein elicits an immune response in a subject which may be therapeutic or partially or fully protective. In one embodiment, the pharmaceutically active peptide or protein is or comprises an immunologically active compound or an antigen or an epitope.

As used herein, the terms "effective amount" and "therapeutically effective amount" are used interchangeably and refer to an amount administered to a subject, either as a single dose or as part of a series of doses, which is effective to produce a desired physiological response or desired therapeutic effect in the subject. Examples of desired therapeutic effects include, without limitation, improvements in the symptoms or pathology, and/or reducing the progression of symptoms or pathology in a subject suffering from an infection, disease, disorder and/or condition; and/or slowing, preventing or delyaing the onset of symptoms or pathology of an infection, disease, disorder and/or condition in a subject susceptible to said infection, disease, disorder and/or condition. The therapeutically effective amount will vary depending on the nature of the formulation used and the type and condition of the recipient. The determination of appropriate amounts for any given composition is within the skill in the art, through standard tests designed to assess appropriate therapeutic levels. Typical and preferred therapeutically effective amounts of the inventive triconjugates and/or polyplexes described herein range from about 0.05 to 1000 mg/kg body weight, and in particular from about 5 to 500 mg/kg body weight.

Thus, in another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, and a RNA, wherein said RNA is preferably non-covalently bound to said conjugate, and wherein said RNA is a pharmaceutically active RNA.

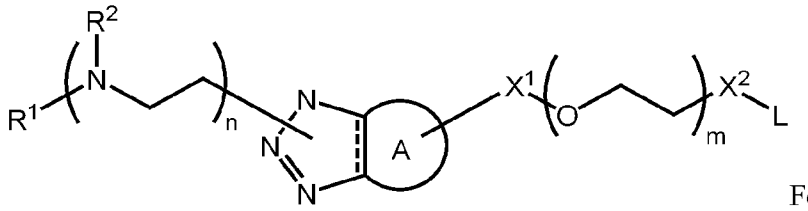


Formula I

wherein A, R^1, R^2, X^1, X^2 and L are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter A, R^1, R^2, X^1, X^2 and L, or collectively to some or all of A, R^1, R^2, X^1, X^2 and L.

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, and a RNA, wherein said RNA is preferably non-covalently bound to said conjugate, and wherein said RNA is a pharmaceutically active RNA

encoding a pharmaceutically active peptide or protein.



Formula I

wherein A, R^1, R^2, X^1, X^2 and L are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter A, R^1, R^2, X^1, X^2 and L, or collectively to some or all of A, R^1, R^2, X^1, X^2 and L.

In a preferred embodiment, said RNA encoding a pharmaceutically active peptide or protein has a size of 100 to about 20000 nucleotides.

In a preferred embodiment, said pharmaceutically active peptide or protein is or comprises an immunologically active compound or an antigen or an epitope. In a preferred embodiment, said pharmaceutically active peptide or protein is or comprises an immunologically active compound or an antigen. In a preferred embodiment, said pharmaceutically active peptide or protein is or comprises an immunologically active compound.

The term "immunologically active compound" relates to any compound altering an immune response, preferably by inducing and/or suppressing maturation of immune cells, inducing and/or suppressing cytokine biosynthesis, and/or altering humoral immunity by stimulating antibody production by B cells, or inducing degranulation of immune cells such as mast cells, eosinophils, neutrophils, cytotoxic T cells or NK cells. In one embodiment, the immune response involves stimulation of an antibody response (usually including immunoglobulin G (IgG)) and/or a cellular response including but not limited to responses by T cells, dendritic cells (DCs), macrophages, natural killer (NK) cells, natural killer T cells (NKT) cells, and γδ T cells. Immunologically active compounds may possess potent immunostimulating activity including, but not limited to, antiviral and antitumor activity, and can also down-regulate other aspects of the immune response, for example shifting the immune response away from a Th2 immune response, which is useful for treating a wide range of Th2 mediated diseases, or, if appropriate, shifting the immune response away from a Th1 immune response.

The term "antigen" covers any substance that will elicit an immune response. In particular, an "antigen" relates to any substance that reacts specifically with antibodies or T-lymphocytes (T-cells). The term "antigen" comprises any molecule which comprises at least

one epitope, preferably against which an immune response can be generated. Preferably, an antigen in the context of the present invention is a molecule which, optionally after processing, induces an immune reaction, which is preferably specific for the antigen, including wherein the immune reaction may be both a humoral as well as a cellular immune reaction. The antigen is preferably presented by a cell, preferably by an antigen presenting cell, in the context of MHC molecules, which results in an immune reaction against the antigen. Antigens include or may be derived from allergens, viruses, bacteria, fungi, plants, parasites and other infectious agents and pathogens or an antigen may also be a tumor antigen. In preferred embodiments, the antigen is a surface polypeptide, i.e. a polypeptide naturally displayed on the surface of a cell, a pathogen, a bacterium, a virus, a fungus, a plant, a parasite, an allergen, or a tumor. The antigen may elicit an immune response against a cell, a pathogen, a bacterium, a virus, a fungus, a plant, a parasite, an allergen, or a tumor.

In one embodiment, an antigen is a self-antigen or a non-self-antigen. In another embodiment, said non-self-antigen is a bacterial antigen, a virus antigen, a fungus antigen, an allergen or a parasite antigen. It is preferred that the antigen comprises an epitope that is capable of eliciting an immune response in a target organism. For example, the epitope may elicit an immune response against a bacterium, a virus, a fungus, a parasite, an allergen, or a tumor. In some embodiments the non-self-antigen is a bacterial antigen.

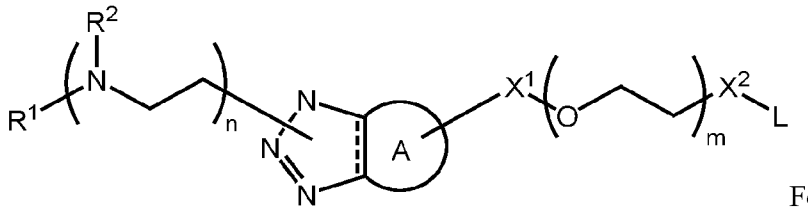
In some embodiments the non-self-antigen is a virus antigen. In some embodiments the non-self-antigen is a polypeptide or a protein from a fungus. In some embodiments the non-self-antigen is a polypeptide or protein from a unicellular eukaryotic parasite.

In some embodiments the antigen is a self-antigen, particularly a tumor antigen. Tumor antigens and their determination are known to the skilled person. In the context of the present invention, the term "tumor antigen" or "tumor-associated antigen" relates to proteins that are under normal conditions specifically expressed in a limited number of tissues and/or organs or in specific developmental stages, for example, the tumor antigen may be under normal conditions specifically expressed in stomach tissue, preferably in the gastric mucosa, in reproductive organs, e.g., in testis, in trophoblastic tissue, e.g., in placenta, or in germ line cells, and are expressed or aberrantly expressed in one or more tumor or cancer tissues. In this context, "a limited number" preferably means not more than 3, more preferably not more than 2. The tumor antigens in the context of the present invention include, for example, differentiation antigens, preferably cell type specific differentiation antigens, i.e., proteins that are under normal conditions specifically expressed in a certain cell type at a certain differentiation stage,

cancer/testis antigens, i.e., proteins that are under normal conditions specifically expressed in testis and sometimes in placenta, and germ line specific antigens. The tumor antigen is preferably associated with the cell surface of a cancer cell and is preferably not or only rarely expressed in normal tissues. Preferably, the tumor antigen or the aberrant expression of the tumor antigen identifies cancer cells. The tumor antigen that is expressed by a cancer cell in a subject, e.g., a patient suffering from a cancer disease, is preferably a self-protein in said subject. In preferred embodiments, the tumor antigen is expressed under normal conditions specifically in a tissue or organ that is non-essential, i.e., tissues or organs which when damaged by the immune system do not lead to death of the subject, or in organs or structures of the body which are not or only hardly accessible by the immune system. Preferably, the amino acid sequence of the tumor antigen is identical between the tumor antigen which is expressed in normal tissues and the tumor antigen which is expressed in cancer tissues. In a preferred embodiment, said term "tumor antigen" refers to a constituent of cancer cells which may be derived from the cytoplasm, the cell surface and the cell nucleus, preferably it refers to those antigens which are produced intracellularly or as surface antigens on tumor cells.

In a preferred embodiment, said nucleic acid is a pharmaceutically active nucleic acid. A "pharmaceutically active nucleic acid" is a nucleic acid that encodes a pharmaceutically active peptide or protein or is pharmaceutically active in its own, e.g., it has one or more pharmaceutical activities such as those described for pharmaceutically active proteins, e.g., immunostimulatory activity.

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, and a nucleic acid, wherein said nucleic acid is preferably non-covalently bound to said conjugate, and wherein said nucleic acid is a pharmaceutically active nucleic acid.

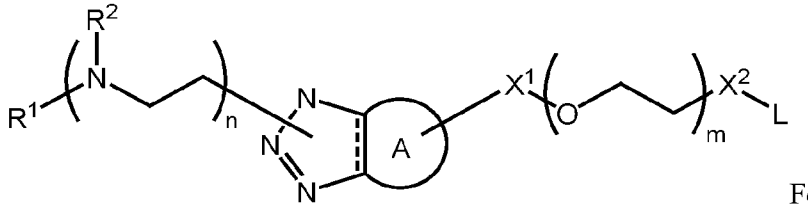


Formula I

wherein A, R^1, R^2, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter A, R^1, R^2, X^1, X^2, L,m and n, or collectively to some or all of A, R^1, R^2, X^1, X^2 L, m and n.

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, and a nucleic acid, wherein said nucleic acid is preferably

non-covalently bound to said conjugate, and wherein said nucleic acid is a pharmaceutically active nucleic acid encoding a pharmaceutically active peptide or protein.



Formula I

wherein A, R^1, R^2, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter A, R^1, R^2, X^1, X^2, L,m and n, or collectively to some or all of A, R^1, R^2, X^1, X^2 L, m and n.

Examples of said pharmaceutically active peptides or proteins include, but are not limited to, cytokines and derivatives thereof such as cytokine-fusions (like albumin-cytokine fusions) and immune system proteins such as immunologically active compounds (e.g., interleukins, colony stimulating factor (CSF), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), erythropoietin, tumor necrosis factor (TNF), interferons, integrins, addressins, selectins, homing receptors, T cell receptors, chimeric antigen receptors (CARs), immunoglobulins including antibodies or bi-, tri-, or multispecific antibodies, e.g., for immune stimulation or production of neutralizing antibodies in case of viral/bacterial infection, soluble major histocompatibility complex antigens, immunologically active antigens such as bacterial, parasitic, or viral antigens, allergens, autoantigens, antibodies), hormones (insulin, thyroid hormone, catecholamines, gonadotrophines, trophic hormones, prolactin, oxytocin, dopamine, bovine somatotropin, leptins and the like), growth hormones (e.g., human grown hormone), growth factors (e.g., epidermal growth factor, nerve growth factor, insulin-like growth factor and the like), growth factor receptors, enzymes (tissue plasminogen activator, streptokinase, cholesterol biosynthestic or degradative, steriodogenic enzymes, kinases, phosphodiesterases, methylases, de-methylases, dehydrogenases, cellulases, proteases, lipases, phospholipases, aromatases, cytochromes, adenylate or guanylaste cyclases, neuramidases, lysosomal enzymes and the like), receptors (steroid hormone receptors, peptide receptors), binding proteins (growth hormone or growth factor binding proteins and the like), transcription and translation factors, tumor growth suppressing proteins (e.g., proteins which inhibit angiogenesis), structural proteins (such as collagen, fibrin, fibrinogen, elastin, tubulin, actin, and myosin), blood proteins (thrombin, serum albumin, Factor VII, Factor VIII, insulin, Factor IX, Factor X, tissue plasminogen activator, protein C, Von Willebrand factor, antithrombin III, glucocerebrosidase, erythropoietin granulocyte colony stimulating factor

(GCSF) or modified Factor VIII, anticoagulants and the like.

In a preferred embodiment, said pharmaceutically active peptide or protein is selected from cytokines and derivatives thereof such as cytokine-fusions (like albumin-cytokine fusions) and immune system proteins such as immunologically active compounds (e.g., interleukins, colony stimulating factor (CSF), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), erythropoietin, tumor necrosis factor (TNF), interferons, integrins, addressins, seletins, homing receptors, T cell receptors, chimeric antigen receptors (CARs), immunoglobulins including antibodies or bispecific antibodies, e.g., for immune stimulation or production of neutralizing antibodies in case of viral/bacterial infection, soluble major histocompatibility complex antigens, immunologically active antigens such as bacterial, parasitic, plant or viral antigens, allergens, autoantigens, antibodies), hormones (insulin, thyroid hormone, catecholamines, gonadotrophines, trophic hormones, prolactin, oxytocin, dopamine, bovine somatotropin, leptins and the like), growth hormones (e.g., human grown hormone), growth factors (e.g., epidermal growth factor, nerve growth factor, insulin-like growth factor and the like), growth factor receptors, enzymes (tissue plasminogen activator, streptokinase, cholesterol biosynthestic or degradative, steriodogenic enzymes, kinases, phosphodiesterases, methylases, de-methylases, dehydrogenases, cellulases, proteases, lipases, phospholipases, aromatases, cytochromes, adenylate or guanylate cyclases, neuramidases, lysosomal enzymes and the like), receptors (steroid hormone receptors, peptide receptors), binding proteins (growth hormone or growth factor binding proteins and the like), transcription and translation factors, tumor growth suppressing proteins (e.g., proteins which inhibit angiogenesis), structural proteins (such as collagen, fibrin, fibrinogen, elastin, tubulin, actin, and myosin), blood proteins (thrombin, serum albumin, Factor VII, Factor VIII, insulin, Factor IX, Factor X, tissue plasminogen activator, protein C, Von Willebrand factor, antithrombin III, glucocerebrosidase, erythropoietin granulocyte colony stimulating factor (GCSF) or modified Factor VIII, anticoagulants and the like.

In a preferred embodiment, said pharmaceutically active peptide or protein is a immunologically active compound. In a preferred embodiment, said pharmaceutically active peptide or protein is a immunologically active compound selected from interleukins, colony stimulating factor (CSF), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), erythropoietin, tumor necrosis factor (TNF), interferons, integrins, addressins, seletins, homing receptors, T cell receptors, chimeric antigen receptors (CARs), immunoglobulins including antibodies or bispecific antibodies, e.g., for

immune stimulation or production of neutralizing antibodies in case of viral/bacterial infection, soluble major histocompatibility complex antigens, immunologically active antigens such as bacterial, parasitic, plant or viral antigens, allergens, autoantigens and antibodies. In another preferred embodiment, said pharmaceutically active peptide or protein is an interleukin. In another preferred embodiment, said pharmaceutically active peptide or protein is a colony stimulating factor (CSF). In another preferred embodiment, said pharmaceutically active peptide or protein is a granulocyte colony stimulating factor (G-CSF). In another preferred embodiment, said pharmaceutically active peptide or protein is a granulocyte-macrophage colony stimulating factor (GM-CSF). In another preferred embodiment, said pharmaceutically active peptide or protein is erythropoietin. In another preferred embodiment, said pharmaceutically active peptide or protein is tumor necrosis factor (TNF). In another preferred embodiment, said pharmaceutically active peptide or protein is an interferons. In another preferred embodiment, said pharmaceutically active peptide or protein is an integrin. In another preferred embodiment, said pharmaceutically active peptide or protein is an addressin. In another preferred embodiment, said pharmaceutically active peptide or protein is a selectin. In another preferred embodiment, said pharmaceutically active peptide or protein is an immunologically active antigen, preferably selected from bacterial, parasitic, plant or viral antigens, allergens, autoantigens and antibodies. In another preferred embodiment, said pharmaceutically active peptide or protein is a bacterial antigen. In another preferred embodiment, said pharmaceutically active peptide or protein is a parasitic antigen. In another preferred embodiment, said pharmaceutically active peptide or protein is a plant antigen. In another preferred embodiment, said pharmaceutically active peptide or protein is a viral antigen. In another preferred embodiment, said pharmaceutically active peptide or protein is an allergen. In another preferred embodiment, said pharmaceutically active peptide or protein is an autoantigen. In another preferred embodiment, said pharmaceutically active peptide or protein is an antibody.

In another preferred embodiment, said pharmaceutically active peptide or protein is selected from interleukin-2, interleukin-4, interleukin-7, interleukin-12, interleukin-15, interferon-α, interferon-β, interferon-γ, colony stimulating factor, granulocyte-macrophage stimulating factor, anti-angiogenic agents, tumor suppressor genes, tumor antigens, viral antigens and bacterial antigens.

In a preferred embodiment, said pharmaceutically active peptide or protein is selected from a cytokine, a growth factor, a hormone, an enzyme, a tumor antigen, a viral antigen, bacterial antigen, an autoantigen, or an allergen.

In a preferred embodiment, said pharmaceutically active peptide or protein comprises, preferably consists of a cytokine.

The term "cytokine", as used herein, refers to a category of small proteins (about 5-20 kDa) that are important in cell signalling. Their release has an effect on the behavior of cells around them. Cytokines are involved in autocrine signalling, paracrine signalling and endocrine signalling as immunomodulating agents. Cytokines include chemokines, interferons, interleukins, lymphokines, and tumour necrosis factors but generally not hormones or growth factors (despite some overlap in the terminology). Cytokines are produced by a broad range of cells, including immune cells like macrophages, B lymphocytes, T lymphocytes and mast cells, as well as endothelial cells, fibroblasts, and various stromal cells. A given cytokine may be produced by more than one type of cell. Cytokines act through receptors and are especially important in the immune system; cytokines modulate the balance between humoral and cell-based immune responses, and they regulate the maturation, growth, and responsiveness of particular cell populations. Some cytokines enhance or inhibit the action of other cytokines in complex ways.

In a preferred embodiment, said said pharmaceutically active peptide or protein is a cytokine selected from an interleukin, an interferon and a chemokine.

In a preferred embodiment, said pharmaceutically active peptide or protein is an interleukin. In a further preferred embodiment, said pharmaceutically active peptide or protein is an interleukin selected from the group consisting of IL-2, IL-7, IL-12, IL-15, and IL-21.

In a further preferred embodiment, said pharmaceutically active peptide or protein is interleukin-2 (IL-2).

Interleukin-2 (IL-2), a key cytokine with pleiotropic effects on the immune system, is produced mainly by antigen-stimulated $CD4^+$ T cells, as well as by $CD8^+$ T cells, natural killer (NK) and dendritic cells (DC). IL-2 promotes the differentiation of naïve CD4 T cells into T helper-1 (Th1) and T helper-2 (Th2) cells and is required for the maintenance of $CD4^+$ regulatory T cells (Tregs). Furthermore, IL-2 promotes CD8 T cell and NK cell cytotoxicity (Liao W et al, Immunity, 2013, 38(1):13-25).

The IL-2 receptor is composed of the three subunits IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132). IL-2Rα is unique to IL-2 and is expressed by several immune cells

including Tregs, activated CD4 and CD8 T cells, B cells and mature Dendritic cells (Wrangle JM et al, J Interferon Cytokine Res, 2018, 38(2):45-68). Binding of IL-2 to the IL-2Rβγ or IL-2Rαβγ complex leads to the recruitment of Janus family tyrosine kinases (JAK1, JAK3), phosphorylation of signal transducer and activator of transcription (STAT1, STAT3, STAT5) and activation of major downstream signaling pathways, which regulate survival, proliferation, differentiation, activation, cytokine production in different types of immune cells (Wrangle JM et al, J Interferon Cytokine Res, 2018, 38(2):45-68).

Recombinant IL-2 protein was approved by FDA in 1998 for treatment of metastatic melanoma and renal cancer. Although IL-2 mediates tumor regression, it fails to improve patients' survival and is associated with severe toxicity (Wrangle JM et al, J Interferon Cytokine Res, 2018, 38(2):45-68; Jiang T et al., Oncoimmunology, 2016, 5(6):e1163462). Due to rapid elimination and metabolism via the kidney, IL-2 has a short serum half-life of several minutes. Thus, to achieve an optimal immune-modulatory effect, IL-2 should be given in a high dose, which will inevitably result in severe toxicities.

Therefore, the present invention to target the delivery of mRNA or plasmid DNA encoding IL2 protein will allow its protein expression at the tumor site. This will enable the activation of the immune cells at the tumor microenvironment and allows to induce an anti-tumorigenic effect with potential limited toxicity.

In a preferred embodiment, said pharmaceutically active peptide or protein is an interferon.

In a preferred embodiment, said pharmaceutically active peptide or protein is an interferon, wherein said interferon is a type-I interferon.

In a preferred embodiment, said pharmaceutically active peptide or protein is an interferon, wherein said interferon is a type-II interferon.

In a preferred embodiment, said pharmaceutically active peptide or protein is an interferon, wherein said interferon is interferon-α (IFN-α), interferon-β (IFN-β), or interferon-γ (IFN-γ).

In a preferred embodiment, said pharmaceutically active peptide or protein is an interferon, wherein said interferon is interferon-α (IFN-α).

In a preferred embodiment, said pharmaceutically active peptide or protein is an interferon, wherein said interferon is interferon-β (IFN-β).

Type-I interferons (IFNs) were originally identified by their anti-viral effects; however, they play important roles in other diseases, including cancer and multiple sclerosis. IFNs have

pleiotropic anti-cancer effects, acting on cancer cells both directly and indirectly. Indirect effects include activation of immune effector cells and ablation of the tumor vasculature (Borden, E. C., Nat Rev Drug Discov, 2019, 18:219–234).

IFNs are a subset of the class-2 α-helical cytokines that have been found in all vertebrates. There are numerous human Type-I-IFNs, including thirteen IFN-α cytokines, one IFN-β, and several other single gene products not yet well characterized (Musella M et al., Oncoimmunology, 2017 6(5): e1314424).

In their canonical signaling pathway, Type-I-IFNs bind to the heterodimeric transmembrane IFN-α/β receptor (IFNAR), which activates the Janus Kinase–Signal Transducer and Activator of Transcription (JAK-STAT) pathway. This cascade induces the transcription of several hundred IFN-stimulated genes (ISGs), resulting in multilayered cellular responses (Schneider WM et al., Annu Rev Immunol, 2014, 32:513-545). These include protein synthesis (both cellular and viral), autophagy, apoptosis, angiogenesis, and immune cell modulation (Borden, E. C., Nat Rev Drug Discov, 2019, 18:219–234). Type I IFNs modulate the activity of both innate and adaptive immune cells, including dendritic cells, CD8+ T cells, CD4+ T cells, Regulatory T cells, and NK cells (Boukhaled GM et al., Annu Rev Pathol, 2021, 16:167-198).

Type I IFNs have been widely used, alone and in combination with other immunotherapeutic agents, to treat solid and hematologic malignancies (Borden, E. C., Nat Rev Drug Discov, 2019, 18:219–234). IFN-α2 was the first human immunotherapeutic approved by the US Food and Drug Administration (FDA) for cancer treatment and is still commonly combined with IL-2 in immunotherapeutic regimens for metastatic renal-cell carcinomas and cutaneous melanoma. IFN-β exerts antiviral and antiproliferative properties similar to those of IFN-α and is available as a preparation derived from natural fibroblasts (IFN-β 1a) or in recombinant form (IFN-β 1b). Nonetheless, systemic administration of IFNs has been associated with severe toxicities. Therefore, optimal concentrations within the tumor bed after administration of tolerable doses of IFNs cannot be achieved (Young PA et al., Semin Oncol, 2014 41(5):623-636).

Beyond cancer treatment, IFN-β is the standard treatment for Multiple Sclerosis (MS). To date, five IFN-β drugs have been approved for the treatment of relapsing forms of MS (Filipi M et al., Int J MS Care, 2020, 22(4):165-172). Furthermore, IFN-β is used in Japan for the treatment of hepatitis C.

Targeted delivery of the nucleic acids encoding for IFN-β is expected to reduce the

associated systemic toxicity. Moreover, targeted expression should lead to high, localized IFN-β concentrations in the desired tissue.

In a preferred embodiment, said pharmaceutically active peptide or protein is an interferon, wherein said interferon is IFN-γ.

Interferon-gamma (IFN-γ), a type II IFN, is a pleiotropic molecule which has antiproliferative, pro-apoptotic and antitumor immunomodulatory mechanisms of action (Castro F et al., Frontier in Immunology, 2018, 9:847). IFNγ is produced by the immune cells, including activated T cells and natural killer (NK) cells. IFNγ exerts its antitumor effects through the activation JAK-STAT pathway that leads to the expression of IFNγ-stimulated genes (ISGs) (Chen Y et al., Journal of Pancreatology, 2023, 6(1):8-17; Ding H et al., Biomed Pharmacother, 2022, 155:113683). IFNγ plays a role in maturation of NK cells, enhancement of CD8 T cell cytotoxicity, stimulation of Th1 polarization, inhibition of Th2 and Th17 differentiation, upregulation of MHC class I and II in APCs, maturation of Dendritic Cells, and the induction of M1 macrophages (Chen Y et al., Journal of Pancreatology, 2023, 6(1):8-17; Ding H et al., Biomed Pharmacother, 2022, 155:113683). The direct inhibitory effects of IFNγ on tumor cells include cell cycle arrest, cell senescence, apoptosis and autophagic cell death. Moreover, it has been demonstrated that IFNγ mediates inhibition of tumor associated fibroblasts in TME and induces an anti-angiogenic effect. Tumor cells limit the production of IFNγ by cytotoxic CD8 T cell by imposing nutrient deprivation or rewiring the cellular metabolism of T cells (Chen Y et al., Journal of Pancreatology, 2023, 6(1):8-17; Ding H et al., Biomed Pharmacother, 2022, 155:113683). Overcoming primary or acquired resistance to IFNγ-induced therapies remains a great clinical challenge (Chen Y et al., Journal of Pancreatology, 2023, 6(1):8-17; Ding H et al., Biomed Pharmacother, 2022, 155:113683). Several clinical trials investigating recombinant IFNγ have shown some improvement in patient survival. However, most studies failed due to toxicity (Chen Y et al., Journal of Pancreatology, 2023, 6(1):8-17; Ding H et al., Biomed Pharmacother, 2022, 155:113683). Targeting the delivery of IFNγ mRNA and expressing it directly in tumor cells can reduce the systemic toxicity and modulate the tumor microenvironment.

In a further preferred embodiment, said pharmaceutically active peptide or protein is a hormone.

In a further preferred embodiment, said pharmaceutically active peptide or protein is human erythropoietin (EPO).

The human erythropoietin (EPO) protein is a hormone that stimulates the production of red blood cells (erythropoiesis) in the bone marrow by binding to the EPO receptor of blood cell precursors, the proerythroblasts, stimulating their differentiation and inhibiting their apoptosis (McGraw K et al., Vitam Horm, 2017, 105:79-100). In adults, EPO is mainly produced by peritubular cells in the kidneys and to a much smaller extent by the liver, spleen, bone marrow, lung and brain (McGraw K et al., Vitam Horm, 2017, 105:79-100; Jelkmann W et al., Transfus Med Hemother, 2013, 40(5):302-309).

Recombinant human EPO (rhEPO) is used in the treatment of anemia associated with chronic kidney disease, HIV infection and chemotherapy and in perioperative therapies (Jelkmann W et al., Transfus Med Hemother, 2013, 40(5):302-309). Repeated administration of rhEPO is associated with high immunogenicity caused by the development of neutralizing antibodies against EPO (Casadevall N et al., N Engl J Med, 2002, 346(7):469-475; Behler CM et al., J Med Case Rep, 2009, 3:7335; Praditpornsilpa K et al., Nephrol Dial Transplant, 2009, 24(5):1545-1549; Rahbar M et al., J Nephropathol, 2017, 6(1):25-29). Recombinant proteins often have different patterns of glycosylation from the endogenously expressed protein, leading to the development of neutralizing antibodies against the rhEPO (Susantad T et al., Sci Rep, 2021, 11(1):1491). These antibodies can also target the endogenous EPO protein (Casadevall N et al., N Engl J Med, 2002, 346(7):469-475).

We have developed a novel approach to selectively deliver mRNAs which encode therapeutic proteins, such as EPO, and enable their endogenous expression in the targeted tissue cells in a living organism. This would allow a more physiological approach/natural source, as opposed to the systemic administration of recombinant EPO. This could also significantly improve the efficacy and safety as compared to the use of recombinant protein-based therapies, like rhEPO in the treatment of anemia. It could reduce immunogenicity as proteins will be expressed with the correct post-translational modifications.

In a further preferred embodiment, said pharmaceutically active peptide or protein is a bacterial antigen.

In a further preferred embodiment, said pharmaceutically active peptide or protein is a viral antigen.

In a further preferred embodiment, said pharmaceutically active peptide or protein is a tumor antigen.

In a further preferred embodiment, said pharmaceutically active peptide or protein is a plant antigen.

In a further preferred embodiment, said pharmaceutically active peptide or protein is Diphtheria toxin (DT).

In a further preferred embodiment, said pharmaceutically active peptide or protein is Diphtheria toxin catalytic domain A (DT-A).

Diphtheria toxin (DT) is one of the most studied bacterial exotoxins. DT is secreted by a non-encapsulated, non-motile, Gram-positive bacillus, *Corynebacterium diphtheriae*. DT is a single polypeptide chain comprising two major domains: the catalytic domain A (DT-A) and B subunit (DT-B). DT-A catalyses inactivation of elongation factor 2 through ADP-ribosylation, thereby blocking protein synthesis and cell death in the target cells (Falnes PO et al, EMBO J, 1998, 17(2):615-625). DT-B includes the translocation and receptor-binding regions and promotes the binding of the toxin to cells and the entry of the A chain into the cytosolic compartment, leading to cell death (Sharma NC et al., Nature Reviews Disease Primers, 2019, 5(1):81).

Several approaches utilizing DT as potential anti-cancer therapies have been examined in pre-clinical studies and clinical trials (Shafiee F et al., Front Microbiol, 2019, 10:2340). These include, antibody-conjugated DT, ligand-targeted DT as well as gene therapies, whereby, the gene encoding the DT is delivered, to produce the toxin *in vivo*. Some of the DT agents that have reached the clinical trials are Ontak™ and Tagraxofusp™ (Frankel AE et al., Biomedicines, 2019, 7(1):6; Frankel AE et al., Blood, 2007, 110(11):894).

As most cells possess the DT cell surface receptor, a non-targeted full-length DT could lead to significant toxicity. There is a need to discover a strategy harnessing the power of the DT in a targeted manner to cancer cells while minimizing off-target effects.

As a low level of DT-A is sufficient for cell killing, the development of specific targeting strategies for DT-A could result in fewer adverse effects on normal cells and tissues while achieving efficient cell killing (Yamaizumi, M et al., Cell, 1978, 1:245–250).

The present invention of targeted delivery of mRNA encoding DT-A will allow its expression at the tumor site and should result in higher efficiency in eradicating tumor cells with reduced systemic toxicity. Moreover, in immunized cancer patients, this strategy may offer additional benefits due to the pre-existing immunity to diphtheria toxin. The immune system could recognize and respond to the DT protein more efficiently, leading to a stronger immune response against the cancer cells. This could potentially increase the efficacy of the treatment.

In a further preferred embodiment, said pharmaceutically active peptide or protein is a receptor binding domain (RBD) of a coronavirus (CoV), or a fragment thereof.

In a further preferred embodiment, said pharmaceutically active peptide or protein is the receptor binding domain (RBD), preferably the receptor binding motif (RBM), of a spike (S) protein of a human coronavirus (HCoV), or a fragment thereof, wherein said HCoV is selected from SARS-CoV-2, SARS-CoV, MERS-CoV.

The spike protein (S) is a type I transmembrane protein expressed on the surface of coronaviruses that mediates the entrance of the virus by interacting with receptors on the target cells (angiotensin-converting enzyme 2, ACE2) (Walls AC et al., Cell, 2020, 181(2):281-292.e6). Coronavirus S proteins are composed of three copies of an S1 subunit and three copies of an S2 subunit. Within the S1 subunit, an N-terminal domain (NTD) and a receptor-binding domain (RBD) are present (Walls AC et al., Cell, 2020, 181(2):281-292.e6).

During the recent COVID-19 pandemic, monoclonal antibodies isolated from patients infected with the newly identified coronavirus (SARS-CoV-2) were characterized. The RBD was found to be the main target of neutralizing antibodies, but also the NTD and the quaternary structure of the trimer were recognized by strong neutralizing antibodies (Barnes CO et al., Cell, 2020, 182(4):828-842.e16; Heinz FX et al., NPJ Vaccines, 2021, 6(1):104). This suggests that a properly folded S protein is needed to induce a potent immune response.

mRNA-based vaccines encoding the S protein of SARS-CoV-2 have been developed, and these have been shown to induce strong and durable immune responses (Widge AT et al., N Engl J Med, 2021, 384(1):80-82; Sahin U et al., Nature, 2020, 586(7830):594-599 and Erratum in Nature, 2021, 590(7844):E17). Selectively delivering the mRNA encoding the spike protein to cancer cells would induce the immune system in the tumor microenvironment to target and destroy the transfected cancer cells. In patients already immunized against the protein, a particularly effective immune response against the cancer cells would be expected.

In a further aspect, the present invention provides for the use of pharmaceutical compositions as described herein and comprising the inventive polyplexes which polyplexes comprises said pharmaceutically active nucleic acid encoding a pharmaceutically active peptide or protein for the therapeutic or prophylactic treatment of various diseases, in particular diseases in which provision of said peptide or protein to a subject results in a therapeutic or prophylactic effect. For example, provision of an antigen or epitope which is derived from a virus may be useful in the treatment of a viral disease caused by said virus. Provision of a tumor antigen or epitope may be useful in the treatment of a cancer disease wherein cancer cells express said tumor antigen. Provision of a cytokine or a cytokine-fusion may be useful to modulate tumor

microenvironment. Provision of cytokines, hormones or growth factors can be used for the treatment of non-oncology related diseases.

In a further aspect, the present invention provides a pharmaceutical composition comprising an inventive compositon, an inventive conjugate, preferably said conjugate of Formula I* or of Formula I, or an inventive polyplex as described herein, and a pharmaceutically acceptable salt thereof.

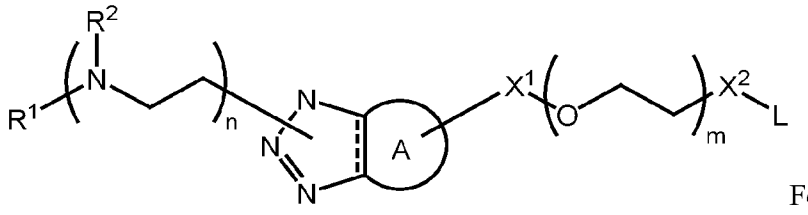
Negatively Charged Polyanions Used to Form Polyplexes

In some embodiments, the triconjugates of the present disclosure can form polyplexes with polyanions and anionic polymers, such as nucleic acids. For example, at physiological pH (e.g., pH 7.4), the LPEI fragment of a triconjugate of the present invention can be at least partially protonated and can carry a net positive charge. In contrast, polyanions such as nucleic acids can be at least partially deprotonated at physiological pH and can carry a net negative charge. Accordingly, in some embodiments co-incubation of a triconjugate of the present invention with a negatively charged polymer and polyanion such as a nucleic acid, and preferably a RNA, further preferably a dsRNA such as poly(IC) or a ssRNA such as mRNA or a pDNA will result in a polyplex (e.g., held together by electrostatic interaction).

In some embodiments, the nucleic acid can be intrinsically cytotoxic and/or immunostimulatory (e.g., polyinosinic:polycytidylic acid, also known as poly(IC)).

Thus, in a further aspect, the present invention provides a polyplex comprising a composition as described herein and a polyanion such as a nucleic acid, preferably polyinosinic:polycytidylic acid poly(IC). In some embodiments, said polyanion is a nucleic acid. In some embodiments, said polyanion is a nucleic acid, wherein said nucleic acid is a RNA or DNA. In another embodiment, said polyanion is a RNA. In another embodiment, said polyanion is a dsRNA. In a further preferred embodiment, said polyanion is poly(IC).

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, and poly(IC), wherein said poly(IC) is preferably non-covalently bound to said conjugate

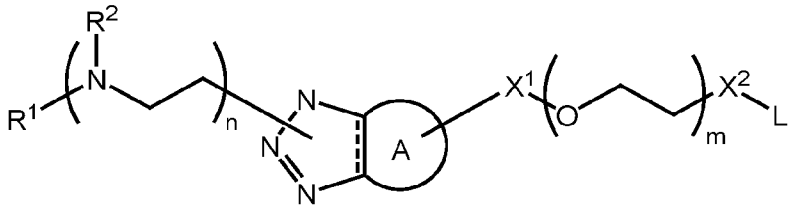


Formula I

wherein A, R^1, R^2, X^1, X^2, L, m and n are as defined herein, preferably as defined in any

embodiment described herein, be it individually related to each parameter A, R^1, R^2, X^1, X^2, L,m and n, or collectively to some or all of A, R^1, R^2, X^1, X^2 L, m and n.

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof, and polyinosinic:polycytidylic acid (poly(IC)), wherein said poly(IC) is preferably non-covalently bound to said conjugate:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating units m of 25 to 100, preferably of a discrete number of repeating units m of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

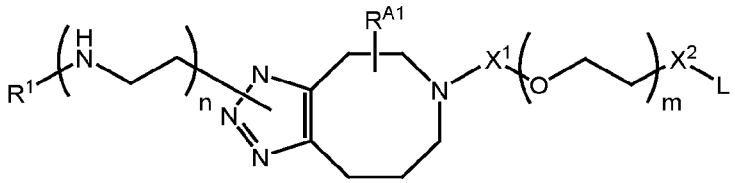
X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

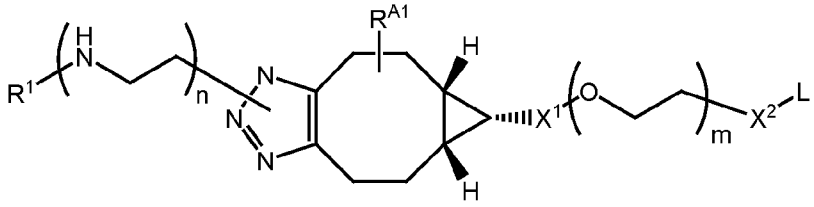
L is a targeting fragment, wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$.

In a preferred embodiment, said Ring A is cyclooctene, succinimide, or 7- to 8-membered heterocycloalkenyl, wherein the heterocycloalkenyl comprises one or two heteroatoms selected from N, O and S, and wherein each cyclooctene or heterocycloalkenyl is optionally substituted at any position with one or more R^{A1}, wherein preferably R^{A1} is oxo or fluorine, or wherein two R^{A1} combine to form one or more fused phenyl rings, preferably one or two fused phenyl rings, wherein each phenyl ring is optionally substituted with one or more $-SO_3H$ or $-OSO_3H$.

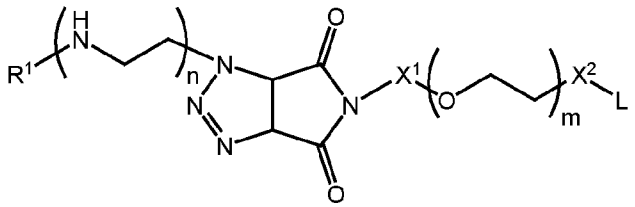
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



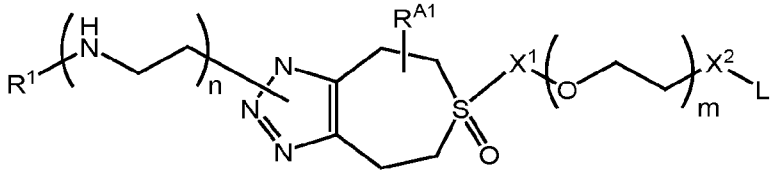
Formula IA,



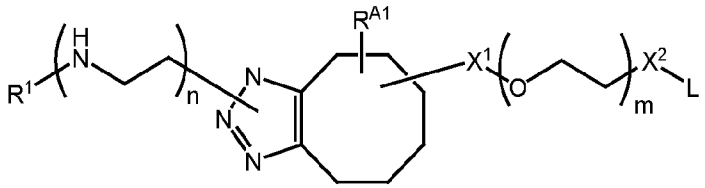
Formula IB,



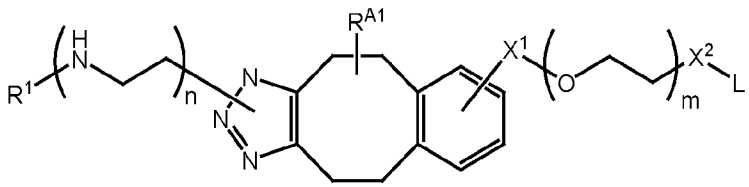
Formula IC,



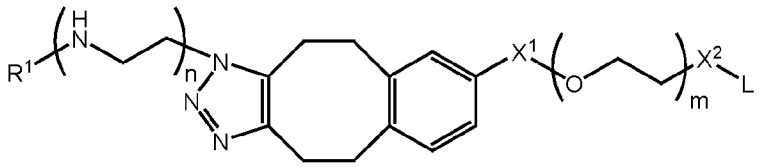
Formula ID,



Formula IE,



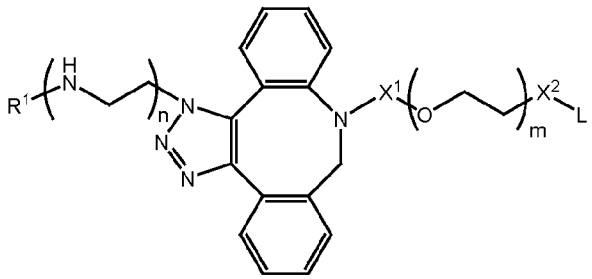
Formula IH, and



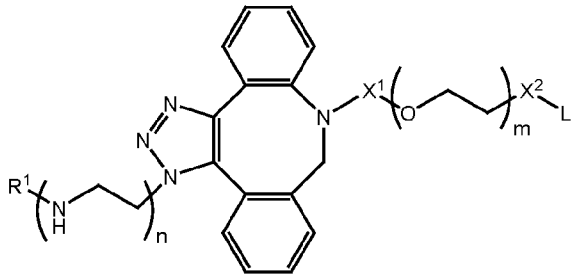
Formula IH-1,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

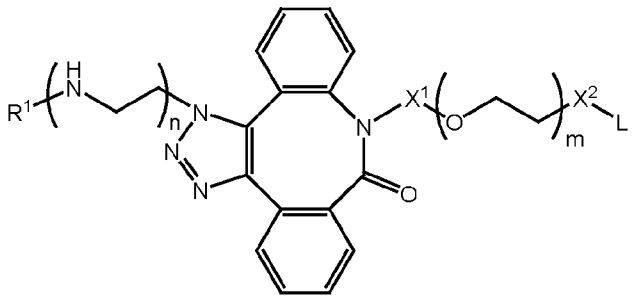
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



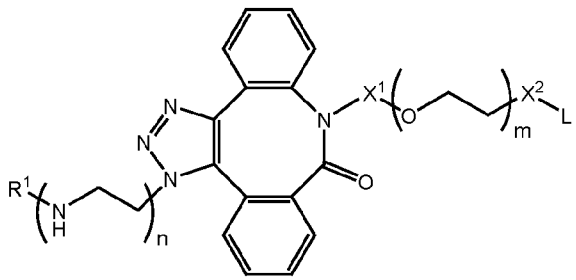
Formula IA-3,



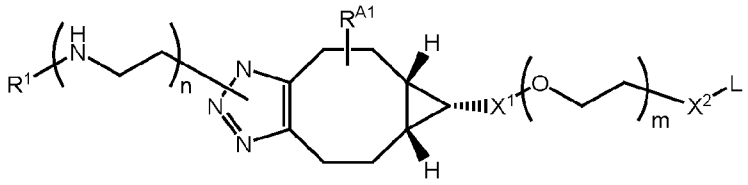
Formula IA-4,



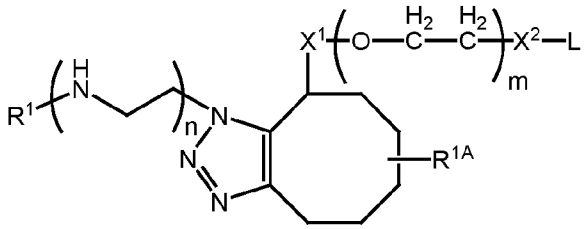
Formula IA-9,



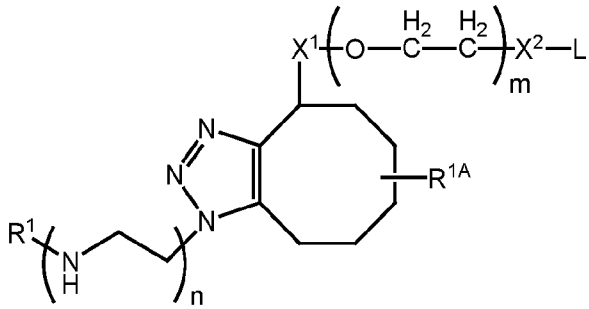
Formula IA-10,



Formula IB,



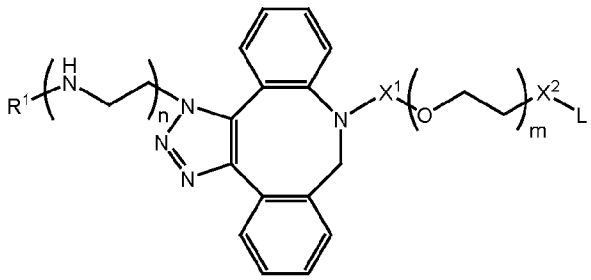
Formula IE-13, and



Formula IE-14,

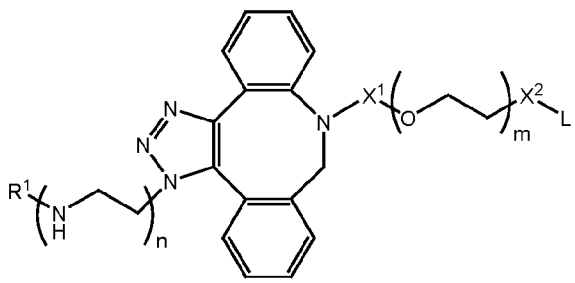
wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IA-3,

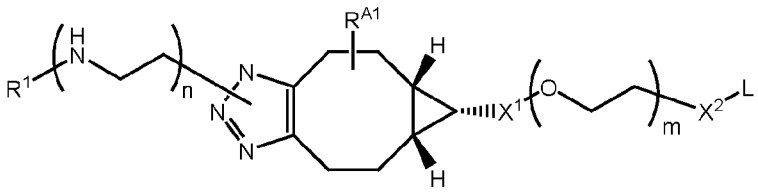
and



Formula IA-4,

wherein R^1, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, X^1, X^2, L, m and n, or collectively to some or all of R^1, X^1, X^2, L, m and n.

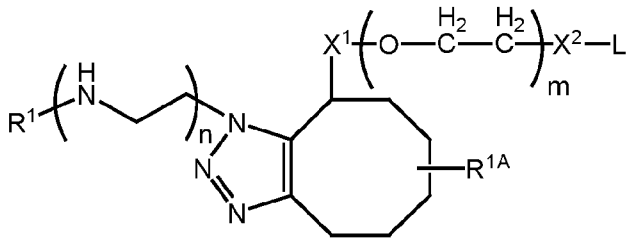
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



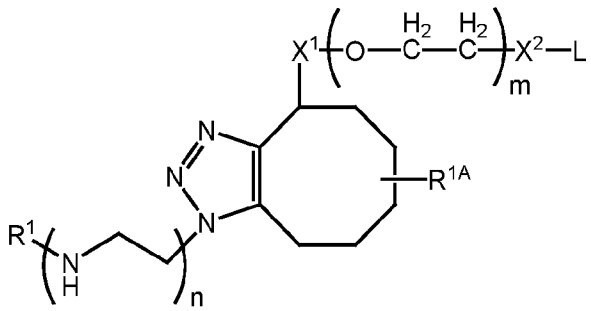
Formula IB,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IE-13, and



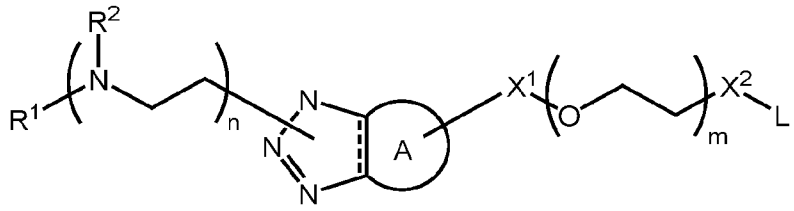
Formula IE-14,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment, said targeting fragment comprises or preferably consists of the DUPA residue (HOOC-$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-). In a further very preferred embodiment, said targeting fragment consists of the DUPA residue (HOOC$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-), wherein both chiral C-atoms having (*S*)-configuration, as depicted in formula **1***.

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer

thereof, and poly(IC), wherein said poly(IC) is preferably non-covalently bound to said conjugate:



Formula I

wherein:

═══ is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating units m of 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

X^1 is a divalent covalent linking moiety;

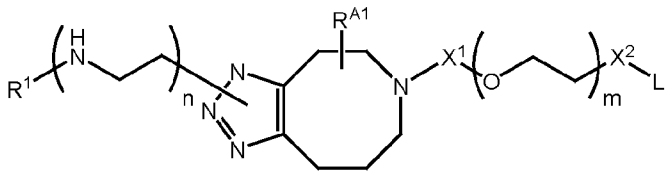
X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor wherein said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$.

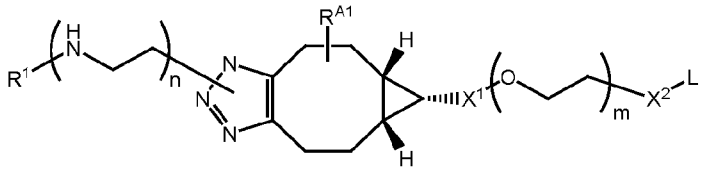
In a preferred embodiment, said Ring A is cyclooctene, succinimide, or 7- to 8-membered heterocycloalkenyl, wherein the heterocycloalkenyl comprises one or two heteroatoms selected from N, O and S, and wherein each cyclooctene or heterocycloalkenyl is optionally substituted at any position with one or more R^{A1}, wherein preferably R^{A1} is oxo or fluorine, or wherein two R^{A1} combine to form one or more fused phenyl rings, preferably one

or two fused phenyl rings, wherein each phenyl ring is optionally substituted with one or more $-SO_3H$ or $-OSO_3H$.

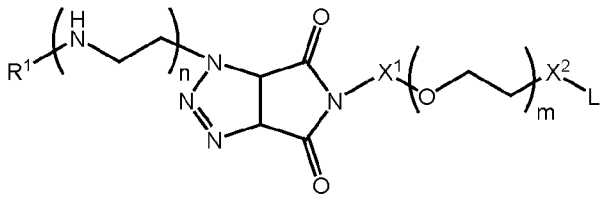
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



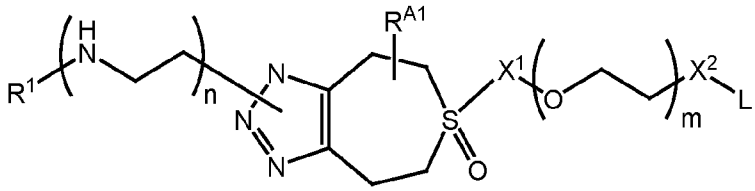
Formula IA,



Formula IB,



Formula IC,



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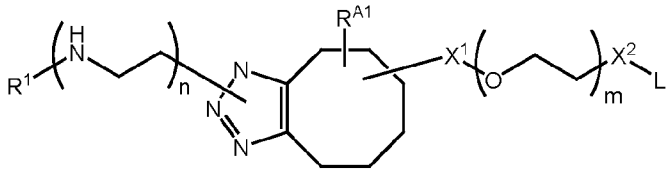
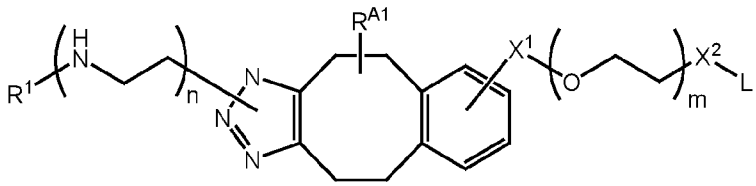
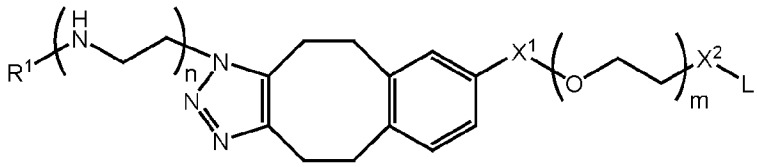


Formula IE,



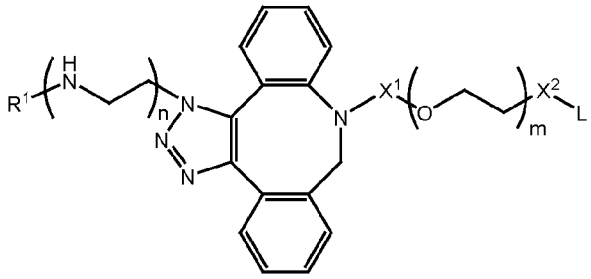
Formula IH, and



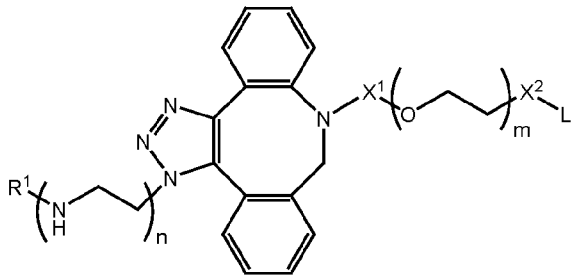
Formula IH-1,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

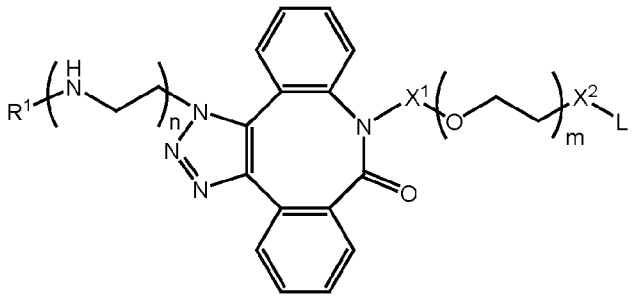
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



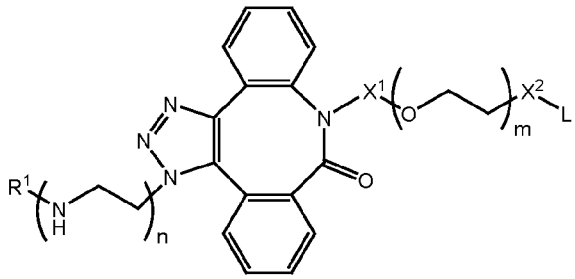
Formula IA-3,



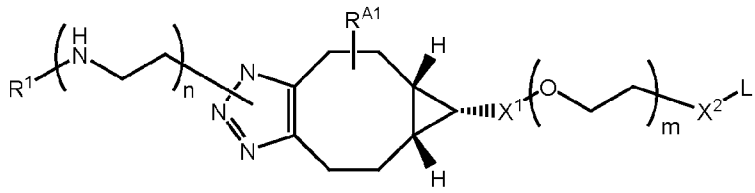
Formula IA-4,



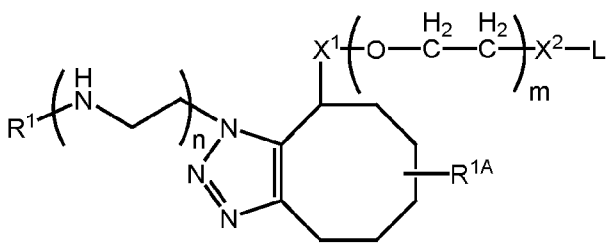
Formula IA-9,



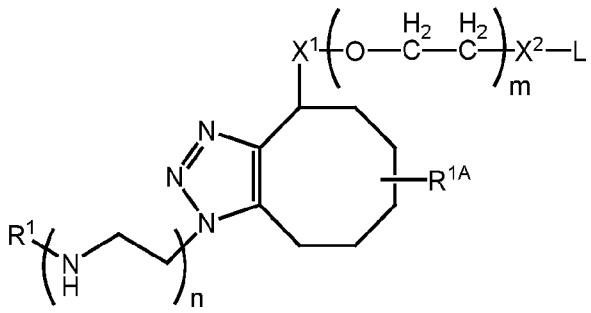
Formula IA-10,



Formula IB,



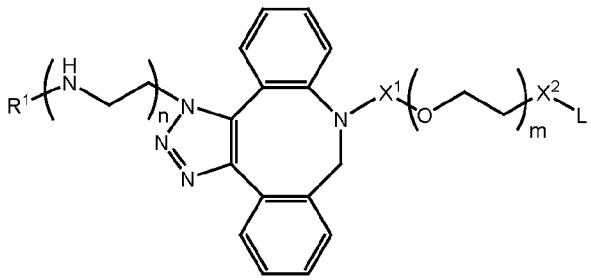
Formula IE-13, and



Formula IE-14,

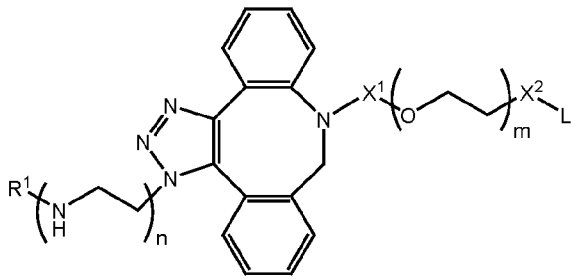
wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IA-3,

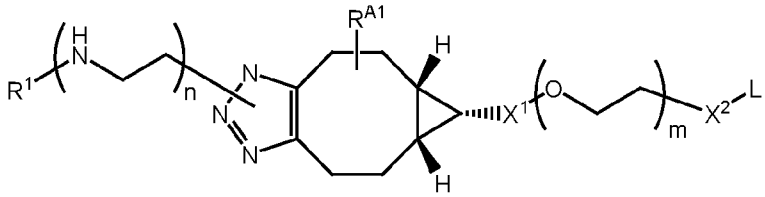
and



Formula IA-4,

wherein R^1, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, X^1, X^2, L, m and n, or collectively to some or all of R^1, X^1, X^2, L, m and n.

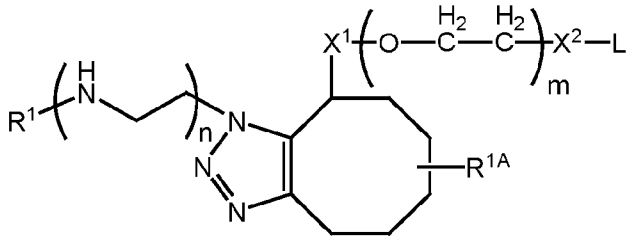
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



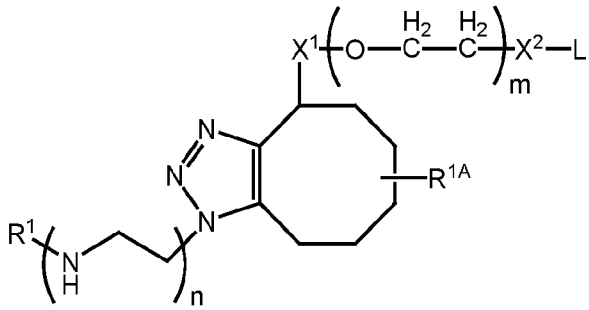
Formula IB,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IE-13, and



Formula IE-14,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment, said targeting fragment comprises or preferably consists of the DUPA residue (HOOC-$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-). In a further very preferred embodiment, said targeting fragment consists of the DUPA residue (HOOC$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-), wherein both chiral C-atoms having (*S*)-configuration, as depicted in formula **1***.

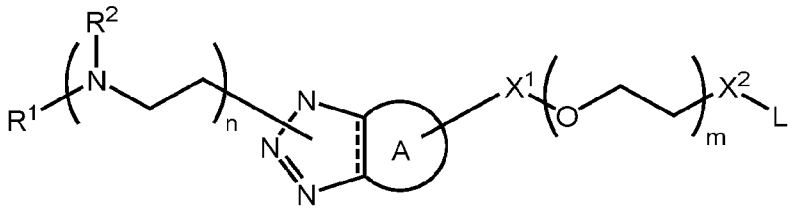
In a preferred embodiment, said poly(IC) are composed of RNA strands, wherein at least 50%, preferably at least 60% of each strand comprises at least 15 and at most 8000 ribonucleotides, preferably at most 5000 ribonucleotides In a preferred embodiment, said poly(IC) are composed of RNA strands, wherein at least 50%, preferably at least 60% of each strand comprises at least 22, preferably at least 45 ribonucleotides. In certain embodiments, at least 50%, preferably at least 60% of each strand has a number of ribonucleotides within the range of 20 to 300.

In some embodiments, said poly(IC) are composed of RNA strands each comprising at least 22, preferably at least 45 ribonucleotides. In certain embodiments, each strand has a number of ribonucleotides within the range of 20 to 300.

In another aspect, the present invention provides a polyplex comprising a conjugate as described herein, preferably said conjugate of Formula I* or of Formula I, and a polyanion such as a nucleic acid, preferably polyinosinic:polycytidylic acid poly(IC). In some embodiments,

said poly(IC) are composed of RNA strands each comprising at least 22, preferably at least 45 ribonucleotides. In certain embodiments, each strand has a number of ribonucleotides within the range of 20 to 300.

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof, and a nucleic acid, wherein said nucleic acid is preferably a mRNA or a pDNA, and wherein further preferably said nucleic acid is a pharmaceutically active nucleic acid, wherein said pharmaceutically active nucleic acid is a nucleic acid that encodes a pharmaceutically active peptide or protein:



Formula I

wherein:

═══ is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating units m of 25 to 100, preferably of a discrete number of repeating units m of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen $-SO_3H$, or $-OSO_3H$;

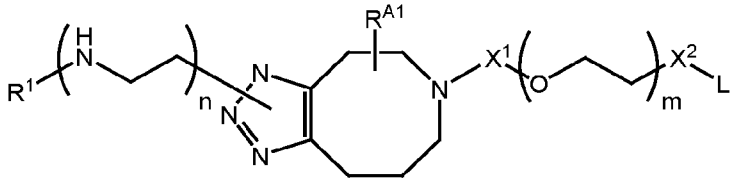
X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

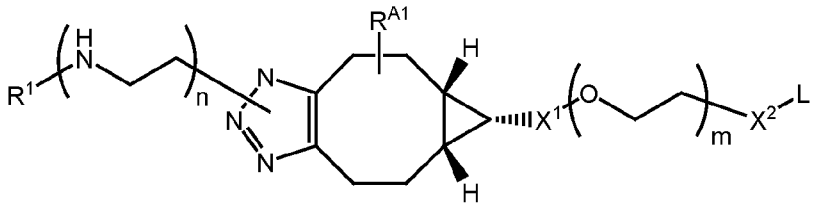
L is a targeting fragment, wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, wherein said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$.

In a preferred embodiment, said Ring A is cyclooctene, succinimide, or 7- to 8-membered heterocycloalkenyl, wherein the heterocycloalkenyl comprises one or two heteroatoms selected from N, O and S, and wherein each cyclooctene or heterocycloalkenyl is optionally substituted at any position with one or more R^{A1}, wherein preferably R^{A1} is oxo or fluorine, or wherein two R^{A1} combine to form one or more fused phenyl rings, preferably one or two fused phenyl rings, wherein each phenyl ring is optionally substituted with one or more $-SO_3H$ or $-OSO_3H$.

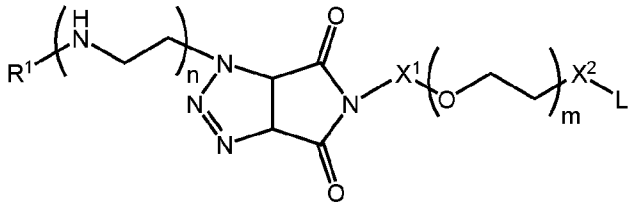
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



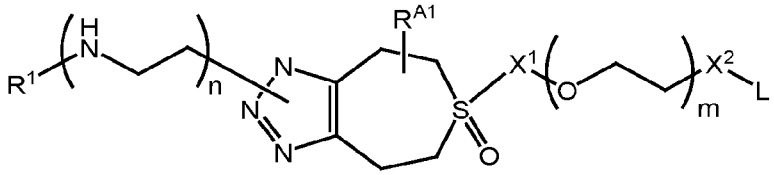
Formula IA,



Formula IB,



Formula IC,



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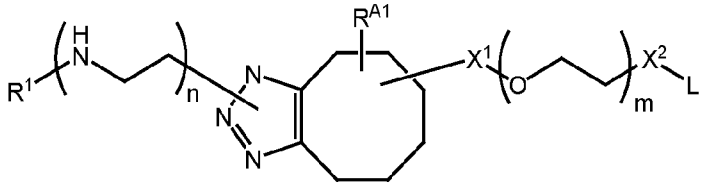
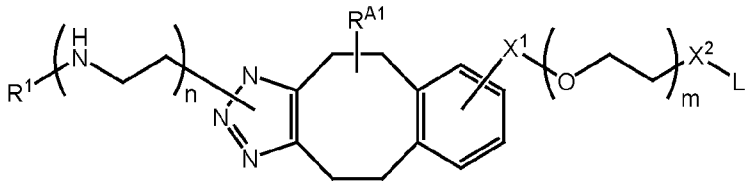
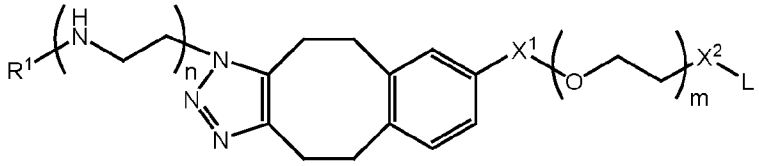


Formula IE,



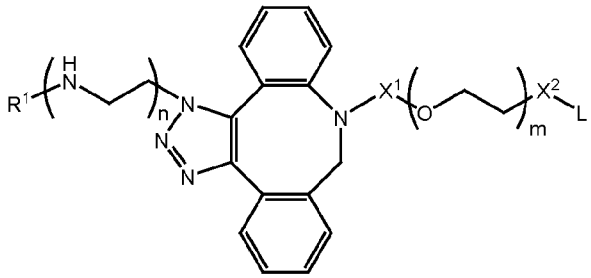
Formula IH, and



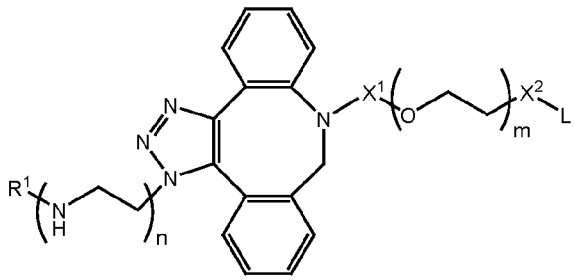
Formula IH-1,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

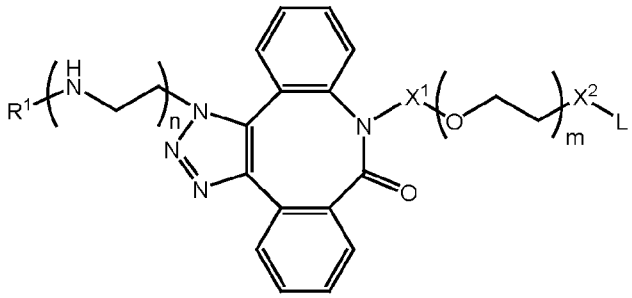
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IA-3,



Formula IA-4,



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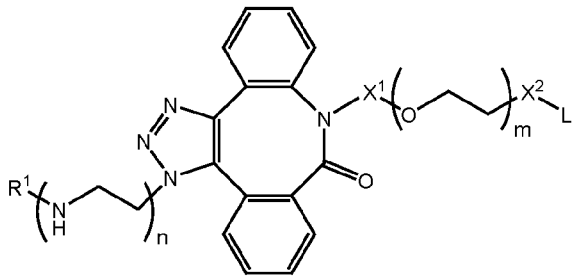

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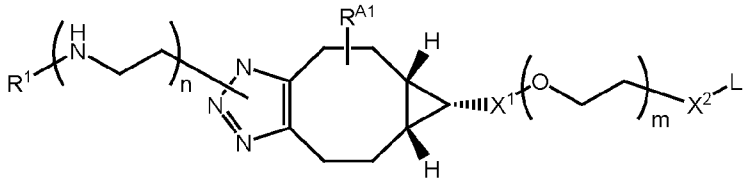
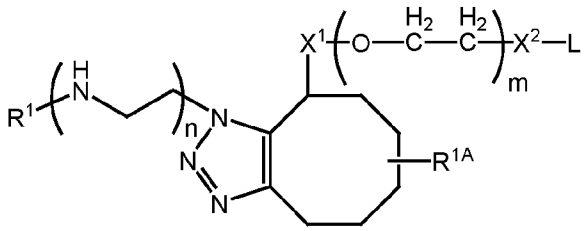
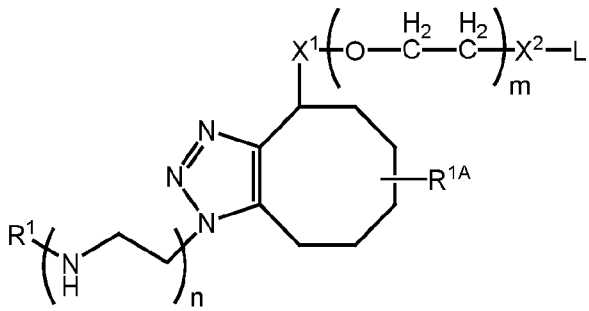


Formula IB,



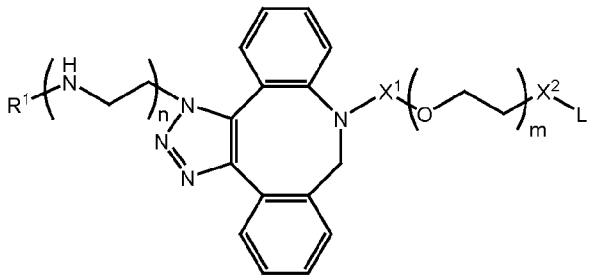
Formula IE-13, and



Formula IE-14,

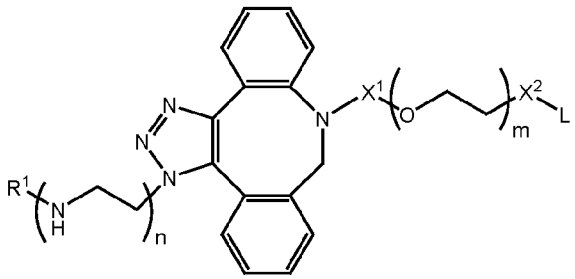
wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IA-3,

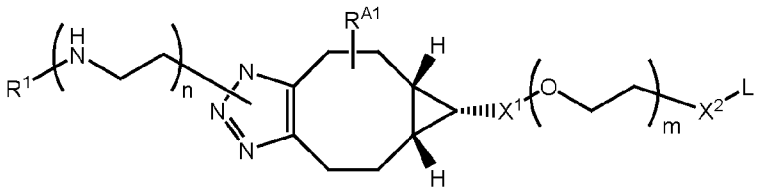
and



Formula IA-4,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

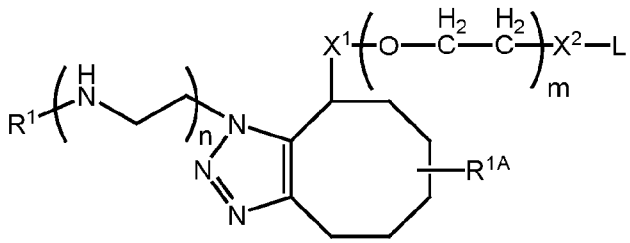
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



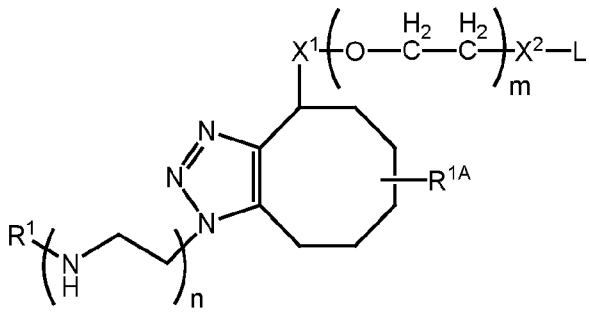
Formula IB,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IE-13, and



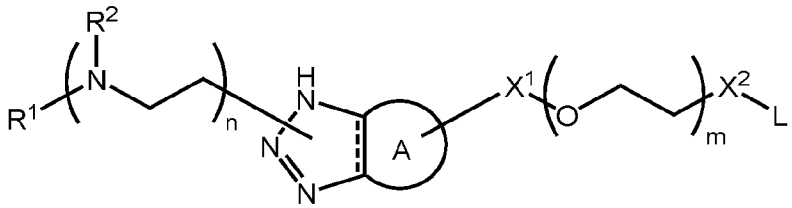
Formula IE-14,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment, said targeting fragment comprises or preferably consists of

the DUPA residue (HOOC-$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-). In a further very preferred embodiment, said targeting fragment consists of the DUPA residue (HOOC$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-), wherein both chiral C-atoms having (*S*)-configuration, as depicted in formula **1***.

In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof, and a nucleic acid, wherein said nucleic acid is preferably a mRNA or a pDNA, and wherein further preferably said nucleic acid is a pharmaceutically active nucleic acid, wherein said pharmaceutically active nucleic acid is a nucleic acid that encodes a pharmaceutically active peptide or protein:



Formula I

wherein:

═══ is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating units m of 36;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said -$(NR^2$-CH_2-$CH_2)_n$– is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

X^1 is a divalent covalent linking moiety;

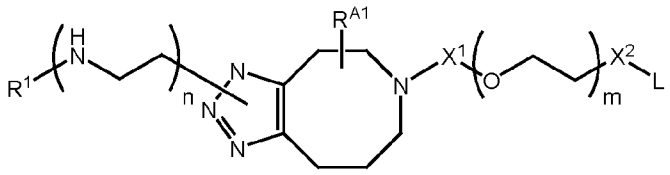
X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA, and wherein further preferably said targeting fragment is

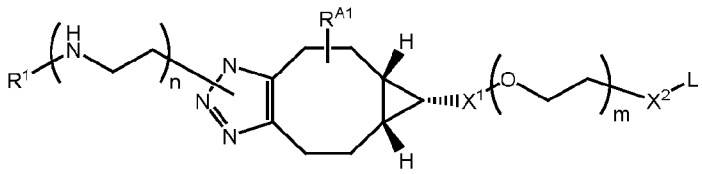
capable of binding to a cell surface receptor wherein said cell surface receptor is PSMA. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$.

In a preferred embodiment, said Ring A is cyclooctene, succinimide, or 7- to 8-membered heterocycloalkenyl, wherein the heterocycloalkenyl comprises one or two heteroatoms selected from N, O and S, and wherein each cyclooctene or heterocycloalkenyl is optionally substituted at any position with one or more R^{A1}, wherein preferably R^{A1} is oxo or fluorine, or wherein two R^{A1} combine to form one or more fused phenyl rings, preferably one or two fused phenyl rings, wherein each phenyl ring is optionally substituted with one or more $-SO_3H$ or $-OSO_3H$.

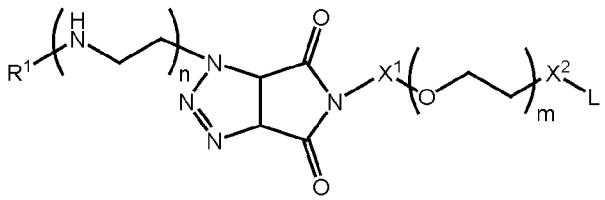
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



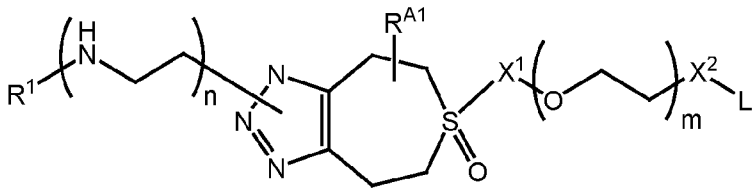
Formula IA,



Formula IB,



Formula IC,



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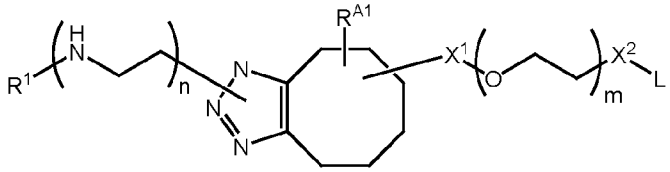
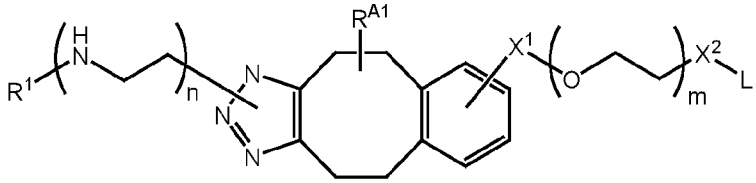
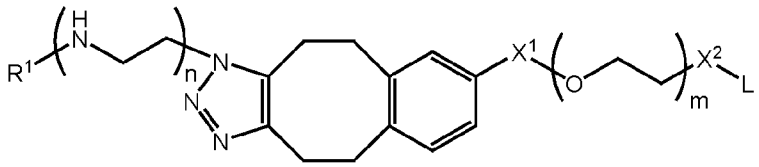


Formula IE,



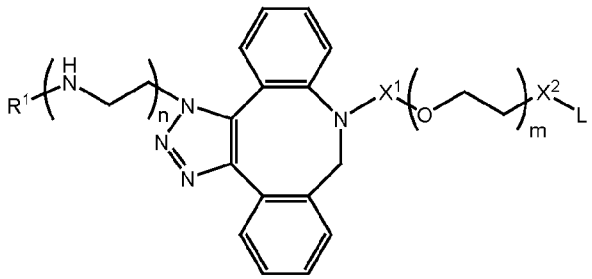
Formula IH, and



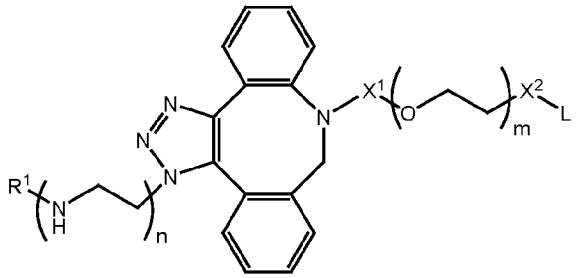
Formula IH-1,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

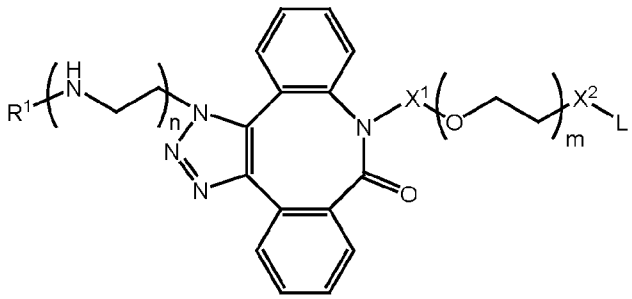
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



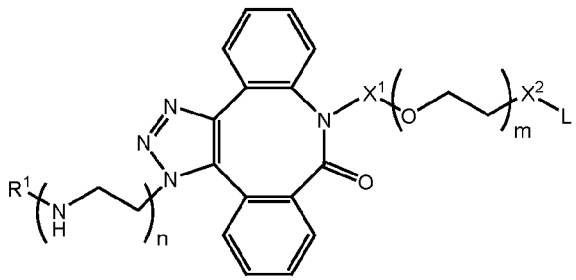
Formula IA-3,



Formula IA-4,



Formula IA-9,



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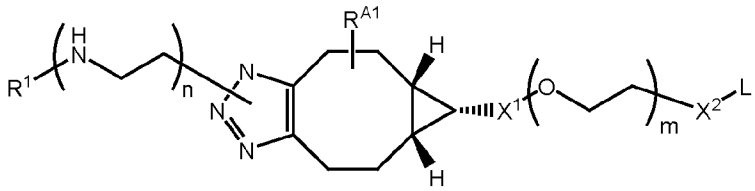
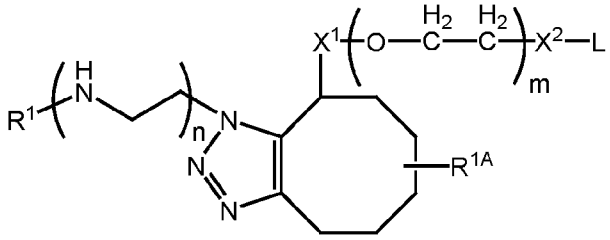
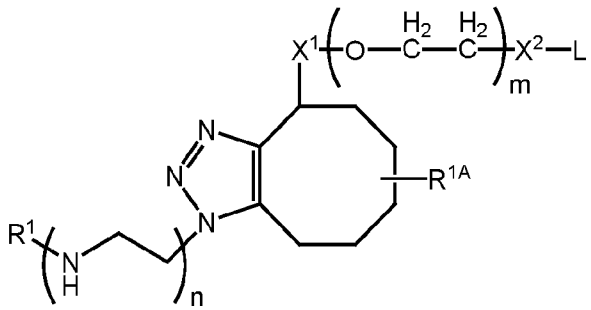


Formula IB,



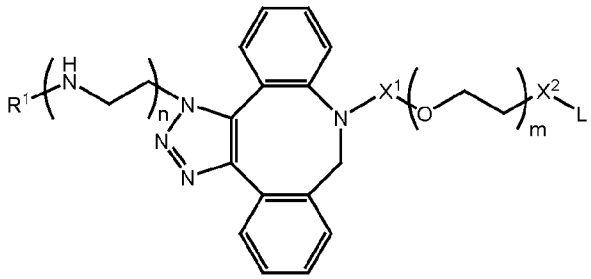
Formula IE-13, and



Formula IE-14,

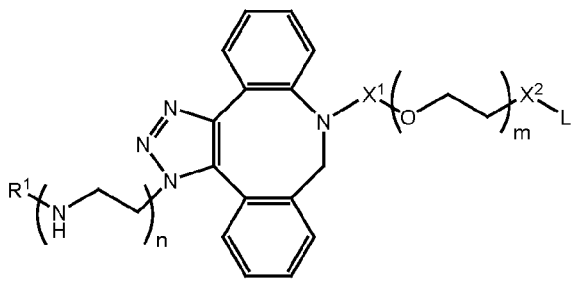
wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IA-3,

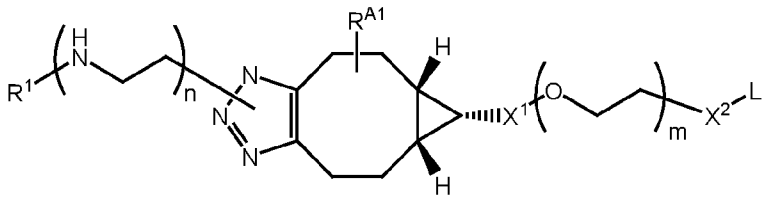
and



Formula IA-4,

wherein R^1, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, X^1, X^2, L, m and n, or collectively to some or all of R^1, X^1, X^2, L, m and n.

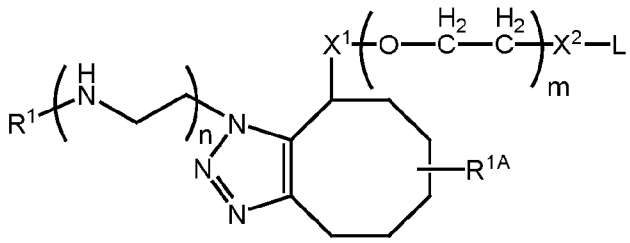
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



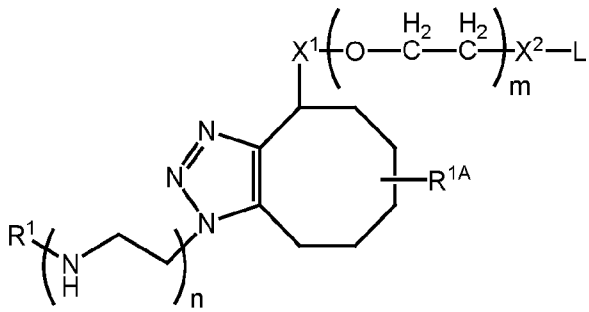
Formula IB,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IE-13, and



Formula IE-14,

wherein R^1, R^{A1}, X^1, X^2, L, m and n are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2, L, m and n, or collectively to some or all of R^1, R^{A1}, X^1, X^2, L, m and n.

In a preferred embodiment, said targeting fragment comprises or preferably consists of the DUPA residue (HOOC-$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-). In a further very preferred embodiment, said targeting fragment consists of the DUPA residue (HOOC$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-), wherein both chiral C-atoms having (*S*)-configuration, as depicted in formula **1***. Synthesis and

Characterization of Polyplexes

The present invention relates to polyplexes comprising a linear conjugate (e.g., a linear conjugate comprising LPEI, PEG, and a targeting fragment such as the DUPA residue) polyplexed with a polyanion such as a nucleic acid like a double stranded RNA (dsRNA such as poly(IC)). As shown in the Examples, polyplexes can be prepared by incubating the inventive triconjugates together with polyanions and nucleic acids such as poly(IC). In some embodiments, polyplexes can form spontaneously (e.g., within an hour or within 30 minutes) by combining the inventive triconjugates with poly(IC) in a solution of HEPES-buffered glucose at pH 7-7.4 (e.g., at room temperature), or in 5% glucose, or in HEPES buffered saline (HBS) pH 7.2, or in an acetate solution at pH 4-4.5 containing 5% glucose e.g., at room temperature).

The particle size distribution such (reported as the z-average diameter and PDI) and ζ-potential of the polyplexes can be measured by dynamic light scattering (DLS) and electrophoretic mobility, respectively. DLS measures the light scatter intensity fluctuations of polyplexes caused by the Brownian motions and calculates hydrodynamic diameter (nm) using the Stokes-Einstein equation. Zeta potential (ζ-potential) measures the electrokinetic potential of the polyplexes.

In some embodiments, the z-average diameter and ζ-potential can be modified as a function of the N/P ratio, defined as the ratio of nitrogen atoms in LPEI to phosphorous atoms in nucleic acid such as poly(IC). In some preferred embodiments, the z-average diameter of an inventivepolyplex is below about 300 nm, more preferably below about 250 nm, yet more preferably below about 200 nm. Without wishing to be bound by theory, polyplexes with z-average diameters below about 200 nm are believed to be well-tolerated *in vivo* (e.g., exhibit high biodistribution and clearance) and are typically stable and not prone to aggregate formation.

In some preferred embodiments, the N/P ratio of the polyplexes is at least 2, at least 2.4, at least 2.5, at least 3, at least 3.5, is at least about 4, at least 4.5, at least 5, or at least 6. In some preferred embodiments, the N/P ratio is 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 9, 10, 11 or 12. As shown herein, the N/P ratios mentioned above can provide polyplexes of acceptable size and stability for said polyplexes containing polyanions, preferably nucleic acids.

In a preferred embodiment, said polyplexes of the invention have a mono- or bi-modal diameter distribution, preferably a monomodal diameter distribution. Preferably, said monomodal diameter distribution is within the sub-micrometer range.

In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 350 nm. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 300 nm. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 250 nm. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 210 nm. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 200 nm. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 180 nm. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 150 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between 350 nm and 50 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between 350 nm and 70 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between 350 nm and 100 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between 300 nm and 50 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between 300 nm and 70 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between 300 nm and 100 nm. In another more preferred embodiment, said polyplexes have a z-average diameter of between 250 nm and around 50 nm. In another more preferred embodiment, said polyplexes have a z-average diameter of between 250 nm and around 70 nm. In another more preferred embodiment, said polyplexes have a z-average diameter of between 250 nm and around 100 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between around 200 nm and around 50 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between around 200 nm and around 70 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between around 200 nm and around 100 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between around 180 nm and around 50 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between around 180 nm and around 70 nm. Preferably, said polyplexes have a mono-modal diameter distribution, preferably within the sub-micrometer range.

In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 350 nm, and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 300 nm, and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 250 nm,

and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 220 nm, and the N/P ratio of the polyplexes is at least 2.4, more preferably at least 3, yet more preferably at least 4. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 200 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 180 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 150 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between 350 nm and 100 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment, said polyplexes have a z-average diameter of between 300 nm and 100 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another more preferred embodiment, said polyplexes have a z-average diameter of between 250 nm and around 100 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment, said polyplexes have a z-average diameter of between around 200 nm and around 100 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. Preferably, said polyplexes have a mono-modal diameter distribution, preferably within the sub-micrometer range.

In a preferred embodiment, the composition of the invention has a polydispersity index (PDI) of 0.7 or less. More preferably, said PDI is 0.5 or less, e.g. between 0.5 and 0.05. Again more preferably, said PDI is 0.35 or less, e.g. between 0.35 and 0.05. In another preferred embodiment, said PDI is 0.25 or less, e.g. between 0.25 and 0.05. In another preferred embodiment, said PDI is 0.2 or less, e.g. between 0.2 and 0.05. In another preferred embodiment said PDI is less than 0.2, e.g. between 0.19 and 0.05. In another more preferred embodiment said PDI is between 0.2 and 0.1. In another preferred embodiment said PDI is between 0.25 and 0.1. Preferably, said polyplexes have a mono-modal diameter distribution, preferably within the sub-micrometer range.

In a preferred embodiment, the composition of the invention has a polydispersity index (PDI) of 0.7 or less, and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4. More preferably, said PDI is 0.5 or less, e.g. between 0.5 and 0.05. Again more preferably, said PDI is 0.35 or less, e.g. between 0.35 and 0.05, and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4. In another preferred embodiment, said PDI is 0.25 or less, e.g. between 0.25 and 0.05, and the N/P ratio of the polyplexes is at least 2.4, more preferably at least 3, yet

more preferably at least 4. In another preferred embodiment, said PDI is 0.2 or less, e.g. between 0.2 and 0.05, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment said PDI is less than 0.2, e.g. between 0.19 and 0.05, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another more preferred embodiment said PDI is between 0.2 and 0.1. In another preferred embodiment said PDI is between 0.25 and 0.1, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. Preferably, said polyplexes have a mono-modal diameter distribution, preferably within the sub-micrometer range.

In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 350 nm, the PDI is 0.5 or less and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 350 nm, the PDI is 0.4 or less and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 300 nm, the PDI is 0.4 and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 250 nm, the PDI is 0.2 or less and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 220 nm, the PDI is 0.2 or less, and the N/P ratio of the polyplexes is at least 2.4, more preferably at least 3, yet more preferably at least 4. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 200 nm, the PDI is 0.2 or less, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 180 nm, the PDI is 0.2 or less, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 150 nm, the PDI is 0.2 or less, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment, said polyplexes have a z-average diameter of between 350 nm and 100 nm, the PDI is 0.2 or less, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment, said polyplexes have a z-average diameter of between 300 nm and 100 nm, the PDI is 0.2 or less, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another more preferred embodiment, said polyplexes have a z-average diameter of between 250 nm and around 100 nm, the PDI is 0.2 or less, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment, said polyplexes have a z-average diameter of between around 200 nm and around 100 nm, the PDI is 0.2 or less, and the

N/P ratio of the polyplexes is at least 3, preferably at least 4. Preferably, said polyplexes have a mono-modal diameter distribution, preferably within the sub-micrometer range.

In a preferred embodiment, the composition of the invention has a zeta potential of greater than or equal to 12, 14 or 18 mV, e.g. between 18 mV and 50 or 60 mV. In a preferred embodiment, the composition of the invention has a zeta potential of greater than or equal to 12 mV, e.g. between 12 mV and 60 mV. In a preferred embodiment, the composition of the invention has a zeta potential of greater than or equal to 18 mV, e.g. between 18 mV and 45 mV. In another preferred embodiment, the composition of the invention has a zeta potential of greater than or equal to 18 mV, e.g. between 18 mV and 42 mV. In another preferred embodiment, the composition of the invention has a zeta potential between 20 mV and 50 mV. In another preferred embodiment, the composition of the invention has a zeta potential between 20 mV and around 45 mV. In another preferred embodiment, the composition of the invention has a zeta potential between 20 mV and around 42 mV. In another preferred embodiment, the composition of the invention has a zeta potential between around 20 mV and around 40 mV. Preferably, said polyplexes have a mono-modal diameter distribution, preferably within the sub-micrometer range.

In a preferred embodiment, the composition of the invention has a zeta potential of greater than or equal to 18 mV, preferably between 18 mV and 50 mV, and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4. In a more preferred embodiment, the composition of the invention has a zeta potential of greater than or equal to 18 mV, preferably between 18 mV and 45 mV, and the N/P ratio of the polyplexes is at least 2.4, more preferably at least 3, yet more preferably at least 4. In another preferred embodiment, the composition of the invention has a zeta potential of greater than or equal to 18 mV, e.g. between 18 mV and 42 mV, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment, the composition of the invention has a zeta potential between 20 mV and 50 mV, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another preferred embodiment, the composition of the invention has a zeta potential between 30 mV and around 40 mV, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another more preferred embodiment, the composition of the invention has a zeta potential between 18 mV and around 40 mV, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. In another even more preferred embodiment, the composition of the invention has a zeta potential between around 20 mV and around 40 mV, and the N/P ratio of the polyplexes is at least 3, preferably at least 4. Preferably, said polyplexes have a mono-modal diameter distribution,

preferably within the sub-micrometer range.

In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 350 nm, and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4, and the composition of the invention has a zeta potential of between 18 mV and 50 mV. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 300 nm, and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4, and the composition of the invention has a zeta potential of between 20 mV and 50 mV. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 250 nm, and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4, and the composition of the invention has a zeta potential of between 20 mV and 50 mV. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 220 nm, and the N/P ratio of the polyplexes is at least 2.4, more preferably at least 3, yet more preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 200 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 180 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 150 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4 and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of between 350 nm and 100 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of between 300 nm and 100 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another more preferred embodiment, said polyplexes have a z-average diameter of between 250 nm and around 100 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of between around 200 nm and around 100 nm, and the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV.

Preferably, said polyplexes have a mono-modal diameter distribution, preferably within the sub-micrometer range.

In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 350 nm, the PDI is between 0.5 and 0.05, the N/P ratio of the polyplexes is at least 2, preferably at least 2.4, and the composition of the invention has a zeta potential of between 18 mV and 50 mV. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 300 nm, the PDI is between 0.5 and 0.05, and the N/P ratio of the polyplexes is at least 2, preferably at least 2.4, and the composition of the invention has a zeta potential of between 18 mV and 50 mV. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 250 nm, the PDI is between 0.35 and 0.05, the N/P ratio of the polyplexes is at least 2, preferably at least 2.4, and the composition of the invention has a zeta potential of between 18 mV and 50 mV. In a preferred embodiment, said polyplexes have a z-average diameter of less than or equal to about 220 nm, the PDI is 0.3 or less, e.g. between 0.3 and 0.05, the N/P ratio of the polyplexes is at least 2.4, more preferably at least 3, yet more preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 200 nm, the PDI is 0.2 or less, e.g. between 0.2 and 0.05, the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 180 nm, the PDI is 0.2 or less, e.g. between 0.2 and 0.05, and the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of less than or equal to 150 nm, the PDI is 0.2 or less, e.g. between 0.2 and 0.05, the N/P ratio of the polyplexes is at least 3, preferably at least, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of between 350 nm and 100 nm, the PDI is 0.2 or less, e.g. between 0.2 and 0.05, the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of between 300 nm and 100 nm, the PDI is 0.2 or less, e.g. between 0.2 and 0.05, the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 25 mV and 45 mV. In another more preferred embodiment, said polyplexes have a z-average

diameter of between 250 nm and around 100 nm, the PDI is 0.2 or less, e.g. between 0.2 and 0.05, the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. In another preferred embodiment, said polyplexes have a z-average diameter of between around 200 nm and around 100 nm, the PDI is 0.2 or less, e.g. between 0.2 and 0.05, the N/P ratio of the polyplexes is at least 3, preferably at least 4, and the composition of the invention has a zeta potential of between 18 mV and 45 mV. Preferably, said polyplexes have a mono-modal diameter distribution, preferably within the sub-micrometer range.

In some embodiments, the polyplex has a z-average diameter below about 200 nm. In some embodiments, the N/P ratio of the polyplex is between about 3 and about 10, preferably wherein the N/P ratio of the polyplex is between about 4 and about 7. In some embodiments, the N/P ratio of the polyplex is about 4, 5 or 7. In some preferred embodiments, the polyplexes of the present disclosure have a ζ-potential between about 15 and about 70 mV, between about 20 and about 70 mV; preferably between about 15 and about 50 mV; preferably between about 15 and about 40 mV.

Activity of the Polyplexes

The present invention relates to polyplexes of conjugates comprising LPEI, PEG, and targeting fragments such as DUPA or folic acid or folate ligands, and of polyanions capable of acting as cytotoxic and/or immunostimulatory agents such as nucleic acids including dsRNA, typically and preferably poly(IC).

The triconjugate:nucleic acid polyplexes disclosed herein, in particular the triconjugate:poly(IC) polyplexes have high potency and selectivity to deliver nucleic acids such as poly(IC) to cells that have high surface expression of a cell surface receptor such as PSMA. As shown in the Examples below, the triconjugates of the present invention hereby serve as vectors for said polyanions and nucleic acids such as poly(IC).

It has been shown that conjugates (and the resulting polyplexes) that contain the targeting fragment 2-[3-(1,3-dicarboxypropyl) ureido] pentanedioic acid (DUPA), can be taken up at greater concentrations in cells that exhibit high expression of prostate-specific membrane antigen (PSMA) including conjugates (and the resulting polyplexes) that contain the targeting fragment folic acid or folate ligands. One of skill in the art will appreciate that the conjugates of the present invention can be effectively modified with a variety of targeting fragments to enable selective uptake of the conjugates into specific cell types.

In preferred embodiments, the inventive polyplexes comprising poly(IC) show high biological potency. As shown, the inventive polyplexes comprising poly(IC) provides high cytotoxicity as well as high immunostimulatory activity as evidenced by high IP-10 secretion. Moreover, the Examples herein demonstrate that the inventive polyplexes were significantly more cytotoxic and active in high PSMA expressing cells than in cells that expressed PSMA at low levels, and thus shows a very high degree of selectivity. Thus, in preferred embodiments, the inventive polyplexes selectively cause cell death in cells that express high levels of a particular cell surface receptor, preferably wherein the inventive polyplexes comprise a targeting fragment that selectively targets the cell surface receptor, wherein said cell surface receptor is PSMA. Moreover, the presented results demonstrate that LPEI-*l*-PEG$_n$-PSMA:poly(IC) induces potent and selective decrease in cell survival in PSMA overexpressing cells. Little to no significant cell death was observed in LNCaP cells when poly(IC) was replaced by poly(Glu).

The same very high degree of selectivity is true for the inventive polyplexes comprising mRNA or pDNA, preferably for such mRNA or pDNA encoding peptides or proteins of interest, in particular such mRNA or pDNA encoding pharmaceutically active petides or proteins. Moreover, such inventive polyplexes do not only selectively deliver such nucleic acids encoding peptides or proteins of interest, in particular pharmaceutically active peptides or proteins, to the targeted cells, in particular cancer cells, but furthermore, said delivery results in high expression and efficient protein translation as well as secretion of the encoded peptides or proteins of interest, preferably said pharmaceutically active proteins.

Polyplexes for Use in Treating Disease

In one aspect, the present invention provides compositions comprising polyplexes described herein for use in the treatment of a disease or disorder. In another aspect, the present invention provides the use of polyplexes described herein for use in the manufacture of a medicament for the treatment of a disease or disorder. In another aspect, the present invention provides a method of treating a disease or disorder in a subject in need thereof, the method comprising administering to the subject an effective amount of a polyplex as described herein.

In one aspect, the present invention provides compositions comprising polyplexes described herein for use in the treatment of disease or disorder such as cancer, preferably prostate cancer. In another aspect, the present invention provides the use of polyplexes described herein for use in the manufacture of a medicament for the treatment of a disease or

disorder such as a cancer, preferably prostate cancer. In another aspect, the present invention provides a method of treating a disease or disorder such as a cancer, preferably prostate cancer, in a subject in need thereof, the method comprising administering to the subject an effective amount of a polyplex as described herein.

In some embodiments, the cancer can be characterized by cells that express, highly express or overexpress one or more cell surface receptors and/or antigens. Without wishing to be bound by theory, the triconjugates and/or polyplexes of the present invention can be targeted to a particular cell type (e.g., cancer cell type) by selecting an appropriate targeting fragment and coupling the appropriate targeting fragment to the PEG fragment to form a targeted triconjugate as described above. The cell surface receptor is preferably prostate-specific membrane antigen (PSMA).

In some embodiments, the cancer can be characterized by cells that have increased expression (e.g., overexpression or high expression) of prostate-specific membrane antigen. In some preferred embodiments, cancers characterized by cells that have increased expression of prostate-specific membrane antigen (PSMA) can be treated with polyplexes comprising a PSMA-targeting fragment such as DUPA. In certain embodiments, the cancer characterized by PSMA-overexpressing cells is prostate cancer and/or metastases thereof. In a preferred embodiment, said cancer is prostate cancer.

In some embodiments, cancer-associated neovasculature can be characterized by increased expression (e.g., overexpression or high expression) of PSMA (see., e.g., Van de Wiele *et al.*, *Histol Histopathol.*, (2020); **35**(9):919-927). In some preferred embodiments, cancers characterized by neovasculature that has increased expression of prostate-specific membrane antigen (PSMA) can be treated with polyplexes comprising a PSMA-targeting fragment such as DUPA. In some preferred embodiments, the cancers characterized by association with PSMA-overexpressing neovasculature are glioblastoma, breast cancer, bladder cancer and/or metastases thereof.

Equivalents

While the present invention has been described in conjunction with the specific embodiments set forth above, many alternatives, modifications and other variations thereof will be apparent to those of ordinary skill in the art. All such alternatives, modifications, and variations are intended to fall within the scope and spirit of the present invention.

EXAMPLES

The invention is further illustrated by the following examples and synthesis schemes, which are not to be construed as limiting this invention in scope or spirit to the specific procedures herein described. It is to be understood that the examples are provided to illustrate certain embodiments and that no limitation to the scope of the invention is intended thereby. It is to be further understood that resort may be had to various other embodiments, modifications, and equivalents thereof which may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or scope of the appended claims.

Abbreviations used in the following examples and elsewhere herein are:

ACN	Acetonitrile
Aoc	8-aminooctanoic acid
Aq.	aqueous
BCN	Bicyclononyne
D	Dispersity
DBCO	Dibenzocyclooctyne
DCM	Dichloromethane
DIEA	N,N-Diisopropylethylamine (Hünig's Base)
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol (reducing agent)
DUPA	2-[3-(1,3-dicarboxypropyl)ureido]pentanedioic acid
EGF	Epidermal growth factor
ELSD	Evaporative light scattering detector
Endo-BCN	(1alpha,8alpha,9beta)-bicyclo[6.1.0]non-4-yne
Epsilon (ε)	Extinction coefficient
Eq	Equivalent
FLuc mRNA	Firefly Luc messenger RNA
HATU	*O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorphosphate
HBG	HEPES buffered glucose solution
hEGF	Human epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

LC	Liquid chromatography
LPEI	Linear polyethyleneimine
MADOPA	N^{10}-Methyl-4-amino-4-deoxypteroic acid
MAL	Maleimide
MCC	4-(N-maleimidomethyl)-cyclohexane-1-carboxy linker
MTX	Methotrexate
NHS	N-hydroxysuccinimide
OPSS	Orthopyridyl disulfide
PEG	Polyethylene glycol
PDI	Polydispersity Index
Poly(IC) *or* pIC *or* p(IC)	Polyinosinic:polycytidylic acid
Poly(Glu)	Poly-L-glutamic acid sodium salt
PyBOP	Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate
RENCA	Renal Carcinoma
RP-HPLC	Reversed phase high pressure liquid chromatography
RP-HPLC-MS	Reversed phase high pressure liquid chromatography mass spectrometry
qTOF MS	Quadrupole time of flight mass spectrometry
SPDP	(succinimidyl 3-(2-pyridyldithio)propionate)
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
TFF	Tangential flow filtration
TIS	Triisopropylsilane

Unless otherwise noted, the following polymer naming conventions are used herein. Linear (i.e., unbranched) polymers are denoted with "*l*" and random (i.e., branched) polymers are denoted with "*r*". Conjugates are further identified using an abbreviation for each fragment of the conjugate (e.g., PEG or LPEI) and/or targeting group (e.g., DUPA) in the orientation in which they are connected. Subscripts, when used, after each fragment within the conjugate indicate the number of monomer units (e.g., LPEI or PEG units) in each fragment. The linking moieties, and in particular the divalent covalent linking moiety Z of Formula I* connecting the LPEI and PEG fragments (e.g., a 1,2,3-triazole or a 4,5-dihydro-1H-[1,2,3]triazole) are defined by the reactive groups that formed the linking moieties and the divalent covalent linking moiety

Z of Formula I*, respectively. For example, the conjugate abbreviated "LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA" is an unbranched (i.e., linear) conjugate comprising LPEI connected to a 36-unit PEG chain through a 1,2,3 triazole formed by the reaction of an azide comprised by the LPEI fragment and DBCO comprised by the PEG fragment, while the terminal end of the PEG fragment is bonded to DUPA.

Analytical Methods, Materials, and Instrumentation. Unless otherwise noted, reagents and solvents were used as received from commercial suppliers. Starting materials are either commercially available or made by known procedures in the reported literature or as illustrated. α-Hydrogen-ω-azido-poly(iminoethylene) (H-$(NC_2H_5)_n$-N_3; LPEI-N_3) ULTROXA® (MW = 22 KDa; dispersity ≤ 1.25) and α-Methyl-ω-azido-poly(iminoethylene) (CH_3-$(NC_2H_5)_n$-N_3; Me-LPEI-N_3) ULTROXA® (MW = 25.3 KDa; dispersity ≤ 1.25) were obtained from AVROXA BV (Belgium). DBCO-amine (Compound 35) was purchased from BROADPHARM Inc (USA) (Product No. BP-22066; $C_{18}H_{16}N_2O$; Mw 276.3), NHS-PEG_{36}-OPSS was purchased from Quanta Biodesign Ltd, (USA) (Product No. 10867; Mw 1969.3). DBCO-PEG_{24}-TFP (Product No. PEG6760, $C_{77}H_{118}F_4N_2O_{28}$; Mw 1595.75), DBCO-$PEG_{24}$-MAL (Product No. JSI-A2405-004, $C_{76}H_{122}N_4O_{29}$; Mw 1555.79), CliCr®-beta-Ala-NH_2 (Product No. RL-4190), HOOC-dPEG_{36}-NH_2 (Product No. PEG3340, CAS No. 196936-04-6) all from IRIS BIOTECH GMBH (Germany). Low molecular weight (LMW) poly(IC) was purchased from Dalton Pharma Services (Canada). Poly(Glu) (MW range: 50-100 KDa) was obtained from Sigma Aldrich. DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (($C_{57}H_{71}N_{11}O_{16}S$; Mw 1198.3; SEQ ID NO:4), DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Maleimide ($C_{60}H_{72}N_{12}O_{16}$; Mw 1217.3; SEQ ID NO:5, were synthesized by CBL Patras S.A. (Greece). Folic acid (Product No. F7876) and N^{10}-methyl-4-amino-4-deoxypteroic acid (Product No. 861553) were purchased from Sigma-Aldrich. Cysteamine 4-methoxytrityl resin (Novabiochem®; Product No. 8.56087.0001) was purchased from Merck KGaA. SCO-PEG_3-NH_2 (Product No. SC-8301) was purchased from Sichem GMBH. Cell lines were obtained from ATCC®. Cell lines used herein were A431 (No. CRL-1555); MCF7 (No. HTB-22); LNCaP (No. CRL-1740); and PC-3 (No. CRL-1435). Acetate buffer was 50 mM sodium acetate (aq.) supplemented with 5% glucose at pH 4-4.5. HEPES buffer was HEPES at a concentration of 20 mM (aq.) at a pH of 7-7.4. mRNA were purchased from TriLink Biotechnologies, USA or Tebubio GmbH, Germany: Luc mRNA (Trilink Biotechnologies, L-7602) comprising SEQ ID NO:6 (mRNA Luc ORF); Capped (CleanCap AG, TriLink) and 5'UTR, 3'UTR, poly A optimized for optimal translational efficiency. Human IL-2 mRNA (Trilink Biotechnologies, WOTL83314) comprising (SEQ ID NO:7 (mRNA hIL-

2 ORF); Capped (CleanCap AG, TriLink); Fully substituted with Pseudo U; 5'UTR, 3'UTR, poly A optimized for optimal translational efficiency. Human IFNβ mRNA (Tebubio, TTAP-122022) comprising (SEQ ID NO:9 (mRNA hIFNβ-2 ORF); Capped (Enzymatic capping with same performance as CleanCap AG, Tebubio); Fully substituted with N1methylspeudo U; 5'UTR, 3'UTR, poly A optimized for optimal translational efficiency. Diphtheria toxin (DT) catalytic domain A (DT-A) mRNA (Tebubio, TTAP-012023 comprising SEQ ID NO:15 (mRNA DT-A ORF); Capped (Enzymatic capping with same performance as CleanCap AG, Tebubio); Fully substituted with N1methylspeudo U; 5'UTR, 3'UTR, poly A optimized for optimal translational efficiency. The following plasmid DNA were used: pGreenFire1-CMV Plasmid (SBI, Cat#TR011PA-1); plasmid hIL-2 (InvivoGen, Cat#pUNO1-hIL02); plasmid hIFNβ (Sino Biological, pCMV3-hIFNβ).

UV spectrophotometry of samples comprising DBCO. Measurements of DBCO content of reagent solution and conjugated samples were performed on a microplate reader (Spectramax Paradigm, Molecular Devices) using Brand® pureGrade UV-transparent microplates at 309 nm. UV absorption of a 100 mL buffered solution was measured and the absorbance of the sample was corrected by subtracting the absorbance of buffer solution alone (blank). ε (309 nm) of DBCO was 12,000 $cm^{-1} \cdot M^{-1}$. The concentration of total DBCO was calculated using this formula:

$$c(\text{DBCO})\ [\text{mol/L}] = A_{309}\ [\text{AU}] / (\varepsilon_{309}\ [\text{L*mol}^{-1}\text{*cm}^{-1}] \text{*} 0.28\ \text{cm}).$$

UV spectrophotometry of samples comprising DUPA. For measurements of DUPA content, UV spectrophotometry was performed on a microplate reader (Spectramax Paradigm, Molecular Devices) at 280 nm. 100 µL of solution were analysed in Brand puregrade 98 UVtransp F as well as 100 µL of the appropriate buffer (blank). The absorbance of the sample was corrected for the blank. ε (280 nm) of DUPA was (theoretically determined): ε (280 nm) = 11'000 $cm^{-1} \cdot M^{-1}$. The concentration of DUPA was calculated using this formula: c(DUPA) [mol/L] = A280 [AU]/ (ε [$L*mol^{-1}*cm^{-1}$]*0,28 cm).

RP-HPLC-coupled Mass Spectrometry. Samples were analyzed by LC-MS using an Agilent 1260 Infinity II HPLC system or an Agilent UHPLC 1290 system.

The Agilent 1260 Infinity II HPLC system was connected to an Agilent iFunnel 6550B qTOF equipped with an Agilent Jet Stream electrospray ionization (AJS ESI) source. The sample was separated on a Phenomenex Aeris Widepore column XB-C8 – 3.6µm, 100x2.1mm (P/N: 00D-4481-AN) at 40°C. 1-5 µL were injected and elution was achieved with the eluent

gradient shown in Table 1 with a flowrate of 0.3 mL/min, where solvent A was 100% H_2O with 0.1% HCOOH and solvent B 100% ACN with 0.1% HCOOH. The AJS ESI source was operated with a capillary voltage of 3000 V and a nozzle voltage of 1000 V with a drying gas temperature of 200°C and a flow rate of 14 L/min, nebulizing gas pressure of 20 psig, and a sheath gas temperature of 325°C and flow rate of 12 L/min. MS data were acquired in the positive ion mode in the range of 100-3200 m/z in the standard mass range at 4Ghz high resolution mode between 2 and 12 min. The fragmentor and octupole RF voltages were set at 380, 750 V respectively.

Table 1. Eluent Gradient for RP-HPLC-MS using Agilent 1260 Infinity II HPLC System

Time [min]	A [%]	B [%]
0.00	95.00	5.00
1.00	95.00	5.00
8.00	50.00	50.00
9.00	5.00	95.00
13.00	5.00	95.00

The Agilent UHPLC 1290 system comprised an Agilent 1290 binary pump (G4220A), Agilent 1290 HiP Sampler (G4226A), Agilent 1290 Column compartment (G1316C), Agilent 1290 DAD UV modules (G4212A), and Agilent Quadrupole LC/MS (6130) at 40 °C using a Phenomenex BioZen column XB-C8 (3.6 µm, 150 × 2.1mm (00F-4766-AN) equipped with a pre-column filter of the same material (AJ0-9812). 5 µL of sample were injected. The flow was 0.4 mL/min. Signal was monitored at 210 nm, 215 nm, 240 nm and 280 nm. The mobile phases were: A) H_2O with 0.1% (vol.) HCOOH and B) ACN. The eluent gradient used is given in Table 2.

Table 2. Eluent Gradient for RP-HPLC-MS using Agilent UHPLC 1290 System

Time [min]	A [%]	B [%]
0.00	95.00	5.00
1.00	95.00	5.00
8.00	50.00	50.00
9.00	5.00	95.00
11.00	5.00	95.00

Analytical RP-HPLC. RP-HPLC experiments were performed on an Agilent UHPLC 1290 system comprising an Agilent 1290 binary pump (G4220A), Agilent 1290 HiP Sampler (G4226A), Agilent 1290 Column Compartment (G1316C), and Agilent 1290 DAD UV

(G4212A) modules at 40 °C using a Phenomenex BioZen™ XB-C8 column (3.6 µm, 150 × 2.1mm (00F-4766-AN) equipped with a pre-column filter of the same material (AJ0-9812). 20 µL of sample were injected. The flow was 0.4 mL/min. Signal was monitored at 210 nm, 214 nm, 220 nm, 230 nm, 240 nm and 280 nm. The mobile phases were A) H_2O + 0.1% TFA (vol.) and B) ACN + 0.1% TFA (vol.). The eluent gradient used is given in Table 3.

Table 3. Eluent Gradient for Analytical RP-HPLC

Time [min]	A [%]	B [%]
0.00	95.00	5.00
1.00	95.00	5.00
8.00	50.00	50.00
9.00	5.00	95.00
11.00	5.00	95.00

Preparative RP-HPLC. Preparative RP-HPLC experiments were performed on a Waters preparative system or a PuriFlash RP preparative system.

The Waters system comprised a Waters 515 HPLC Pump, Waters 2545 Binary Gradient Module, Waters 2777C Sampler, Waters Fraction Collector III and Waters 2487 Dual λ Absorbance Detector module using a Phenomenex Kinetex 5 mm XB-C18 column (100Å, 100 x 21.0 mm, 00D-4605-P0-AX) equipped with a Phenomenex SecurityGuard PREP Cartridge Core-shell C18 pre-column (15 x 21.2 mm, G16-007037). The flow rate was 35 mL/min and the signal was monitored at 240 nm. The fractions collector collected from 0.1 min to 30 min volumes of ~8 mL/tube (88% total filling) according to the following profile: Eluent A: H_2O with 0.1%(vol.) TFA. Eluent B: CAN with 0.1% (vol) TFA. The eluent gradient used is given in Table 4.

Table 4. Eluent Gradient for Preparative RP-HPLC Using Waters Preparative System

Time [min]	A [%]	B [%]
0.00	90.00	10.00
30.00	50.00	50.00
35.00	2.00	98.00
36.00	2.00	98.00
38.00	90.00	10.00

The PuriFlash system comprised an Interchim Inc. PuriFlash 1 Serie system comprising an injector, pump, detector and fraction collector using a Phenomenex Kinetex 5 mm XB-C18 column (100Å, 100 x 21.0mm, 00D-4605-PO-AX) equipped with a Phenomenex SecurityGuard PREP Cartridge Core-shell pre-column (C18 15 x 21.2 mm, G16-007037).

When injecting (from 00 s to 04 s), the flow rate was 10 mL/min and then was 35 mL/min until the end of run. The signal was monitored at 210 nm. The mobile phases were: Eluent A: H_2O with 0.1% (vol) TFA. Eluent B: ACN with 0.1% (vol.) TFA. The eluent gradient used is given in Table 5.

Table 5. Eluent Gradient for Preparative RP-HPLC Using PuriFlash Preparative System

N°	Time	Flow [mL/min]	A [%]	B [%]
01	00 s	10.0	90	10
02	01 s	10.0	90	10
03	04 s	10.0	90	10
04	01:03	10.0	89	11
05	01:06	10.0	89	11
06	01:35	10.0	88	12
07	01:38	35.0	88	12
08	30:00	35.0	50	50
09	35:00	35.0	02	98
10	36:00	35.0	02	98
11	38:00	35.0	90	10
12	40:00	35.0	90	10

Copper Assay. The copper assay provides the concentration in mg/mL of total LPEI present in the solution (Ungaro *et al.*, *J. Pharm. Biomed. Anal.* 31; 143-9 (2003)). A stock solution of copper reagent (10x) was prepared by dissolving 23.0 mg of $CuSO_4 \cdot 5H_2O$ in 10.0 mL acetate buffer (100 mM; pH 5.4). This stock solution was stored at 4 °C. Prior to analysis, this reagent was diluted ten-fold with acetate buffer (100 mM pH 5.4) and used directly. As a control, a solution of known concentration of LPEI (in vivo-jetPEI; 150mM nitrogen concentration; Polyplus 201-50G) was used. 6.7 µL aliquots of the in vivo-jetPEI solution were prepared in plastic tubes and frozen for use as control samples which were freshly thawed and diluted 15x with Milli-Q water (93.3 µL) prior to use.

The solutions of experimental samples and control samples were dispensed in a UV-compatible 96 well microplate (BRANDplates, pureGrade) as shown in Table 6 and were measured in triplicate.

Table 6. Solutions Used in Copper Assay.

Sample	Sample volume [µl]	Water volume [µl]	$CuSO_4$ volume [µl]
In vivo-Jet LPEI (15x; Control)	8	92	100
LPEI-/-PEG-[Targeting Fragment]	8	92	100

A blank consisting of 100 µL water and 100 µL $CuSO_4$ reagent was also measured in

triplicate and the mean absorbance of the blank was subtracted from the absorbance values recorded for the experimental samples and the control sample. Solutions were left to react for 20 minutes at room temperature and their absorbance was then measured at 285 nm in a microplate reader (Spectramax Paradigm, Molecular Devices). Individual measurements were validated if the absorbance values were in the calibration range and were otherwise further diluted. Individual measurements were not validated if the coefficient of variation of the measurement was greater than 10.0% but were instead repeated. The measurement run was validated if the value of the control was within 10% of 150 mM. Concentrations were calculated using the following formula using the calibration slope $k = 0.0179$:

$$c(\text{LPEI total})\ [\text{mg/L}] = (A_{\text{corr, average}}\ [\text{AU}] / k\ [\text{L*mg}^{-1}]) * (200/8) * \text{dilution factor}$$

Lyophilization. Lyophilization was performed on a freeze-drying device from Christ (Alpha 2-4 LP Plus). Because of the presence of acetonitrile in some samples, the samples were cooled for about three minutes with liquid nitrogen at -196 °C before lyophilization.

Samples were lyophilized at -82 °C (condenser temperature) and 100 mbar (75 Torr). The time of lyophilization was adjusted based on the properties of the lyophilized compound.

Buffer Exchange general method. For preparation of triconjugates in a HEPES buffer, the resuspended TFA-lyophilisate solution was pH adjusted with NaOH to pH 6.5 before exchanging the buffer with 20 mM HEPES at pH 7.2.

For preparation of triconjugates in an acetate buffer, the resuspended TFA-lyophilisate solution was pH adjusted with NaOH to pH 4.5 before exchanging the buffer with 50 mM acetate at pH 4.3.

Detailed buffer exchange procedures that are compound specific are also provided below:

Tangential flow filtration (TFF) 2 kDa purification:

For the removal of TFA from DBCO-PEG$_{36}$-DUPA (Compound 18) • TFA salt, tangential flow filtration was performed on a Sartorius Slice Cassette composed of a peristaltic pump (Sartorius Stedim / Tandem Model 1082 / SciLog, Inc.) with Masterflex ® PharMed® tubing (Ref. 06508-15) and Hydrosart membrane with a molecular weight cut-off (MWCO) of 2 kDa and a surface of 200 cm^2 (Sartorius Stedim / Sartocon Slice 200 / Ref.: 3051441901E-SG / Lot: 90279123). The membrane was stored in 20-24% aq. EtOH.

The following TFF parameters were used: TMP: 2.0 bars; flow rate feed: 428 mL/min; flow rate permeate: 28 g/min.

For step-wise TFF, (1)169 mL of DBCO-PEG$_{36}$-DUPA (Compound 18) solution were supplemented with 81 mL of 15 mM acetate pH 5.5. The solution was filtrated down to 50 mL by TFF. (2) The resulting 50 mL were supplemented with 250 mL of 15 mM acetate pH 5.5. The solution was filtrated down to 50 mL by TFF. (3) The resulting 50 mL were supplemented with 250 mL of 15 mM acetate pH 5.5. The solution was filtrated down to 50 mL by TFF. (4) The resulting 50 mL were supplemented with 250 mL of 15 mM acetate pH 5.5. The solution was filtrated down to 50 mL by TFF. (5) The resulting 50 mL were supplemented with 250 mL of 15 mM acetate pH 5.5. The solution was filtrated down to 50 mL by TFF. (6) The resulting 50 mL were supplemented with 250 mL of 15 mM acetate pH 5.5. The solution was filtrated down to 50 mL by TFF.

Tangential flow filtration (TFF) 10 kDa purification:

For the removal of TFA from LPEI-*l*-[N$_3$:DBCO]-PEG$_{36}$-DUPA (Compounds 12a and 12b) • TFA salt, tangential flow filtration experiments were performed on a Sartorius Slice Cassette composed of a peristaltic pump (Sartorius Stedim / Tandem Model 1082 / SciLog, Inc.) with Masterflex ® PharMed® tubing (Ref. 06508-15) and Hydrosart membrane with a molecular weight cut-off (MWCO) of 10 kDa with a surface of 200 cm^2 (Sartorius Stedim / Sartocon Slice 200 / Ref.: 3051443901E-SG / Lot: 01181123). The membrane was stored in 20-24% aq. EtOH.

The following TFF parameters were used: TMP: 1.6 bars; flow rate feed: 517 mL/min; flow rate permeate: 155 g/min.

For step-wise TFF, (1) 30 mL of LPEI-*l*-[N$_3$:DBCO]-PEG$_{36}$-DUPA (Compounds 12a and 12b) • TFA salt solution were supplemented with 220 mL of 20 mM HEPES pH 7.2. The solution was filtrated down to 50 mL by TFF. (2) The resulting 50 mL were supplemented with 250 mL of 20 mM HEPES pH 7.2. The solution was filtrated down to 50 mL by TFF. (3) The resulting 50 mL were supplemented with 250 mL of 20 mM HEPES pH 7.2. The solution was filtrated down to 50 mL by TFF. (4) The resulting 50 mL were supplemented with 250 mL of 20 mM HEPES pH 7.2. The solution was filtrated down to 50 mL by TFF. (5) The resulting 50 mL were supplemented with 250 mL of 20 mM HEPES pH 7.2. The solution was filtrated down to 50 mL by TFF.

Polyplex Sizing Measurements and Characterization.

Triconjugates (e.g., LPEI-*l*-[N$_3$:DBCO]-PEG$_{36}$-DUPA) were complexed with nucleic acids (e.g., poly(IC)) to form polyplexes (e.g., LPEI-*l*-[N$_3$:DBCO]-PEG$_{36}$-DUPA:poly(IC)). The N/P ratio of the polyplexes, as referred herein, corresponds to the molar

ratio of the nitrogen (N) content of the triconjugate to the phosphorus (P) content of poly(IC) measured prior to preparing polyplexes by mixing at the specified N/P ratio. Polyplex size distribution and ζ-potential were measured by DLS and ELS according to Hickey *et al.*, *J. Control. Release*, 2015, 219, 536-47. The size of the polyplexes was measured by DLS with a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., UK), working at 633 nm at 25 °C and equipped with a backscatter detector (173°), for example in HBG buffer (20 mM HEPES, 5% glucose, pH 7.2). Each sample was measured in triplicate. Briefly, polyplexes in HBG buffer or 5% glucose were transferred into a quartz cuvette, typically and preferably using particle RI of 1.59 and absorption of 0.01 in HBG or 5% glucose (wt/vol) at 25° C with viscosity of (0.98 mPa.s or 1.078 mPa.s) and RI of 1.330. Measurements were made using a 173° Backscatter angle of detection previously equilibrated to 25° C for at least 30 sconds, typically and preferably 60 seconds in triplicate, each with automatic run duration, without delay between measurements. Each measurement was performed seeking optimum position with an automatic attenuation selection. Data was analyzed using a General-Purpose model with normal resolution. The calculations for particle size and PDI are determined according to the ISO standard document ISO 22412:2017. The ζ-potential of polyplexes was measured by phase-analysis light scattering (PALS) (for example in HBG buffer at 25 °C), and/or electrophoretic light scattering (ELS) as described by instrument supplier (https://www.malvernpanalytical.com/en/products/technology/light-scattering/electrophoretic-light-scattering). Briefly, polyplex samples in the indicated formulation buffer (e.g. 5% glucose) were transferred into a folded capillary cell and measured in 3-5 replicates. For nanoparticle material, settings of polystyrene latex were used: R.I. of 1.59 and absorption of 0.01. For dispersant, the experimentally determined viscosity of the formulation buffer were used (e.g. R.I. of 1.33 and viscosity 1.078 mPa.s for 5% glucose). Measurements were performed after at least 30 s incubation at 25°C using the auto mode.

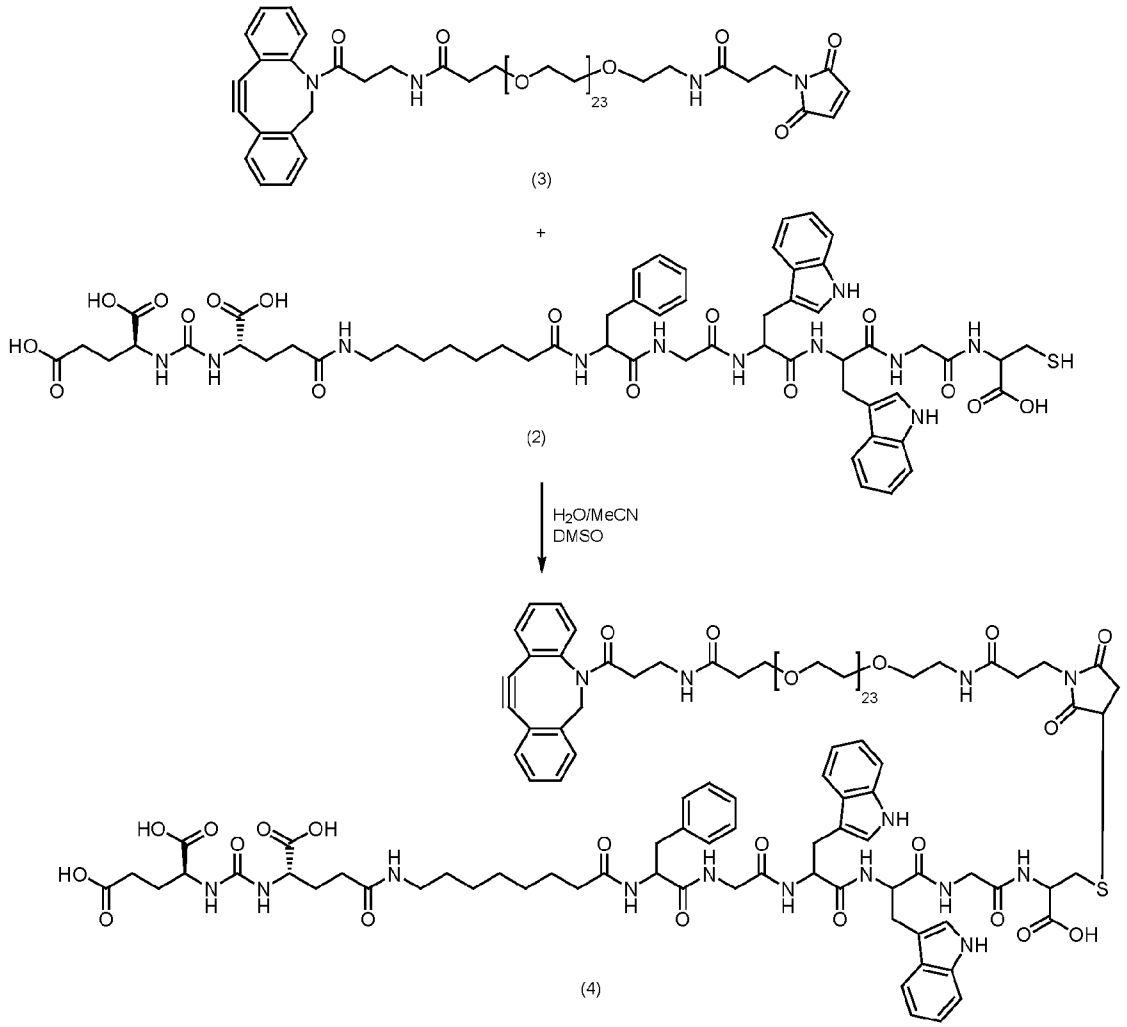
EXAMPLE 1

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA (COMPOUNDS 1a AND 1b)

LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA was synthesized as a mixture of regioisomers 1a and 1b in two steps according to the schemes below. In the first step, DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; SEQ ID NO:4) (prepared analogously as described in WO2015/173824 A1 and WO2019/063705 A1) was coupled to dibenzoazacyclooctyne-24(ethylene glycol)-maleimide (DBCO-PEG_{24}-MAL; Compound 3) by Michael addition to prepare DBCO-PEG_{24}-

DUPA (Compound 4). In the second step, DBCO-PEG$_{24}$-DUPA (Compound 4) was conjugated to LPEI-N$_3$ to produce LPEI-*l*-[N$_3$:DBCO]-PEG$_{24}$-DUPA (Compounds 1a and 1b).

Step 1: Synthesis of DBCO-PEG$_{24}$-DUPA (Compound 4)

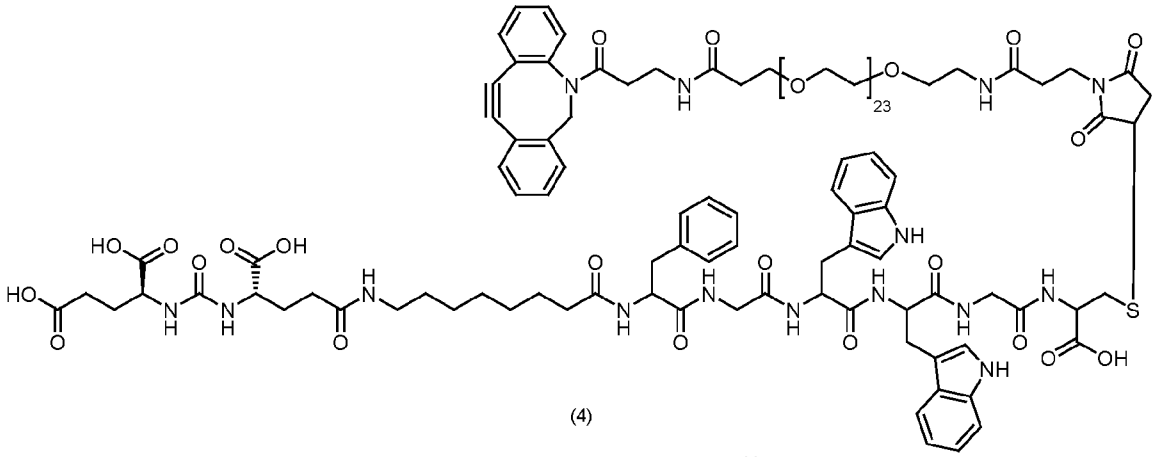


18.06 mg (crude mass) of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; 15 µmol pure theoretical peptide content) were weighed in a 50 mL Falcon tube and dissolved in 9 mL H_2O/25% ACN (2.0 mg/mL stock solution). The solution was sonicated for about 15 seconds to help dissolve the DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2). The pH of the solution was adjusted to 3.5 with 8.5 µL 6 M HCl.

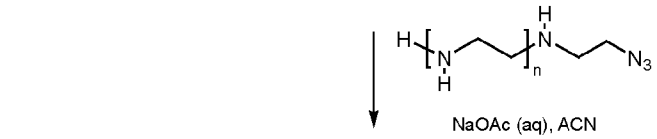
21.38 mg (crude mass) of DBCO-PEG$_{24}$-MAL (Compound 3; 13 µmol pure product) were weighed in a 1.5 mL Eppendorf tube and dissolved in 650 µL DMSO (20 mM pure product). In the 50 mL Falcon tube containing the Compound 2 solution (15 µmol, 1.5 eq), 500 µL of the DBCO-PEG$_{24}$-MAL (Compound 3) stock solution (10 µmol, 1.0 eq) were added. The reaction mixture was protected from light and incubated on a Stuart rotator (20 rpm) for about

20 hours (RT). The reaction was monitored by C8-RP-HPLC and was continued up to complete conversion of DBCO-PEG$_{24}$-MAL (Compound 3). The identity of the DBCO-PEG$_{24}$-DUPA (Compound 4) produced by the reaction was confirmed by LC-MS (C8-RP-HPLC coupled with ESI-qTOF MS) analysis ((M+2H)2+]/2=1377.16, monoisotopic mass [Da] measured 2752.30, monoisotopic mass [Da] calculated 2752.30). The reaction was not quenched or purified and was used directly in Step 2.

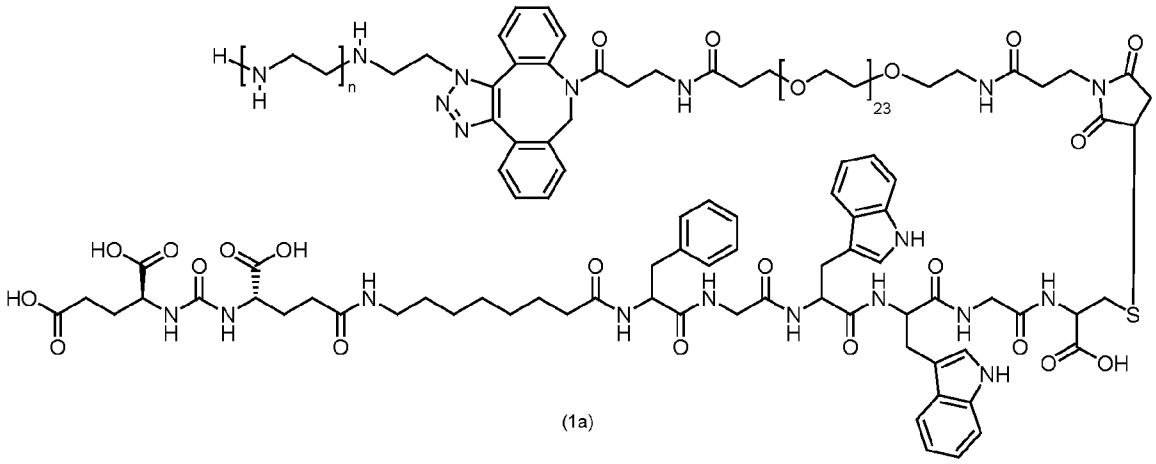
Step 2: Synthesis of LPEI-*l*-[N$_3$:DBCO]-PEG$_{24}$-DUPA (Compounds 1a and 1b)



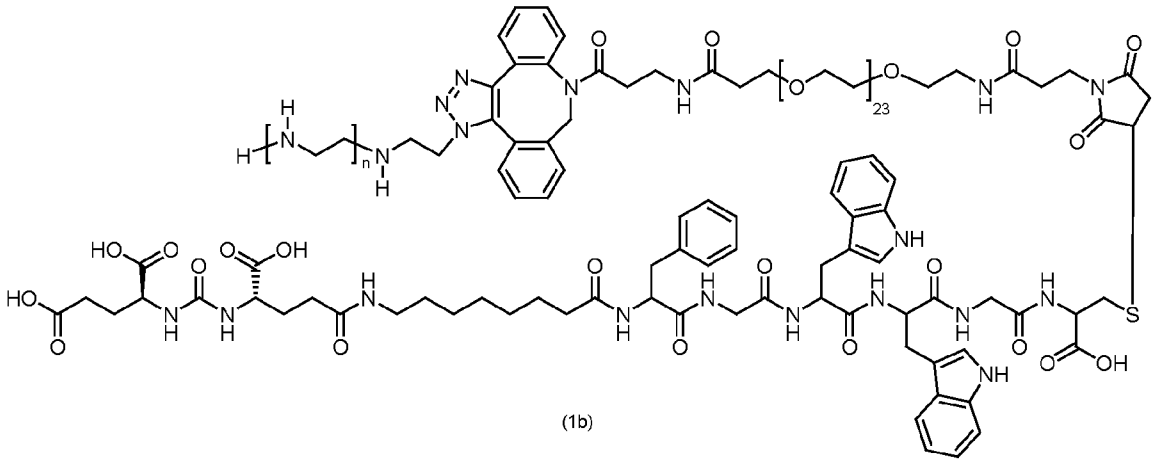
(4)



NaOAc (aq), ACN



(1a)



(1b)

201.8 mg (crude mass) of LPEI-N_3 were weighed in a 15 mL Falcon tube and dissolved in 8 mL of 50 mM acetate buffer, pH 4.0. The pH of the solution was adjusted to 3.5 with 375 µl of 6 M HCl, heated to 70 °C, and sonicated for about three minutes to fully dissolve the LPEI particles. The solution was assayed using the copper assay and a concentration of 17.8 mg/mL total LPEI (0.811 mM) was measured (74% assay of LPEI-N_3).

8.3 mL of LPEI-N_3 solution (7 µmol, 1.0 eq) were transferred to a 50 mL Falcon tube and mixed with 6.5 mL of the DBCO-PEG_{24}-DUPA (Compound 4) preparation of Step 1 (7 µmol, 1.0 eq). As the reaction mixture became cloudy, 2 mL of acetonitrile were added (about 22% ACN final volume). The solution was degassed with argon for about 30 seconds.

The mixture of LPEI-N_3 and DBCO-PEG_{24}-DUPA was incubated for about 70 hours (RT) on a Stuart rotator (20 rpm), protected from light, and monitored by RP-C8-HPLC. After about three hours, white precipitates were visible in the solution and the reaction mixture gave a sweet, fruity odour.

Prior to preparative separation, the reaction mixture (~16 mL) was diluted with 20 mL of H_2O containing 0.1% TFA to reduce the acetonitrile percentage to about 10%. The solution was centrifugated for 5 min at 15,000 g) and the supernatant was purified using the PuriFlash Preparative RP-HPLC system.

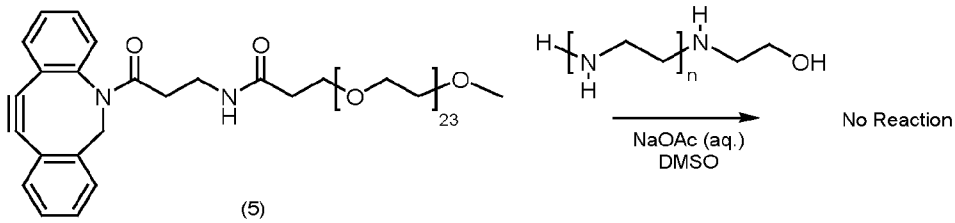
The pooled fractions containing pure Compounds 1a and 1b were lyophilized, weighed, and analyzed by RP-HPLC, copper assay, and UV spectrophotometry at 280 nm. 28 mg of LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA (Compounds 1a and 1b), each with a LPEI:DUPA ratio of 1:1 and no further impurities was isolated (7% overall yield in LPEI). The retention time of the LPEI-*l*-[N_3:DBCO]-PEG_{24}- DUPA (Compounds 1a and 1b) in the analytical RP-HPLC analysis was 5.4-6.4 min with a maximum at 5.5 min.

EXAMPLE 2

NO CYCLOADDITION REACTION BETWEEN LPEI-OH AND DBCO-PEG$_{23}$-OCH$_3$

To demonstrate the chemospecificity of the click-coupling reaction between an azide-modified LPEI fragment and a PEG fragment modified with an activated alkyne, a non-azide containing LPEI was treated with DBCO-PEG$_{23}$-OCH$_3$ (Compound 5) at pH 4 under the conditions set forth above in Example 1, Step 2.

Step 1: Treatment of DBCO-PEG$_{23}$-OCH$_3$ with LPEI-OH



(5)

11.1 mg (crude mass) of non-azide-modified LPEI (α-methyl-ω-hydroxy-poly(iminoethylene), CH$_3$(NC$_2$H$_5$)$_n$-OH, 21KDa, ChemCon GmbH, CAS No. 9002-98-6) were weighed in a 1.5 mL Eppendorf tube and dissolved in 400 µL of 50 mM acetate, pH 4.0. 26 µL of 6 M HCl were added to help dissolve and to adjust to pH 4. The concentration as measured by copper assay was 25.7 mg/mL (1.22 mM pure product). 400 µL of the LPEI solution (0.49 µmol, 1.0 eq) were transferred in a 1.5 mL Eppendorf tube and 29 µL of DBCO-PEG$_{23}$-OCH$_3$ (Compound 5) solution (0.60 µmol, 1.3 eq) were added to the reaction mixture. The solution was incubated at 40°C for about 67 hours and monitored for product formation using analytical RP-HPLC. No product was evident at pH 4.

No reaction was observed using analytical RP-HPLC monitoring over 18 hours at room temperature. At higher pH 5, evidence of a product was observed by analytical RP-HPLC, which was characterized as the hydroamination reaction product from coupling of the LPEI polyimine with the activated alkyne (F. Pohlki & S. Doye *The catalytic hydroamination of alkynes* Chem. Soc. Rev. 32. 104-114(2003)).

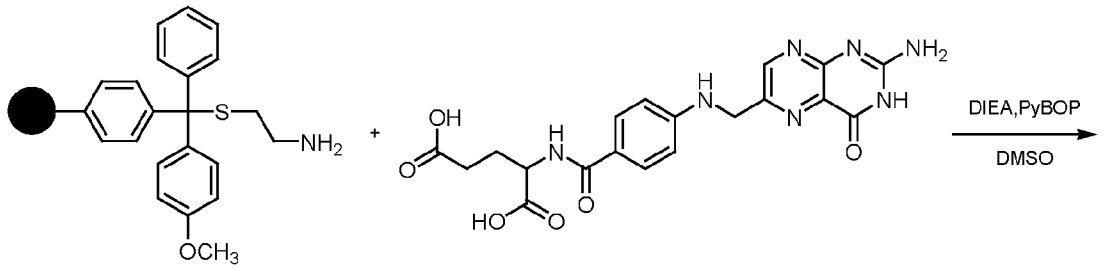
EXAMPLE 3

SYNTHESIS OF LPEI-*l*-[N$_3$:DBCO]-PEG$_{24}$-Folate (COMPOUNDS 6a AND 6b)

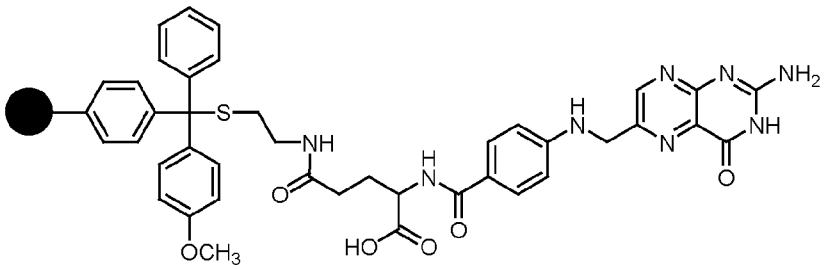
LPEI-*l*-[N$_3$:DBCO]-PEG$_{24}$-Folate was synthesized as a mixture of regioisomers 6a and 6b in a multi-step procedure according to the schemes below. In the first step, folic acid (Compound 7) was functionalized at the gamma-Glu residue with a cysteamine spacer using a

solid phase synthesis approach, analogous to that described by Atkinson *et al.*, (*J. Biol. Chem.* **276**(30) 27930-35 (2001)). The resultant folate-thiol (Compound 10) was coupled to dibenzoazacyclooctyne-24(ethylene glycol)-maleimide (DBCO-PEG_{24}-MAL; Compound 3) by Michael addition. In a next step, DBCO-PEG_{24}-Folate (Compound 11) was added to LPEI-N_3 in a [2+3] cycloaddition reaction to produce LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate (Compounds 6a and 6b).

Step 1: Folic Acid Loading to Solid Phase Resin



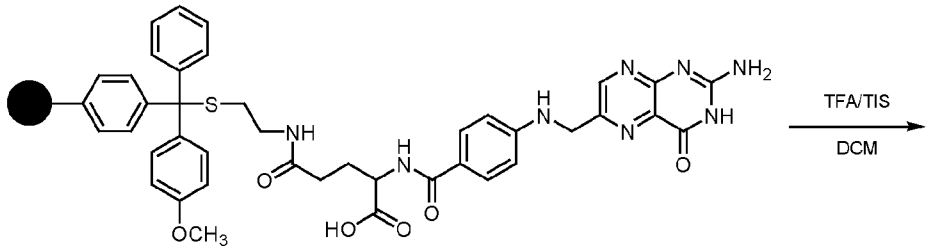
(8) (7)



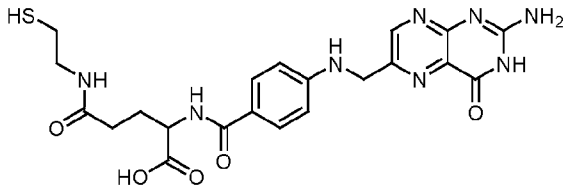
(9)

20 mL of DMSO was heated at 50°C in a 50 mL Erlenmeyer and folic acid (Compound 7; 881.4 mg, 2.0 mmol, 5.0 eq) was slowly added under magnetic stirring. Dry cysteamine 4-methoxytrityl resin (Compound 8; 397.3 mg, 0.4 mmol, 1.0 equiv., 1.01 mmol/g) was added to a 50 mL Erlenmeyer flask and the previously prepared folic acid solution was added to the resin followed by the addition of DIEA (1018 µL, 6.0 mmol, 15.0 equiv) and PyBOP (1084.0 mg, 2.0 mmol, 5.0 equiv). The reaction mixture was stirred four hours at room temperature then transferred to a glass column and filtered over a glass frit and washed with DMSO (7 x 10 mL), DMF (5 x 10 mL), DCM (5 x 10 mL) and MeOH (5 x 10 mL). A TNBSA (picrylsulfonic acid) colour test on the sampled resin confirmed the absence of free amine.

Step 2: Cleavage of the Folate-thiol from the Resin



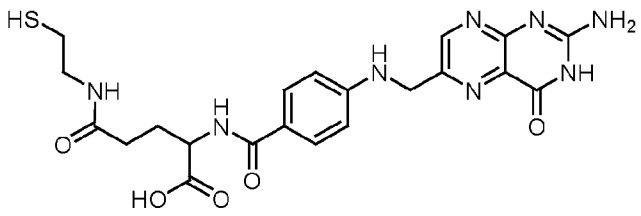
(9)



(10)

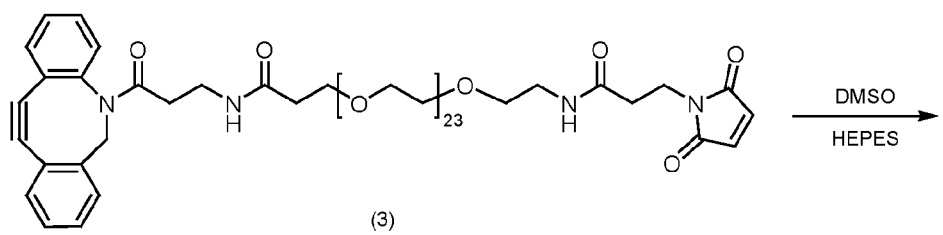
10 mL of DCM/TFA/TIS (92/3/5 v/v/v) was added to the folate-modified resin (Compound 9) of Step 1 in the glass column and the mixture was kept for 30 min with occasional swirling of the flask. The resin was filtered and washed (10 mL DCM/TFA (95/5 v/v) and the filtrate and washings were recovered and concentrated under reduced pressure. After concentration, the mixture was separated in two phases and the light phase was discarded. Crude product was precipitated by addition of 30 mL cold diethyl ether and washed twice with diethyl ether. The folate-SH (Compound 10) crude product was dried overnight under reduced pressure and confirmed by mass spectrometry. The thiol content of the crude Compound 10 was measured by Ellman's test yielding a positive result for free thiol. Mass spectrometry (ESI): $C_{21}H_{24}N_8O_5S$ $[M-H]^-$ 499.54, found 499.2.

Step 3: Synthesis of DBCO-PEG_{24}-Folate (Compound 11)



(10)

+



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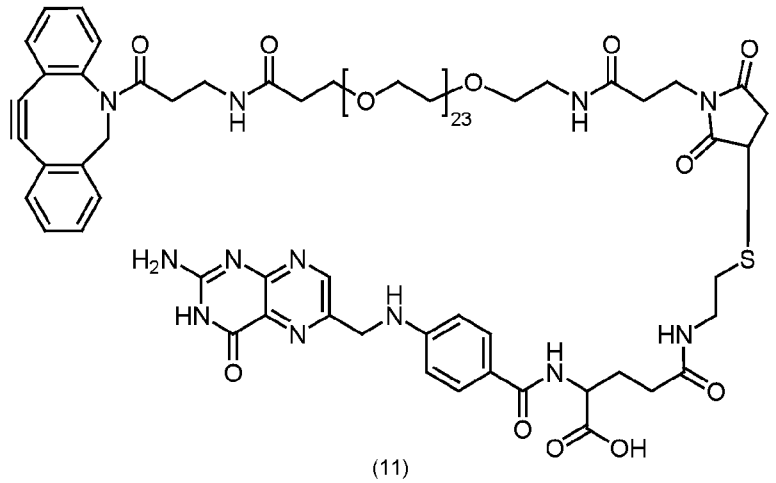
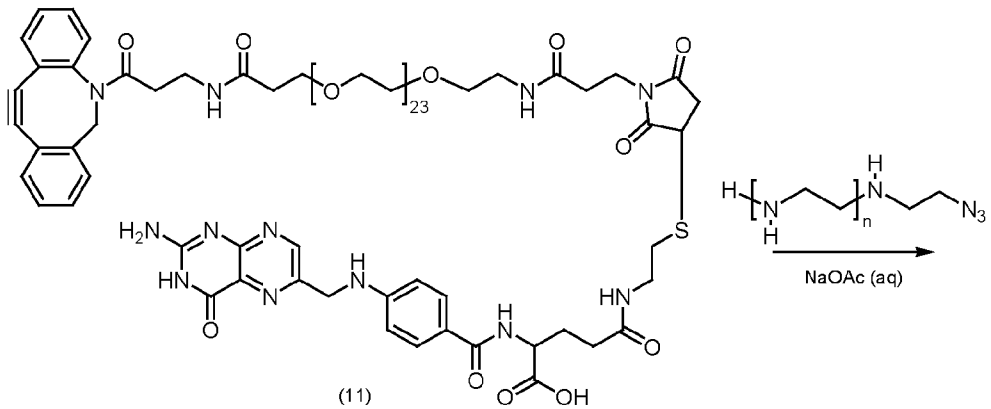


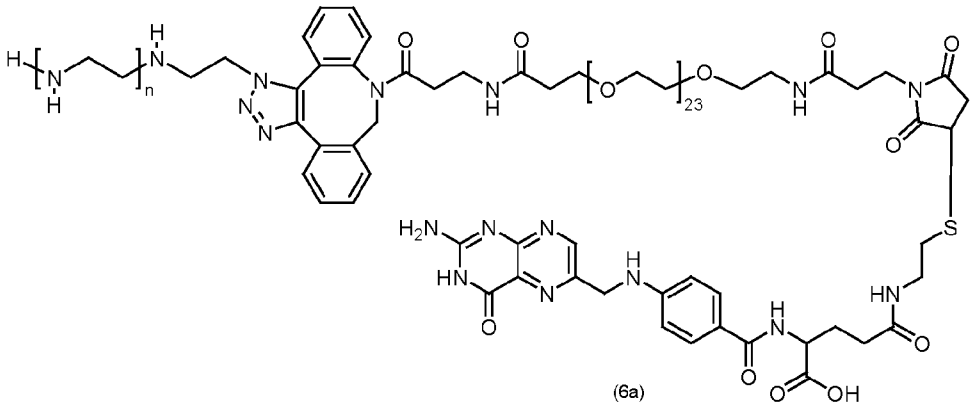
(11)

The folate-thiol (Compound 10) of Step 2 (16.0 mg, 29.4 µmol, 1.7 eq) was dissolved in 8 mL DMSO in a round-bottom flask (2.0 mg/mL stock solution). The solution was sonicated to completely dissolve Compound 10 and diluted with 72 mL of 20 mM HEPES (pH 7.4). DBCO-PEG_{24}-MAL (Compound 3; *see* Example 1) (29.1 mg, 17.5 µmol, assay 93.6%, 1.0 eq) was weighed in a 1.5 mL Eppendorf tube and dissolved in 875 µL DMSO (20 mM pure product stock solution). To the 80 mL round-bottom flask containing folate-thiol (Compound 10) solution (29.4 µmol, 1.7 eq), the DBCO-PEG_{24}-MAL (Compound 3) stock solution (13 µmol, 1.0 eq) was added slowly under magnetic stirring. The reaction mixture was kept at room temperature and protected from light for about one hour. DBCO-PEG_{24}-Folate (Compound 11) was purified by preparative chromatography using a Puriflash system and was confirmed by mass spectrometry. Mass spectrometry (ESI): $[M+3H]^{3+}$ 2056.32, found 686.2.

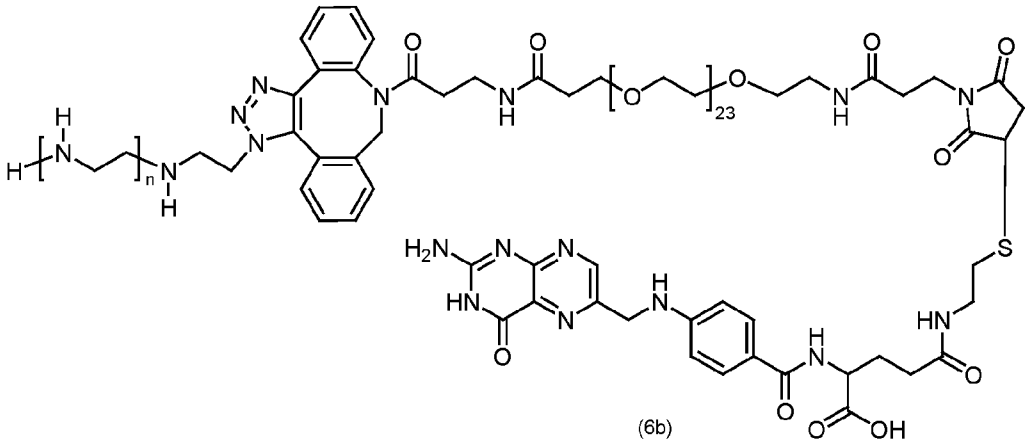
Step 4: Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate (Compounds 6a and 6b)



(11)



(6a)



(6b)

LPEI-N_3 stock (203.9 mg) was weighed in a 15 mL Falcon tube and dissolved in 8 mL of 50 mM acetate buffer (pH 4.0). The solution was acidified, heated to 70°C, sonicated to fully dissolve LPEI particles and adjusted to pH 4.0 with a total of 340 µL of 6 M HCl. The copper assay was performed on the solution to determine the total LPEI content of the LPEI-N_3 solution. LPEI-N_3 solution (8.3 mL, 6.7 µmol, 1.0 eq) was transferred to a 50 mL Falcon tube and mixed with 1.5 mL of DBCO-PEG_{24}-Folate solution (Compound 11; 7 µmol, 1.0 eq). The reaction mixture was degassed with argon and incubated for about 20 hours on a thermoshaker (40°C) and protected from light.

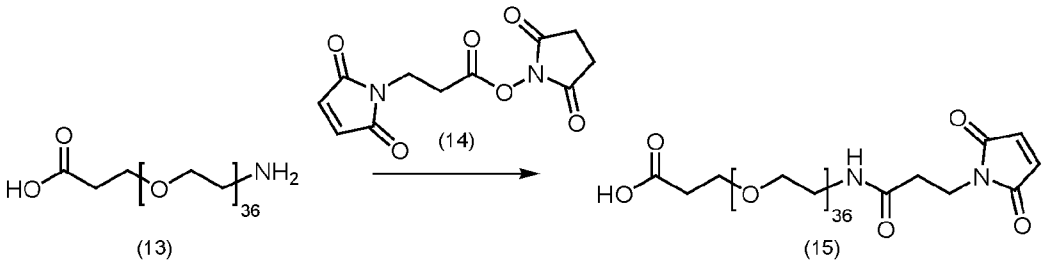
Crude LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate was purified by preparative chromatography using a Puriflash system and isolated as a mixture of regioisomers 6a and 6b. Pooled fractions were measured for total LPEI content using the copper assay and for folate content by spectrophotometry (360 nm, ε = 6'765 $M^{-1}cm^{-1}$). Yield: 19 mg in LPEI content (copper assay); LPEI/folate ratio 1:1.

EXAMPLE 4

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (COMPOUNDS 12a AND 12b)

LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA was synthesized as a mixture of regioisomers 12a and 12b according to the schemes below. In a first step, HOOC-PEG_{36}-NH_2 (Compound 13) was coupled to N-succinimidyl 3-maleimidopropionate (Compound 14) by amine formation to produce HOOC-PEG_{36}-MAL (Compound 15). In a next step, HOOC-PEG_{36}-MAL (Compound 15) was coupled to DBCO-NH_2 (Compound 16) by amine formation to produce DBCO-PEG_{36}-MAL (Compound 17). In a next step, DBCO-PEG_{36}-MAL (Compound 17) was coupled to DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; SEQ ID NO:4) by a Michael addition to produce DBCO-PEG_{36}-DUPA (Compound 18). In a next step, DBCO-PEG_{36}-DUPA (Compound 18) was coupled to LPEI-N_3 by a [2+3] cycloaddition to produce LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA as a mixture of regioisomers 12a and 12b.

Step 1: Synthesis of HOOC-PEG_{36}-MAL (Compound 15)

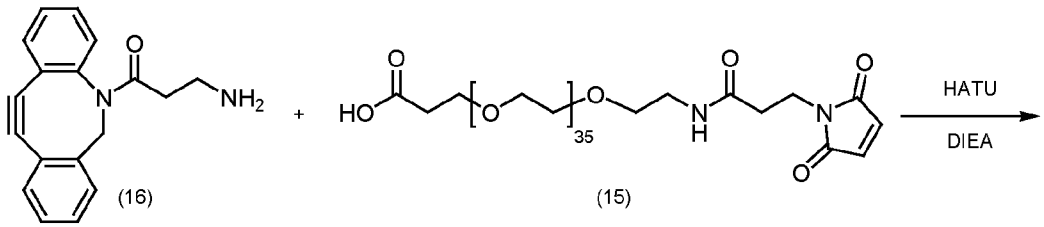


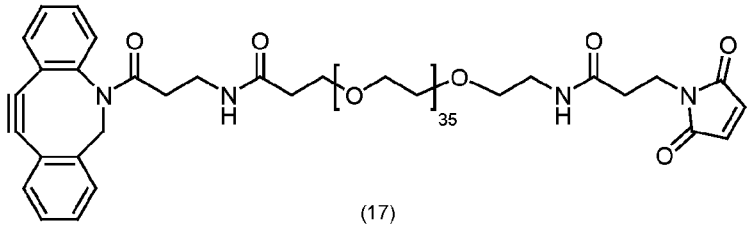
Stock solutions were prepared as follows: HOOC-PEG_{36}-NH_2 (Compound 13) was weighed (364.4 mg, 218 µmol, 1.0 eq) in a 50 mL Falcon tube and 5.0 mL of DCM were added to yield a 44 mM stock solution. N-succinimidyl 3-maleimidopropionate (Compound 14) was weighed (83.0 mg, 312 µmol) in a 5.0 mL Eppendorf tube and 3.0 mL of DCM were added to yield a 104 mM stock solution.

To the HOOC-PEG_{36}-NH_2 containing Falcon tube, DIEA (55.6 µL, 327 µmol, 1.5 eq) and 2.308 mL (240 µmol, 1.1 eq) of N-succinimidyl 3-maleimidopropionate stock solution were added. The reaction mixture was incubated on a Stuart rotator (RT, 15 rpm, protected from light) and monitored by RP-C_8-HPLC. After 30 minutes, all the HOOC-PEG_{36}-NH_2 had reacted. After a total of two hours the reaction mixture (~7.3 mL) was purified by precipitation: 30 mL of n-hexane were added and the mixture was vortexed for a few seconds and centrifugated (10 min; 4'400 rpm). A yellow oil was recovered and dried overnight (25°C, 10 mbar). 458 mg (crude mass) of a white-yellowish material (crude HOOC-PEG_{36}-MAL;

Compound 15) were recovered and analyzed by RP-C8-HPLC; qTOF mass spectrometry (calculated monoisotopic mass: 1'825.02 Da; measured: 1'825.02 Da).

Step 2: Synthesis of DBCO-PEG$_{36}$-MAL (Compound 17)





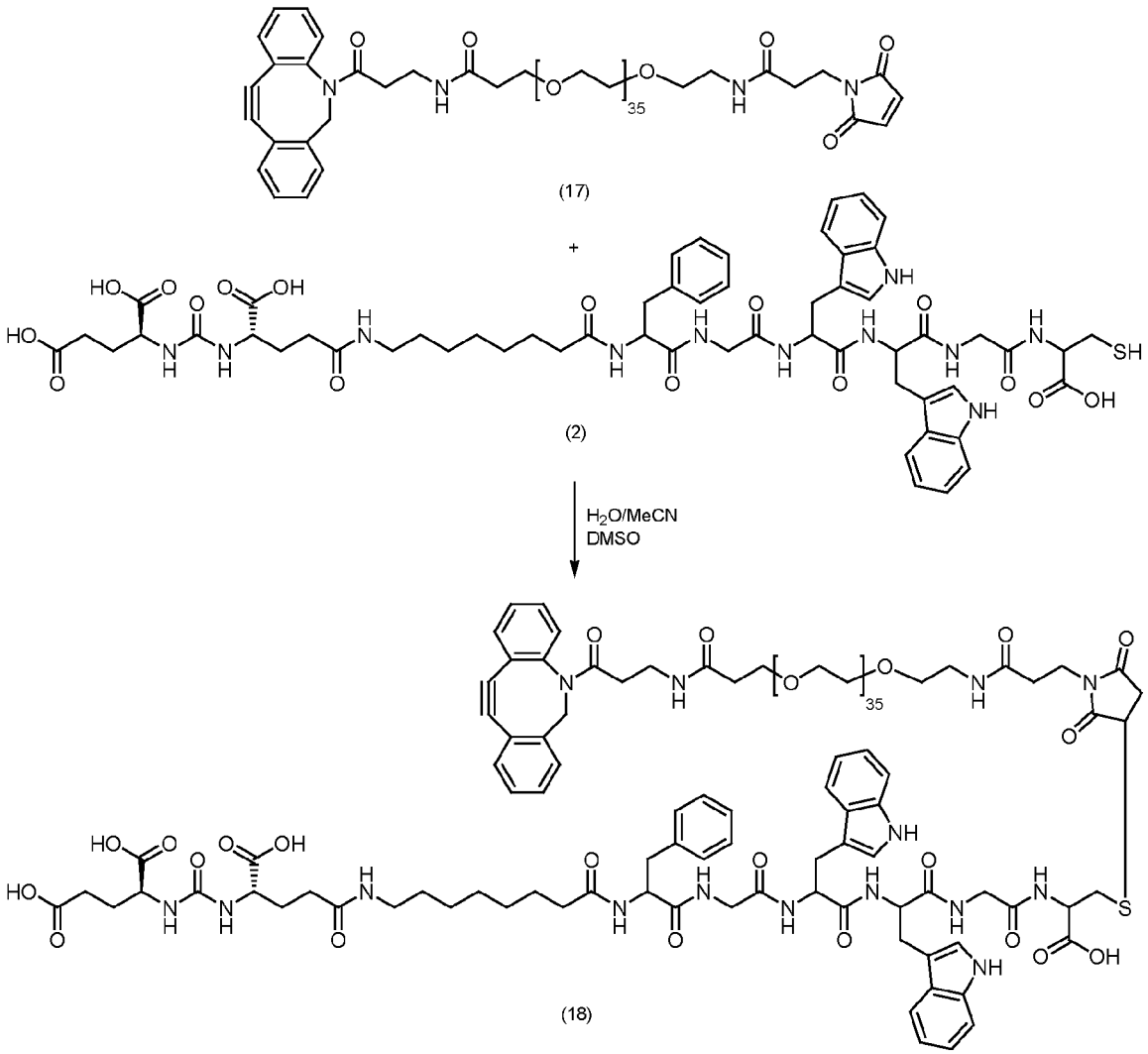
(17)

A stock solution of HOOC-PEG$_{36}$-MAL was prepared by dissolving 458 mg (crude mass) of HOOC-PEG$_{36}$-MAL (Compound 15) in 4.0 mL DCM. For the stoichiometry calculations, it was assumed that the crude mass was pure HOOC-PEG$_{36}$-MAL (246 µmol, 1.0 eq). A stock solution of DBCO-NH$_2$ (Compound 16) was prepared by weighing 84.0 mg of DBCO-NH$_2$ (246 µmol) in a 5.0 mL Eppendorf tube followed by the addition of 1.0 mL of DMF to yield a 304 mM stock solution. A stock solution of HATU was prepared by weighing 82.5 mg of HATU (217 µmol) in a 5.0 mL Eppendorf. 1.0 mL of DMF were added to yield a 217 mM stock solution.

To the HOOC-PEG$_{36}$-MAL (Compound 15), 1.0 mL (221 µmol, 0.9 eq) of HATU stock solution were added. The solution was stirred on a Stuart rotator for about one minute. DIEA (75 µL, 442 µmol, 2.0 eq) were added and the solution was stirred for about 3 minutes followed by the addition of DBCO-NH$_2$ (Compound 16; 728 µL, 221 µmol, 0.9 eq) stock solution. The reaction mixture was incubated on a Stuart rotator (15 rpm, RT, light protected) and was monitored by RP-C$_8$-HPLC. After one hour of incubation, additional DBCO-NH$_2$ solution (80 µL, 25 µmol, 0.1 eq) was added to the reaction mixture to ensure complete consumption of HOOC-PEG$_{36}$-MAL. After 3 hours the reaction mixture (~5.9 mL) was purified by precipitation. n-Hexane (30 mL) was added on the reaction mixture, vortexed and centrifugated (10 min; 4'400 rpm). The supernatant was discarded and 20 mL of cold diethyl ether were added. The precipitate was recovered and dried overnight in a vacuum-drying oven (25°C, 10 mbar). DBCO-PEG$_{36}$-MAL (Compound 17), was recovered as a light yellow solid (542 mg)

and analysed for purity by RP-C_8-HPLC and qTOF mass spectrometry (calculated monoisotopic mass: 2'083.13 Da ; measured: 2'083.14 Da).

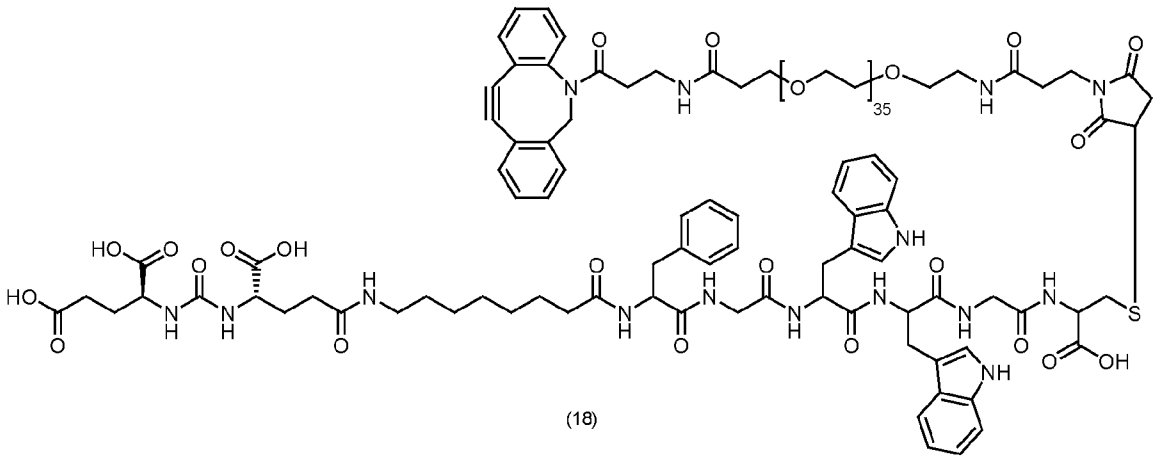
Step 3: Synthesis of DBCO-PEG_{36}-DUPA (Compound 18)



A stock solution of DBCO-PEG_{36}-MAL (Compound 17) was prepared by dissolving 548 mg in a 50 mL Falcon tube and dissolving in 10 mL DMSO (26.3 mM stock solution). A stock solution of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; SEQ ID NO:4) was prepared by weighing 318 mg in a 250 mL round-bottom flask equipped with a magnetic stirrer. Acetate buffer (15 mM, 159 mL, pH 5.2) was added and the mixture was agitated for a few minutes until complete dissolution of Compound 2. The solution was adjusted to pH 5.5 with 350 µL of 5 M NaOH. DBCO-PEG_{36}-MAL stock solution (10 mL, 263 µmol, 1.0 eq) was slowly added to the Compound 2 solution (265 µmol, 1.0 eq,) and the reaction mixture was stirred and protected from light. The reaction was monitored with RP-C8 HPLC. After one hour the excess of Compound 2 was removed by TFF (2 kDa MWCO membrane). The solution (~169 mL) was

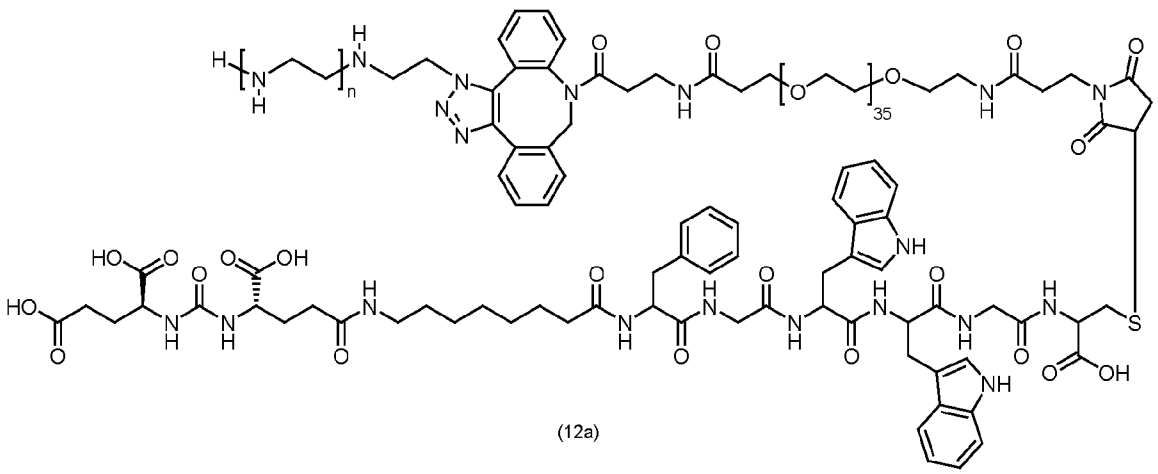
ultrafiltered using TFF against 15 mM acetate buffer (pH 4.8). The recovered solution (~55 mL) was lyophilized for about 48 hours on a freeze-drying device and the lyophilisate was analyzed by RP-C8-HPLC. Residual impurities were removed by precipitation. 500 mg of the lyophilized material were dissolved in 6 mL DMF in a 50 mL Falcon tube. To the slightly turbid solution, cold diethyl ether (30 mL) was added, and a precipitate was formed, collected and washed with cold diethyl ether (30 mL) and dried in a vacuum oven overnight (25°C; 10 mbar) to give 270 mg DBCO-PEG$_{36}$-DUPA (Compound 18). qTOF mass spectrometry (calculated monoisotopic mass: 3'280.60 Da; measured: 3'280.64 Da)

Step 4: Synthesis of LPEI-*l*-[N_3:DBCO]-PEG$_{36}$-DUPA (Compounds 12a and 12b)



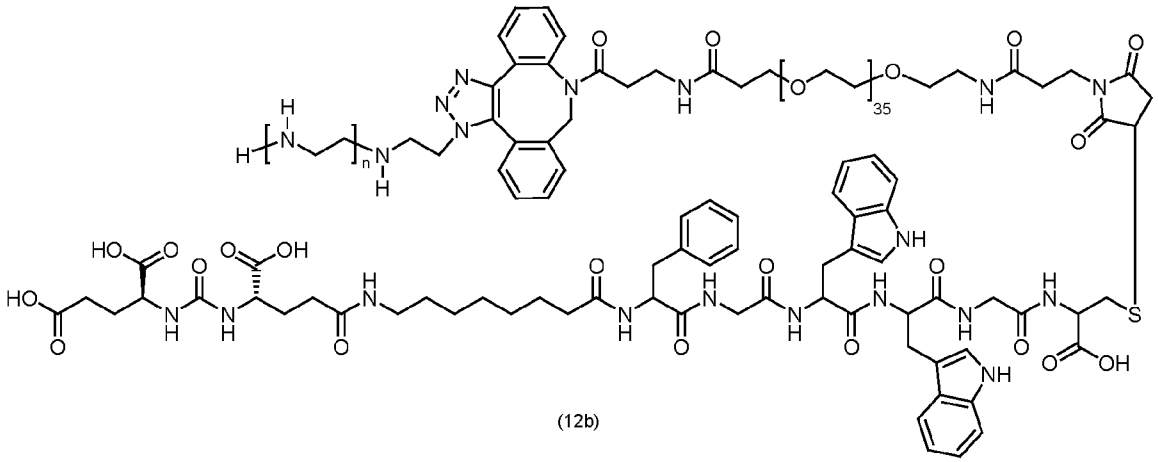
(18)

NaOAc (aq.)



(12a)

+



(12b)

LPEI-N_3 1013 mg (crude mass) were weighed in a 50 mL Falcon tube and dissolved in 35.0 mL of 50 mM acetate buffer, pH 4.0. The solution was acidified and sonicated for 10 minutes to fully dissolve the LPEI-N_3 and the final pH was adjusted to pH 4.0. A concentration of 22.1 mg/mL in total LPEI amine (1.0 mM) was determined by copper assay (corresponding to a content in LPEI-N_3 of 82% of the crude mass). A stock solution of DBCO-PEG_{36}-DUPA (Compound 18) was prepared by dissolving 219 mg of DBCO-PEG_{36}-DUPA in a 50 mL Falcon tube with 20.0 mL of 50 mM acetate buffer. The pH of the solution was adjusted to pH 4.0 by adding 1 M HCl. The concentration in DBCO was determined by spectrophotometry at 309 nm with Nanodrop One C and was measured at 2.0 mM. DBCO-PEG_{36}-DUPA solution (~21 mL, 40 µmol) was slowly added to the magnetically stirred solution of the LPEI solution (37 mL, 38 µmol, 1.0 eq). The mixture was stirred for 72 hours at room temperature and protected from light. The reaction mixture (~60 mL) was supplemented with acetonitrile (10% ACN final volume) and with TFA (1% TFA final volume). The solution turned cloudy but became clear after adjusting the pH to pH 3.5 with 5 M NaOH. Purification was by preparative RP-C_{18} - HPLC. Pooled fractions of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA were recovered as a mixture of regioisomers 12a and 12b. The fractions were lyophilized to give 830 mg lyophilisate as a TFA salt, 34% weight LPEI content by Cu assay). The pooled fractions containing purified products were analyzed by RP-HPLC, copper assay, and UV spectrophotometry at 280 nm. An LPEI:DUPA molar ratio of 1:1 was determined.

Step 5: Preparation of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 12a and 12b) HEPES salt

To exchange TFA by HEPES, 421 mg (crude mass) of lyophilized LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA-TFA salt (w_{LPEI} = 34%, ~143 mg in total LPEI) were dissolved in 30 mL 20 mM HEPES pH 7.2 in a 50 mL Falcon tube. The pH was adjusted to pH 6.0 with 11 µL 5 M NaOH

and 7 µL 6 M HCl. TFF was performed against 20 mM HEPES pH 7.2 with a total dilution of 10'757x. About 45 mL of LPEI-*l*-[N3:DBCO]-PEG_{36}-DUPA HEPES salt solution were recovered after TFF. Copper assay and RP-C8-HPLC were performed on the final LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 12a and 12b) HEPES salt solution (~45 mL) and a concentration of 2.7 mg/mL total LPEI (ratio LPEI/DUPA = 1/1.1) was measured. The yield recovery after TFF was calculated to be 85% based on the total LPEI content.

Step 6: Preparation of LPEI-*l*-[N3:DBCO]-PEG_{36}-DUPA (Compounds 12a and 12b) acetate salt

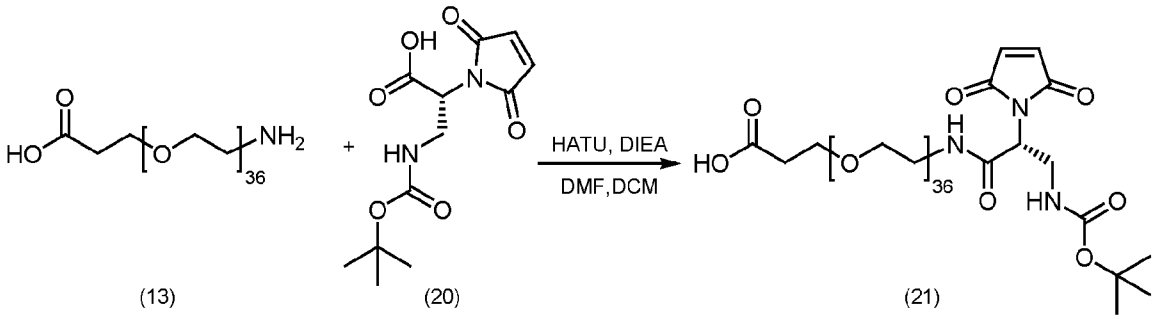
Lyophilized LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA-TFA salt (4.9 mg, w_{LPEI} = 34%, ~1.7 mg in total LPEI) was dissolved in 0.8 mL 50 mM acetate pH 4.3 in a 1.5 mL Eppendorf tube. The pH was adjusted to pH 4.5 with 3.0 µL 5 M NaOH. Two centrifugal filters (Amicon Ultra – 0.5 mL, 3kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA-TFA salt solution each. They were centrifugated one time at 14'000 g for 30 minutes to remove buffer and then 3 times against 400 µL 50 mM acetate, pH 4.3 at 4°C. A concentrated solution of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA-acetate salt (177 µL) was recovered after buffer exchange and supplemented with 0.45 mL 50 mM acetate, pH 4.3. Copper assay and analytical RP-C_8-HPLC was performed on the LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 12a and 12b) acetate salt solution (~0.6 mL) and a concentration of 2.0 mg/mL total LPEI was determined.

EXAMPLE 5

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA (COMPOUNDS 19a AND 19b)

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA was synthesized as a mixture of regioisomers 19a and 19b according to the schemes below. In the first step, HOOC-PEG_{36}-NH_2 (Compound 13) was condensed with Mal-L-Dap(Boc)-OH (Compound 20) to give HOOC-PEG_{36}-(Boc)-MAL (Compound 21). Compound 21 was subsequently condensed with DBCO-NH_2 (Compound 16) and deprotected to give DBCO-PEG_{36}-(NH_2)-MAL (Compound 22). Compound 22 was reacted with DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2) via Michael Addition and cyclized with LPEI-N_3 to produce compounds 19a and 19b.

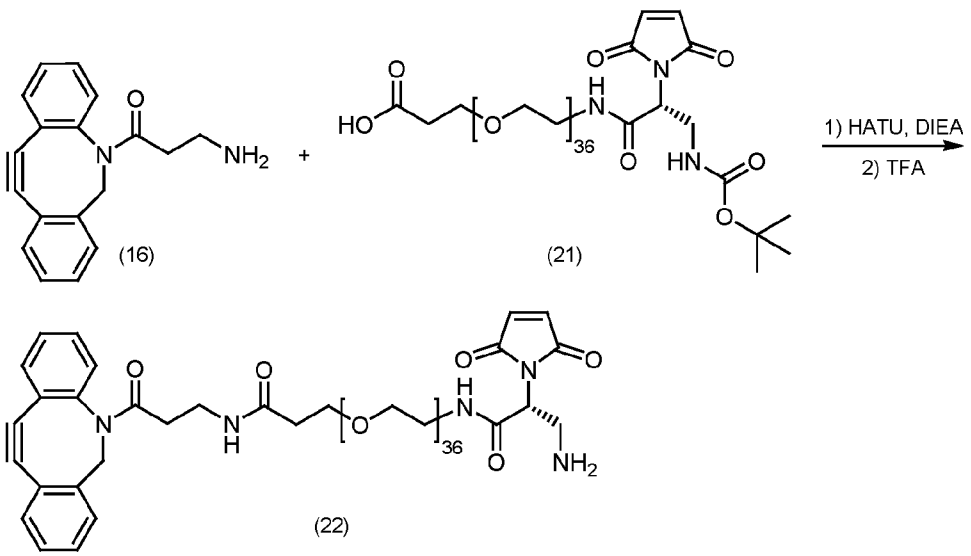
Step 1. Synthesis of HOOC-PEG_{36}-(Boc)-MAL (Compound 21)



(13) (20) (21)

A solution Mal-L-Dap(Boc)-OH (N-α-Maleimido-N-β-t-butyloxycarbonyl-L-2,3-diaminopropionic acid DCHA salt; Compound 20; 50 µmol, 1.1 eq, 294 mM) in DCM (0.17 mL) was mixed with a solution of HATU (45 µmol, 0.9 eq, 217 mM) in DMF (0.207 mL). To the resulting mixture 17 µL of DIEA (100 µmol, 2.0 eq) were added. Finally, HOOC-PEG_{36}-NH_2 (Compound 13, 50 µmol, 1.0 eq, 248 mM) as a solution in DCM (0.20 mL) was added. The reaction mixture was incubated on a Stuart rotator at room temperature and the reaction was monitored by RP-C_8-HPLC. After 1.5 hours, an additional 0.2 eq of Mal-L-Dap(Boc)-OH was added. After a further one and half hours, 5.0 mL of n-hexane were added to induce precipitation and the reaction mixture was centrifuged. The precipitate was washed with 4.5 mL cold diethyl ether. A solid (77 mg) containing crude HOOC-PEG_{36}-(Boc)-MAL (Compound 21) was recovered and analyzed by HPLC – ESI^+ qTOF mass spectrometry (calculated monoisotopic mass: 1940.08 Da; measured: 1940.10 Da). The crude Compound 21 was used without further purification in the next step.

Step 2. Synthesis of DBCO-PEG_{36}-(NH_2)-MAL (Compound 22)



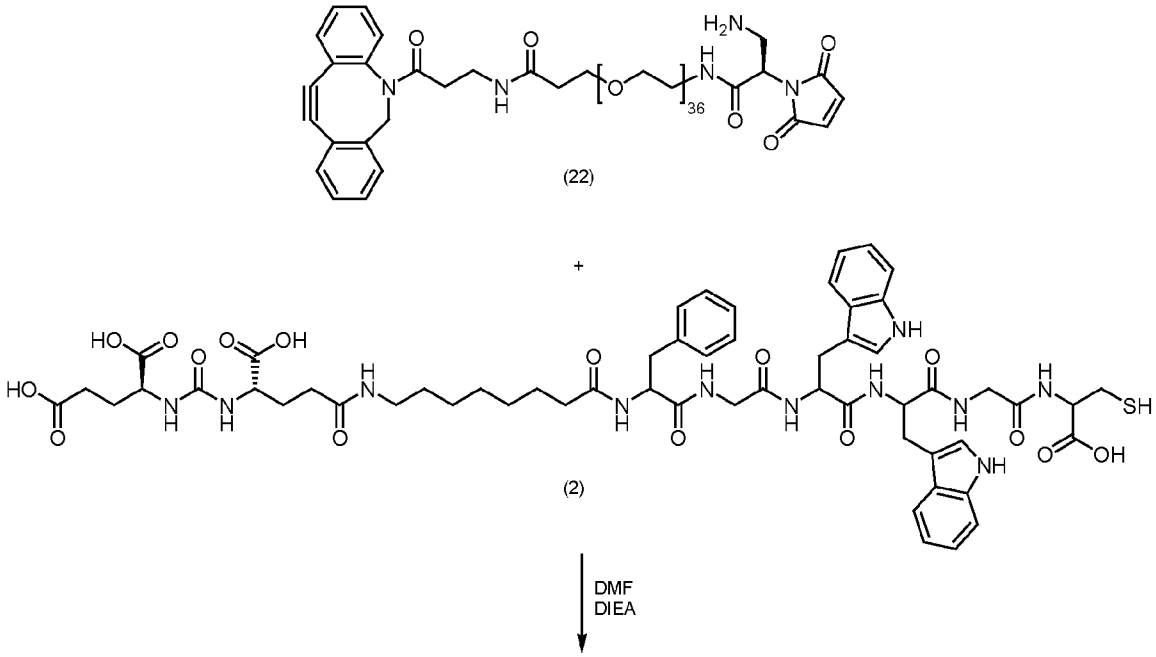
(16) (21) (22)

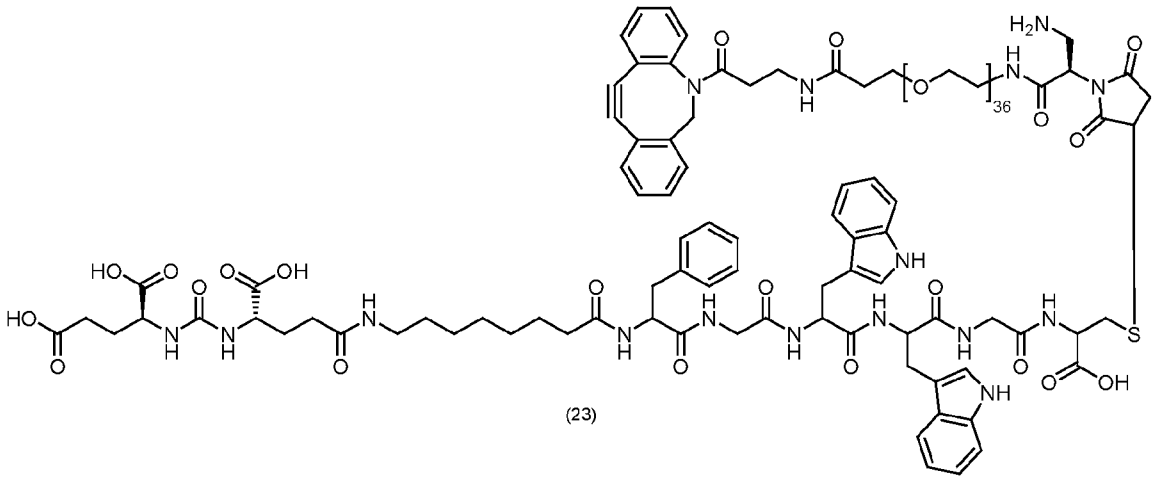
HATU (35 µmol, 0.9 eq, 208 mM) in DMF (169 µL) was added to a solution of HOOC-PEG_{36}-(Boc)-MAL (Compound 21; 39 µmol, 1.0 eq, 98 mM) in DCM (400 mL). The solution

was mixed on a Stuart rotator for one minute followed by the addition of DIEA (13 µL, 78 µmol, 2.0 eq) and a solution DBCO-NH_2 (Compound 16; 20 µmol, 0.5 eq, 370 mM) in DMF (53 µL). The reaction mixture was incubated on a Stuart rotator at room temperature and was monitored by RP-C_8-HPLC. At 20 minutes into reaction, an additional amount of DBCO-NH_2 (8 µmol, 0.2 eq) in DMF (22 µL) was added. After a total of 45 min, 4.5 mL cold diethyl ether were added. The precipitate was further washed with 4.5 mL cold diethyl ether. Crude DBCO-PEG_{36}-(Boc)-MAL was isolated as a yellow solid (92 mg) and analyzed by HPLC – ESI^+ qTOF MS (calculated monoisotopic mass: 2198.20 Da; measured: 2198.20 Da) and dissolved without purification in 2.7 mL DCM and 40 µL TFA.

The Boc group deprotection of DBCO-PEG_{36}-(Boc)-MAL was monitored by RP-C_8-HPLC. Upon completion, n-hexane (2.5 mL) was added and the precipitate was washed with 4.5 mL cold diethyl ether. The recovered solid material (DBCO-PEG_{36}-(NH_2)-MAL; Compound 22) was analyzed by HPLC – ESI^+ qTOF mass spectrometry (calculated monoisotopic mass: 2098.14 Da; measured: 2098.14 Da).

Step 3. Synthesis of DBCO-PEG_{36}-[(NH_2)MAL-S]-DUPA (Compound 23)

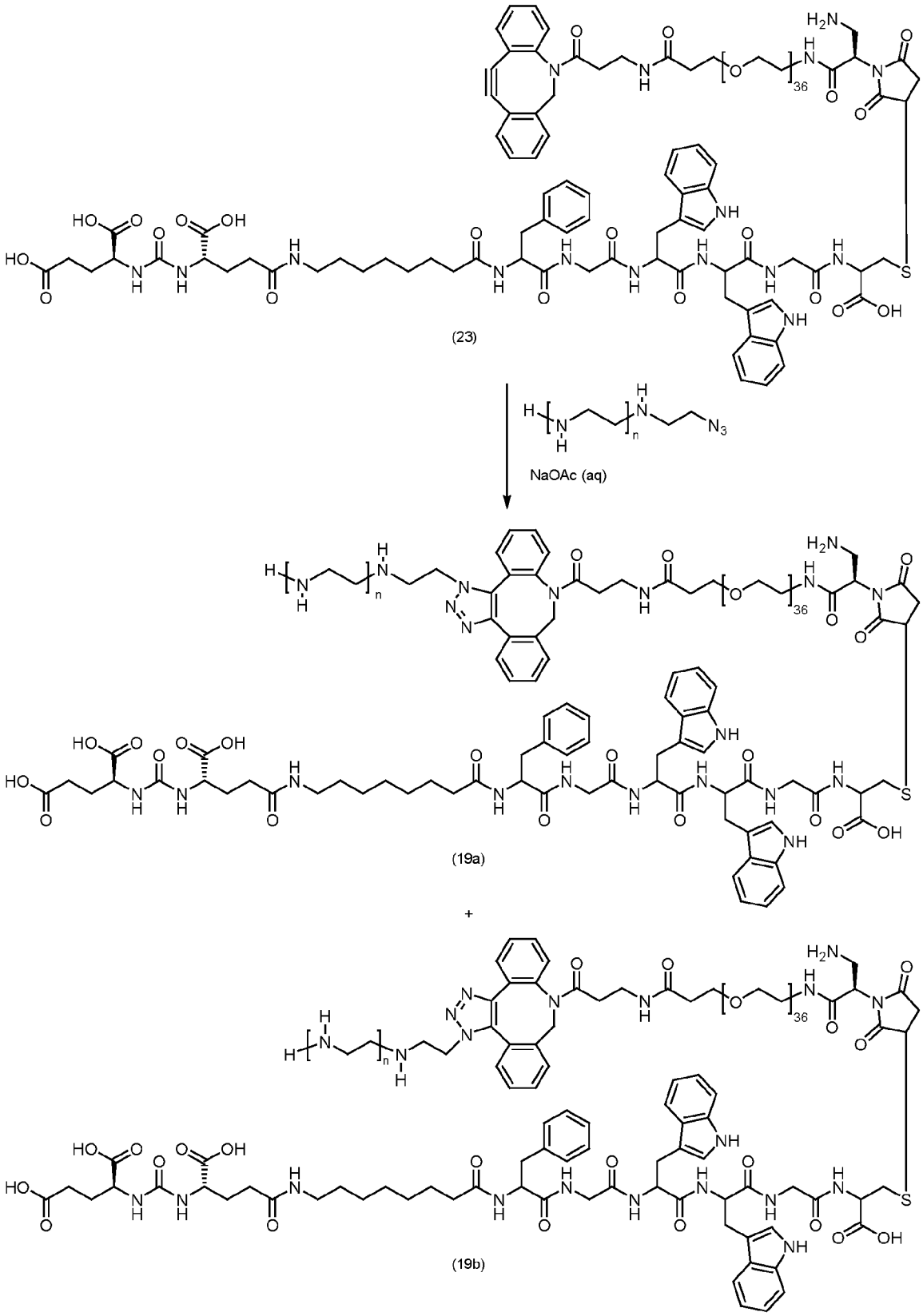




(23)

A solution of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; SEQ ID NO:4) (20 µmol, 0.5 eq, 142 mM) in DMF (141 µL) was added to 400 µL of a solution of DBCO-PEG_{36}-(NH_2)-MAL (Compound 22; 39 µmol, 1.0 eq, 98 mM) in DMF and 10 µL of DIEA (59 µmol, 3.0 eq). The reaction mixture was incubated on a Stuart rotator at room temperature and monitored by RP-C8-HPLC. After one hour, cold diethyl ether (4.5 mL) was added and the product precipitated. The precipitate was washed with 4.5 mL cold diethyl ether, dissolved in 1.0 mL DMSO and supplemented with a mixture of 1% TFA/H_2O: 1% TFA ACN (14 mL 9:1 v/v). The pH was adjusted to 6.0 to ensure that the solution was clear. The solution of DBCO-PEG_{36}-[(NH_2)MAL-S]-DUPA (Compound 23) was purified using RP-C_{18} preparative HPLC and the pooled fractions were lyophilized. The lyophilisate was analyzed by RP-HPLC-ELSD and RP-HPLC – ESI^+ qTOF mass spectrometry (DBCO-PEG_{36}-[(NH_2)MAL-S]-DUPA calculated monoisotopic mass: 3313.64 Da (maleimide ring opened); measured: 3313.66 Da).

Step 4. Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA (Compounds 19a and 19b)



LPEI-N_3 solution (2.3 mL, 2.3 µmol, 1.5 eq, 1.0 mM) in 50 mM acetate buffer pH 4.0 was slowly added to 4.0 mL solution of DBCO-PEG_{36}-[(NH_2)MAL-S]-DUPA (Compound

23; 1.5 µmol, 1.0 eq, 0.37 mM). After 70 hours, the reaction mixture was supplemented with 0.78 mL acetonitrile and 78 µL TFA. LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA was isolated as a mixture of regioisomers 19a and 19b using RP-C_{18} preparative HPLC. Pooled fractions were lyophilized to give 38 mg of a fluffy white solid which was characterized by RP-C_8-HPLC, copper assay and spectrophotometry at 280 nm for determination of the DUPA content. The lyophilisate had a weight percentage in LPEI of 32% w/w and a LPEI to DUPA ratio of 1/1.1.

Step 5. Preparation of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA (Compounds 19a and 19b) HEPES salt

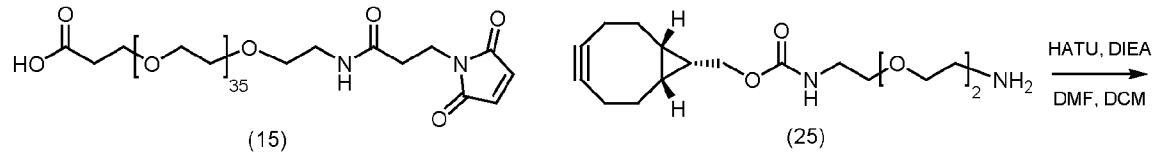
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA (Compounds 19a and 19b) TFA salt (21.9 mg, w_{LPEI} = 32%, 7.0 mg in total LPEI) were dissolved in 1.2 mL 20 mM HEPES pH 7.5. Three centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)-MAL-S]-DUPA solution each, centrifuged one time at 14'000 g for 30 minutes and then three times after addition of 400 uL 20 mM HEPES, pH 7.2. Approximately 261 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA-HEPES salt solution were recovered and supplemented with 2.4 mL 20 mM HEPES, pH 7.2. The concentration of the solution was determined by copper assay to be 2.2 mg/mL in total LPEI.

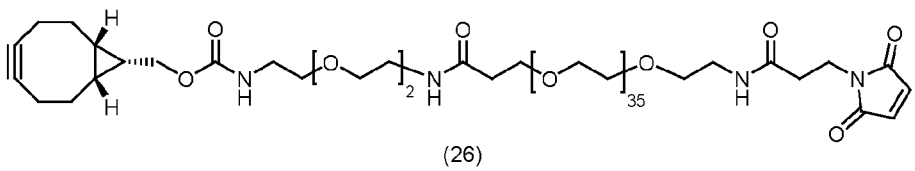
EXAMPLE 6

SYNTHESIS OF LPEI-*l*-[N_3:BCN]-PEG_{36}[MAL-S]-DUPA (COMPOUND 24)

LPEI-*l*-[N_3:BCN]-PEG_{36}[MAL-S]-DUPA (Compound 24) was synthesized according to the schemes below. Endo-BCN-PEG_{36}-MAL (Compound 26) was prepared by condensing HOOC-PEG_{36}-MAL (Compound 15) with endo-BCN-PEG_2-NH_2 (Compound 25). In a next step, Compound 26 was condensed with Compound 2, and the resulting endo-BCN-PEG_{36}-[MAL-S]-DUPA (Compound 27) was reacted with LPEI-N_3 to give Compound 24.

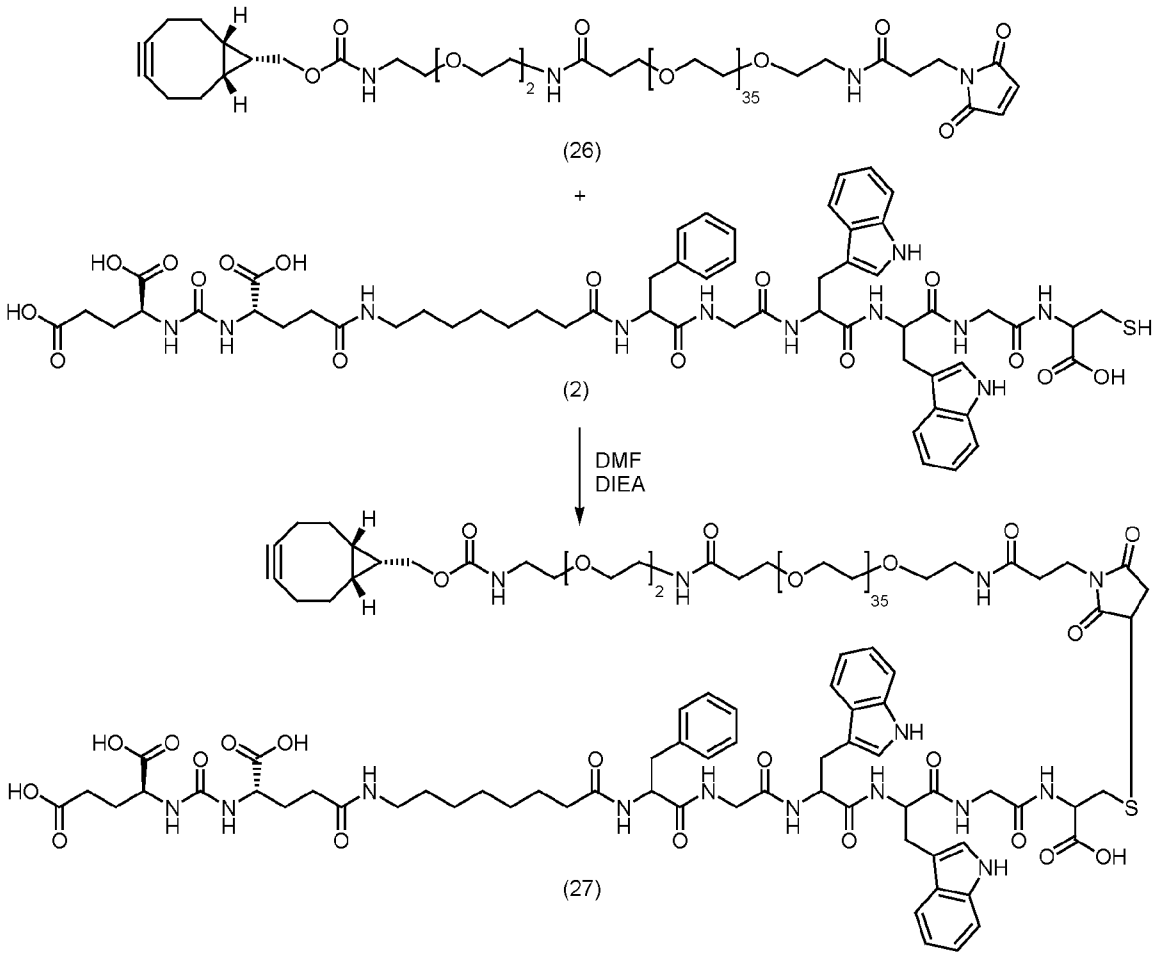
Step 1. Synthesis of *endo*-BCN-PEG_{36}-MAL (Compound 26)





A solution of HATU (20 µmol, 0.9 eq, 123 mM) solution (165 µL) was added to a solution of HOOC-PEG_{36}-MAL (Compound 15; see Example 4; 23 µmol, 1.0 eq, 58 mM) in DCM (400 µL) and DIEA (7.7 µL, 45 µmol, 2.0 eq). To the reaction mixture was added *endo*-BCN-PEG_2-NH_2 (Compound 25; 18 µmol, 0.8 eq, 145 mM) as a solution in DCM (124 µL) and the reaction was monitored by RP-C_8-HPLC. Further amounts of *endo*-BCN-PEG_2-NH_2 (2x 0.2 eq) were added at 20 min intervals. After an additional one hour, n-hexane (4.5 mL) was added to the reaction mixture. The resulting precipitate was separated by centrifugation and washed with 4.5 mL cold diethyl ether and dried under vacuum. Crude *endo*-BCN-PEG_{36}-MAL (Compound 26; 61 mg) was isolated and analysed by RP-C_8-HPLC coupled with ESI^+-qTOF mass spectrometry (Calculated monoisotopic mass: 2'131.21 Da; measured: 2'131.22 Da) and used in the next step without further purification.

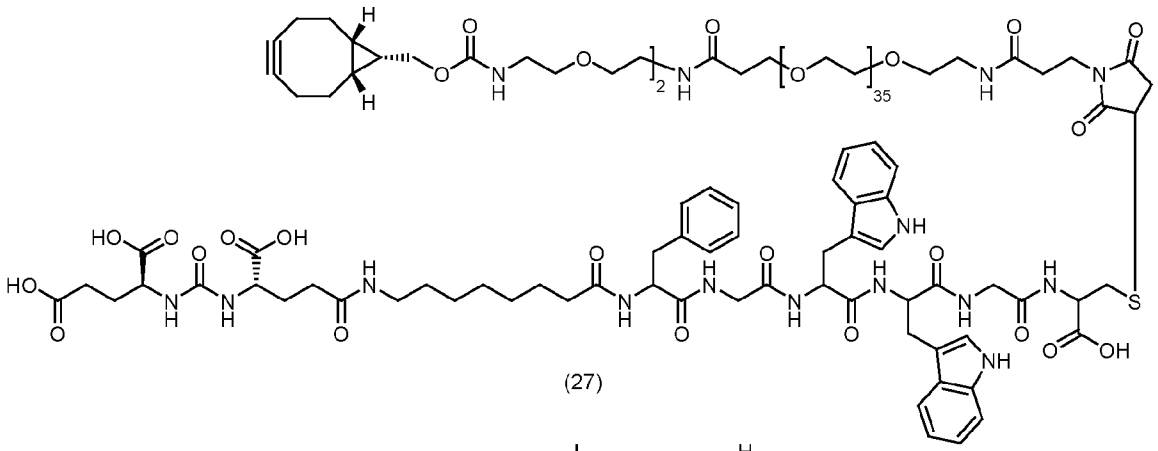
Step 2. Synthesis of *endo*-BCN-PEG_{36}-[MAL-S]-DUPA (Compound 27)



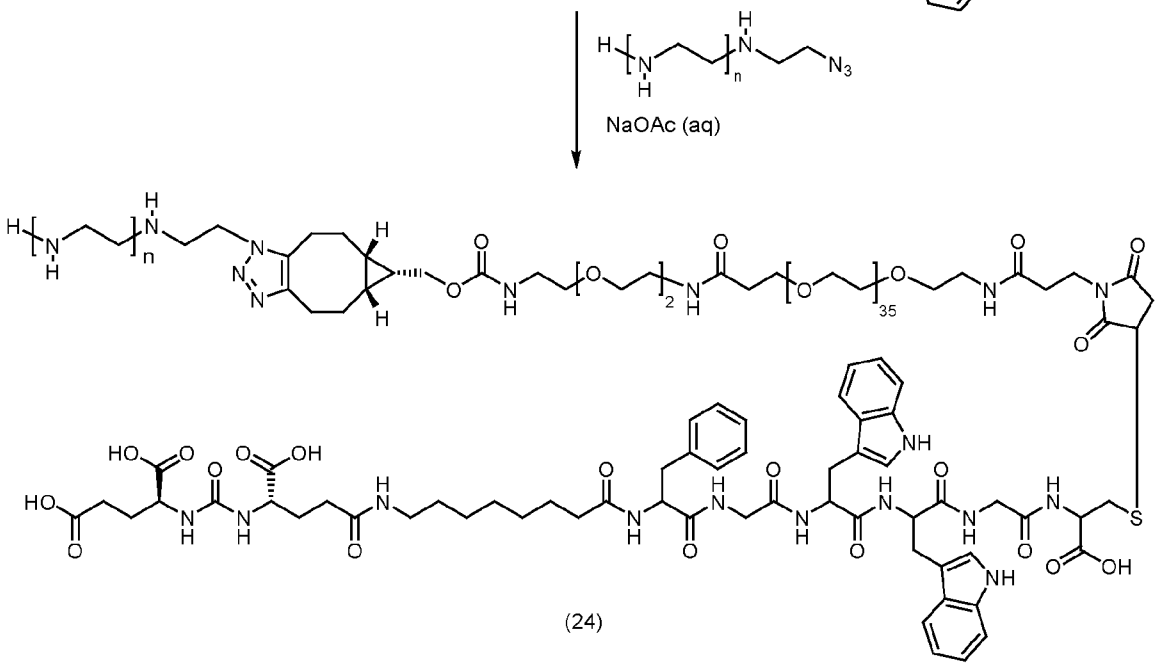
A solution of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; SEQ ID NO:4) (21 µmol, 1.1 eq) in DMF (239 µL) was slowly added to a mixture containing *endo*-BCN-PEG_{36}-MAL (Compound 26; 400 µmol, 1.0 eq, 48 mM) and DIEA (7 µL, 42 µmol, 2.0 eq) in DMF.

After one hour, cold diethyl ether (4.5 mL) was added. The precipitated solid was filtered, washed with cold diethyl ether, and dried to give 70 mg of *endo*-BCN-PEG$_{36}$-[MAL-S]-DUPA (Compound 27). A sample was analyzed by HPLC ESI$^+$ qTOF mass spectrometry (*endo*-BCN-PEG$_{36}$-[MAL-S]-DUPA: calculated monoisotopic mass: 3328.69 Da; measured: 3328.72 Da).

Step 3. Synthesis of LPEI-*l*-[N$_3$:BCN]-PEG$_{36}$-[MAL-S]-DUPA (Compound 24)



(27)



(24)

endo-BCN-PEG$_{36}$-[MAL-S]-DUPA (Compound 27; 3.8 µmol, 1.5 mM, 1.0 eq) in acetate buffer (50 mM, 2.5 mL, pH 4.0) was slowly added to a solution of LPEI-N$_3$ (4.1 µmol, 1.1 eq, 22 mg/mL) in acetate buffer (50 mM, 4.2 mL, pH 4.0). The mixture was shaken for about 70 hrs at room temperature on a Stuart rotator and protected from light. To the reaction mixture were added 3.0 mL 50 mM acetate buffer, pH 4.0, followed by acetonitrile (1.0 mL) and TFA (100 µL). The resultant mixture was filtered (0.45 µm PA membrane) and purified using RP-C$_{18}$ preparative chromatography. Pooled fractions containing LPEI-*l*-[N$_3$:BCN]-PEG$_{36}$-[MAL-

S]-DUPA (Compound 24) were lyophilized to give 61 mg lyophilized product and characterized by analytical RP-C_8 HPLC, copper assay and spectrophotometry at 280 nm for determination of the DUPA content. The product was found to have a weight percentage in LPEI of 31%w/w as determined by Cu assay.

Step 4. Preparation of LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA (Compound 24) HEPES salt

24.8 mg of LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA (Compound 24) TFA salt (w_{LPEI} = 31%, ~7.7 mg in total LPEI) were dissolved in 1.2 mL 20 mM HEPES pH 7.2. The pH was adjusted to pH 7.3. Three centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA solution each. They were centrifugated one time at 14'000 g for 30 minutes and then three times after addition of 400 µL 20 mM HEPES, pH 7.2 at 20°C. About 263 µL of the concentrated solution of LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA HEPES salt were recovered after buffer exchange and were supplemented with 2.4 mL 20 mM HEPES, pH 7.2. The concentration of the solution was determined by copper assay to be 2.3 mg/mL in total LPEI.

Step 5. Preparation of LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA (Compound 24) Acetate salt

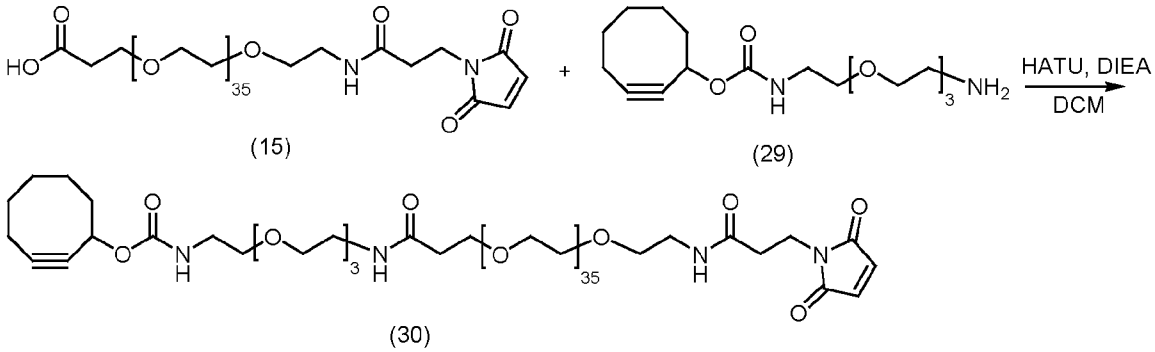
5.5 mg of LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA (Compound 24) TFA salt (w_{LPEI} = 31%, ~1.7 mg in total LPEI) were dissolved in 0.8 mL 50 mM acetate, pH 4.0. Two centrifugal filters (Amicon Ultra – 0.5 mL, 3kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA solution each. They were centrifuged one time at 14'000 g for 30 minutes and then three times after addition of 400 µL 50 mM acetate, pH 4.3. About 144 µL of LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA acetate salt solution were recovered and supplemented with 0.6 mL 50 mM acetate, pH 4.3. The concentration of the solution was determined by copper assay to be 2.2 mg/mL in total LPEI.

EXAMPLE 7

SYNTHESIS OF LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA (COMPOUNDS 28a AND 28b)

LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA was synthesized as a mixture of regioisomers 28a and 28b according to the schemes below. SCO-PEG_{36}-MAL (Compound 30) was prepared by condensing HOOC-PEG_{36}-MAL (Compound 15) with SCO-PEG_3-NH_2 (Compound 29). Compound 30 was reacted with Compound 2 via Michael Addition, and the resulting SCO-PEG_{36}-[MAL-S]-DUPA (Compound 31) was reacted with LPEI-N_3 to synthesize Compounds 28a and 28b.

Step 1. Synthesis of SCO-PEG$_{36}$-MAL (Compound 30)

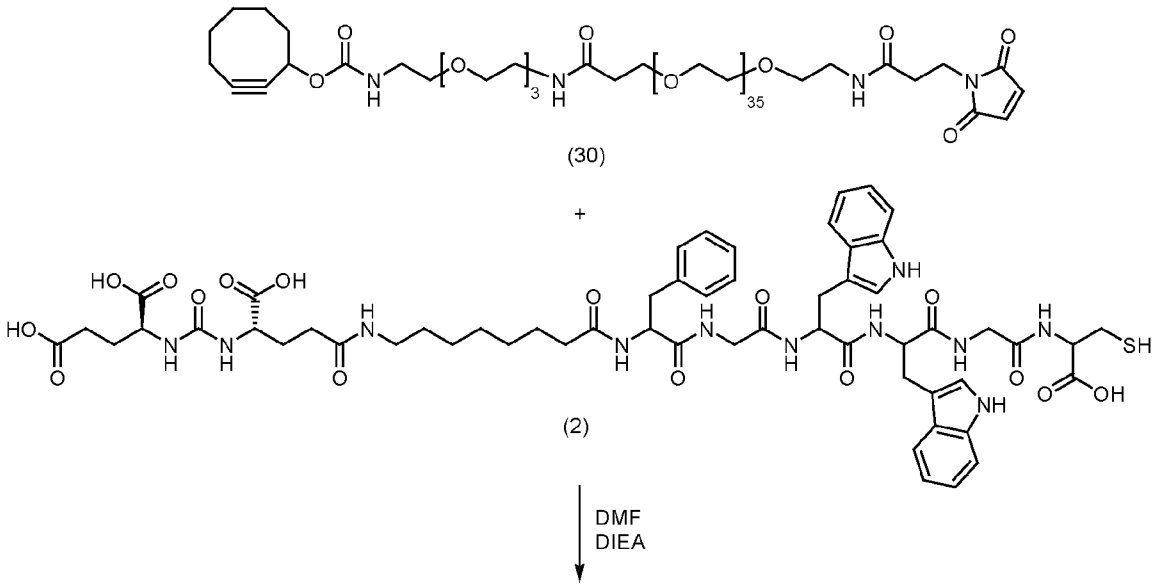


(15) (29)

(30)

A solution of HATU (25 µmol, 0.9 eq, 147 mM) in DMF (69 µL) was added to HOOC-PEG$_{36}$-MAL (Compound 15; 28 µmol, 1.0 eq, 70 mM) in DCM followed by DIEA (9.6 µL, 56 µmol, 2.0 eq). To the reaction mixture was added a solution of SCO-PEG$_3$-NH$_2$ (Compound 29; 22 µmol, 0.8 eq, 137 mM) in DCM (166 µL). The reaction was placed on a Stuart rotator and reaction progress was monitored by RP-C$_8$-HPLC. After 10 min, HATU (0.1 eq) and two additional lots of SCO-PEG$_3$-NH$_2$ (0.2 eq and 0.1 eq) were added to the reaction mixture. After a total of 1hr 30 min, 4.5 mL of n-hexane were added. The precipitated solid was washed with 4.5 mL cold diethyl ether and dried. SCO-PEG$_{36}$-MAL (Compound 30) was isolated as a yellow solid (69 mg) and characterized by analytical RP-C$_8$-HPLC and ESI$^+$ qTOF mass spectrometry (calculated monoisotopic mass: 2149.2 Da; measured: 2149.2 Da).

Step 2. Synthesis of SCO-PEG$_{36}$-[MAL-S]-DUPA (Compound 31)



(30)

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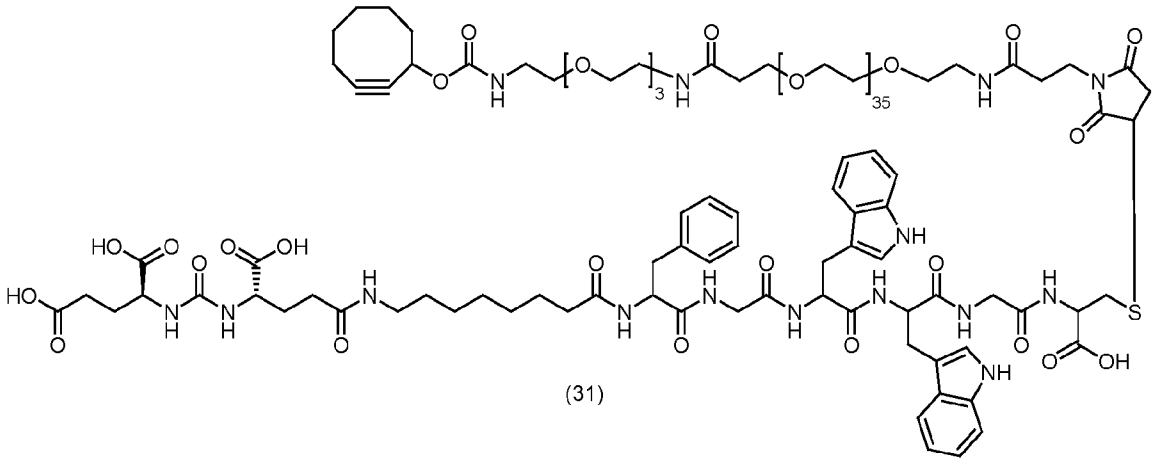
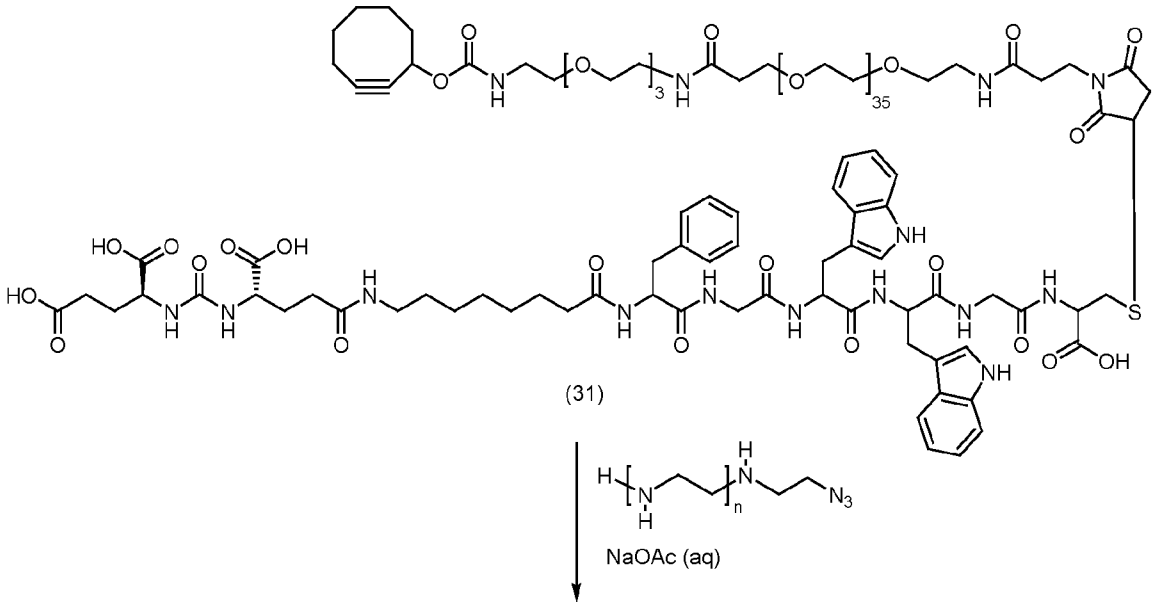
(2)

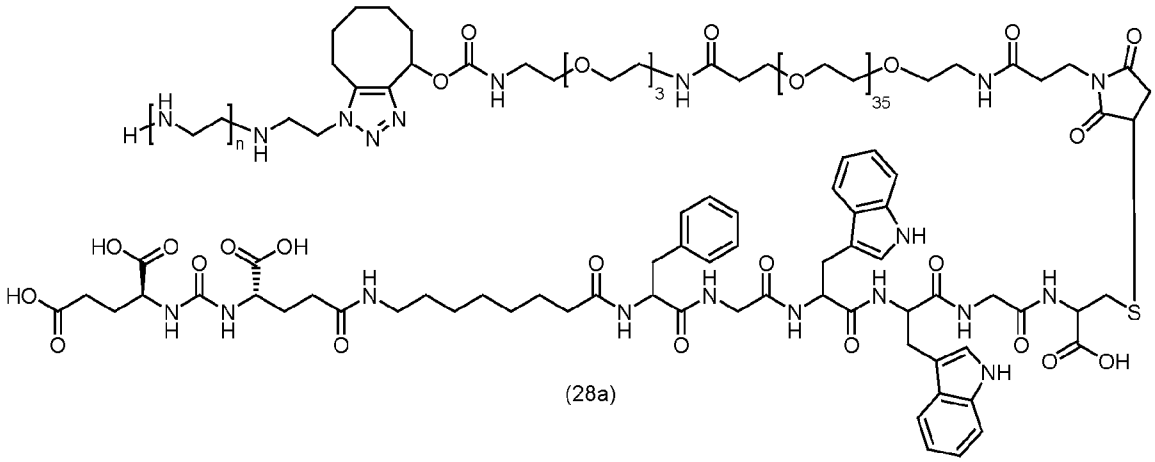


(31)

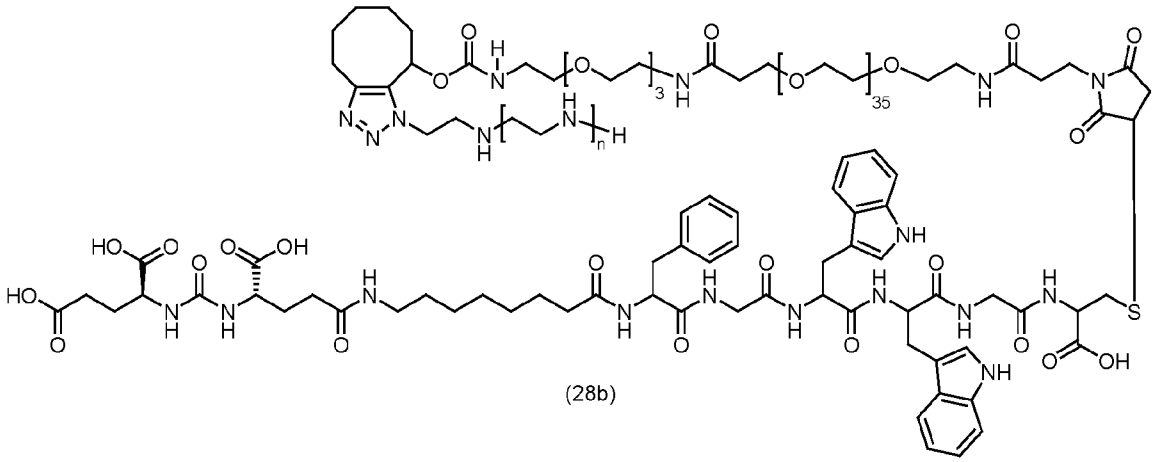
A solution of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; SEQ ID NO:4) (15 µmol, 0.5 eq, 100 mM) in DMF (150 µL) and DIEA (10 µL, 62 µmol, 2.0 eq) were added to a solution of SCO-PEG$_{36}$-MAL (Compound 30; 31 µmol, 1 eq, 78 mM) in DMF. The reaction mixture was placed on a Stuart rotator. After 10 min a further amount of Compound 2 (30 µL, 3 µmol, 0.1 eq) was added. After one hour cold diethyl ether was added and the resultant precipitate was washed with 4.5 mL of cold diethyl ether and dried. The solid (98 mg) was resuspended in 0.5 mL DMSO and diluted with 7.5 mL H_2O (+1% TFA)/CAN (+1% TFA) (9:1 v/v) and purified by prepRP-C_{18}-HPLC. Pooled fractions of SCO-PEG$_{36}$-[MAL-S]-DUPA (Compound 31) were lyophilized and analyzed by HPLC-ESI$^+$ qTOF mass spectrometry (SCO-PEG$_{36}$-[MAL-S]-DUPA calculated monoisotopic mass: 3346.70 Da; measured: 3346.71 Da).

Step 3: Synthesis of LPEI-*l*-[N_3:SCO]-PEG$_{36}$-[MAL-S]-DUPA (Compounds 28a and 28b)





(28a)



(28b)

LPEI-N_3 solution (4.2 mL, 5 µmol, 1.0 eq) in 50 mM acetate buffer pH 4.0 was slowly added to 5.0 mL of a SCO-PEG_{36}-[MAL-S]-DUPA (Compound 31) solution (5 µmol, 1.0 eq, 1 mM) in 50 mM acetate buffer pH 4.0. The mixture was incubated for about 90 hours at room temperature on a Stuart rotator and protected from light. Acetonitrile (1 mL) and TFA (100 µL) were added to the reaction mixture for preparative RP-C_{18} HPLC purification. Pooled fractions were lyophilized to give 66 mg LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA as a mixture of regioisomers 28 and 28b. The lyophilized solid was characterized by analytical RP-C_8 HPLC, copper assay and spectrophotometry at 280 nm. A weight percentage in LPEI of 26% w/w was determined by copper assay for the lyophilized solid.

Step 4. Preparation of LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA (Compounds 28a and 28b) HEPES salt

23.2 mg of LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA (Compounds 28a and 28) TFA salt (w_{LPEI} = 26%, 6.0 mg in total LPEI) were dissloved in 1.2 mL 20 mM HEPES pH 7.4. Three centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA solution each, centrifuged one time at 14'000 g for 30

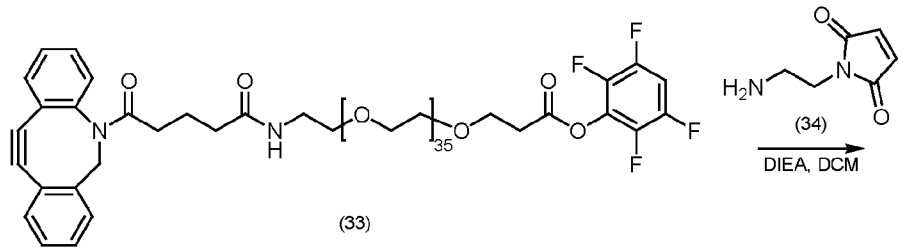
minutes and then three times after addition of 400 µL 20 mM HEPES, pH 7.2. About 276 µL of LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA HEPES salt solution were recovered and supplemented with 2.4 mL 20 mM HEPES, pH 7.2. The concentration of the solution was determined by copper assay to be 2.1 mg/mL in total LPEI.

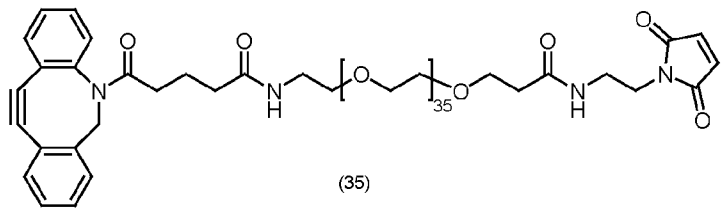
EXAMPLE 8

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]CONH-PEG_{36}-[MAL-S]-DUPA COMPOUNDS 32a AND 32b)

LPEI-*l*-[N_3:DBCO]CONH-PEG_{36}-[MAL-S]-DUPA was synthesized as a mixture of regioisomers 32a and 32b according to the schemes below. DBCO-[CONH]-PEG_{36}-MAL (Compound 35) was prepared by condensing DBCO-[CONH]-PEG_{36}-TFP (Compound 33) with NH_2-MAL (Compound 34). The resulting DBCO-[CONH]-PEG_{36}-MAL (Compound 35) was condensed with Compound 2 and reacted with LPEI-N_3 to give Compounds 32a and 32b.

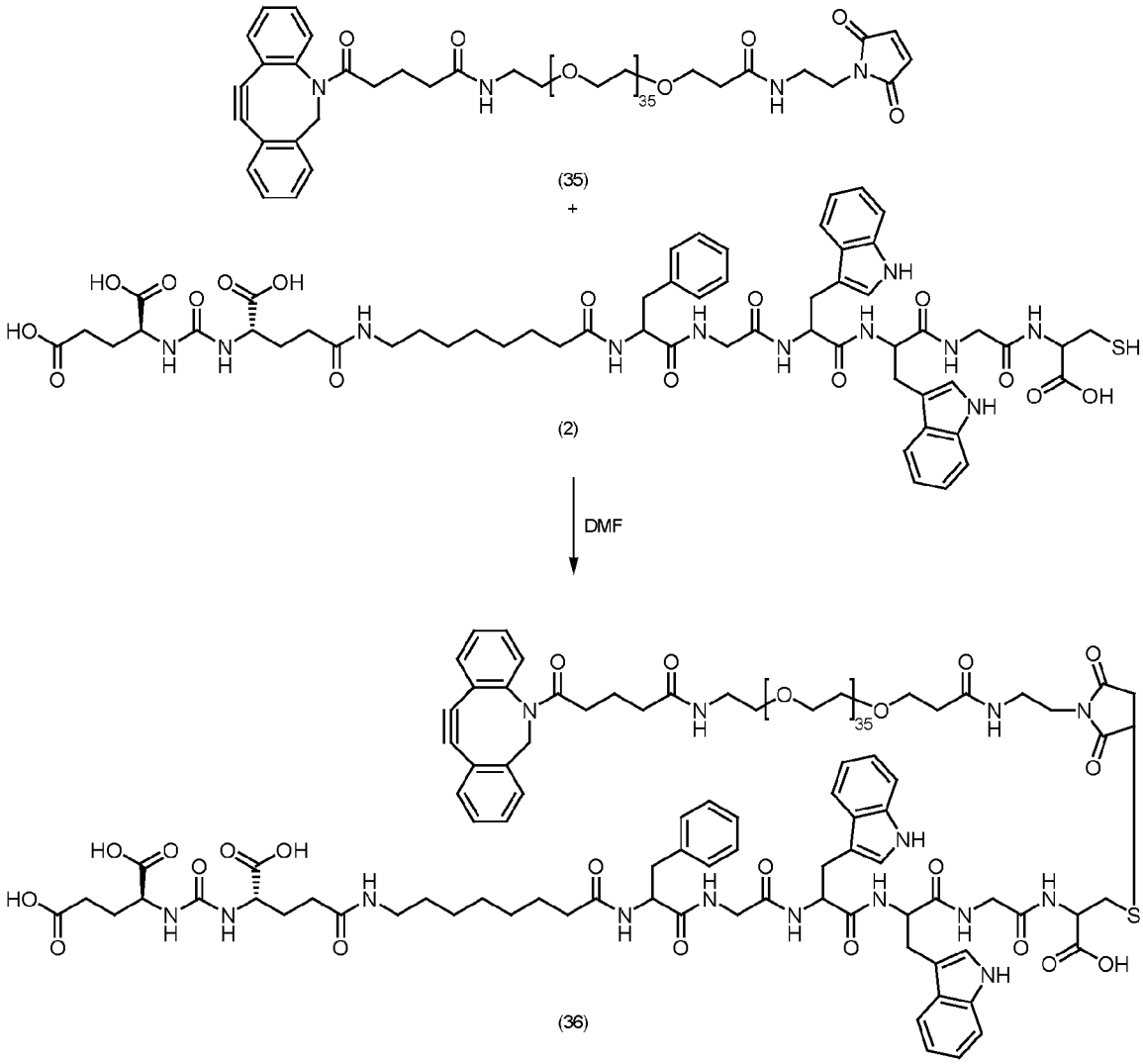
Step 1. Synthesis of DBCO-[CONH]-PEG_{36}-MAL (Compound 35)





A solution of DBCO-[CONH]-PEG_{36}-TFP (Compound 33; 24 µmol, 1.0 eq, 60 mM) in DCM (0.40 mL) was mixed with a solution of NH_2-MAL (Compound 34; 26 µmol, 1.1 eq, 480 mM) in DMF (55 µL) and DIEA (8 µL, 48 µmol, 2.0 eq). The reaction mixture was incubated on a Stuart rotator at room temperature and the reaction was monitored by RP-C_8-HPLC. After two hours, n-hexane (4.5 mL) was added and the product was precipitated. The precipitate was washed with 4.5 mL cold diethyl ether. Recovered material was analyzed by RP-HPLC – ESI^+ qTOF mass spectrometry. The solid contained DBCO-[CONH]-PEG_{36}-MAL (Compound 35; calculated monoisotopic mass: 2097.15 Da; measured: 2097.16 Da).

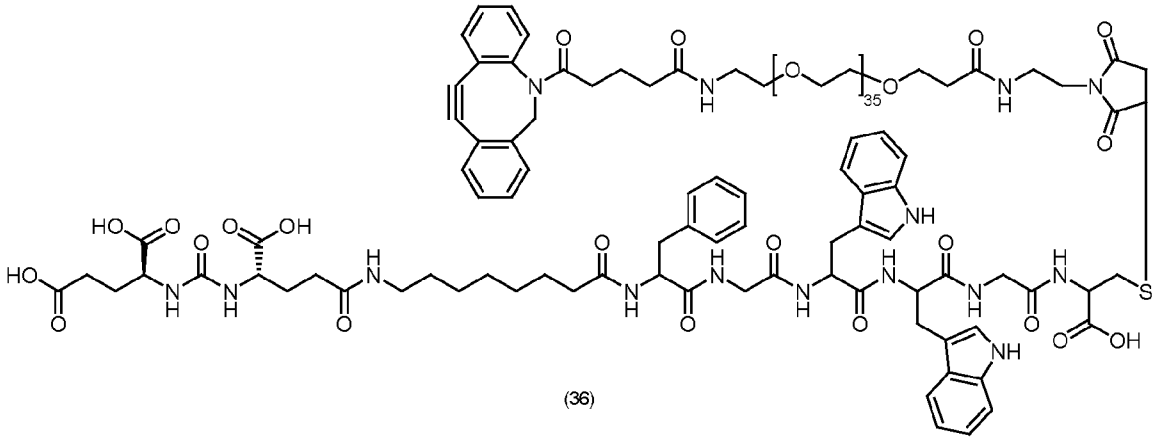
Step 2. Synthesis of DBCO-[CONH]-PEG$_{36}$-[MAL-S]-DUPA (Compound 36)



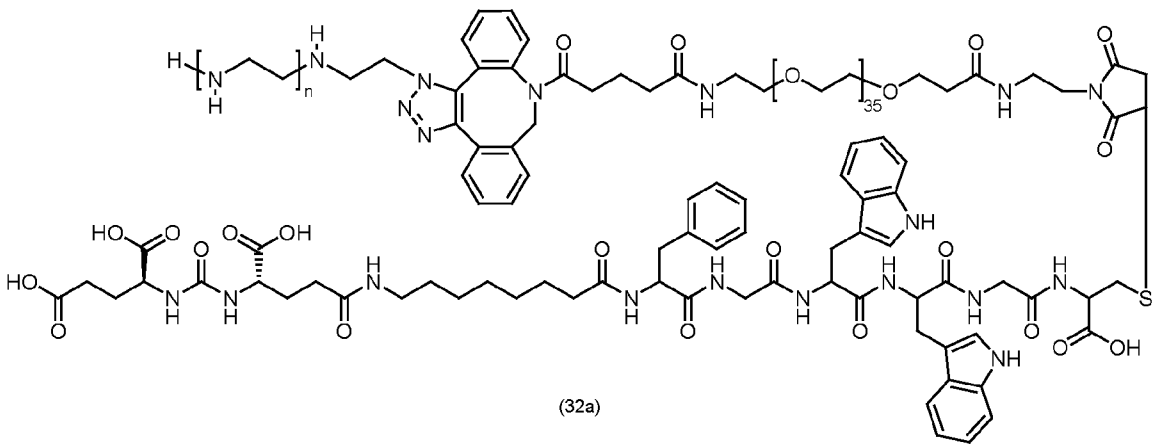
A solution of DBCO-[CONH]-PEG$_{36}$-MAL (Compound 35; 24 µmol, 1.0 eq, 120 mM) in DMF (0.20 mL) was mixed with a DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; SEQ ID NO:4) (17 µmol, 0.7 eq, 123 mM) in DMF (137 µL). The reaction mixture was incubated on a Stuart rotator at room temperature and protected from light. After 15 min, an additional amount of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (39 µL, 5 µmol, 0.2 eq) was added. At 40 min into reaction, an additional amount of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (14 µL, 1.7 µmol, 0.07 eq) was added. After a further one hour mixing, cold diethyl ether (4.5 mL) was added. The precipitate was washed with cold diethyl ether (4.5 mL). The precipitate was dissolved in DMSO (0.5 mL) and was supplemented with H_2O (6.75 mL) and acetonitrile (0.75 mL). DBCO-PEG$_{36}$-[CONH]-DUPA (Compound 36) was isolated following RP-C$_{18}$ preparative HPLC and lyophilization of pooled fractions. The lyophilisate was analyzed by RP-

HPLC-ELSD and RP-HPLC – ESI^+ qTOF mass spectrometry (Solid DBCO-[CONH]-PEG_{36}-[MAL-S]-DUPA (Compound 36; 36 mg) calculated monoisotopic mass: 3294.64 Da; measured: 3294.65 Da).

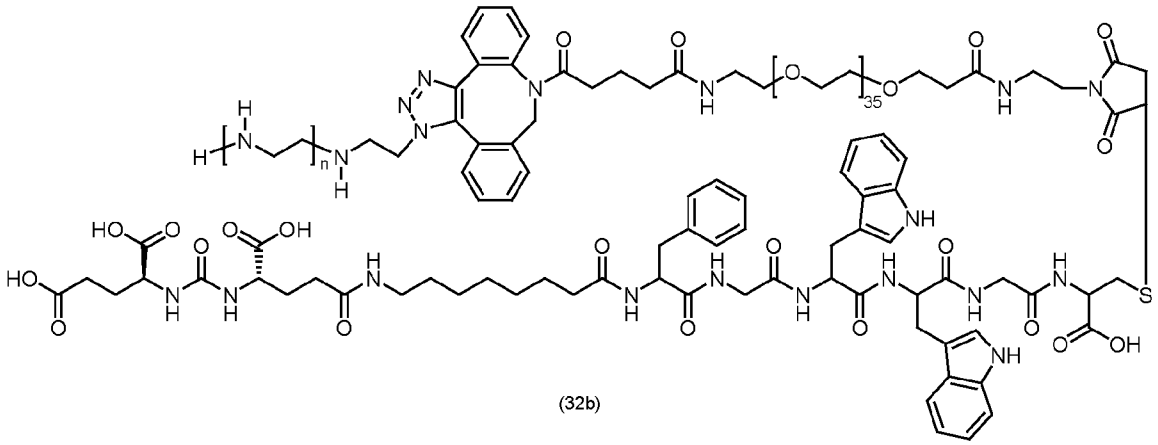
Step 3. Synthesis of LPEI-*l*-[N_3;DBCO]CONH-PEG_{36}-[MAL-S]-DUPA (Compounds 32a and 32b)



(36)



(32a)



(32b)

LPEI-N_3 solution (4.2 mL, 5 µmol, 1.0 eq, 1.2 mM) in 50 mM acetate buffer pH 4.0 was slowly added to 2.4 mL of a solution of DBCO-[CONH]-PEG_{36}-[MAL-S]-DUPA (Compound 36; 5 µmol, 1.0 eq, 2.0 mM). The mixture was incubated at room temperature on a Stuart rotator and monitored by RP-C_8-HPLC. After 70 hours, the reaction mixture was supplemented with acetonitrile (0.73 mL) and TFA (74 µL) and isolated using RP-C_{18} preparative HPLC. The pooled fractions were lyophilized to give LPEI-*l*-[N_3:DBCO]-CONH-PEG_{36}-[MAL-S]-DUPA (87 mg) as a mixture of regioisomers 32a and 32b and as a fluffy white solid. The lyophilizate was characterized by RP-C_8-HPLC, copper assay and spectrophotometry at 280 nm for determination of the DUPA content. The lyophilisate had a weight percentage in LPEI of 30% w/w and a LPEI to DUPA ratio of 1/1.1.

Step 4. Preparation of LPEI-*l*-[N_3:DBCO]-CONH-PEG_{36}-[MAL-S]-DUPA (Compounds 32a and 32b) HEPES salt

LPEI-*l*-[N_3:DBCO]-CONH-PEG_{36}-[MAL-S]-DUPA (Compounds 32a and 32b) TFA salt (20.8 mg, w_{LPEI} = 30%, 6.2 mg in total LPEI) was dissolved in 1.2 mL 20 mM HEPES pH 7.2. Three centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:DBCO]-CONH-PEG_{36}-[MAL-S]-DUPA solution each, centrifugated one time at 14000 g for 30 minutes and then three times after addition of 400 µL 20 mM HEPES, pH 7.2. About 246 µL of LPEI-*l*-[N_3:DBCO]-CONHPEG_{36}-[MAL-S]-DUPA-HEPES salt solution were recovered and supplemented with 2.4 mL 20 mM HEPES, pH 7.2. The concentration of the solution was determined by copper assay to be 2.1 mg/mL in total LPEI.

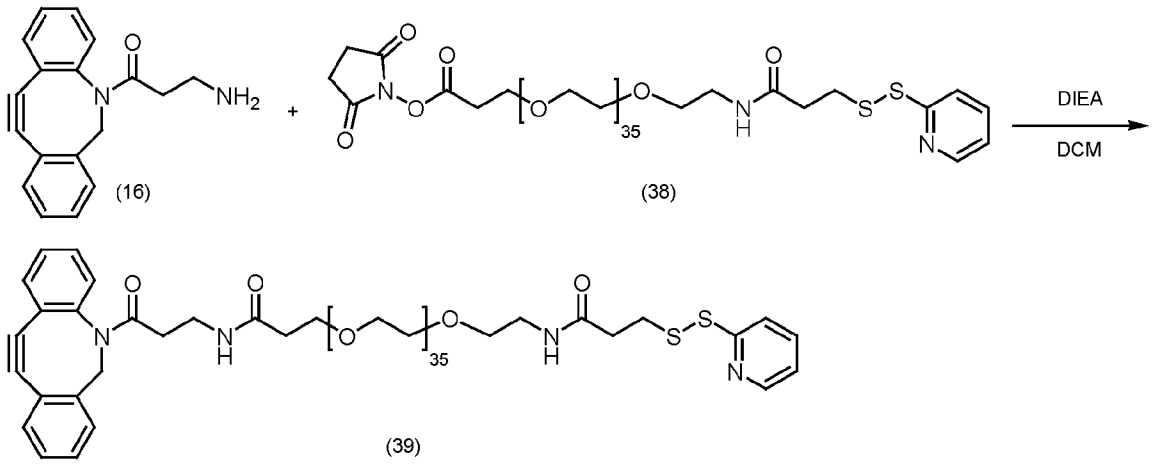
EXAMPLE 9

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA (COMPOUNDS 37a

AND 37b)

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA was prepared as a mixture of regioisomers 37a and 37b according to the schemes below. DBCO-PEG_{36}-SH (Compound 40) was prepared by condensing DBCO-NH_2 (Compound 16) with NHS-PEG_{36}-OPSS (Compound 38) and subsequent reduction. Compound 40 was then condensed with DUPA-MAL (Compound 41) and reacted with LPEI-N_3 to give Compounds 37a and 37b.

Step 1. Synthesis of DBCO-PEG_{36}-OPSS (Compound 39)

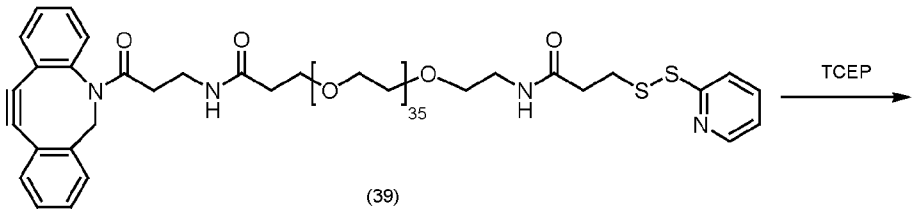


(16) (38)

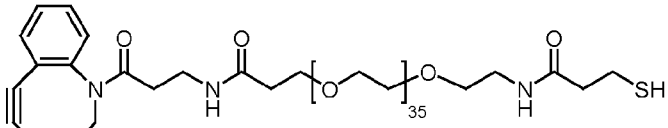
(39)

A solution of NHS-PEG_{36}-OPSS (Compound 38; 49 µmol, 1.0 eq, 123 mM) in DCM (0.40 mL) was mixed with a solution containing DBCO-NH_2 (Compound 16; 54 µmol, 1.1 eq, 357 mM and DIEA (17 µL, 100 µmol, 2.0 eq)) in DMF (151 µL). The reaction mixture was incubated on a Stuart rotator at room temperature and the reaction was monitored by RP-C_8-HPLC. After 15 min, an additional amount of DBCO-NH_2 (5 µmol, 0.1 eq, 357 mM) was added. After a total of 30 minutes, 4.5 mL of n-hexane were added. The resulting precipitate was filtered, centrifuged, and washed with 4.5 mL cold diethyl ether. Solid DBCO-PEG_{36}-OPSS (Compound 39) was recovered and analyzed by HPLC – ESI+ qTOF mass spectrometry (calculated monoisotopic mass: 2129.10 Da; measured: 2129.12 Da) and used in the next step without further purification.

Step 2. Synthesis of DBCO-PEG$_{36}$-SH (Compound 40)



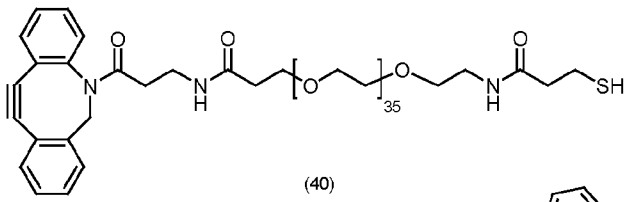
(39)



(40)

A solution of DBCO-PEG$_{36}$-OPSS (Compound 39; 4.8 µmol, 1.0 eq, 12 mM assuming 100% purity) in DMSO (0.40 mL) was mixed with a solution of TCEP (5.8 µmol, 1.2 eq, 127 mM) in 20 mM HEPES pH 7.4 (45 µL). The reaction mixture was incubated on a Stuart rotator at room temperature and the reaction was monitored by RP-C$_8$-HPLC. The reaction mixture comprising DBCO-PEG$_{36}$-SH (Compound 40) was used without further purification in the next step.

Step 3. Synthesis of DBCO-PEG$_{36}$-[S-MAL]-DUPA (Compound 42)



(40)

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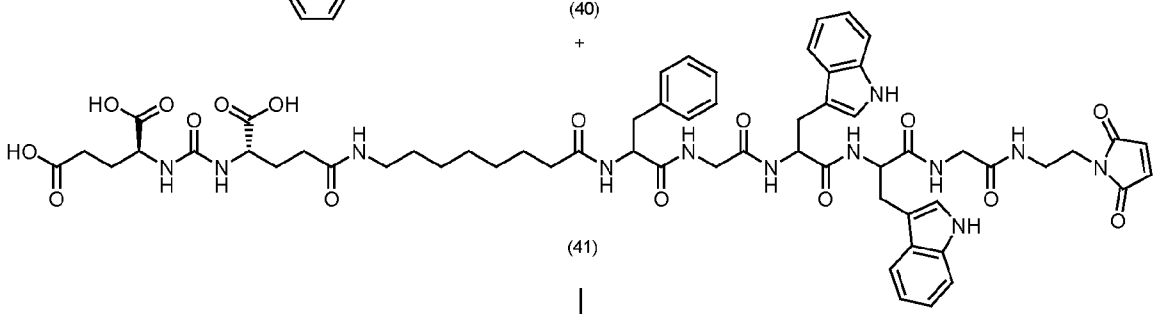
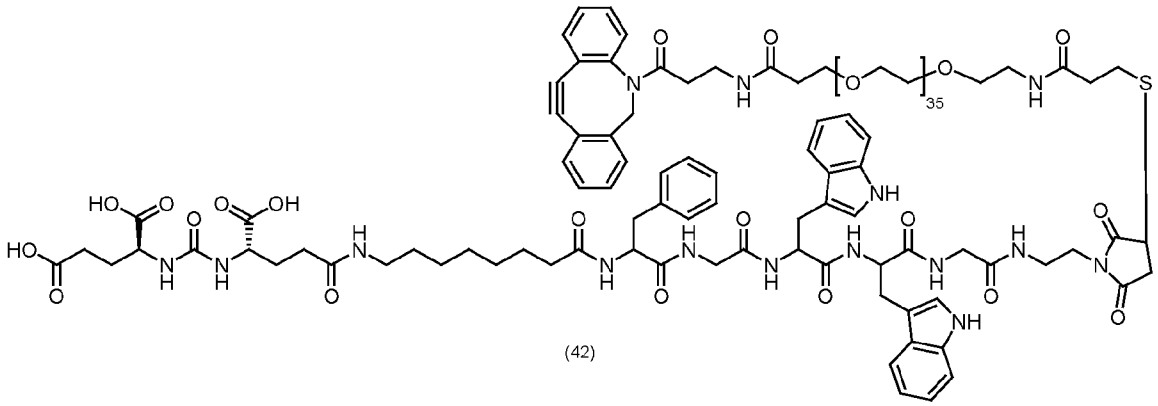


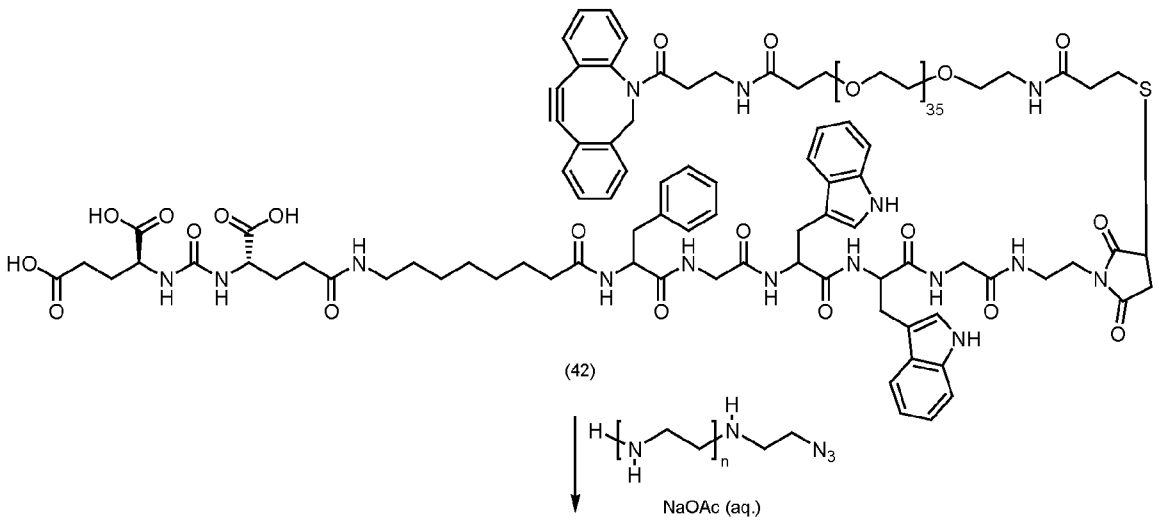
(41)

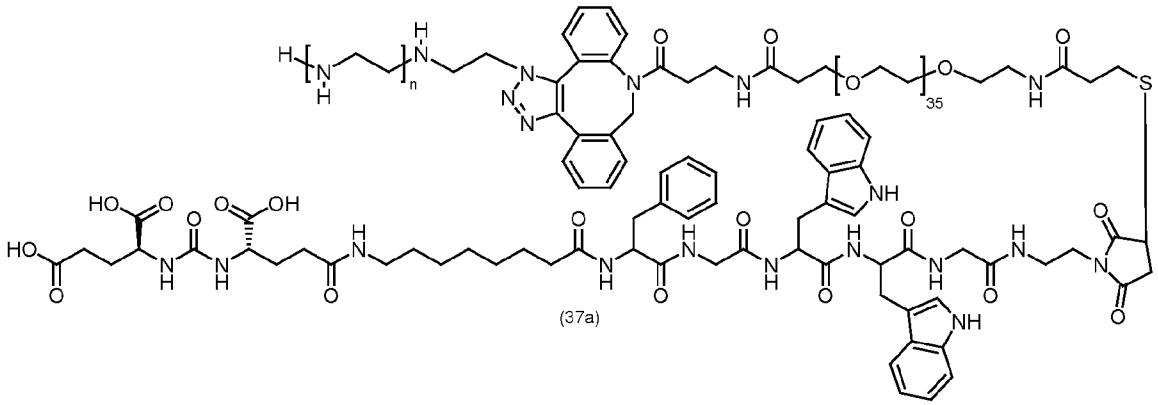


(42)

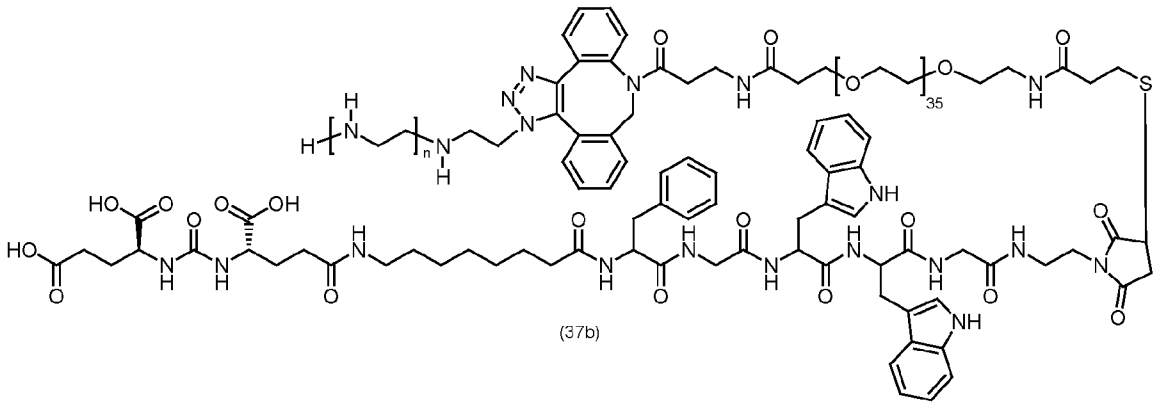
A solution of DUPA-MAL (Compound 41; 4.0 µmol, 1.0 eq, 2.5 mM) in 20 mM HEPES pH 7.4 (1.6 mL) was added to the solution of DBCO-PEG$_{36}$-SH (Compound 40; 364 µL, 4.0 µmol, 1.0 eq) prepared in Step 2 and the reaction mixture was incubated on a Stuart rotator at room temperature and monitored by RP-C$_8$-HPLC. After 15 min, an additional amount of DUPA-MAL (320 µL, 0.3 µmol, 0.1 eq) was added. After a total of 30 minutes, DBCO-PEG$_{36}$-[S-MAL]-DUPA (Compound 42) was isolated following preparative RP-C$_{18}$ HPLC and lyophilization of pooled fractions. The lyophilizate was analyzed by RP-HPLC-ELSD and RP-HPLC – ESI$^+$ qTOF mass spectrometry (DBCO-PEG$_{36}$-[S-MAL]-DUPA (7 mg) calculated monoisotopic mass: 3236.62 Da; measured: 3236.65 Da).

Step 4. Synthesis of LPEI-*l*-[N$_3$:DBCO]-PEG$_{36}$-[S-MAL]-DUPA (Compounds 37a and 37b)





(37a)



(37b)

LPEI-N_3 solution (2.5 mL, 2.0 µmol, 1.0 eq) in 50 mM acetate buffer pH 4.0 was slowly added to 4.0 mL of a solution of DBCO-PEG_{36}-[S-MAL]-DUPA (Compound 42; 2.5 µmol, 1.2 eq, 1 mM) in 50 mM acetate buffer pH 4.0. The mixture was incubated at room temperature on a Stuart rotator and protected from light. After 20 hours, the reaction mixture was supplemented with acetonitrile (0.78 mL) and TFA (79 µL). LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA was isolated as a mixture of regioisomers 37a and 37b using RP-C_{18} preparative HPLC and characterized by analytical RP-C_8-HPLC, copper assay and spectrophotometry at 280 nm for determination of the DUPA content. The lyophilisate had a weight percentage in LPEI of 28% w/w and a LPEI to DUPA ratio of 1/1.08.

Step 5. Preparation of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA (Compound 37a and 37b) HEPES salt

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA (Compound 37a and 37b) TFA salt (24.9 mg, w_{LPEI} = 28%, 7.0 mg in total LPEI) was dissolved in 0.8 mL 20 mM HEPES pH 7.2. Two centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA solution each, centrifugated one time at 14000 g for 30 minutes and then three times after addition of 400 µL 20 mM HEPES, pH 7.2. About 269 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA (Compound 37a and 37b) HEPES salt solution

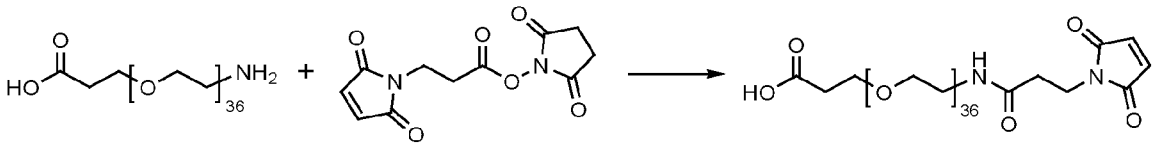
were recovered and supplemented with 2.4 mL 20 mM HEPES, pH 7.2. The concentration of the solution was determined by copper assay to be 2.5 mg/mL in total LPEI.

EXAMPLE 10

SYNTHESIS OF Me-LPEI-l-[N_3:BCN]-PEG_{36}-DUPA (COMPOUND 43)

Me-LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA (Compound 43) was synthesized according to the schemes below. In a first step, HOOC-PEG_{36}-NH_2 (Compound 13) was coupled to N-succinimidyl 3-maleimidopropionate (Compound 14) by amide formation to produce HOOC-PEG_{36}-MAL (Compound 15). *Endo*-BCN-PEG_{36}-MAL (Compound 26) was prepared by condensing HOOC-PEG_{36}-MAL (Compound 15) with *endo*-BCN-PEG_2-NH_2 (Compound 25). In a next step, Compound 26 was condensed with Compound 2, and the resulting *endo*-BCN-PEG_{36}-[MAL-S]-DUPA (Compound 27) was reacted with Me-LPEI-N_3 to give Compound 43.

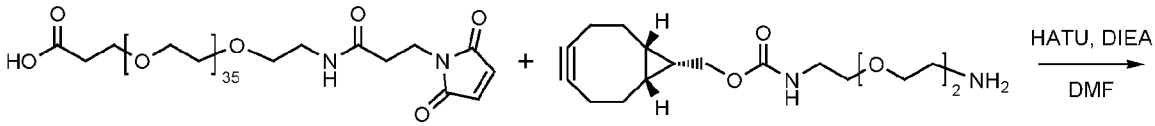
Step 1: Synthesis of HOOC-PEG_{36}-MAL (Compound 15)



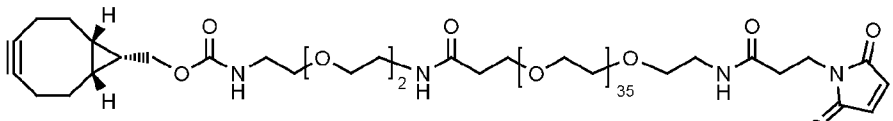
(13) (14) (15)

A solution of HOOC-PEG_{36}-NH_2 (Compound 13, 94 µmol, 1.0 eq, 234 mM) in DCM (0.40 mL) was mixed with a solution of N-succinimidyl 3-maleimidopropionate (Compound 14, 85 µmol, 0.9 eq, 184 mM) in DCM (0.83 mL). The reaction mixture was shaken on a Stuart rotator at room temperature and the reaction was monitored by RP-C_8 HPLC. At one hour into reaction, an additional amount of N-succinimidyl 3-maleimidopropionate (137 µL, 14 µmol, 0.15 eq) was added and at 1h 15 min into reaction, an additional amount of N-succinimidyl 3-maleimidopropionate (83 µL, 9 µmol, 0.1 eq) was added. After a total of 1.5 hours, 4.5 mL of cold diethyl ether were added to induce precipitation followed by centrifugation. The precipitate was washed with 4.5 mL cold diethyl ether and 183 mg of HOOC-PEG_{36}-MAL (Compound 15) were recovered (calculated monoisotopic mass: 1825.03 Da; measured: 1825.02 Da).

Step 2. Synthesis of *endo*-BCN-PEG_{36}-MAL (Compound 26)



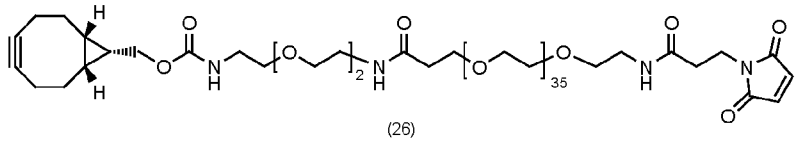
(15) (25)



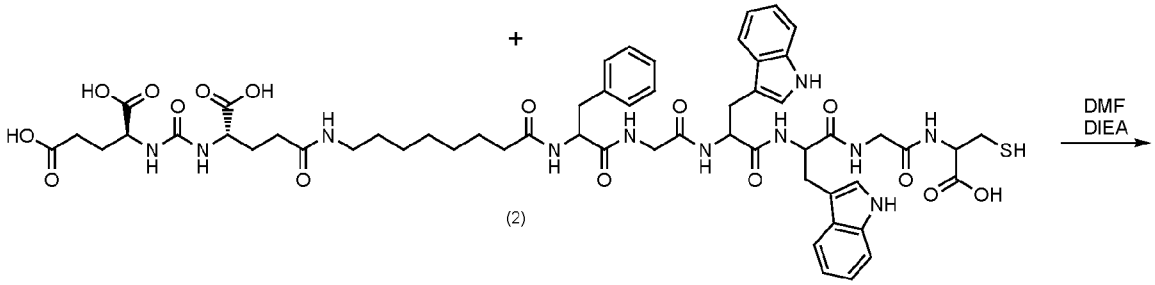
(26)

A solution of HOOC-PEG$_{36}$-MAL (Compound 15, 40 µmol, 1.0 eq, 99 mM) in DCM (0.40 mL) was mixed with a solution of HATU (36 µmol, 0.9 eq, 325 mM) in DMF (111 µL). The mixture was stirred for one minute and DIEA (14 µL, 80 µmol, 2.0 eq) was added. The mixture was stirred for three minutes and was mixed with a solution of *endo*-BCN-PEG$_2$-NH$_2$ (Compound 25, 32 µmol, 0.8 eq, 370 mM) in DCM (86 µL). The reaction mixture was shaken on a Stuart rotator at room temperature and the reaction was monitored by RP-C$_8$-HPLC. At 15 minutes into reaction, an additional amount of *endo*-BCN-PEG$_2$-NH$_2$ (54 µL, 20 µmol, 0.5 eq) was added and at 30 minutes into reaction, a further amount of *endo*-BCN-PEG$_2$-NH2 (32 µL, 12 µmol, 0.3 eq) was added. After a total of 45 minutes, 4.5 mL of n-hexane were added to induce precipitation followed by centrifugation. The precipitate was washed with 4.5 mL cold diethyl ether and 103 mg of *endo*-BCN-PEG$_{36}$-MAL (Compound 26) solid material were recovered (calculated monoisotopic mass: 2131.21 Da; measured: 2131.22 Da).

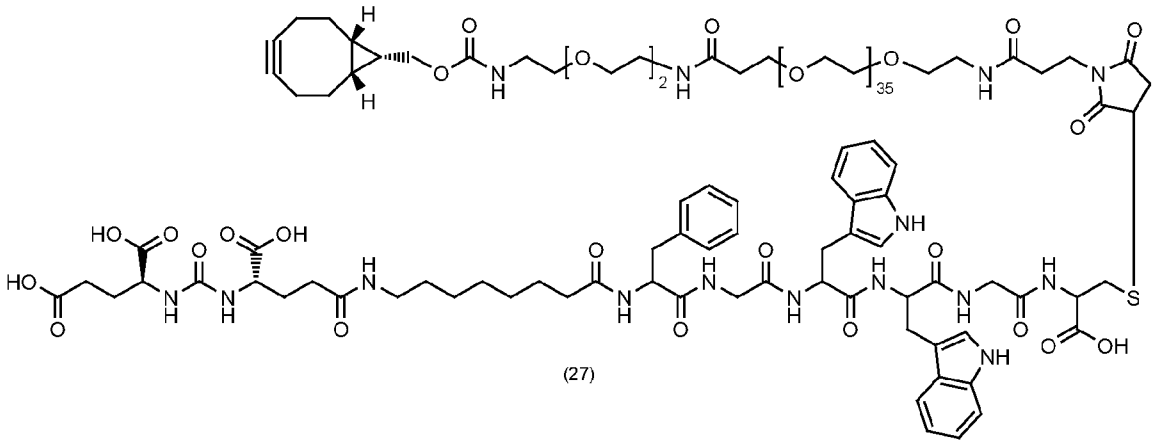
Step 3. Synthesis of *endo*-BCN-PEG$_{36}$-[MAL-S]-DUPA (Compound 27)



(26)



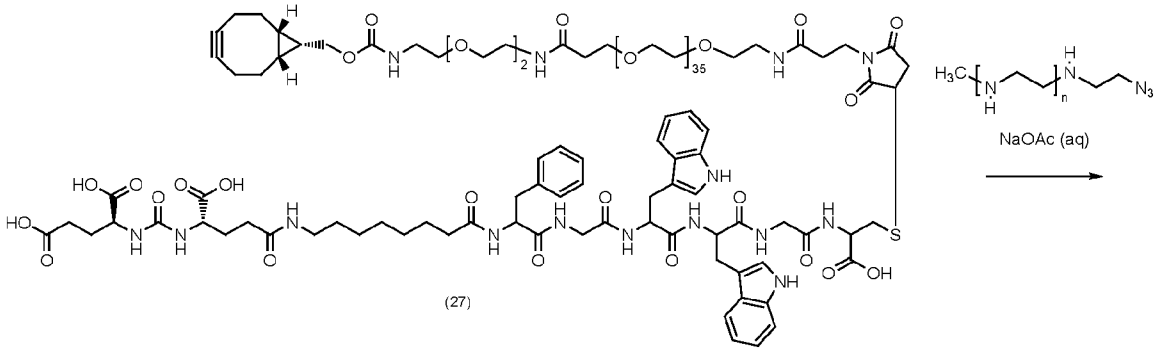
(2)



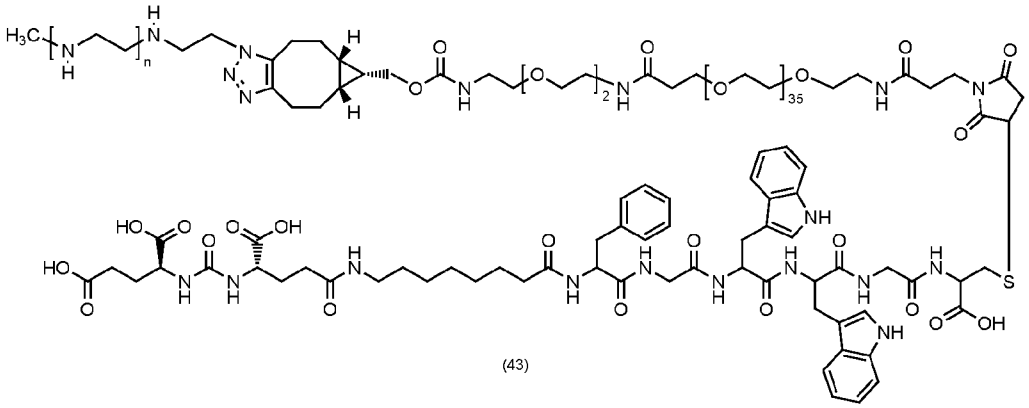
(27)

A solution of *endo*-BCN-PEG$_{36}$-MAL (Compound 26, 19 µmol, 1.0 eq, 93 mM) in DMF (0.20 mL) was mixed with a solution of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2, 13 µmol, 0.7 eq, 77 mM) in DMF (173 µL) and DIEA (6 µL, 38 µmol, 2.0 eq). The reaction mixture was shaken on a Stuart rotator at room temperature and the reaction was monitored by RP-C$_8$ HPLC. At 1 hour into reaction, an additional amount of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (26 µL, 2 µmol, 0.1 eq) was added. After a total of 2 hours the mixture was purified using RP-C$_{18}$ preparative chromatography and pooled fractions containing *endo*-BCN-PEG$_{36}$-[MAL-S]-DUPA (Compound 27) were lyophilized to give 12 mg lyophilized product. The lyophilisate was analyzed by RP-HPLC-ELSD and RP-HPLC – ESI$^+$ qTOF mass spectrometry. The solid mainly contained *endo*-BCN-PEG$_{36}$-[MAL-S]-DUPA (calculated monoisotopic mass: 3328.69 Da; measured: 3328.71 Da).

Step 4. Synthesis of Me-LPEI-*l*-[N$_3$:BCN]-PEG$_{36}$-DUPA (Compound 43)



(27)



(43)

Me-LPEI-N_3 solution (4.7 µmol, 1.0 eq) in acetate buffer (50 mM, 3.2 mL, pH 4.0) was slowly added to a solution of *endo*-BCN-PEG_{36}-[MAL-S]-DUPA (Compound 27, 3.3 µmol, 0.7 eq, 1.1 mM) in acetate buffer (50 mM, 3.0 mL, pH 4.0). The mixture was shaken for about 45 hrs at room temperature on a Stuart rotator and protected from light. To the reaction mixture were added acetonitrile (0.66 mL) and TFA (70 µL) and the resultant mixture was purified using RP-C_{18} preparative chromatography. Me-LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA (Compound 43) was lyophilized to give 55 mg lyophilized product and characterized by analytical RP-C_8 HPLC, copper assay and spectrophotometry at 280 nm for determination of the DUPA content. The product was found to have a weight percentage in LPEI of 28%w/w and a LPEI to DUPA ratio of 1/0.90.

Step 5. Preparation of Me-LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA (Compound 43) HEPES salt

24.8 mg of Me-LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA (Compound 43) TFA salt (w_{LPEI} = 28%, 6.9 mg in total LPEI) were dissolved in 0.8 mL 20 mM HEPES pH 7.2. Two centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of Me-LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA solution each. They were centrifuged one time at 14000 g for 30 minutes and then three times after addition of 400 µL 20 mM HEPES, pH 7.2. About 253 µL of concentrated solution were recovered and supplemented with 2.5 mL 20 mM HEPES, pH 7.2. The concentration of the solution was determined by copper assay to be 2.3 mg/mL in total LPEI (LPEI/DUPA ratio = 1/0.97.

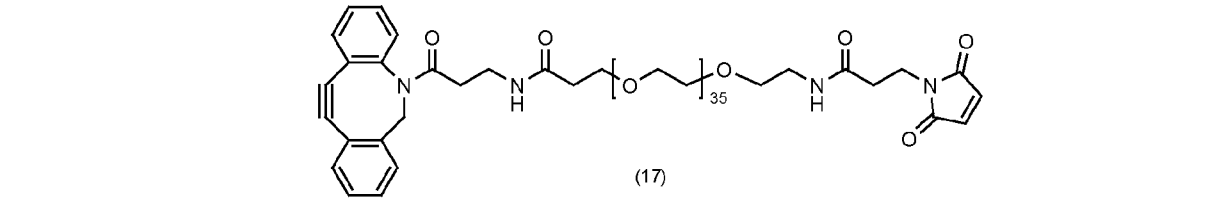
EXAMPLE 11

SYNTHESIS OF Me-LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (COMPOUNDS 44a AND 44b)

Me-LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (44a and 44b) was synthesized according to the schemes below. In a first step, DBCO-PEG_{36}-MAL (Compound 17) was coupled to DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; SEQ ID NO:4) by a Michael addition to produce

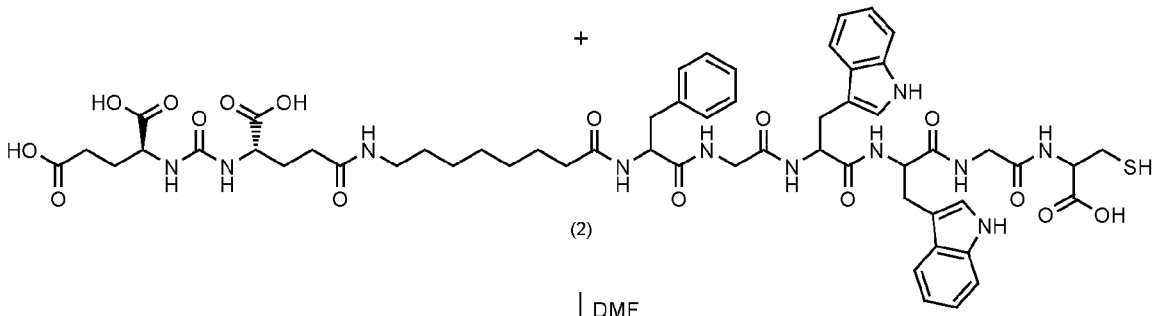
DBCO-PEG$_{36}$-DUPA (Compound 18). In a next step, DBCO-PEG$_{36}$-DUPA (Compound 18) was coupled to Me-LPEI-N$_3$ by a [2+3] cycloaddition to produce Me-LPEI-*l*-[N$_3$:DBCO]-PEG$_{36}$-DUPA as a mixture of regioisomers 44a and 44b.

Step 1: Synthesis of DBCO-PEG$_{36}$-DUPA (Compound 18)



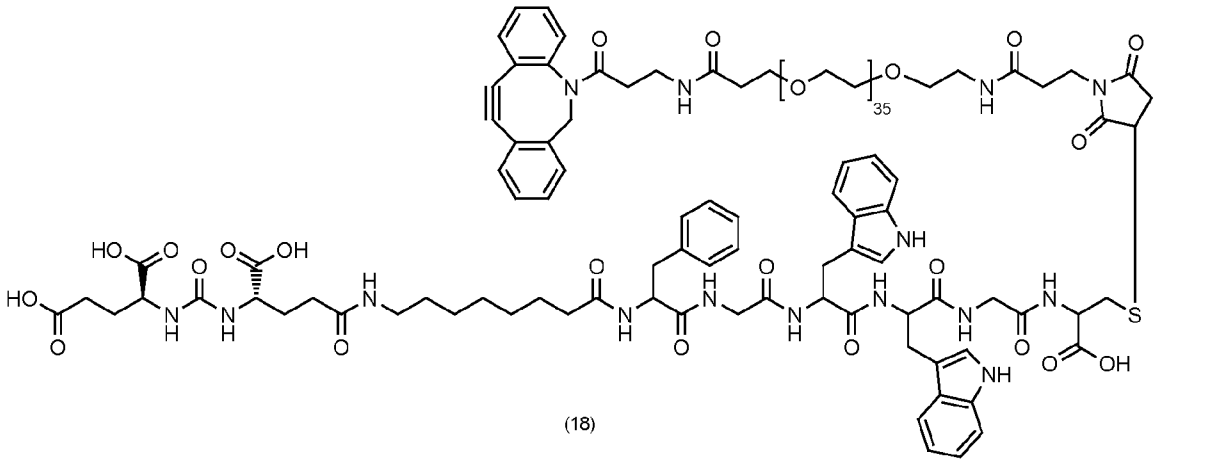
(17)

+



(2)

DMF
DIEA

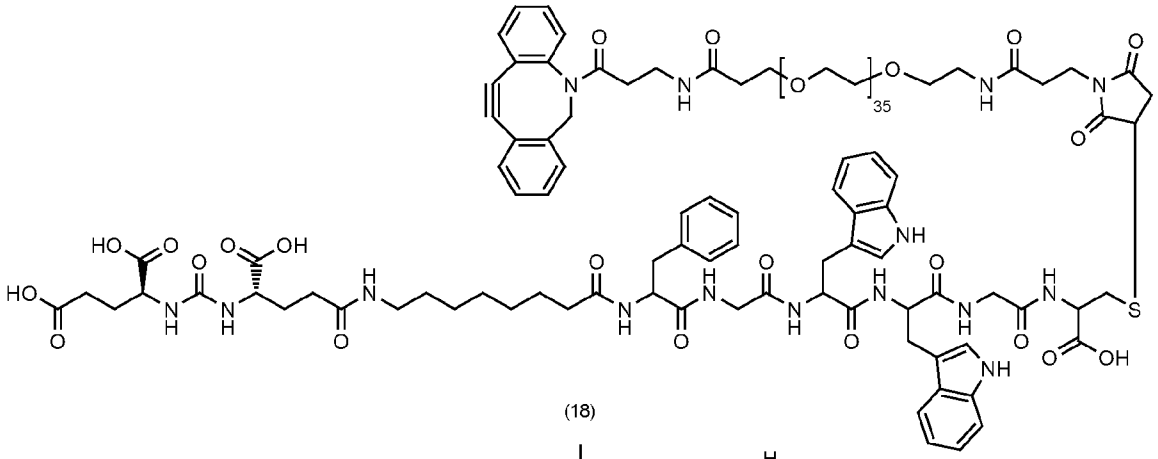


(18)

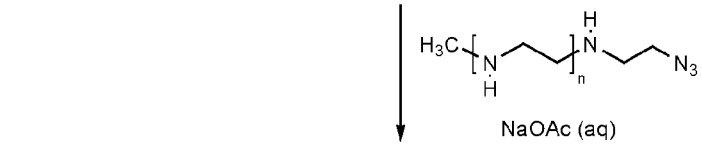
A solution of DBCO-PEG$_{36}$-MAL (Compound 17, 10 µmol, 1.0 eq, 49 mM) in DMF (0.20 mL) was mixed with a solution of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; SEQ ID NO:4) (10 µmol, 1.0 eq, 82 mM) (151 µL) and DIEA (3 µL, 20 µmol, 2.0 eq) in DMF. The mixture was shaken for about 30 minutes at room temperature on a Stuart rotator and protected from light and the reaction was monitored by RP-C$_8$ HPLC. The resultant mixture was purified using RP-C$_{18}$ preparative chromatography and pooled fractions containing DBCO-PEG$_{36}$-DUPA were lyophilized to give 20 mg of DBCO-PEG$_{36}$-DUPA (Compound 18). A sample was analyzed by analytical RP-HPLC-ELSD and HPLC – ESI$^+$ qTOF mass

spectrometry (calculated monoisotopic mass: 3280.61 Da; measured: 3280.64 Da)

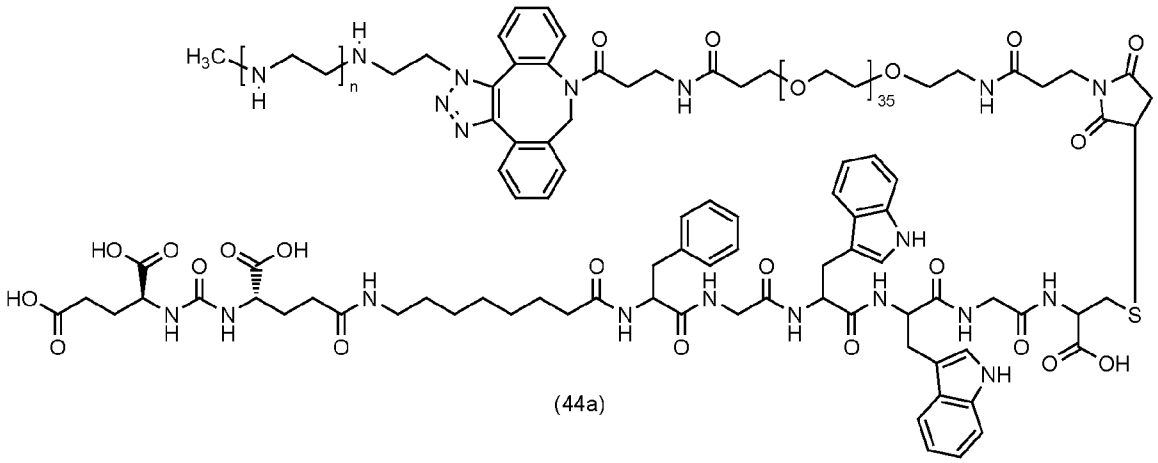
Step 2: Synthesis of Me-LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 44a and 44b)



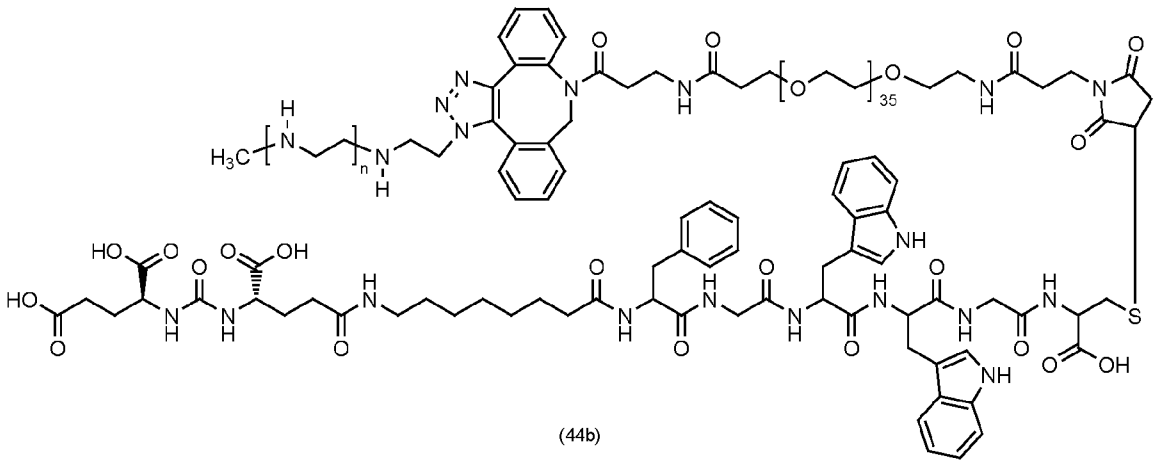
(18)



NaOAc (aq)



(44a)



(44b)

Me-LPEI-N_3 solution (4.5 µmol, 1.3 mM, 1.0 eq) in acetate buffer (50 mM, 3.2 mL, pH 4.0) was slowly added to a solution of DBCO-PEG_{36}-DUPA (Compound 18, 6.6 µmol, 1.5 eq,

2.2 mM) in acetate buffer (50 mM, 3.0 mL, pH 4.0). The mixture was shaken for about 20 hrs at room temperature on a Stuart rotator and protected from light. To the reaction mixture were added acetonitrile (0.70 mL) and TFA (70 µL). The resultant mixture was purified using RP-C_{18} preparative chromatography and pooled fractions containing Me-LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 44a and 44b) were lyophilized to give 70 mg lyophilized product and characterized by RP-C_8 HPLC, copper assay and spectrophotometry at 280 nm for determination of the DUPA content. The product was found to have a weight percentage in LPEI of 28%w/w and a LPEI to DUPA ratio of 1/1.17.

Step 3. Preparation of Me-LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 44a and 44b) HEPES salt

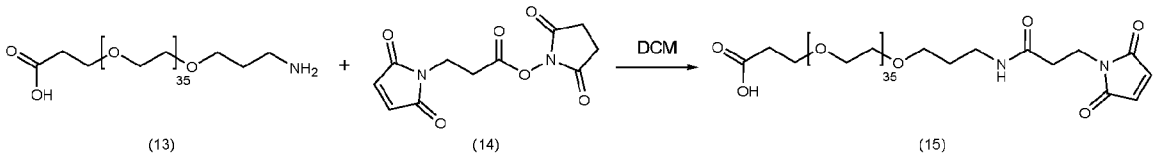
25.0 mg of Me-LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 44a and 44b) TFA salt (w_{LPEI} = 28%, 7.0 mg in total LPEI) were dissolved in 0.8 mL 20 mM HEPES pH 7.2. The pH was adjusted to pH 7.2. Two centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of Me-LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA solution each. They were centrifuged one time at 14000 g for 30 minutes and then three times after addition of 400 µL 20 mM HEPES, pH 7.2. About 254 µL of the concentrated solution were recovered after buffer exchange and were supplemented with 2.5 mL 20 mM HEPES, pH 7.2. The concentration of the solution was determined by copper assay to be 2.3 mg/mL in total LPEI and an LPEI to DUPA molar ratio of = 1/1.19 was determined.

EXAMPLE 12

SYNTHESIS OF LPEI-*l*-[N_3:CliCr®]-PEG_{36}-DUPA (COMPOUND 45)

LPEI-*l*-[N_3:CliCr®]-PEG_{36}-DUPA (Compound 45) was synthesized according to the schemes below. In a first step, CliCr®-beta-Ala-NH_2 (Compound 46) was coupled to HOOC-PEG_{36}-MAL (Compound 15) to produce CliCr®-PEG_{36}-MAL (Compound 47). Subsequently, CliCr®-PEG_{36}-MAL (Compound 47) was coupled to DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; SEQ ID NO:4) by a Michael addition to produce CliCr®-PEG_{36}-DUPA (Compound 48). In a final step, CliCr®-PEG_{36}-DUPA (Compound 48) was reacted with LPEI-N_3 in a [2+3] cycloaddition reaction to produce LPEI-*l*-[N_3:CliCr®]-PEG_{36}-DUPA (Compound 45).

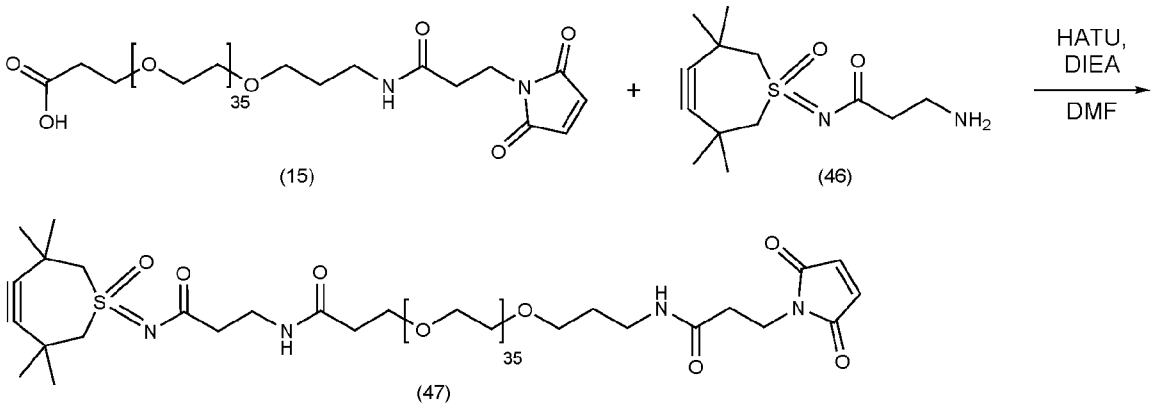
Step 1: Synthesis of HOOC-PEG_{36}-MAL (Compound 15)



(13) (14) (15)

A solution of HOOC-PEG$_{36}$-NH$_2$ (Compound 13, 94 µmol, 1.0 eq, 234 mM) in DCM (0.40 mL) was mixed with a solution of N-succinimidyl 3-maleimidopropionate (Compound 14, 85 µmol, 0.9 eq, 184 mM) in DCM (0.83 mL). The reaction mixture was shaken for one hour on a Stuart rotator at room temperature and the reaction was monitored by RP-C$_8$ HPLC. An additional amount of N-succinimidyl 3-maleimidopropionate (220 µL, 23 µmol, 0.25 eq) was added. After a total of 3.5 hours mixing, 4.5 mL of cold diethyl ether were added to induce precipitation followed by centrifugation. The precipitate was washed with 4.5 mL cold diethyl ether and 183 mg of HOOC-PEG$_{36}$-MAL (Compound 15) were recovered (calculated monoisotopic mass: 1825.03 Da; measured: 1825.02 Da).

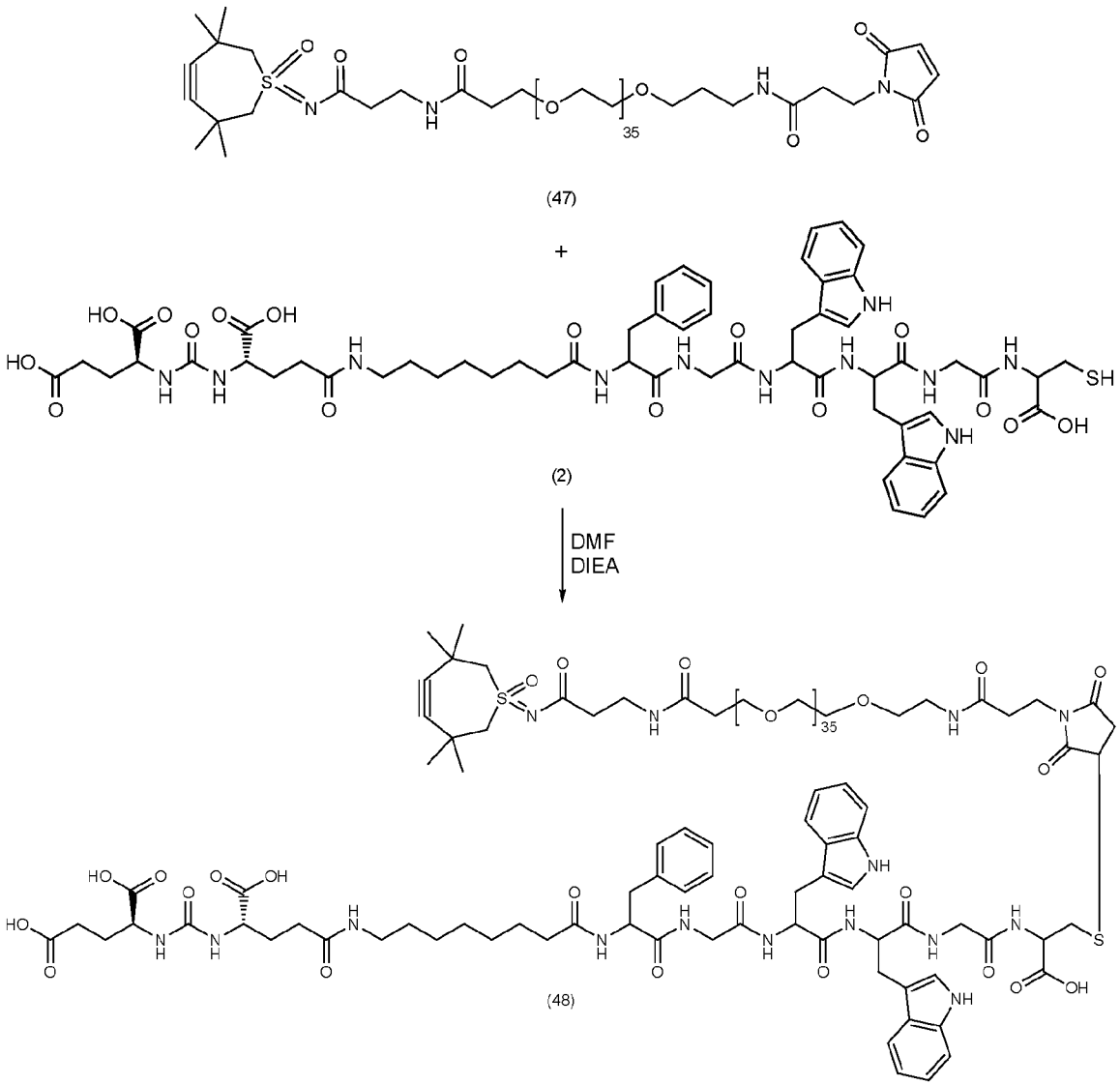
Step 2: Synthesis of CliCr®-PEG$_{36}$-MAL (Compound 47)



(15) (46) (47)

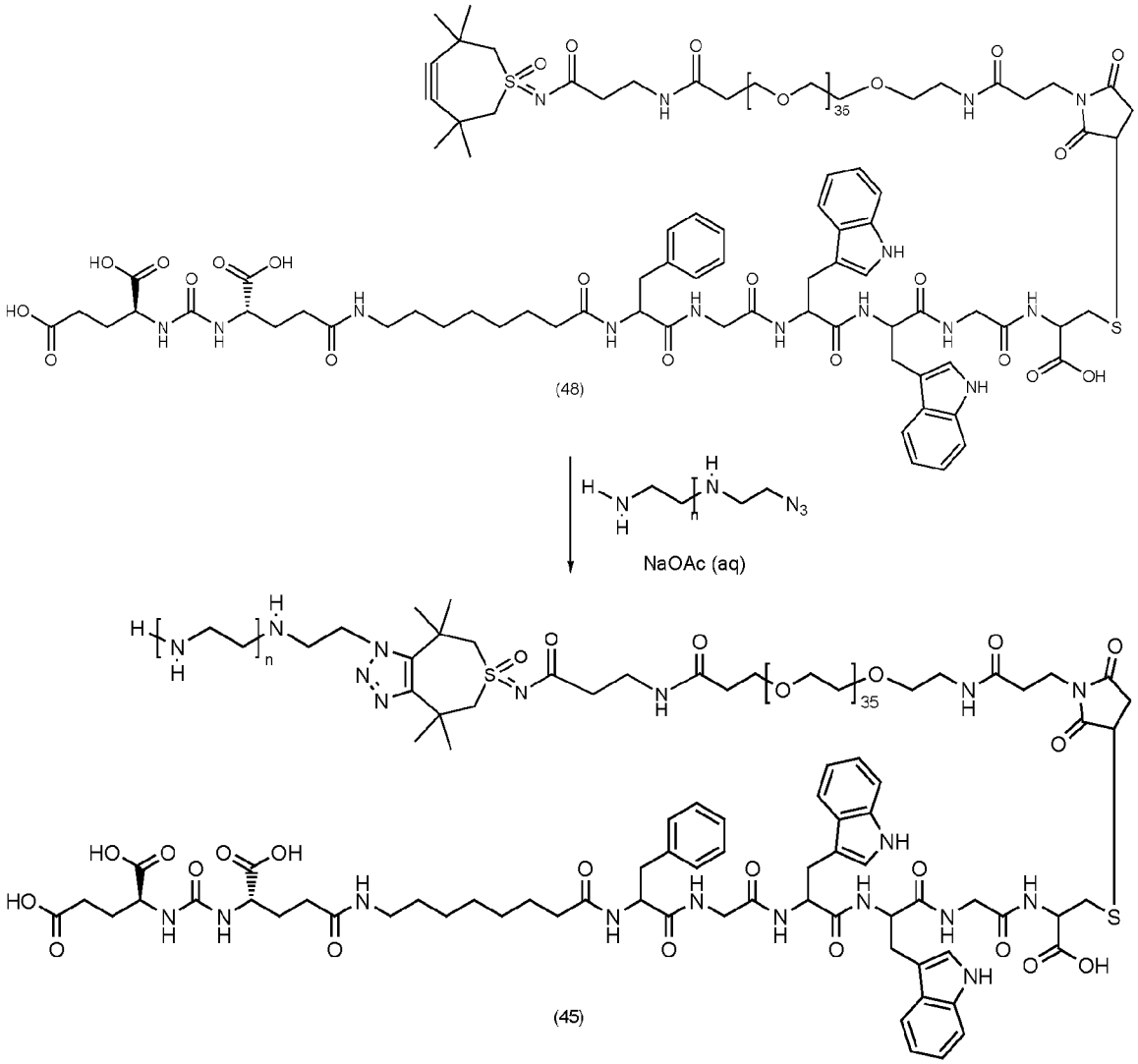
A solution of HOOC-PEG$_{36}$-MAL (Compound 15, 17 µmol, 1.0 eq, 42 mM) in DMF (0.40 mL) was mixed with a solution of HATU (17 µmol, 1.0 eq, 89 mM) and DIEA (6 µL, 34 µmol, 2.0 eq) in DMF (191 µL). A solution of CliCr®-beta-Ala-NH$_2$ (Compound 46, 20 µmol, 1.2 eq, 163 mM) in DMF (123 µL) was then added. The mixture was shaken for about 30 minutes at room temperature on a Stuart rotator and the reaction was monitored by RP-C$_8$ HPLC. The resultant mixture was purified using RP-C$_{18}$ preparative chromatography and pooled fractions containing CliCr®-PEG$_{36}$-MAL were lyophilized to give 11 mg of CliCr®-PEG$_{36}$-MAL (Compound 47). A sample was analyzed by analytical RP-HPLC ELSD and HPLC – ESI$^+$ qTOF mass spectrometry (calculated monoisotopic mass: 2077.15 Da; measured: 2077.17 Da).

Step 3: Synthesis of CliCr®-PEG$_{36}$-DUPA (Compound 48)



A solution of CliCr®-PEG$_{36}$-MAL (Compound 47, 5.3 µmol, 1.0 eq, 13 mM) in DMF (0.40 mL) was mixed with a solution of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 2; SEQ ID NO:4) (5.3 µmol, 1.0 eq, 44 mM) (120 µL) and DIEA (1.8 µL, 11 µmol, 2.0 eq) in DMF. The mixture was shaken for about 30 minutes at room temperature on a Stuart rotator and the reaction was monitored by RP-C_8 HPLC. The resultant mixture was purified using RP-C_{18} preparative chromatography and pooled fractions containing CliCr®-PEG$_{36}$-DUPA were lyophilized to give 11 mg of CliCr®-PEG$_{36}$-DUPA (Compound 48). A sample was analyzed by analytical RP-HPLC ELSD and HPLC – ESI$^+$ qTOF mass spectrometry (calculated monoisotopic mass: 3274.63 Da; measured: 3274.66 Da).

Step 4: Synthesis of LPEI-*l*-[N_3:CliCr®]-PEG$_{36}$-DUPA (Compound 45)



LPEI-N_3 solution (4.1 µmol, 0.84 mM, 1.0 eq) in acetate buffer (50 mM, 5.0 mL, pH 4.0) was slowly added to a solution of CliCr®-PEG_{36}-DUPA (Compound 48, 3.9 µmol, 1.0 eq, 3.9 mM) in acetate buffer (50 mM, 1.0 mL, pH 4.0). The mixture was shaken for about 3 hrs at room temperature on a Stuart rotator. To the reaction mixture were added acetonitrile (0.67 mL) and TFA (67 µL). The resultant mixture was purified using RP-C_{18} preparative chromatography and pooled fractions containing LPEI-*l*-[N_3:CliCr®]-PEG_{36}-DUPA (Compound 45) were lyophilized to give 85 mg lyophilized product and characterized by RP-C_8 HPLC, copper assay and spectrophotometry at 280 nm for determination of the DUPA content. The product was found to have a weight percentage in LPEI of 29%w/w and a LPEI to DUPA ratio of 1/1.0.

Step 5: Preparation of LPEI-*l*-[N_3:CliCr®]-PEG_{36}-DUPA (Compound 45) HEPES salt

27.0 mg of LPEI-*l*-[N_3:CliCr®]-PEG_{36}-DUPA (Compound 45) TFA salt (w_{LPEI} = 29%, 7.8 mg in total LPEI) were dissolved in 0.8 mL 20 mM HEPES pH 7.2. The pH was adjusted to pH 7.2. Two centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with

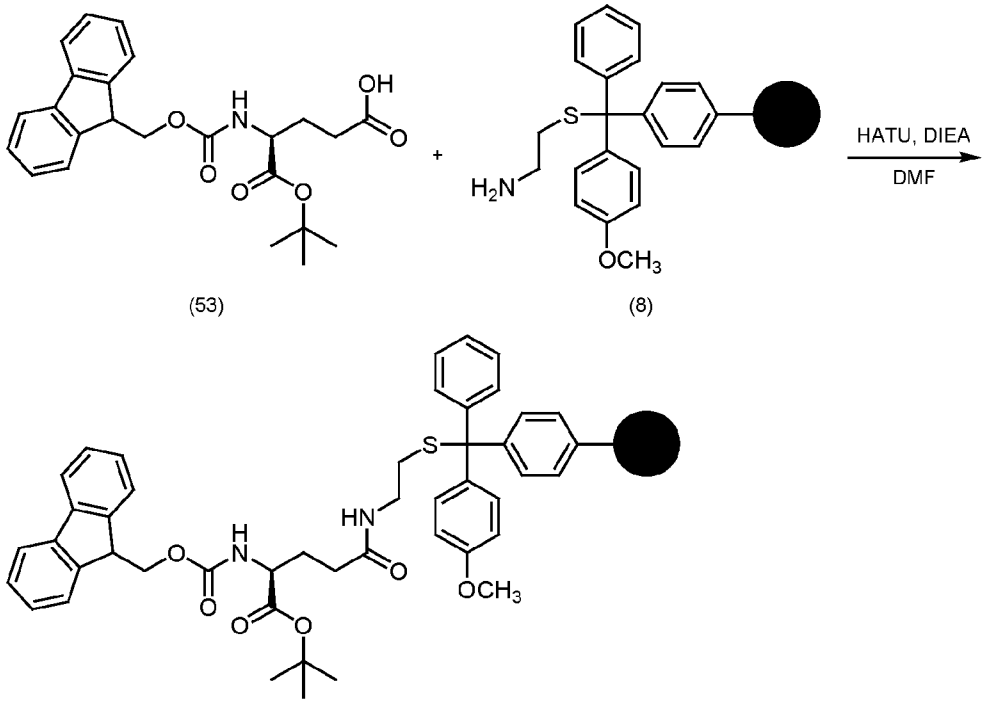
400 µL of LPEI-*l*-[N_3:CliCr®]-PEG_{36}-DUPA solution each. They were centrifuged one time at 14000 g for 30 minutes and then three times after addition of 400 µL 20 mM HEPES, pH 7.2. About 249 µL of the concentrated solution were recovered after buffer exchange and were supplemented with 3.0 mL 20 mM HEPES, pH 7.2. The concentration of the solution was determined by copper assay to be 2.0 mg/mL in total LPEI and an LPEI to DUPA molar ratio of = 1/1.0 was determined.

EXAMPLE 13

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX (COMPOUNDS 49a AND 49b)

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX was synthesized as a mixture of regioisomers 49a and 49b according to the schemes below. Thiol-modified methotrexate MTX-SH (Compound 50) was prepared using solid phase synthesis. Compound 50 was condensed via Michael addition with DBCO-PEG_{36}-MAL (Compound 17), and the resulting DBCO-PEG_{36}-MTX (Compound 51) was reacted with LPEI-N_3 to give Compounds 49a and 49b.

Step 1. Synthesis of Fmoc-Glu-(OtBu)-cysteamine-4-methoxytrityl resin (Compound 52)

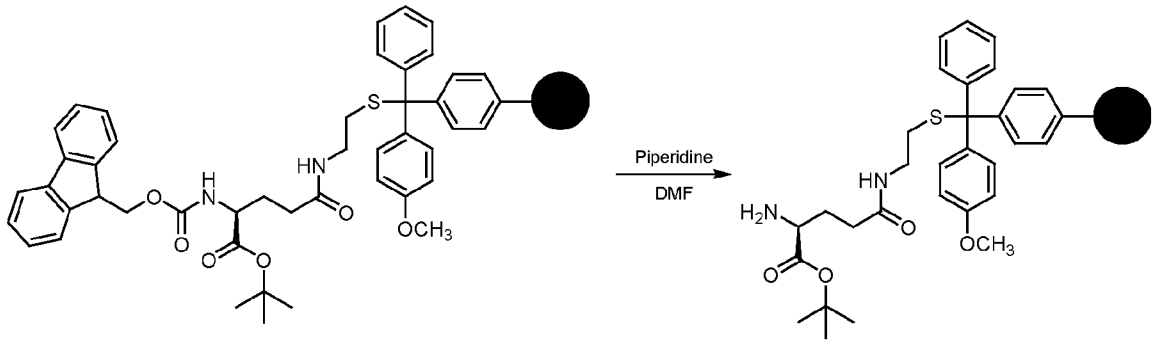


(53) (8) (52)

A solution of Fmoc-Glu-(OtBu) (Compound 53; 242 µmol, 5 eq, 242 mM) in DMF (1

mL) was added to a solution of HATU (246 µmol, 1 eq, 246 mM) in DMF (1 mL) and DIEA (42 µL, 250 µmol, 5 eq). After 3 min the reaction mixture was added to cysteamine 4-methoxytrityl resin (Compound 8; 51.1 mg, 50 µmol, 1.0 eq). The reaction mixture was incubated on a shaker at room temperature. After one hour, the reaction mixture was filtered and the Fmoc-Glu-(OtBu)-cysteamine-4-methoxy trityl resin (Compound 52) was washed with DMF (3 x 10 mL), DCM (3 x 10 mL) and MeOH (3 x 10 mL).

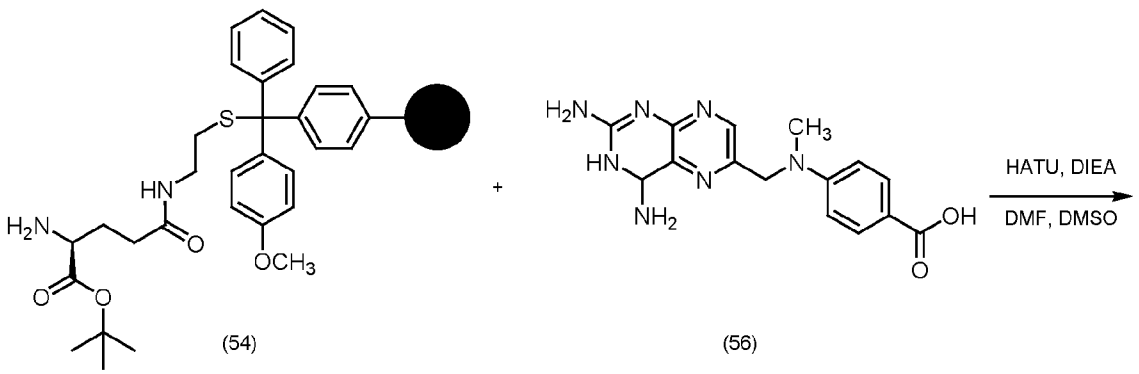
Step 2. Synthesis of Glu-(OtBu)-cysteamine-4-methoxytrityl resin (Compound 54)



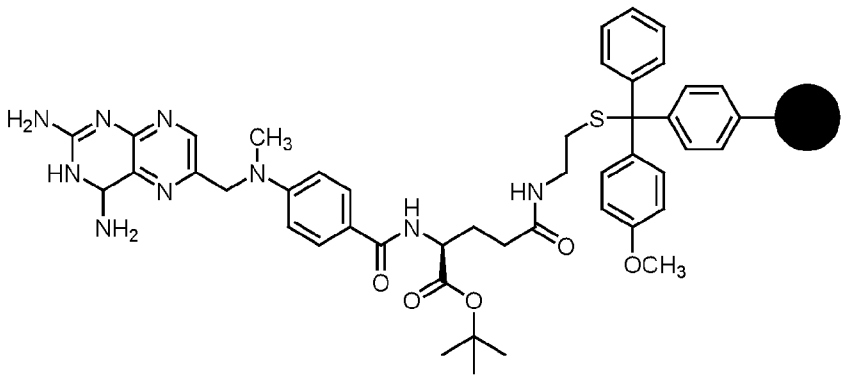
(52) (54)

A solution of 25% piperidine in DMF (5 mL) was added to the Fmoc-Glu-(OtBu)-cysteamine-4-methoxy trityl resin (Compound 52) prepared in Step 1 and the reaction mixture was manually stirred for about 10 minutes. The resin was filtered and washed with DMF (3 x 10 mL), DCM (3 x 10 mL) and MeOH (3 x 10 mL) to give Glu-(OtBu)-cysteamine-4-methoxy trityl resin (Compound 54).

Step 3. Synthesis of MTX-4-methoxy trityl resin (Compound 55)



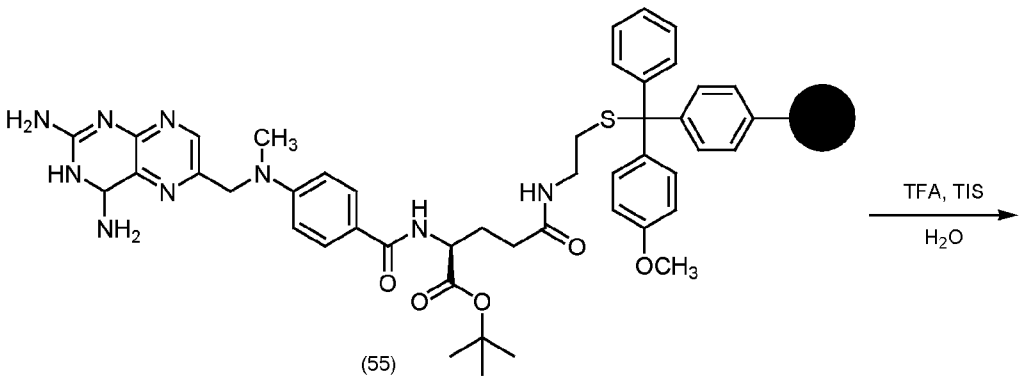
(54) (56)



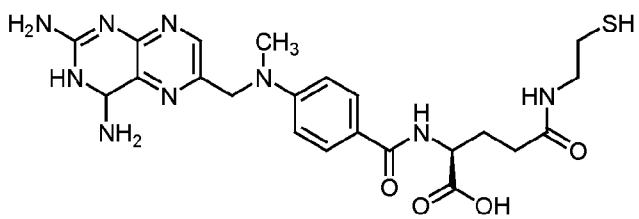
(55)

A solution of N^{10}-Methyl-4-amino-4-deoxypteroic acid (MADOPA; Compound 56; 154 µmol, 3 eq, 17 mM) in DMF/DMSO (2:1) (9 mL) was mixed with a solution of HATU (146 µmol, 3 eq, 146 mM) in DMF (1 mL) and DIEA (25 µL, 147 µmol, 3 eq). The reaction mixture was mixed for 3 minutes and then added to 50 µmol (1 eq) of the Glu-(OtBu)-cysteamine-4-methoxy trityl resin (Compound 54) prepared in Step 2. The reaction mixture was transferred to a glass column with glass frit and was filtered and washed with DMSO (3 x 10 mL), DMF (3 x 10 mL), DCM (3 x 10 mL) and MeOH (3 x 10 mL) to give MTX-4-methoxy trityl resin (Compound 55).

Step 4. Synthesis of MTX-SH (Compound 50)



(55)

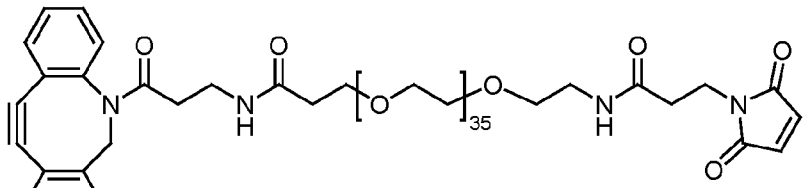


(50)

A solution of TFA/TIS/H_2O (95:2.5:2.5) (4 mL) was added to the MTX-4-methoxy trityl resin (Compound 55) prepared in Step 3. The reaction mixture was incubated for one hour on a shaker at room temperature. The resin was filtered, and the filtrate was recovered and

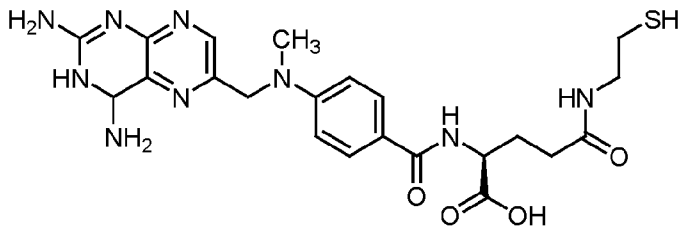
concentrated under nitrogen flow for 15 minutes to evaporate TFA. Cold diethyl ether (10 mL) was added. The resultant precipitate was washed with cold diethyl ether (4.5 mL). A brown-yellowish solid material comprising MTX-SH (Compound 50) was recovered and analyzed by HPLC – ESI^+ single quadrupole mass spectrometry (calculated masses $[M+1]^+$: 514.20 Da, $[M+2]^+$: 257.80 Da; measured masses $[M+1]^+$: 515.0 Da, $[M+2]^+$: 258.00 Da).

Step 5. Synthesis of DBCO-PEG_{36}-MTX (Compound 51)



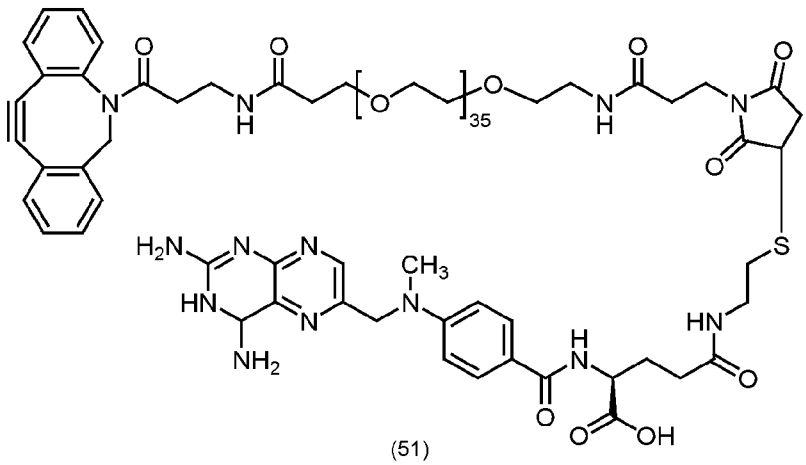
(17)

+



(50)

DMSO, HEPES

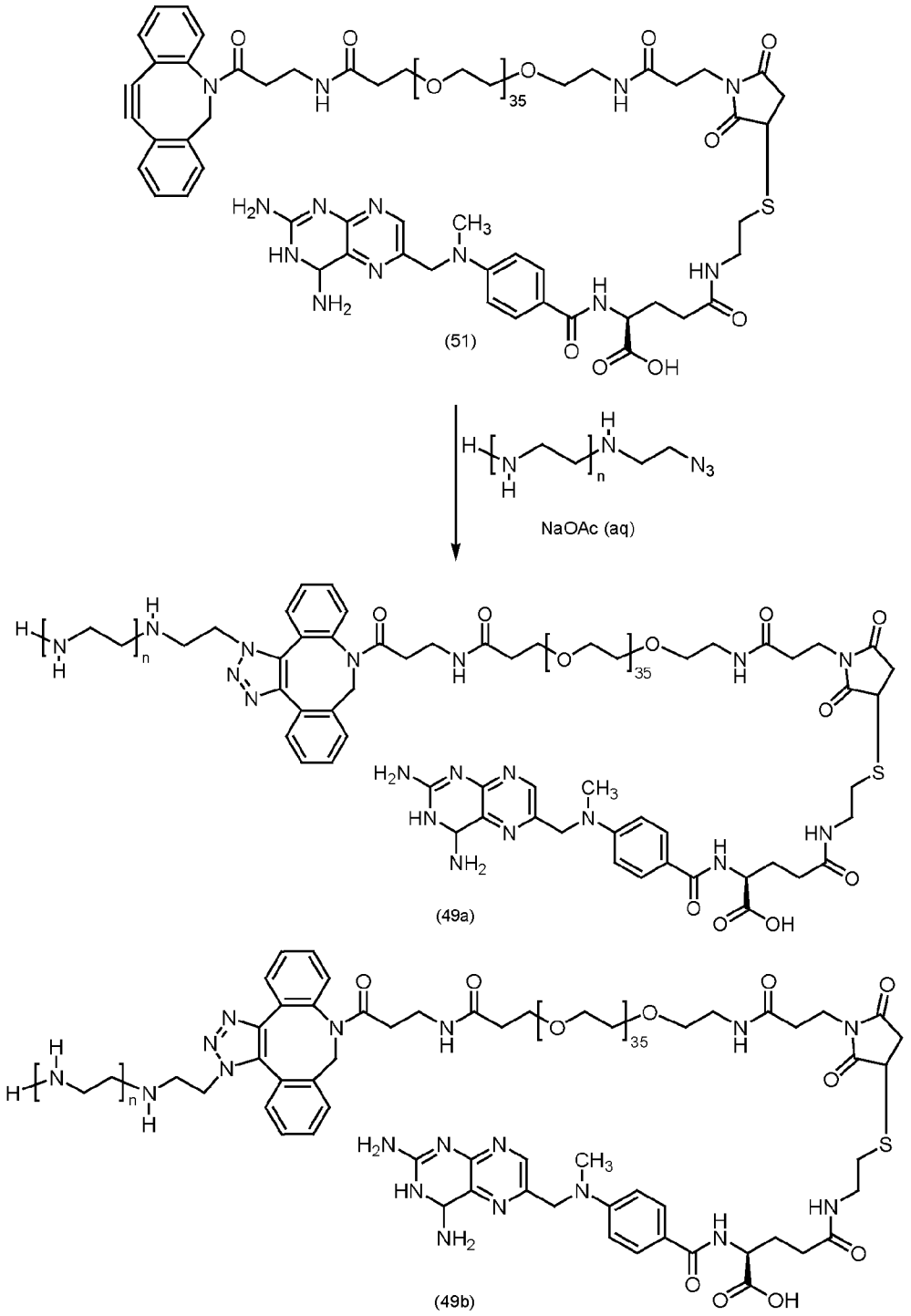


(51)

A solution of MTX-SH (Compound 50; 8 µmol, 0.9 eq, 1.1 mM in thiol) in DMSO/20 mM HEPES pH 7.4 (1:9) (7.0 mL) was mixed with a solution of DBCO-PEG_{36}-MAL (Compound 17; 9 µmol, 1.0 eq, 41 mM) in DMSO (220 µL). The reaction mixture was incubated on a Stuart rotator at room temperature, protected from light and was monitored by

RP-C_8-HPLC. After 1.5 hr acetonitrile (0.8 mL) was added to the reaction mixture. DBCO-PEG_{36}-MTX (Compound 51; 14 mg) was isolated following RP-C_{18} preparative HPLC and lyophilization of pooled fractions and analyzed by HPLC – ESI^+ qTOF mass spectrometry (calculated monoisotopic mass: 2596.32 Da; measured: 2596.35 Da).

Step 6. Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX (Compounds 49a and 49b)



LPEI-N_3 solution (4.2 mL, 5.0 µmol, 0.9 eq, 1.2 mM) in 50 mM acetate buffer pH 4.0

was slowly added to 5.0 mL of a solution of DBCO-PEG_{36}-MTX (Compound 51; 5.4 µmol, 1.0 eq, 1.1 mM) in 50 mM acetate buffer pH 4.0. The reaction mixture was incubated at room temperature on a Stuart rotator, protected from light, and monitored by RP-C_8-HPLC. After twenty hours of reaction, the mixture was supplemented with acetonitrile (1.0 mL) and with TFA (100 µL). LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX was isolated as a mixture of regioisomers 49a and 49b using RP-C_{18} preparative HPLC. Pooled fractions were lyophilized to give 90 mg of a fluffy white-yellow solid which was characterized by RP-C_8-HPLC, copper assay and spectrophotometry at 305 nm for determination of the methotrexate content. The lyophilisate had a weight percentage in LPEI of 34%w/w and a LPEI to methotrexate ratio of 1/1.0.

Step 7. Preparation of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX (Compounds 49a and 49b) HEPES salt

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX (Compounds 49a and 49b) TFA salt (23.8 mg, w_{LPEI} = 34%, 8.1 mg in total LPEI) were dissolved in 0.8 mL 20 mM HEPES pH 7.2. Two centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX solution each, centrifuged one time at 14'000 g for 30 minutes and then three times after addition of 400 µL 20 mM HEPES, pH 7.2. About 250 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX-HEPES salt solution were recovered and supplemented with 2.3 mL 20 mM HEPES, pH 7.2. The concentration of the solution was determined by copper assay to be 2.6 mg/mL in total LPEI.

EXAMPLE 14

POLYPLEX FORMATION, POLYPLEX SIZING AND ZETA POTENTIAL MEASUREMENTS

General Procedure for Polyplex Formation with poly(IC).

For the preparation of preferred polyplexes, the respective triconjugates were complexed with poly(IC) at N/P ratio of 4 in HBG buffer (20 mM HEPES, pH 7.2, 5% glucose, wt/vol). Nitrogen to phosphorus (N/P) ratio was calculated based on the nitrogen content in the LPEI portion of the used triconjugates and the phosphorous content in poly(IC). Hereby, stock solutions of triconjugates such as LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA and poly(IC) were diluted with HBG to the appropriate concentrations for N/P ratio of 4 prior to mixing. The diluted triconjugate solution was added to an equal volume of nucleic acid solution to a final concentration of 0.1875 mg/mL of nucleic acid in the polyplex preparation and mixed

vigorously. The mixture was left and incubated at RT for 30 min for polyplex formation prior to use. In an analogous manner, polyplexes with polyanions such as poly(Glu) were prepared. The polyplexes were typically further characterized with respect to particle size distribution and ζ-potential.

FIG 1 is a DLS back scatter plot taken in triplicate of a Me-LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(IC) polyplex measuring size distribution and ζ-potential in 20 mM HEPES, 5% glucose at pH 7.2, 0.1875 mg/mL, 1.0 mL volume, N/P ratio of 4. The z-average diameter was 130 nm with a polydispersity index (PDI) of 0.134. The ζ-potential was 26.6 mV.

FIG 2 is a DLS back scatter plot taken in triplicate of a Me-LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) polyplex measuring size distribution and ζ-potential in 20 mM HEPES, 5% glucose at pH 7.2, 0.1875 mg/mL, 1.0 mL volume, N/P ratio of 4. The z-average diameter was 140 nm with a polydispersity index (PDI) of 0.132. The ζ-potential was 28.2 mV.

Physico-chemical characterization by DLS of additional polyplexes comprising poly(IC) and triconjugates prepared in the Examples above is shown in Table 7.

Table 7. Physicochemical Characterization data for Triconjugate LPEI-*l*-PEG-DUPA:poly(IC) polyplex at 0.1875 mg/mL, in HBG, pH 7.2, N/P ratio of 4.

Triconjugate	ZEN2112 (Quartz)		DTS1070 DLS*		
	DLS (nm)	**PDI**	**DLS (nm)**	**PDI**	**ζ-pot (mV)**
LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA (Compounds 1a and 1b)	-	-	120	0.125	31.1
LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 12a and 12b)	140	0.120	123	0.138	35.2
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA (Compounds 19a and 19b)	154	0.151	148	0.153	34.5
LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA (Compound 24)	145	0.166	144	0.214	33.9
LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA (Compounds 28a and 28b)	143	0.156	132	0.153	34.0

LPEI-*l*-[N_3:DBCO]CONH-PEG_{36}-[MAL-S]-DUPA (Compounds 32a and 32b)	140	0.120	133	0.147	34.5
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA (Compounds 37a and 37b)	nd	nd	149	0.153	24.7
Me-LPEI-l-[N_3:BCN]-PEG_{36}-DUPA (Compound 43)	nd	nd	130	0.134	26.6
Me-LPEI-l-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 44a and 44b	nd	nd	140	0.132	28.2
LPEI-l-[N_3:CliCr®]-PEG_{36}-DUPA (Compound 45)	nd	nd	141	0.151	26.2

*for DLS and ζ-potential measured in DTS1070 cuvette samples were 2x diluted due to insufficient amount of the sample.

In all tested samples comprising poly(IC) mean Z-average diameter in the range between 120 nm and 154 nm was observed and particles were found to be monodisperse (PDI <0.3).

In all samples positive mean ζ-potential in the range of 24 mV and 35 mV was observed.

General Procedure for Polyplex Formation with mRNA.

For the preparation of preferred polyplexes, the respective triconjugates were complexed with selected mRNAs at various N/P ratios in 5% glucose (wt/vol) or HBS (HEPES-buffered saline pH 7.2). Nitrogen to phosphorus (N/P) ratios were calculated based on the nitrogen content in the LPEI portion of the used triconjugates and the phosphorous content in the mRNA. The concentrations of the triconjugate such as LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (expressed as total LPEI in mg/mL) at each mRNA concentration and N/P ratio are summarized in the table below:

mRNA concentration (mg/mL)	N/P ratio					
	3	4	5	6	8	12
0.10	0.039	0.052	0.065	0.078	0.104	0.156
0.02	0.0078	0.0104	0.013	0.0156	0.0208	0.0312

Hereby, stock solutions of triconjugates such as LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA and mRNA were diluted with 5% glucose or HBS to the appropriate concentrations for the selected N/P ratio prior to mixing. The diluted triconjugate solution was added to an equal volume of

nucleic acid solution to a final concentration of 0.1-0.02 mg/mL of nucleic acid in the polyplex preparation and mixed vigorously. The mixture was incubated at RT for 30 min for polyplex formation prior to use. In an analogous manner, polyplexes with polyanions such as poly(Glu) were prepared. The polyplexes were typically further characterized with respect to particle size distribution and ζ-potential.

Physico-chemical characterization by Dynamic Light Scattering (DLS) of polyplexes comprising various mRNA and the triconjugate LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 12a and 12b) are shown in Tables 8 and 9.

Table 8. Particle size distribution and polydispersity data by DLS for polyplexes comprising various mRNA and the triconjugate LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 12a and 12b) at 0.1 mg/mL in 5% glucose and the indicated N/P ratios.

	DLS: Z-Average (d, nm)			DLS: PDI (Polydispersity Index)		
	N/P ratio			N/P ratio		
	4	6	12	4	6	12
mRNA	Mean	Mean	Mean	Mean	Mean	Mean
IL-2 (725 NT)*	152.4	127.1	140.9	0.042	0.156	0.204
Luc (1929 NT)*	132.4	101.6	114.5	0.120	0.147	0.150
IFN-β (1047 NT)	103.4	98.67	123.4	0.173	0.214	0.236
Diphtheria toxin (1068 NT)	103.4	106.6	118.7	0.197	0.194	0.228

For all measurements, the viscosity value of 1.078 mPa.s was used except for the measurements marked with an asterisk (*), for which the viscosity value of 0.98 mPa.s was used.

Table 9. ζ-potentials of polyplexes polyplexes comprising various mRNA and the triconjugate LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 12a and 12b) at 0.1 mg/mL in 5% glucose and the indicated N/P ratios.

	Zeta potential (mV)		
	N/P ratio		
	4	6	12
mRNA	Mean	Mean	Mean
IL-2 (725 NT)*	19.3	31.4	43.5
Luc (1929 NT)*	27.9	44.7	43.0

IFN-β (1047 NT)	45.44	47.09	46.8
Diphtheria toxin (1068 NT)	44.47	46.99	48.86

For all measurements, the viscosity value of 1.078 mPa.s was used except for the measurements marked with an asterisk (*), for which the viscosity value of 0.98 mPa.s was used.

Physico-chemical characterization by Dynamic Light Scattering (DLS) of polyplexes comprising various mRNA and the triconjugate LPEI-l-[N_3:DBCO]-PEG_{24}-Folate (Compounds 6a and 6b) are shown in Tables 10 and 11.

Table 10. Particle size distribution and polydispersity data by DLS for polyplexes comprising the indicated mRNA and the triconjugate LPEI-*l*-[N3:DBCO]-PEG24-Folate at the indicated N/P ratios.

	DLS: Z-Average (d, nm)			DLS: PDI (Polydispersity Index)		
	N/P ratio			N/P ratio		
	5	8	12	5	8	12
mRNA	Mean	Mean	Mean	Mean	Mean	Mean
Renilla Luc (1212 NT)	106.6	96.7	75.1	0.096	0.155	0.208
Epo (858 NT)	91.4	80.4	nd	0.132	0.135	nd

nd=not determined

Table 11. ζ-potentials of polyplexes comprising various mRNA and the triconjugate LPEI-l-[N3:DBCO]-PEG24-Folate at the indicated N/P ratios.

	Zeta potential (mV)		
	N/P ratio		
	5	8	12
mRNA	Mean	Mean	Mean
Renilla Luc (1212 NT)	25.9	43.0	41.2
Epo (858 NT)	32.6	36.9	nd

nd=not determined

In all tested samples mean Z-average diameter in the range between 75 nm and 153 nm was observed and particles were found to be monodispersed (PDI <0.3).

In all samples positive mean ζ-potential in the range of 19 mV and 49 mV was observed.

EXAMPLE 15

CELL SURFACE EXPRESSION OF PSMA ON PROSTATE CANCER CELL LINES

PSMA expression on the cell surface of different prostate cancer cells (LNCaP, VCaP, PC-3, DU145) was examined by flow cytometry analysis.

Prostate cancer cells (150,000 cells) were stained with PE anti-human PSMA (FOLH1) Antibody (Biolegend Cat. No. 342503) for 1 hour and cell surface expression was measured on live cells using the flow cytometer CytoFLEX S (Beckman Coulter). Zombie NIR (BioLegend Cat. No. 423106)) was used to discriminate live/dead cells. Data were analysed with the FlowJo software (v10.8.1).

FIG. 3 demonstrates PSMA cell surface expression on prostate cancer cell lines based on the mean fluorescence intensity (MFI). LNCaP cells show the highest MFI, indicating highest PSMA expression ($PSMA^{high}$), VCaP showed lower MFI and are therefore considered to express medium levels of PSMA ($PSMA^{medium}$). PC-3 and DU145 showed an even lower MFI and are therefore considered as low PSMA expressing cell ($PSMA^{low}$). The following was considered with respect to PSMA expression on the above indicated cells LNCaP >VCaP>PC3>DU145. The expression levels have been previously described (Ghosh A et al., Cancer Res. 2005, 65(3):727-731; Bakht MK, et al., PNAS USA 2022, 119(4):e2025710119; Staniszewska M, et al., Int J Mol Sci 2021,22(14):7431)

EXAMPLE 16

SELECTIVE DELIVERY OF THE INVENTIVE POLYPLEXES INCREASES MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I (MHC I) CELL SURFACE EXPRESSION ON PSMA-OVEREXPRESSING PROSTATE CANCER CELLS

Major Histocompatibility Complex class I (MHC-I) molecules have a critical function in reporting intracellular changes, such as those caused by viral infections or malignant transformation, to the immune system by presenting endogenous antigens. This facilitates the initiation of a CD8+ T-cell response, which is essential for effective immune surveillance (Cornel AM et al., Cancers, 2020, 12(7):1760). CD8+ T cells are exerting their cytotoxic function following their recognition of peptide bound MHC-I complexes and co-stimulatory signals. Downregulation of Major Histocompatibility Complex I (MHC-I) is one of the mechanisms by which tumor cells avoid immunosurveillance. Therefore, increasing MHC-I expression on the surface of cancer cells can restore anti-tumor immunity (Taylor BC et al.,

Front Immunol 2022, 13:844866). The effect of PSMA targeted poly(IC) delivery on MHC I expression on the cell surface of prostate cancer cells with high PSMA (LNCaP) and with low PSMA expression (DU145) was examined by flow cytometry analysis.

LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes were formulated in 20 mM HEPES with 5% glucose, pH 7.2 at a N/P ratio of 4. Cancer cells (140,000 cells/well in a 12-well plate) were treated for 24 hours with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes at various concentrations of the payload (0.0125 and 0.125 µg/ml). Cells were stained with PE Mouse Anti-Human HLA-ABC Antibody (BD Pharmingen, Cat. No. 555553) for 1 hour and cell surface expression was measured on live cells using the flow cytometer CytoFLEX S (Beckman Coulter). Zombie NIR (BioLegend, Cat. No. 423106) was used to discriminate live/dead cells and data were analysed with the FlowJo software (v10.8.1). Selective delivery of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) at both concentrations used increased the cell surface expression of MHC I on prostate cancer cells with high PSMA expression (LNCaP) as indicated by the increase in MFI compared to the untreated control (FIG 4A). In contrast, delivery of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) did not induce a profound effect on MHC I cell surface expression on cells with low PSMA expression (DU145) (FIG 4B). LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) control polyplexes did not increase MHC I cell surface expression on both cell lines tested (FIG 4A and FIG 4B).

EXAMPLE 17

SELECTIVE DELIVERY OF INVENTIVE POLYPLEXES DECREASES SURVIVAL OF PSMA-OVEREXPRESSING CELLS

Selective delivery of inventive polyplexes decreases survival of prostate cancer cells with differential expression of PSMA. The following polyplexes were formulated in 20 mM HEPES with 5% glucose, pH 7.2 at a N/P ratio of 4:

LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC);

LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(Glu);

LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC)

LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC);

LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu);

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA:poly(IC);

LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA:poly(IC);

LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA:poly(Glu);

LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S-]-DUPA:poly(IC);

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[CONH]-DUPA:poly(IC); and

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA:poly(IC)

Me-LPEI[N_3:DBCO]PEG_{36}-[MAL-S]-DUPA/poly(IC);

Me-LPEI[N_3:DBCO]PEG_{36}-[MAL-S]-DUPA/poly(Glu);

Me-LPEI[N3:BCN]PEG_{36}-[MAL-S]-DUPA/poly(IC); and

Me-LPEI[N3:BCN]PEG_{36}-[MAL-S]-DUPA/poly(Glu).

Cancer cell lines (3000 cells/well) with differential expression of the PSMA receptor (PC-3: low PSMA expression ($PSMA^{low}$); DU145 low PSMA expression; and LNCaP: high PSMA expression ($PSMA^{high}$)) were treated with the listed polyplexes for 72 hours.

Cell survival was analyzed using Cell Titer-Glo (Promega). The concentrations shown as Log(polyplex) reflect the concentrations of poly(Glu) or poly(IC) in the respective polyplexes. The IC_{50} values were calculated in PrismGraphPad using the Sigmoidal, 4PL, X is log(concentration) algorithm.

The results are provided in Tables 12 and 13 and in Figures 5-13.

Table 12 shows the cell survival measured in PC-3 and DU145 cells (low PSMA), as well as in LNCaP (high PSMA) cells as a function of treatment with linear LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) or linear LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) polyplexes as reported above. Moreover, the cell survival data measured in an analogous manner of the prior art branched, random LPEI-*r*-PEG_{2KDa}-DUPA:poly(IC) is provided. The data shows that the linear polyplexes in accordance with the present invention are not only more potent than the prior art random, branched polpylexes, but further show a higher selectivity for the PSMA overexpressing cell line.

Table 12: PSMA expressing cell survival data following treatment with linear and random, branched polyplexes.

polyplex	IC_{50} (µg/mL)		
	PC-3 cells (low PSMA)	DU145 cells (low PSMA)	LNCaP cells (high PSMA)
LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC)	0.24	>0.625	0.020
LPEI-*l*-[N_3:DBCO]-PEG_{36}-	0.22	>0.625	0.020

DUPA:poly(IC)			
LPEI-*r*-PEG_{2KDa}-DUPA:poly(IC)*	>0.625	nd	~ 0.14

*randomly (*r*)substituted analog: data extrapolated from Figure 2A of Langut et al, PNAS (2017) 114(52):13655–13660; nd= not determined

FIG 5A is a plot of cell survival in LNCaP cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(Glu). LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(Glu) was inactive (i.e., no significant cell death was observed at concentrations as high as 0.625 µg/mL), whereas LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) induced a robust decrease in LNCaP cell survival with an IC_{50} of 0.02 µg/mL.

FIG 5B is a plot of cell survival in PC-3 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(Glu). LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(Glu) was inactive (i.e., no significant cell death was observed at concentrations as high as 0.625 µg/mL). LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) inhibited PC-3 cell survival with an IC_{50} value of 0.24 µg/mL.

FIG 5C is a plot of cell survival in DU145 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(Glu). LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(Glu) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) were inactive (i.e., no significant cell death was observed for either polyplex at concentrations as high as 0.625 µg/mL).

PSMA, also known as folate hydrolase 1 (FOLH1), has a role in folate metabolism and internalization as a folate hydrolase (Yao et al., Prostate 2006, 66:867-875; Yao et al., Prostate 2010, 70:305-316) and folate has been validated as a PSMA ligand in PSMA-expressing cells (Patil Y et al., Nanomedicine 2018, 14(4):1407-1416; Flores O et al., Theranostics 2017, 7(9):2477-2494). The folate-conjugated polyplexes were used to test their selective delivery to PSMA-expressing prostate cancer cells and their selective cell death induction.

FIG 5D is a plot of cell survival in LNCaP prostate cancer cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(Glu) polyplexes. LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC) decreased the survival of PSMA high expressing LNCaP prostate cancer cells with an IC_{50} of 0.13 µg/mL. In contrast, delivery of LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(Glu) polyplexes did not have a significant effect on cell survival in LNCaP cells

FIG 5E is a plot of cell survival in DU145 prostate cancer cells with low PSMA cell surface expression as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(Glu) polyplexes. LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(Glu) polyplexes had little to no activity in PSMA low expressing DU145 cancer cells at concentrations as high as 0.625 μg/mL.

FIG 6A is a plot of cell survival in LNCaP cells as a function of treatment with LPEI-*l*-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-PEG_{36}-DUPA:poly(Glu). LPEI-*l*-PEG_{36}-DUPA:poly(Glu) was inactive (i.e., no significant cell death was observed for either polyplex at concentrations as high as 0.625 μg/mL), whereas LPEI-*l*-PEG_{36}-DUPA:poly(IC) induced a robust decrease in LNCaP cell survival with an IC_{50} of 0.02 μg/mL.

FIG 6B is a plot of cell survival in PC-3 cells as a function of treatment with LPEI-*l*-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-PEG_{36}-DUPA:poly(Glu). LPEI-*l*-PEG_{36}-DUPA:poly(Glu) was inactive (i.e., no significant cell death was observed at concentrations as high as 0.625 μg/mL), whereas LPEI-*l*-PEG_{36}-DUPA:poly(IC) inhibited PC-3 cell survival with an IC_{50} value of 0.22 μg/mL.

FIG 6C is a plot of cell survival in DU145 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu). LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) were inactive (i.e., no significant cell death was observed at concentrations as high as 0.625 μg/mL).

FIG 7 is a plot of cell survival in LNCaP cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC), LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu), Me-LPEI-*l*-[N3:DBCO]PEG_{36}-[MAL-S]-DUPA:poly(IC), and Me-LPEI-*l*-[N3:DBCO]PEG_{36}-[MAL-S]-DUPA:poly(Glu) demonstrating that selective delivery of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and Me-LPEI-*l*-[N3:DBCO]PEG_{36}-[MAL-S]-DUPA/poly(IC) similarly decreased the survival of PSMA high expressing LNCaP prostate cancer cells with an IC_{50} of 0.020 and 0.014 μg/mL, respectively. In contrast, delivery of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) and LPEI-*l*-[N3:DBCO]PEG_{36}-[MAL-S]-DUPA/poly(Glu) polyplexes did not have a significant effect on cell survival in LNCaP cells.

FIG 8 is a plot of cell survival in LNCaP cells as a function of treatment with LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA:poly(IC), LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA:poly(Glu), Me-LPEI-*l*-[N3:BCN]PEG_{36}-[MAL-S]-DUPA:poly(IC), and Me-LPEI-*l*-[N3:BCN]PEG_{36}-[MAL-S]-DUPA:poly(Glu) demonstrating that selective delivery of LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA:poly(IC) and Me-LPEI-*l*-[N3:BCN]PEG_{36}-[MAL-S]-

DUPA/poly(IC) similarly decreased the survival of high PSMA expressing LNCaP prostate cancer cells cancer cells with an IC_{50} of 0.013 and 0.016 μg/mL, respectively. In contrast, delivery of LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA:poly(Glu) and Me-LPEI-*l*-[N3:BCN]PEG_{36}-[MAL-S]-DUPA/poly(Glu) polyplexes did not have a significant effect on cell survival in LNCaP cells at concentrations as high as 0.625 μg/mL.

FIG 9 is a plot of cell survival in DU145 prostate cancer cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC), LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu), Me-LPEI[N3:DBCO]PEG_{36}-[MAL-S]-DUPA:poly(IC), and Me-LPEI[N3:DBCO]PEG_{36}-[MAL-S]-DUPA:poly(Glu) demonstrating that LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) or poly(Glu) and Me-LPEI[N3:DBCO]PEG_{36}-[MAL-S]-DUPA:poly(IC) or poly(Glu) were inactive in PSMA low expressing DU145 cancer cells. No significant cell death was detected for either of the polyplexes at concentrations as high as 0.625 μg/mL.

FIG 10 is a plot of cell survival in DU145 prostate cancer cells with low PSMA expression as a function of treatment with LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(IC), LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(Glu), Me-LPEI[N3:BCN]PEG_{36}-[MAL-S]-DUPA:poly(IC), and Me-LPEI-*l*-[N3:BCN]PEG_{36}-[MAL-S]-DUPA:poly(Glu) showing that LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA:poly(IC) or poly(Glu) and Me-LPEI[N3:BCN]PEG_{36}-[MAL-S]-DUPA:poly(IC) or poly(Glu) were inactive in PSMA low expressing DU145 cancer cells. No significant cell death was detected for either of the polyplexes at concentrations as high as 0.625 μg/mL.

FIG 11 is a plot of cell survival in LNCaP cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA:poly(IC); LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(IC); LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-[CONH]-DUPA:poly(IC); and LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA:poly(IC) polyplexes. As shown, the inventive polyplexes demonstrated and induced significant potency in LNCaP cells.

FIG 12 is a plot of cell survival in VCaP prostate cancer cells with intermediate PSMA cell surface expression as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes. The X axis indicates the concentration of poly(IC) or poly(Glu) delivered. LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) decreased the survival of prostate cancer cells with intermediate expression of PSMA, VCaP. In contrast, delivery of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) did not

have a significant effect on cell survival of VCaP cells

FIG 13 is a plot of cell survival in DU145 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA:poly(IC); LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(IC); LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-[CONH]-DUPA:poly(IC); and LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA:poly(IC) polyplexes. No significant cell death was detected for either of the polyplexes accross the tested concentrations in DU145 cells.

Table 13 provides the cell survival measured in DU145 cells (low PSMA) as well as in LNCaP (high PSMA) cells as a function of treatment with linear LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA:poly(IC); LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(IC); LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-[CONH]-DUPA:poly(IC); LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA:poly(IC); Me-LPEI-l-[N_3:BCN]-PEG_{36}-DUPA; and Me-LPEI[N_3:DBCO]PEG_{36}-[MAL-S]-DUPA/poly(IC) polyplexes as reported above.

All of the inventive linear conjugate:poly(IC) polyplexes tested induced a similar selective and significant decrease in the survival of PSMA overexpressing cells, while a much weaker effect on cell survival was observed on PSMA low-expressing cells. The IC_{50}s for DU145 cells were above the highest concentration tested of 0.625 µg/mL.

Table 13: PSMA expressing cell survival data of linear polyplexes

Polyplex	IC_{50} (µg/mL)	
	DU145 cells (low PSMA)	LNCaP cells (high PSMA)
LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC)	>0.625	0.13
LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) (based on compounds 12a and 12b)	>0.625	0.020
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA:poly(IC) (based on compounds 19a and 19b)	>0.625	0.014
LPEI-*l*-[N_3:BCN]-PEG_{36}-[MAL-S]-DUPA:poly(IC) (based on Compound 24)	>0.625	0.013

LPEI-*l*-[N_3:SCO]-PEG_{36}-[MAL-S]-DUPA:poly(IC) (based on Compounds 28a and 28b)	>0.625	0.012
LPEI-*l*-[N_3:DBCO]CONH -PEG_{36}-[MAL-S-DUPA:poly(IC) (based on Compounds 32a and 32b	>0.625	0.016
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA:poly(IC) (based on Compounds 37a and 37b)	>0.625	0.019
Me-LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA (based on Compound 43)	>0.625	0.016
Me-LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA/poly(IC) (based on Compounds 44a and 44b)	>0.625	0.014

EXAMPLE 18

CYTOKINE SECRETION IN PSMA-EXPRESSING CANCER CELL LINES

LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) polyplexes were formulated in 20 mM HEPES with 5% glucose, pH 7.2 at a N/P ratio of 4. Cancer cells (40,000 cells/well in a 96-well plate) with differential expression of PSMA (LNCaP: high PSMA expression; PC-3 and DU145: low PSMA expression) were treated for 6 or 24 hours with LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) polyplexes at various concentrations (0.0625, 0.625 µg/ml). The medium from treated cells was collected and analyzed after 6 hours of transfection for Human IP-10 (CXCL10) and interferon beta (IFN-β) and after 24 hours of transfection for RANTES (CCL5) utilizing ELISA assay (PeproTech (IP-10 and RANTES), InvivoGen (IFN-β)) and detected using a microplate reader Synergy H1 (BioTek).

Treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) polyplexes at the indicated concentrations selectively induces IP-10, RANTES, and IFNβ cytokine release from PSMA overexpressing cells (LNCaP) as compared to low PSMA expressing cells (PC-3 and DU145). The results are shown in FIGs 14A-16C.

FIG 14A is a plot of IP-10 secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) concentration from LNCaP cells and PC-3 cells. In LNCaP cells, LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) induced IP-10 secretion of 382 pg/mL and 1245.67 pg/mL at 0.0625 µg/mL and 0.625 µg/mL, respectively. In PC-3 cells, LPEI-*l*-[N_3:DBCO]-PEG_{24}-

DUPA:poly(IC) induced IP-10 secretion of 11.33 pg/mL and 37.67 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively.

FIG 14B is a plot of IP-10 secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration from LNCaP cells and PC-3 cells. In LNCaP cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced IP-10 secretion of 582.87 pg/mL and 1524.97 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively. In PC-3 cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced IP-10 secretion of 0 pg/mL and 0 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively.

FIG 14C is a plot of IP-10 secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration from LNCaP cells and DU145 cells. In LNCaP cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced IP-10 secretion of 582.87 pg/mL and 1524.97 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively. In DU145 cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced IP-10 secretion of 0 pg/mL and 0 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively.

For FIG14B and 14C, treatment with polyplexes was compared in parallel in LNCaP, PC3 and DU145 in the same experiment. The figures have been separated for ease of viewing and the values for IP-10 secretion in LNCaP cells is the same in both figures.

FIG 15A is a plot of RANTES secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) concentration from LNCaP cells and PC-3 cells. In LNCaP cells, LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) induced RANTES secretion of 514.33 pg/mL and 1368.33 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively. In PC-3 cells, LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) induced RANTES secretion of 0 pg/mL and 24 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively.

FIG 15B is a plot of RANTES secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration from LNCaP cells and PC-3 cells. In LNCaP cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced RANTES secretion of 209.67 pg/mL and 1057 pg/mL at 0 μg/mL and 0.625 μg/mL, respectively. In PC-3 cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced RANTES secretion of 214.33 pg/mL and 210.33 pg/mL at 0 μg/mL and 0.625 μg/mL, respectively.

FIG 15C is a plot of RANTES secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration from LNCaP cells and DU145 cells. In LNCaP cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced RANTES secretion of 209.67 pg/mL and 1057 pg/mL at 0 μg/mL and 0.625 μg/mL, respectively. In DU145 cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-

DUPA:poly(IC) induced RANTES secretion of 207.67 pg/mL and 167.67 pg/mL at 0 μg/mL and 0.625 μg/mL, respectively.

For FIG 15B and 15C, treatment with polyplexes was compared in parallel in LNCaP, PC3 and DU145 in the same experiment. The figures have been separated for the ease of the viewing and the values for RANTES secretion in LNCaP cells is the same.

FIG 16A is a plot of IFN-β secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) concentration in LNCaP cells and PC-3 cells. In LNCaP cells, LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) induced IFN-β secretion of 181.5 pg/mL and 312.3 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively. In PC-3 cells, LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) induced IFN-ß secretion of 0 pg/mL and 40.47 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively.

FIG 16B is a plot of IFN-ß secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration from LNCaP cells and PC-3 cells. In LNCaP cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced IFN-β secretion of 216.27 pg/mL and 606.6 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively. In PC-3 cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced IFN-β secretion of 44.17 pg/mL and 86.57 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively.

FIG 16C is a plot of IFN-β secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration from LNCaP cells and DU145 cells. In LNCaP cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced IFN-β secretion of 216.27 pg/mL and 606.6 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively. In DU145 cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced IFN-β secretion of 4.37 pg/mL and 5 pg/mL at 0.0625 μg/mL and 0.625 μg/mL, respectively.

For 16B and 16C, treatment with polyplexes was compared in parallel in LNCaP, PC3 and DU145 in the same experiment. The figures have been separated for the ease of the viewing and the values for IFN-ß secretion from LNCaP cells is the same.

Treatment with the inventive polyplexes, LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) at two concentrations, 0.0625 μg/mL and 0.625 μg/mL, selectively induces A) IP-10 B) RANTES and C) IFNβ cytokine release, in PSMA overexpressing cells (LNCaP) as compared to low PSMA expressing cells, PC-3 or DU145.

EXAMPLE 19

INDUCTION OF CELL DEATH AND INTERFERON STIMULATED GENE

PATHWAYS IN PSMA-EXPRESSING CANCER CELL LINES

LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes were formulated in 20 mM HEPES with 5% glucose, pH 7.2 at a N/P ratio of 4.

Cancer cells (400,000 cells/well in a 6-well plate) with differential expression of PSMA (LNCaP: high PSMA expression; DU145: low PSMA expression) were treated for 6 hours with LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) or with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes. LNCaP were treated with 0.00625 or 0.0625 µg/mL LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) or with 0.0625 µg/mL LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu). DU145 cells were treated with 0.0625 µg/mL LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) or with 0.0625 µg/mL LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu). Cells were then lysed and protein lysates were loaded on SDS-PAGE (10 µg total protein lysates/lane) followed by Western blotting analysis for the indicated proteins (Cell Signaling; Caspase 3 (9665), Cleaved Caspase 3 (9664), PARP (9542), Cleaved PARP (5625), RIG-1 (3743); MDA5 (Abcam ab126630) and ISG15 (Santa Cruz SC-166755)). GAPDH (Cell Signaling 2118) and beta-Actin (Sigma A5441) were used as protein loading controls.

FIG 17 is a Western Blot imaging analysis showing qualitative levels of Caspase 3, cleaved Caspase 3, PARP, cleaved PARP, RIG-1; MDA5, and ISG15 as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes at 0, 0.0625 and 0.625 µg/mL.

Treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced a selective increase in the expression of proteins that are associated with the interferon-stimulated gene response, e.g., MDA5, RIG-1 and ISG15 and induced apoptotic markers, e.g., cleavage of PARP and Caspase 3, in PSMA overexpressing cells (LNCaP) while no effect was observed in PSMA low expressing cells (DU145). LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) control polyplexes did not induce these signals.

EXAMPLE 20

TARGETED DELIVERY OF POLY(IC) TO HIGH PSMA EXPRESSING CANCER CELL LINES SELECTIVELY INDUCES SELECTIVE PRR DOWN-STREAM SIGNALING

LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes were formulated in 20 mM HEPES with 5% glucose, pH 7.2 at a

N/P ratio of 4.

Cancer cells (400,000 cells/well in a 6-well plate) with differential expression of PSMA (LNCaP: high PSMA expression; DU145: low PSMA expression) were treated with 0.02 or 0.2 μg/mL with LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) and 0.2 μg/mL LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes for 5 and 24 hours. Cells were then lysed and protein lysates were loaded on SDS-PAGE (10 μg total protein lysates/lane) followed by Western blot analysis for the indicated proteins IκBα (Cell Signaling; 4812), Phospho IκBα (Ser32, Cell Signaling; 2859), IRF3 (Cell Signaling; D6I4C; 11904), Phospho IRF3 (Ser396; Cell Signaling; 4D4G; 4947), NFκB p65 (Cell Signaling; L8F6; 6956), Phospho NFκB p65 (Ser536; Cell Signaling; 93H1; 3033), STAT1 (Cell Signaling; 9H2; 9176), Phospho STAT1 (Tyr701; Cell Signaling; 9176), PD-L1 (Cell Signaling; E1L3N; 13684). GAPDH (Cell Signaling: 2118) was used as protein loading controls for the respective analysed protein panels (panel 1: IκBα, Phospho IκBα, IRF3, Phospho IRF3, NFκB p65, Phospho NFκB p65; panel 2: PD-L1).

FIG 18 is an immune blot imaging analysis showing protein levels IκBα and Phospho IκBα, IRF3 and Phospho IRF3, NFκB p65 and Phospho NFκB p65 (upper panel) and PD-L1 (lower panel) as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes at 0, 0.02 and 0.2 μg/mL of the payload.

Treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) for 5 and 24 hours induced a selective increase in protein expression of PD-L1 and in phosphorylation of IκB, IRF3, and NFκB p65 in PSMA high expressing cells (LNCaP). No effect was observed in PSMA low expressing cells (DU145). LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) control polyplexes did not induce these signals.

EXAMPLE 21

POLYPLEX MORPHOLOGY USING SEM

Scanning electron microscopy (SEM) was conducted on a Thermo-Scientific Teneo SEM instrument using the following parameters: beam energy: 1 keV; beam current: 25 pA; image size 1536X1024 pixels; dwell time of 30 μSec (500 nSec x 60 line integrations). The sample was "sputter" coated by 5 nm of Iridium prior to imaging. Polyplexes were formed using Compounds 12a and 12b, i.e., LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) at an N/P 4 ratio and a concentration of 0.1875 mg/mL, in HEPES 20 mM buffer, 5% glucose (HBG), pH 7.2. A drop (20 μL) of the polyplex mixture on a stub, dried under vacuum, was analysed.

The resultant SEM image (FIG 19) shows that polyplexes particles comprising compounds 12a and 12b, i.e., LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) have a uniform morphology of low size dispersity, are spherical in nature and furthermore exhibit particle sizes in a range comparable to those determined by DLS analysis.

EXAMPLE 22

SELECTIVE DELIVERY OF mRNA ENCODING LUCIFERASE USING THE INVENTIVE POLYPLEXES RESULTS IN HIGH EXPRESSION OF LUCIFERASE IN PSMA OVEREXPRESSING CELLS

LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:Luc mRNA polyplexes were formulated in HBS at N/P ratios of 6 and 12. Prostate cancer cell lines with differential expression of PSMA (DU145: low PSMA expression; LNCaP: high PSMA expression) were treated with the polyplexes. Luminescence was measured 24 hours after the transfection and detected using Luminoskan Ascent Microplate Luminometer (Thermo Scientific).

In detail, 15,000 cells of humane prostate cancer cell lines LNCaP (high expression of PSMA) and DU145 (low expression of PSMA) were seeded into 96 well plates in triplicates and grown over night. Luc mRNA (Trilink Biotechnologies, L-7602 comprising SEQ ID NO:6 (mRNA LUC ORF) was formulated with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA in HBS (Hepes Buffered Saline, 20 mM HEPES, 150 mM NaCl, pH 7.3). The mRNA was first diluted with HBS to 0.04 mg/ml for all N/P ratios. LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA was diluted with HBS to 0.0312 mg/ml (N/P 6) and 0.0624 mg/ml (N/P 12). The diluted LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA was added to the diluted mRNA and mixed by pipetting and incubated for 30 minutes at room temperature to form polyplex. The final concentration of mRNA and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA in the polyplexes at the indicated N/P ratios are presented below:

N/P ratio	6	12
Conc of mRNA (mg/ml)	0.02	0.02
Conc of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (mg/ml)	0.0156	0.0312

The polyplexes were serially diluted and added to the cells (using 10X dilution) to obtain the indicated final concentrations of the mRNA (0.25, 0.5 and 1.0 µg/ml) Luciferase activity was measured 24 hours after the transfection with ONE-Glo™ EX Luciferase Assay System (Promega, Cat#E8130) and detected using Luminoskan Ascent Microplate Luminometer (Thermo Scientific).

Cell survival assay: Cell viability was measured by a colorimetric Methylene Blue assay. Briefly, the cells were fixed with 2.5% Glutaraldehyde in PBS (pH 7.4), washed with double distilled water, and then stained with a 1% (wt/vol) solution of methylene blue in borate buffer for 1 hour. The stain was extracted with 0.1 M HCl and the optical density of the stain solution was read at 630 nm in a microplate reader (Synergy H1, Biotek).

Selective delivery of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:Luc mRNA resulted in high expression of Luciferase in LNCaP cells at N/P ratios of 6 and 12 normalized to cell survival (FIG 20). In contrast, much lower expression of Luciferase was obtained in DU145 cells. These results demonstrate the selectivity of delivery of Luc mRNA to cancer cells with high expression of PSMA and efficient translation of a functional luciferase protein.

EXAMPLE 23

SELECTIVE DELIVERY OF HUMAN IL-2 mRNA USING THE INVENTIVE POLYPLEXES RESULTS IN HIGH EXPRESSION OF HUMAN IL-2 IN PSMA OVEREXPRESSING CELLS

LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:hIL-2 mRNA polyplexes were formulated in HBS at N/P ratios of 4, 6 and 12. Prostate cancer cell lines with differential expression of PSMA (DU145: low PSMA expression; LNCaP: high PSMA expression) were treated with the polyplexes. Release of IL-2 into the medium was examined 24 hours after the transfection by IL-2 ELISA.

In detail, 15,000 cells of human prostate cancer cell lines LNCaP (high expression of PSMA) and DU145 (low expression of PSMA) were seeded into 96 well plates in triplicates and grown overnight. Human IL-2 mRNA (Trilink Biotechnologies, WOTL83314 comprising SEQ ID NO:7 (mRNA hIL-2 ORF), 0.958 mg/mL in 1 mM Sodium Citrate, pH 6.4) was formulated with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA in HBS (Hepes Buffered Saline, 20 mM HEPES, 150 mM NaCl, pH 7.3). The mRNA was first diluted with HBS to 0.04 mg/ml for all N/P ratios. LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA was diluted with HBS to 0.0208 mg/ml (N/P 4); 0.0312 mg/ml (N/P 6) and 0.0624 mg/ml (N/P 12). The diluted LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA was added to the diluted mRNA and mixed by pipetting. Polyplexes were formed for 30 minutes at room temperature. The final mRNA and LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA concentrations in the polyplexes are presented below:

N/P ratio	4	6	12

Conc of mRNA (mg/ml)	0.02	0.02	0.02
Conc of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (mg/ml)	0.0104	0.0156	0.0312

The polyplexes were serially diluted and then added to the cells (using 10X dilution) to obtain the indicated final concentrations of the mRNA (0.25, 0.5 and 1 µg/ml). The medium was collected 24 hours after the transfection, frozen at -20 ^{0}C and after thawing was subjected to human IL-2 ELISA (Peprotech, Cat#900-T12). Signal was detected using a Microplate Reader Synergy H (Biotek).

Selective delivery of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:hIL-2 mRNA resulted in high expression of human IL-2 protein (SEQ ID NO:8) by LNCaP cells at all N/P ratios (FIG 21). In contrast much lower expression of IL-2 was obtained in the medium of DU145 cells.

These results demonstrate the selectivity of delivery of IL-2 mRNA to cancer cells with high expression of PSMA and the efficient IL-2 protein translation and secretion.

EXAMPLE 24

SELECTIVE DELIVERY OF HUMAN IFNβ mRNA USING THE INVENTIVE POLYPLEXES RESULTS IN HIGH EXPRESSION OF HUMAN IFNβ IN PSMA OVEREXPRESSING CELLS

LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA:hIFNβ mRNA polyplexes were formulated in HBS at N/P ratios of 4, 6 and 12. Prostate cancer cell lines with differential expression of PSMA (DU145: low PSMA expression; LNCaP: high PSMA expression) were treated with the polyplexes. Release of IFNβ into the medium was examined 24 hours after the transfection by IFNβ ELISA.

In detail, 15,000 cells of human prostate cancer cell lines LNCaP (high expression of PSMA) and DU145 (low expression of PSMA) were seeded into 96 well plates in triplicates and grown overnight. Human IFNβ mRNA (Tebubio, TTAP-122022 comprising SEQ ID NO:9 (mRNA hIFNβ ORF) in RNAse/DNAse free water was formulated with LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA in HBS (HEPES Buffered Saline, 20 mM HEPES, 150 mM NaCl, pH 7.3). The mRNA was first diluted with HBS to 0.04 mg/ml for all N/P ratios. LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA was diluted with HBS to 0.0208 (N/P 4); 0.0312 (N/P 6) and 0.0624 (N/P 12). The diluted LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA was added to the diluted mRNA and mixed by pipetting. Polyplexes were formed for 30 minutes at room temperature. The final mRNA and LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA concentrations in the polyplexes are presented below:

The polyplexes were serially diluted and were added to the cells (10X dilution) to obtain

N/P ratio	4	6	12
mRNA (mg/ml)	0.02	0.02	0.02
LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA (mg/ml)	0.0104	0.0156	0.0312

the indicated final concentrations of the mRNA (0.25, 0.5 and 1.0 µg/ml). The medium was collected 24 hours after the transfection and frozen at -80 ^{0}C and after thawing was subjected to human IFNβ ELISA (InvivoGen, Catalog code: luex-hifnbv2). Signal was detected using a Microplate Reader Synergy H (Biotek).

Selective delivery of LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA:hIFNβ mRNA resulted in high expression of human IFNβ protein (SEQ ID NO:16) by LNCaP cells at all N/P ratios at 1.0 µg/ml concentration (FIG 22). In contrast, much lower expression of IFNβ was obtained in the medium of DU145 cells except the N/P ratio of 12 at the highest concentration (1.0 µg/ml) where higher release of IFNβ can be observed.

These results demonstrate the selective delivery of IFNβ mRNA to cancer cells with high expression of PSMA using LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA and efficient protein translation and secretion.

FIG 22 depicts the levels of secreted human IFNβ from two cell lines with differential PSMA expression: PSMA high expressing LNCaP cells, and PSMA low expressing DU145 cells following transfection with PSMA targeting polyplexes containing hIFNβ mRNA (SEQ ID NO:9 (mRNA hIFNβ ORF). Selective expression of human IFNβ from PSMA high expressing cells is demonstrated.

EXAMPLE 25

SELECTIVE DELIVERY OF mRNA ENCODING DIPHTHERIA TOXIN (DT) CATALYTIC DOMAIN A (DT-A) USING THE INVENTIVE POLYPLEXES RESULTS IN INHIBITION OF PROTEIN BIOSYNTHESIS IN PSMA HIGH EXPRESSING CELLS

DT-A inhibits the enzymatic ADP-ribosylation of elongation factor 2, thereby blocking the translational machinery of target cells. The antibiotic puromycin binds to newly synthesized polypeptide chains which can then be detected by Western blot analysis using an anti-puromycin antibody. Reduction in detection indicates inhibition of protein biosynthesis. The selective inhibition of protein biosynthesis mediated by targeted delivery of mRNA encoding

DT-A and the consequent DT-A protein expression in high PSMA expressing cells was examined by western blot analysis using anti-Puromycin antibody.

LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA:DT-A mRNA polyplexes were formulated in 5% glucose at N/P ratio of 4. Prostate cancer cell lines with differential expression of PSMA (DU145: low PSMA expression; LNCaP: high PSMA expression) were treated with the polyplexes for 24 hours followed by puromycin treatment for 15 minutes.

In detail, human prostate LNCaP (high expression of PSMA) and DU145 (low expression of PSMA) cancer cells were seeded into 6 well plates (400,000 cells/well) and grown overnight. DT-A mRNA (Tebubio, TTAP-012023 comprising SEQ ID NO:15 (mRNA DT-A ORF) was formulated with LPEI[N3:DBCO]PEG36-DUPA in 5% glucose at 0.1 mg/ml at the indicated N/P ratio of 4 with mRNA concentration of 0.1 mg/ml and LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA concnetration of 0.052 mg/ml.

The mRNA was first diluted with 5% glucose to 0.2 mg/ml. LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA was diluted with 5% glucose 0.104 mg/ml (N/P ratio 4). The diluted LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA was added to the diluted mRNA (equal volumes of each) and mixed by pipetting. Polyplexes were allowed to form for 30 minutes at room temperature. The polyplexes were serially diluted and then added to the cells (using 10 X dilution) to obtain the indicated final concentrations of mRNA (0.25, 0.5 and 1.0 µg/ml). After 24 hours of treatment, cells were treated with 5 µg/ml puromycin (Med Chem Express HY-B1743A) for 15 minutes, then harvested and the protein lysates were prepared. 30 µg of each protein lysate were run on 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad) before being transferred to 0.2 µm PVDF membranes (BioRad). Protein biosynthesis inhibition was detected by an anti-Puromycin antibody (Sigma/Merck, MABE343) and GAPDH (Cell signaling 2118) was used as a loading control.

Selective delivery of LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA:DT-A mRNA at N/P ratio of 4 resulted in dose dependent protein biosynthesis inhibition in LNCaP cells (FIG 23). In contrast, no inhibition of protein biosynthesis was observed in DU145 cells. These results demonstrate the selectivity of delivery of DT-A mRNA to cancer cells with high expression of PSMA and efficient expression of functional DT-A protein (SEQ ID NO:17).

FIG 23 depicts protein biosynthesis inhibition by DT-A protein in two cell lines with differential PSMA expression: high PSMA-expressing LNCaP cells, and low PSMA-expressing DU145 cells following transfection with PSMA targeting polyplexes LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA containing SEQ ID NO:17 (mRNA DT-A ORF). Western blot

analysis with an anti-puromycin antibody as probe was utilized to detect inhibition of protein biosynthesis. Selective inhibition of protein biosynthesis in PSMA overexpressing cells is demonstrated.

EXAMPLE 26

SELECTIVE DELIVERY OF SARS-CoV-2 SPIKE mRNA BY THE INVENTIVE POLYPLEXES RESULTS IN HIGH EXPRESSION OF THE SPIKE PROTEIN IN PSMA-HIGH EXPRESSING CELLS

Inventive polyplexes targeting PSMA and containing mRNA encoding the SARS-Cov-2 S protein are formulated in HBS (HEPES Buffered Saline, 20 mM HEPES, 150 mM NaCl, pH 7.3) at N/P ratios of 4, 6, or 12. Cancer cell lines with differential expression of PSMA (LNCaP: high PSMA expression; DU145: no PSMA expression) are transfected with the polyplexes. Cells are harvested 24 hours after transfection and protein expression is determined by western-blot analysis.

In detail, 400,000 cells of human prostate cancer cell lines LNCaP (high expression of PSMA) and DU145 (low expression of PSMA) are seeded into 6 well plates and grown overnight. SARS-CoV-2 S mRNA (Trilink Biotechnologies) is formulated with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA in HBS (Hepes Buffered Saline, 20 mM HEPES, 150 mM NaCl, pH 7.3) at 0.02 mg/ml at the indicated N/P ratios.

N/P ratio	4	6	12
Conc of mRNA (mg/ml)	0.02	0.02	0.02
Conc of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (mg/ml)	0.0104	0.0156	0.0312

The mRNA is first diluted with HBS to 0.04 mg/ml for all N/P ratios. LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA is diluted with HBS to 0.0208 mg/ml (N/P 4); 0.0312 mg/ml (N/P 6) and 0.0624 mg/ml (N/P 12). The diluted LPEI-*l*-[N_3:DBCO]PEG_{36}-DUPA is added to the diluted mRNA and mixed by pipetting and incubated for 30 minutes at room temperature to form polyplexes. The polyplexes are serially diluted and added to the cells (using 10X dilution) to obtain the indicated final concentrations (0.25, 0.5 and 1.0 µg/ml) of the mRNA. Cells are lysed after 24 hours of treatment and lysates are prepared. Protein lysates are run on 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad) before being transferred onto 0.2 µm PVDF membranes (BioRad). S protein expression is detected by an α-Spike antibody (Sino

Biological, Cat#40591-MM42) and β-actin (Sigma Aldrich, Cat#A5441) is used as a loading control.

EXAMPLE 27

SELECTIVE DELIVERY OF PLASMID DNA ENCODING LUCIFERASE UTILIZING THE INVENTIVE POLYPLEXES RESULTS IN HIGH EXPRESSION OF LUCIFERASE IN PSMA OVEREXPRESSING CELLS

LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:pGreenFire-CMV polyplexes were formulated in HBS at N/P ratios of 4 and 6. Prostate cancer cell lines with differential expression of PSMA (DU145: low PSMA expression; LNCaP: high PSMA expression) were treated with the polyplexes. Luminescence was measured 24 hours after the transfection.

In detail, cells were seeded into 96-well plates (15000 cells/well) and grown overnight. pLuc plasmid (pGreenFire-CMV, SBI) was formulated with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA in HBS (HEPES Buffered Saline, 20 mM HEPES, 150 mM NaCl, pH 7.3) at 0.02 mg/ml at the indicated N/P ratios, diluted and added to the cells, to obtain the indicated final concentrations of the plasmid. Triplicate samples were tested for each condition. Luminescence was measured 24 hours after the transfection with the ONE-Glo™ EX Luciferase Assay System (Promega).

Selective delivery of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:pGreenFire-CMV resulted in high luminescence signals in LNCaP cells in a dose dependent manner at N/P 6 (FIG 24). In contrast, much lower expression of luciferase was obtained in DU145 cells.

These results demonstrate the selective delivery of plasmid DNA encoding luciferase to cancer cells with high expression of PSMA and efficient protein expression and activity.

FIG 24 depicts luminescence from human prostate cell lines with differential cell surface expression of PSMA: high-PSMA expressing LNCaP cells, and low PSMA-expressing DU145 cells. The cells were treated with PSMA-targeting polyplexes containing plasmid DNA encoding luciferase. The X axis indicates the concentration of the pGreenFire-CMV in the polyplexes (0.25, 0.5 and 1.0 µg/mL). The Y axis indicates luminescence in arbitrary units (AU). Average and standard deviation from triplicate samples are presented. Selective expression of luciferase after transfection of PSMA overexpressing cells with plasmid DNA encoding luciferase (pGreenFire-CMV) is demonstrated.

EXAMPLE 28

SELECTIVE DELIVERY OF PLASMID DNA ENCODING HUMAN IL2 USING THE INVENTIVE POLYPLEXES RESULTS IN HIGH EXPRESSION OF HUMAN IL2 IN PSMA OVEREXPRESSING CELLS

LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:pUNO1-hIL2 polyplexes were formulated in HBS at N/P ratios of 4, 6 and 12. Prostate cancer cell lines with differential expression of PSMA (DU145: low PSMA expression; C4-2, LNCaP: high PSMA expression (Juzeniene A et al, Cancers 2021, 13(4):779) were treated with the polyplexes. Release of human IL2 into the medium was examined 24 hours after the transfection by human IL-2 ELISA.

In detail, human prostate cancer cells that express high (LNCaP, C4-2) or low (DU145) levels of PSMA were seeded into 96-well plates in triplicates (15,000 cells/well) and grown overnight. Plasmid DNA encoding hIL2 (pUNO1-hIL2, InvivoGen, comprising SEQ ID NO:18) was formulated with LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA in HBS (HEPES Buffered Saline, 20 mM HEPES, 150 mM NaCl, pH 7.3). pUNO1-hIL2 was first diluted with HBS to 0.04 mg/ml for all N/P ratios. LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA was diluted with HBS to 0.0208 mg/ml (N/P ratio of 4); 0.0312 mg/ml (N/P ratio of 6) and 0.0624 mg/ml (N/P ratio of12). The diluted LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA was added to the diluted pUNO1-hIL2 and mixed by pipetting. Polyplex were formed for 30 minutes at room temperature. The final concentrations of plasmid DNA and LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA in the polyplexes were as follows:

N/P ratio	4	6	12
Conc of plasmid (mg/ml)	0.02	0.02	0.02
Conc of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (mg/ml)	0.0104	0.0156	0.0312

The polyplexes were serially diluted and were added to the cells to obtain the indicated final concentrations of the plasmid (0.25, 0.5 and 1.0 µg/ml).

The medium was collected 24 hours after the transfection and stored at -20°C. Medium was thawed and subjected to human IL2 ELISA (Peprotech, Cat#900-T12). Signals from ELISA were detected using a Microplate Reader Synergy H (Biotek). Survival of the cells was measured with CellTiter-Glo (Promega, Cat#G7571) using Luminoskan Ascent Microplate Luminometer (Thermo Labsystems). Normalized IL2 concentrations were obtained by dividing the average of IL2 concentrations by the average luminescence (survival).

Delivery of LPEI-/-[N_3:DBCO]-PEG_{36}-DUPA:pUNO1-hIL2 resulted in high expression of human IL2 by LNCaP and C4-2 cells at all N/P ratios (FIG 25). In contrast, much lower expression of IL2 was obtained in the medium of DU145 cells at all N/P ratios.

These results demonstrate the selective delivery of plasmid DNA encoding human IL2 protein (SEQ ID NO:8) to cancer cells with high expression of PSMA at all N/P ratios as well as efficient translation and secretion of the encoded IL-2 protein.

FIG 25 depicts levels of secreted human IL2 normalized to cell survival, in cell lines with differential PSMA expression: high-expressing LNCaP and C4-2 cells, and low-expressing DU145 cells following transfection with PSMA-targeting polyplexes containing plasmid encoding IL2 protein. The X axis indicates the concentration of pUNO1-hIL2 plasmid DNA (0.25, 0.5 and 1.0 µg/mL) in the polyplexes. The Y axis indicates the concentration of secreted IL-2 normalized to cell survival in Arbitrary Units (AU). The selective expression/secretion of human IL2 after transfection of PSMA overexpressing cells with plasmid DNA encoding hIL-2 is demonstrated.

CLAIMS

1. A composition comprising a conjugate, wherein said conjugate comprises:

a linear polyethyleneimine fragment comprising an alpha terminus and an omega terminus;

a polyethylene glycol fragment comprising a first terminal end and a second terminal end, wherein said polyethylene glycol fragment comprises, preferably consists of, a discrete number m of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60;

wherein the alpha terminus of said polyethyleneimine fragment is an initiation residue;

wherein the omega terminus of the polyethyleneimine fragment is connected to the first terminal end of the polyethylene glycol fragment by a divalent covalent linking group -Z-X^1-, wherein -Z-X^1- is not a single bond and -Z- is not an amide;

wherein the second terminal end of the polyethylene glycol fragment is connected to a targeting fragment by a divalent covalent linking moiety X^2 , and

wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

2. A composition comprising a conjugate, wherein said conjugate is of the Formula I* or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof

R^1-(NR^2-CH_2-CH_2)$_n$-Z-X^1-(O-CH_2-CH_2)$_m$-X^2-L (Formula I*);

wherein

n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

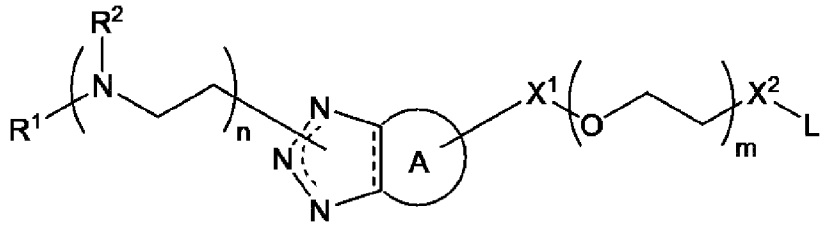
R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$- is H;

X^1 and X^2 are independently divalent covalent linking moieties;

Z is a divalent covalent linking moiety wherein Z-X^1 is not a single bond and Z is not -NHC(O)-;

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

3. The composition of claim 1 or claim 2, wherein said conjugate is of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



Formula I

wherein:

═══ is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating -(O-CH_2-CH_2)- units, wherein said discrete number m of repeating -(O-CH_2-CH_2)- units is any discrete number of 25 to 100, preferably of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or -CH_3;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

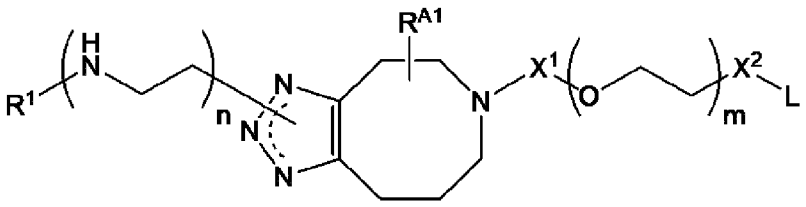
L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

4. The composition of any one of the claims 2 to 3, wherein said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number m of repeating -(O-CH_2-CH_2)- units of 25 to 60, wherein preferably said -(O-CH_2-CH_2)$_m$-moiety consists of a discrete number m of repeating -(O-CH_2-CH_2)- units of 25 to 48, and wherein further preferably said discrete number m of repeating -(O-CH_2-CH_2)- units is 36.

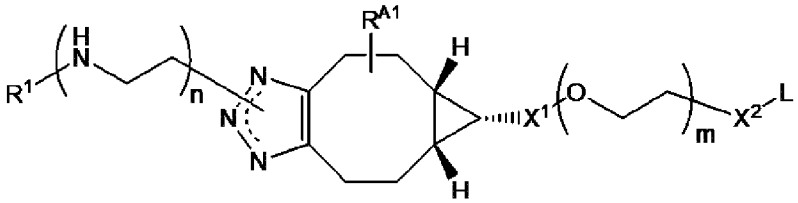
5. The composition of any one of the claims 3 to 4, wherein Ring A is an 8-membered cycloalkenyl, 5-membered heterocycloalkyl, or 7- to 8-membered heterocycloalkenyl, wherein each cycloalkenyl, heterocycloalkyl or heterocycloalkenyl is optionally substituted at any position with one or more R^{A1}.

6. The composition of any one of the claims 3 to 5, wherein Ring A is cyclooctene, succinimide, or 7- to 8-membered heterocycloalkenyl, wherein the heterocycloalkenyl comprises one or two heteroatoms selected from N, O and S, and wherein each cyclooctene or heterocycloalkenyl is optionally substituted at any position with one or more R^{A1}, wherein preferably R^{A1} is oxo or fluorine, or wherein two R^{A1} combine to form one or more fused phenyl rings, preferably one or two fused phenyl rings, wherein each phenyl ring is optionally substituted with one or more -SO_3H or -OSO_3H.

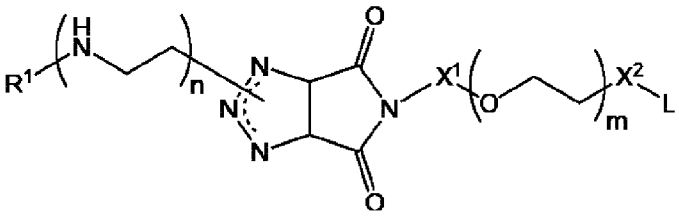
7. The composition of any one of the claims 3 to 6, wherein said conjugate of Formula I is selected from:



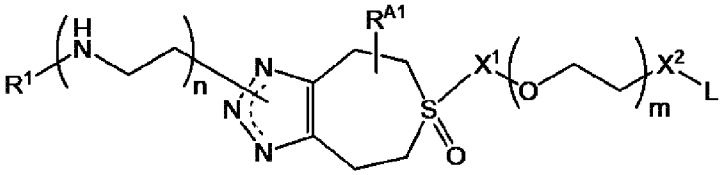
Formula IA,



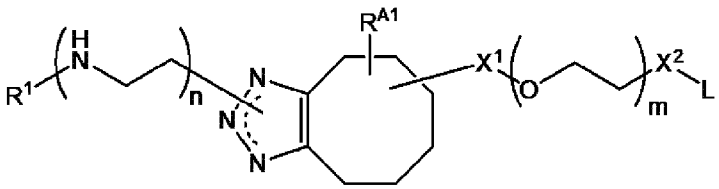
Formula IB,



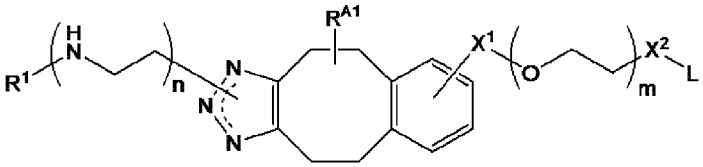
Formula IC,



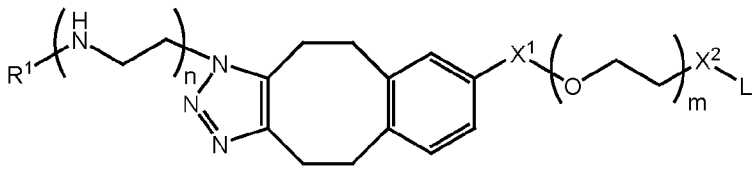
Formula ID,



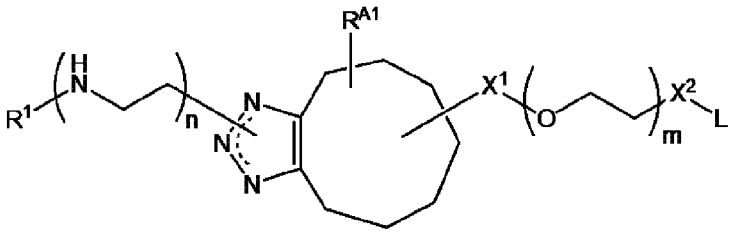
Formula IE,



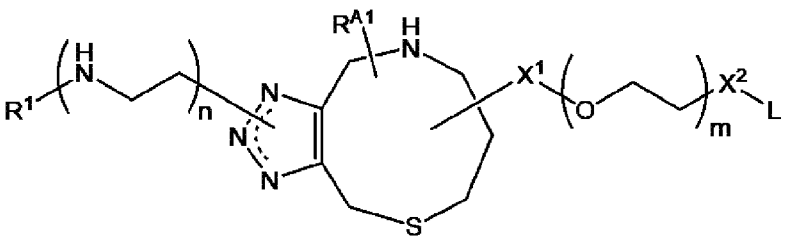
Formula IH,



Formula IH-1,

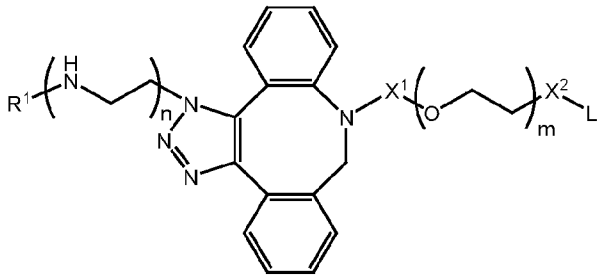


Formula IJ, and

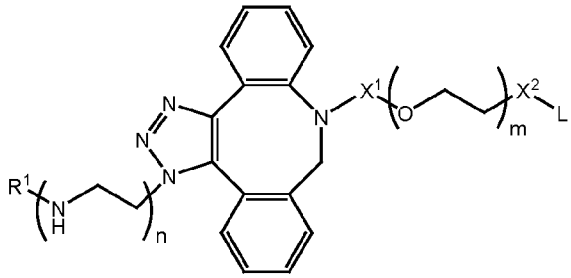


Formula IK.

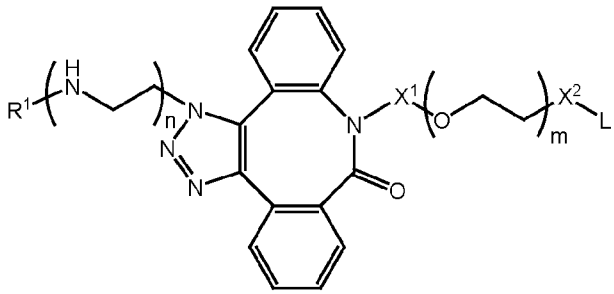
8. The composition of any one of the claims 3 to 7, wherein said conjugate of Formula I is selected from:



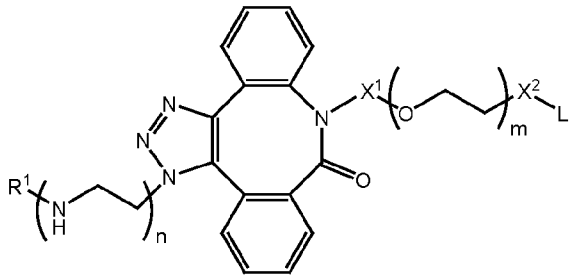
Formula IA-3,



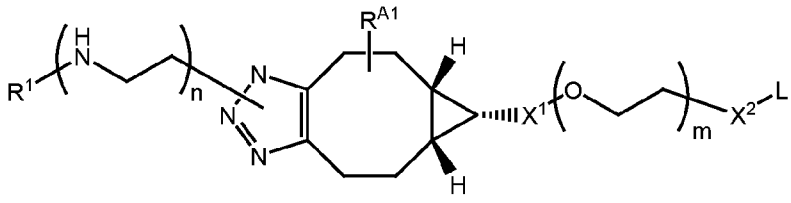
Formula IA-4,



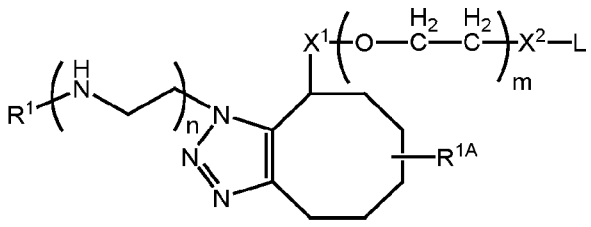
Formula IA-9,



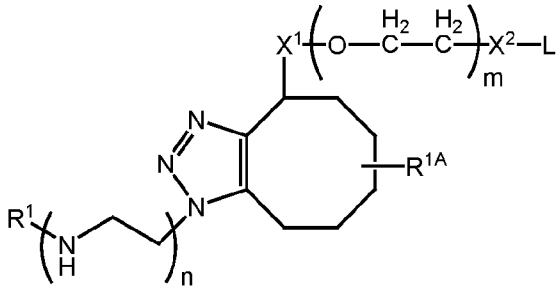
Formula IA-10,



Formula IB,

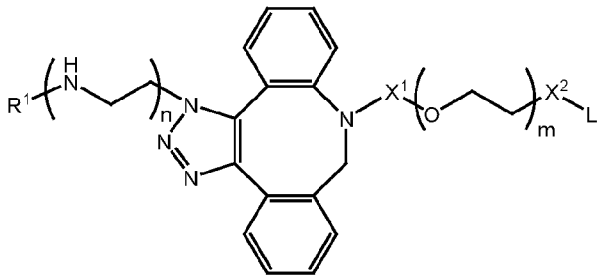


Formula IE-13, and



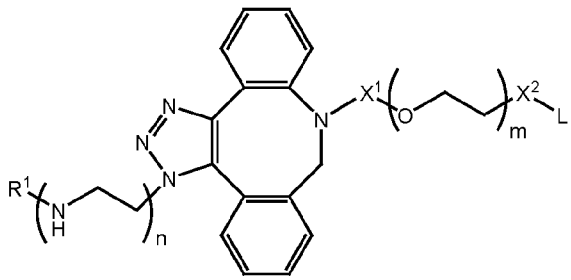
Formula IE-14.

9. The composition of any one of the claims 3 to 8, wherein said conjugate of Formula I is selected from:



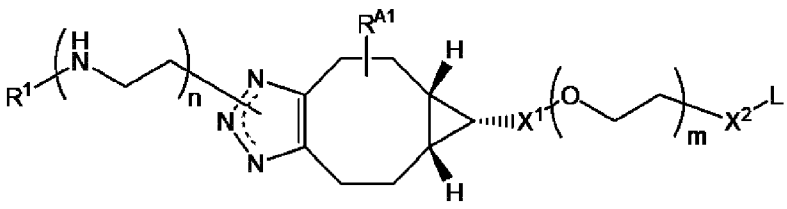
Formula IA-3,

and



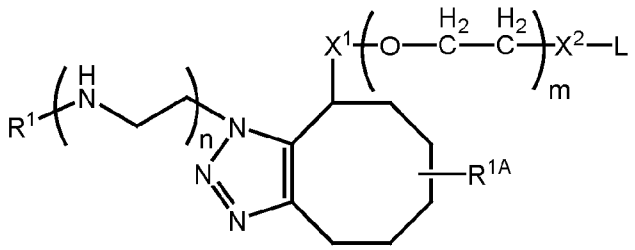
Formula IA-4.

10. The composition of any one of the claims 3 to 8, wherein said conjugate of Formula I is selected from:

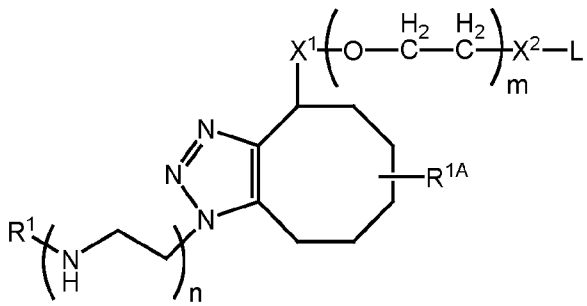


Formula IB.

11. The composition of any one of the claims 3 to 8, wherein said conjugate of Formula I is selected from:

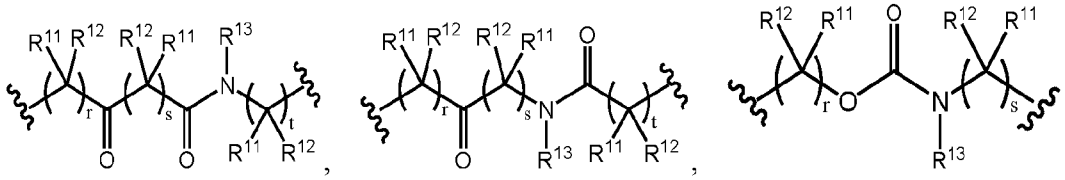


Formula IE-13, and



Formula IE-14.

12. The composition of any one of the claims 3-11, wherein X^1 comprises a group selected from:



wherein:

r is independently, at each occurrence, 0-6, preferably 0, 1, 2, or 5; more preferably 0;

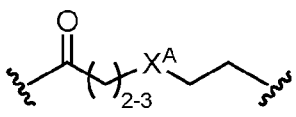
s is independently, at each occurrence, 0-6, preferably 0, 2, 3, or 4; more preferably 2 or 3;

t is independently, at each occurrence, 0-6, preferably 0, 1, 2, 4; more preferably 2;

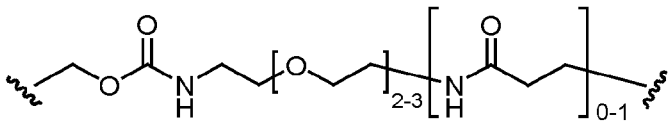
R^{11} and R^{12} are independently, at each occurrence, selected from -H and -C_1-C_2 alkyl, preferably -H; and

R^{13} is -H; preferably wherein the wavy line nearest to the integer "r" is a bond to Ring A and the wavy line nearest to the integer "s" or "t" is a bond to $-[OCH_2\text{-}CH_2]_m-$.

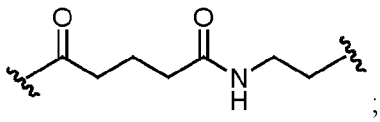
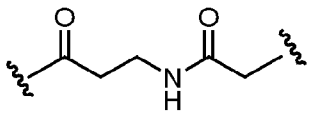
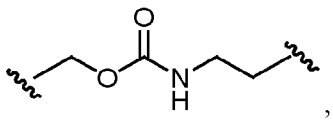
13. The composition of any one of the claims 3-11, wherein X^1 is selected from:



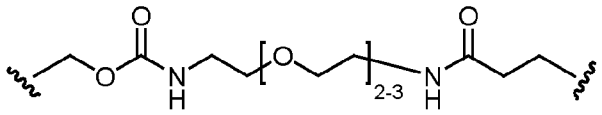
, wherein X^A is -NHC(O)- or -C(O)NH-; and

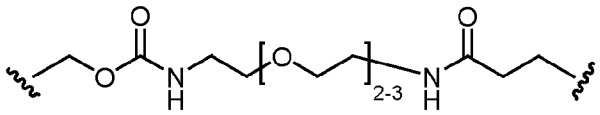

; preferably wherein the wavy line on the left side is a bond to Ring A and the wavy line on the right side is a bond to –[OCH_2-CH_2]$_m$–.

14. The composition of any one of claims 3-11, wherein X^1 is selected from:

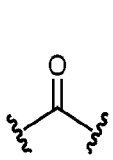
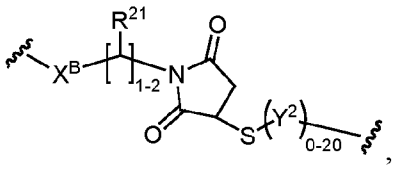
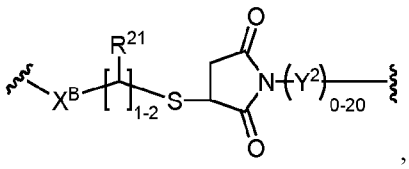

;

;

,

and




; preferably wherein the wavy line on the left side is a bond to Ring A and the wavy line on the right side is a bond to –[OCH_2-CH_2]$_m$–.

15. The composition of any one of claims 3-14, wherein X^2 is selected from:


,

, and

,

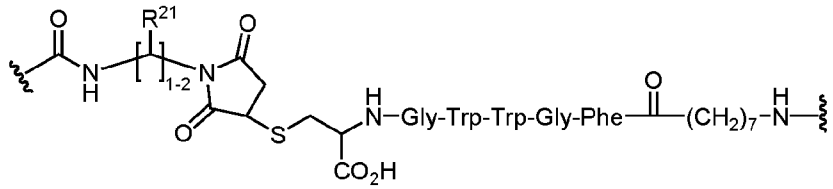
wherein X^B is -C(O)NH- or -NH-C(O)-;

wherein each occurrence of Y^2 is independently selected from a chemical bond, -$CR^{21}R^{22}$-, NR^{23}-, -O-, -S-, -C(O)-, an amino acid residue, a divalent phenyl moiety, a divalent carbocyle moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl and divalent heteroaryl is optionally substituted with one or more R^{23}, and wherein each divalent heterocycle moiety is optionally substituted with one or more R^{24};

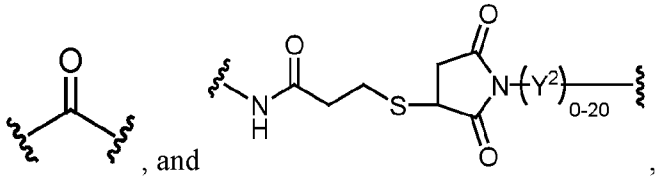
R^{21}, R^{22}, and R^{23} are each independently, at each occurrence, -H, -SO_3H, -NH_2, -CO_2H, or C_1-C_6 alkyl, wherein each C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, -CO_2H, -NH_2, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl; and

R^{24} is independently, at each occurrence, -H, -CO_2H, C_1-C_6 alkyl, or oxo; preferably wherein the wavy line on the left side is a bond to –[OCH_2-CH_2]$_m$– and the wavy line on the right side is a bond to L.

16. The composition of any one of claims 3-14, wherein X^2 is selected from:



(SEQ ID NO: 10),



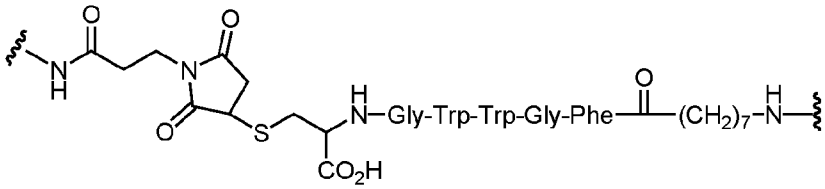
, and ,

wherein each occurrence of Y^2 is independently selected from a chemical bond, -$CR^{21}R^{22}$-, NR^{23}-, -O-, -S-, -C(O)-, an amino acid residue, a divalent phenyl moiety, a divalent carbocyle moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl and divalent heteroaryl is optionally substituted with one or more R^{23}, and wherein each divalent heterocycle moiety is optionally substituted with one or more R^{24};

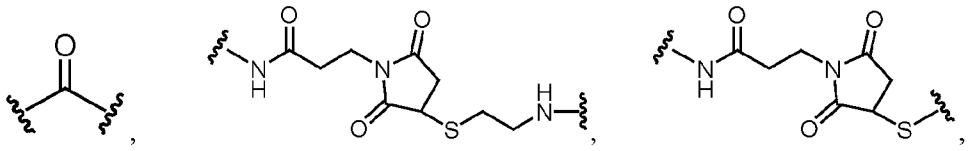
R^{21}, R^{22}, and R^{23} are each independently, at each occurrence, -H, -SO_3H, -NH_2, -CO_2H, or C_1-C_6 alkyl, wherein each C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, -CO_2H, -NH_2, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl; and

R^{24} is independently, at each occurrence, -H, -CO_2H, C_1-C_6 alkyl, or oxo; preferably wherein the wavy line on the left side is a bond to –$[OCH_2$-$CH_2]_m$– and the wavy line on the right side is a bond to L.

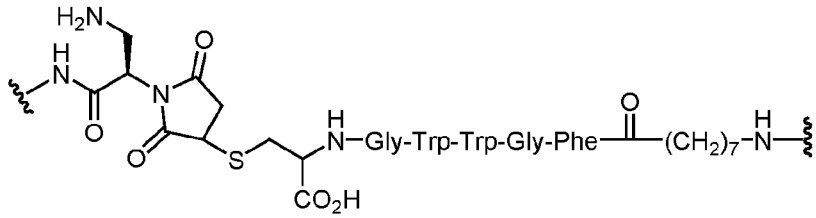
17. The composition of any of claims 3-14, wherein X^2 is selected from:



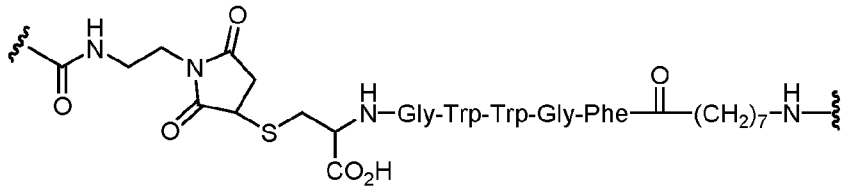
(SEQ ID NO: 11),



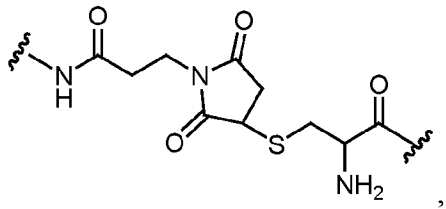
, , ,



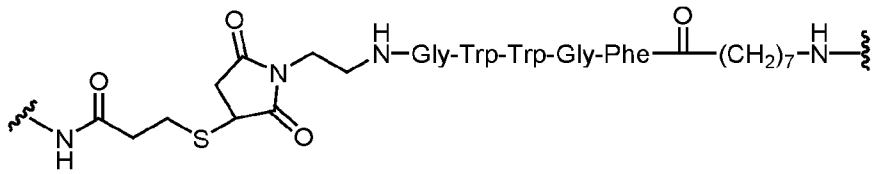
(SEQ ID NO: 12),



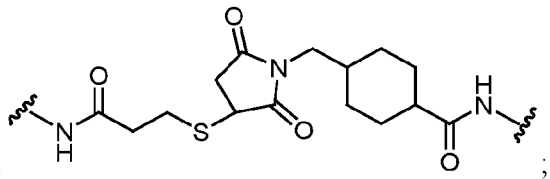
(SEQ ID NO: 13),



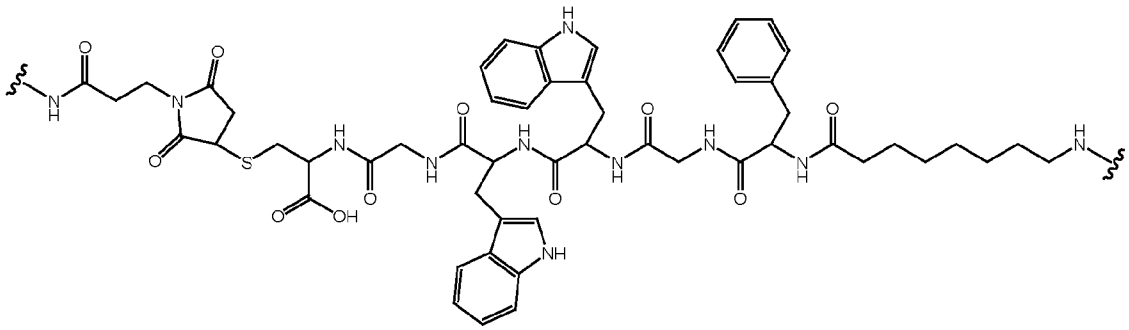
,



(SEQ ID NO: 14),

and  ; preferably wherein the wavy line on the left side is a bond to $–[OCH_2\text{-}CH_2]_m–$ and the wavy line on the right side is a bond to L.

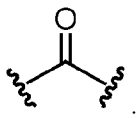
18. The composition of any one of claims 3-14, wherein X^2 is



;

preferably wherein the wavy line on the left side is a bond to $–[OCH_2\text{-}CH_2]_m–$ and the wavy line on the right side is a bond to L.

19. The composition of any one of claims 3-14, wherein X^2 is



20. The composition of any one of the preceding claims, wherein said targeting fragment L is capable of binding to a cell surface receptor expressing PSMA, wherein preferably said targeting fragment is capable of specifically binding to a cell surface receptor expressing PSMA.

21. The composition of claim 20, wherein said cell surface receptor is a transmembrane protein, preferably a transmembrane protein of type II.

22. The composition of claim 20 or claim 21, wherein said cell surface receptor is prostate specific membrane antigen (PSMA).

23. The composition of any one of the preceding claims, wherein said targeting fragment L is capable of binding to a cell surface receptor expressing PSMA, and wherein said targeting fragment is a peptide, a protein, a small molecule ligand, a saccharide, an oligosaccharide, an oligonucleotide, a lipid, an amino acid, an antibody, an antibody fragment, an aptamer or an affibody.

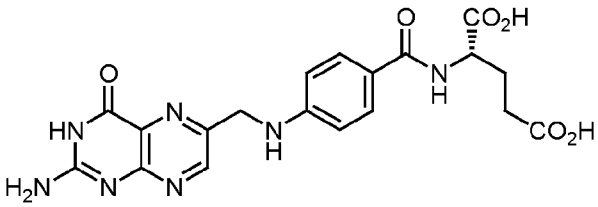
24. The composition of any one of the preceding claims, wherein said targeting fragment L is selected from a PSMA antibody, a PSMA aptamer and a small-molecule PSMA targeting fragment, preferably wherein said small-molecule PSMA targeting fragment is a DUPA residue or a folate ligand.

25. The composition of any one of the preceding claims, wherein said targeting fragment L is a small-molecule PSMA targeting fragment.

26. The composition of claim 25, wherein said small-molecule PSMA targeting fragment is a urea based PSMA peptidase inhibitor

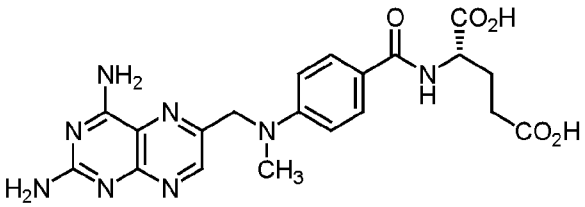
27. The composition of claim 25, wherein said small-molecule PSMA targeting fragment is a folate ligand.

28. The composition of claim 27, wherein said folate ligand is folic acid:



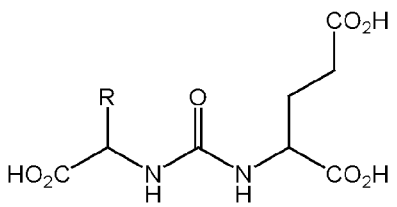
wherein either the *alpha* carboxylate group or the *gamma* carboxylate group of said folic acid serves as the point of covalent attachment to the X^2 linking moiety, and wherein preferably the *gamma* carboxylate group of said folic acid serves as the point of covalent attachment to the X^2 linking moiety.

29. The composition of claim 27 wherein said folate ligand is methotrexate:



wherein either the *alpha* carboxylate group or the *gamma* carboxylate group of said folic acid serves as the point of covalent attachment to the X^2 linking moiety, and wherein preferably the *gamma* carboxylate group of said folic acid serves as the point of covalent attachment to the X^2 linking moiety.

30. The composition of any one of the preceding claims, wherein said targeting fragment L is of formula **1**:

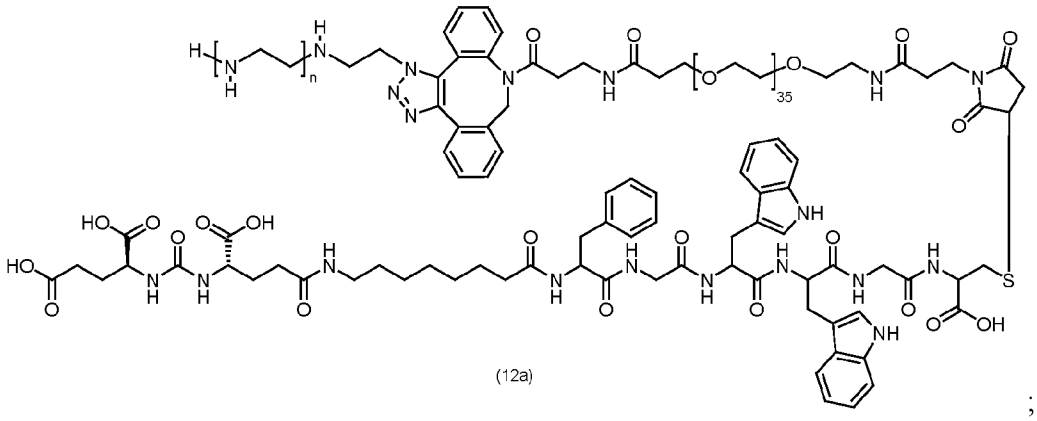


1,

wherein R is $C_{1\text{-}6}$-alkyl substituted one or more times, preferably one time, with OH, SH, NH_2, or COOH, wherein one of said NH_2, OH or SH or COOH group serves as the point for covalent attachment to the X^2 linking moiety, and wherein the alkyl group can optionally be interrupted by N(H), S or O.

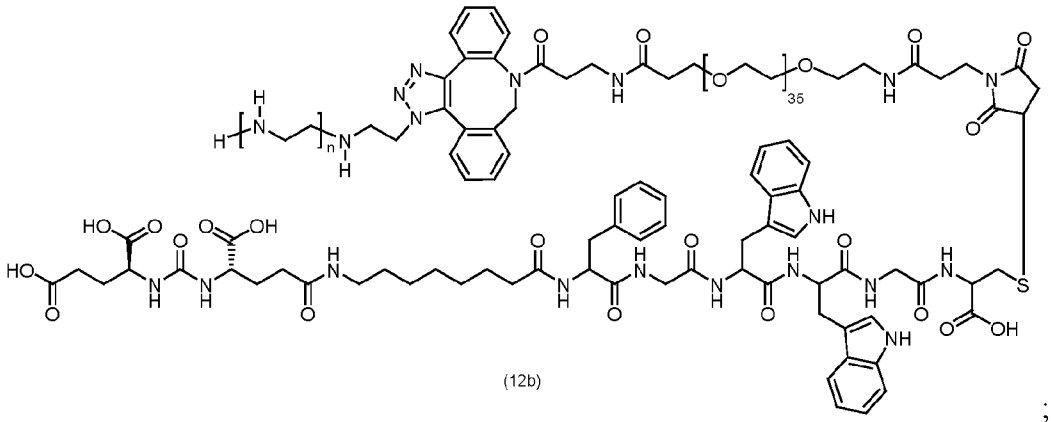
31. The composition of any one of the preceding claims, wherein said targeting fragment L is the DUPA residue (HOOC(CH_2)$_2$-CH(COOH)-NH-CO-NH-CH(COOH)-(CH_2)$_2$-CO-).

32. The composition of claim 1, wherein said conjugate is selected from Compound 12a, Compound 12b, Compound 19a, Compound 19b, Compound 24, Compound 28a, Compound 28b, Compound 32a, Compound 32b, Compound 37a, Compound 37b, Compound 43, Compound 44a, Compound 44b, Compound 45, Compound 49a, and/or Compound 49b.



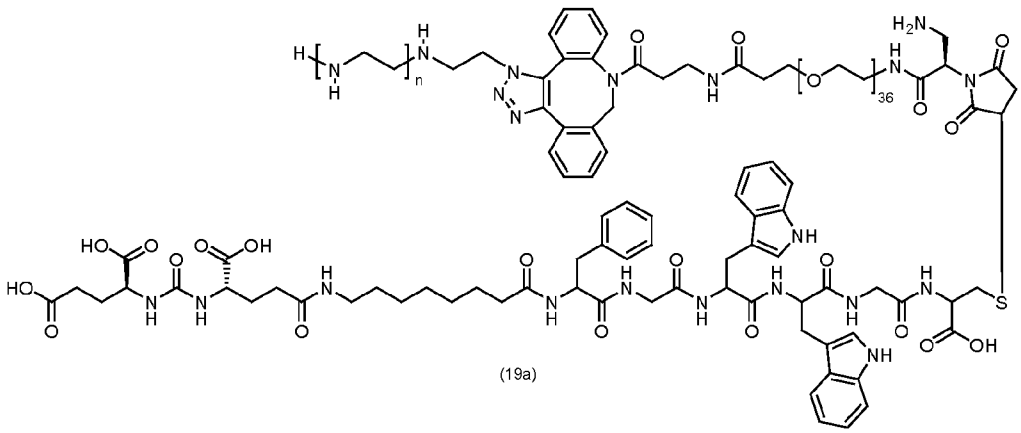
(12a)

;



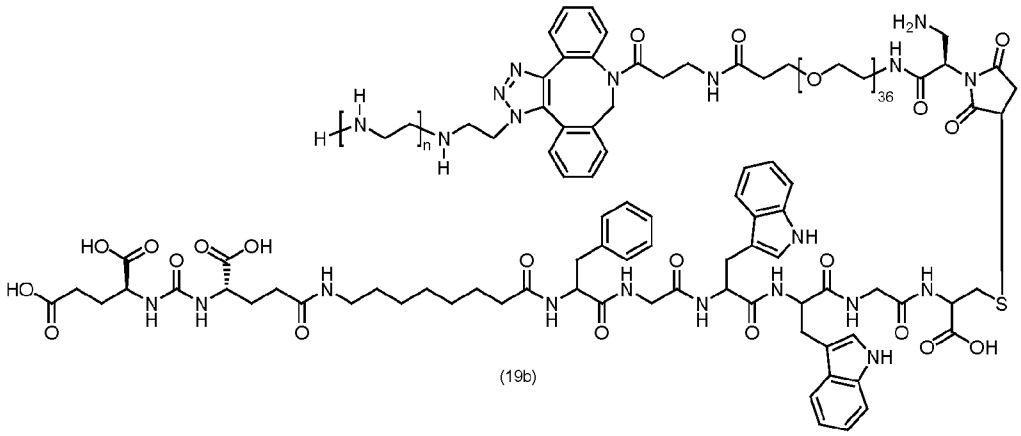
(12b)

;



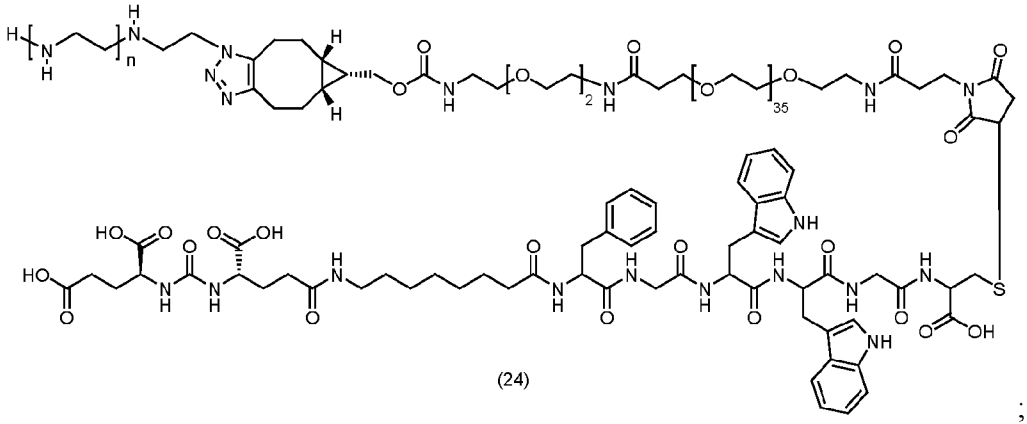
(19a)

;



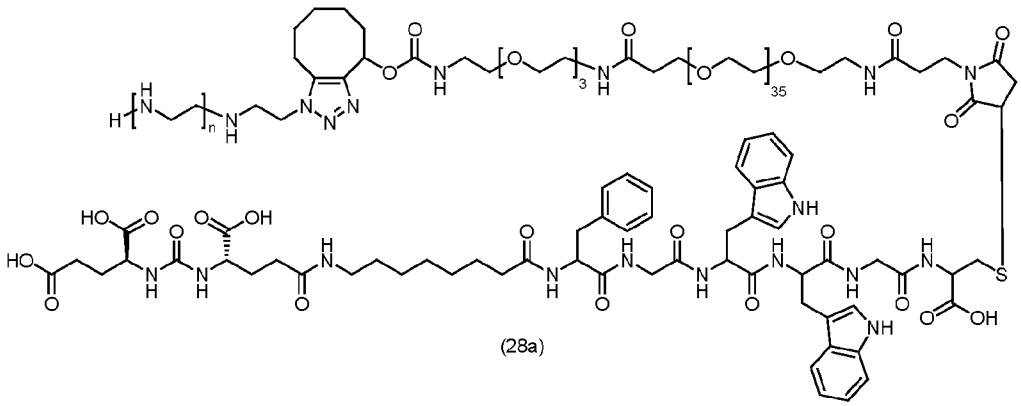
(19b)

;



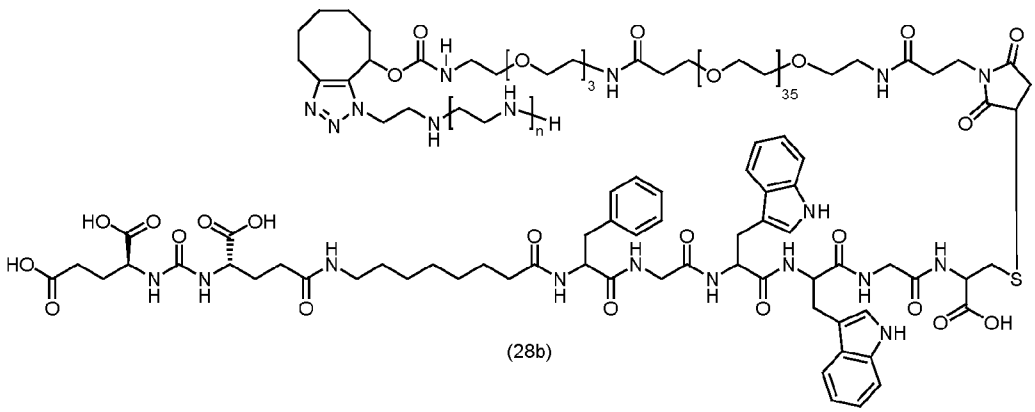
(24)

;



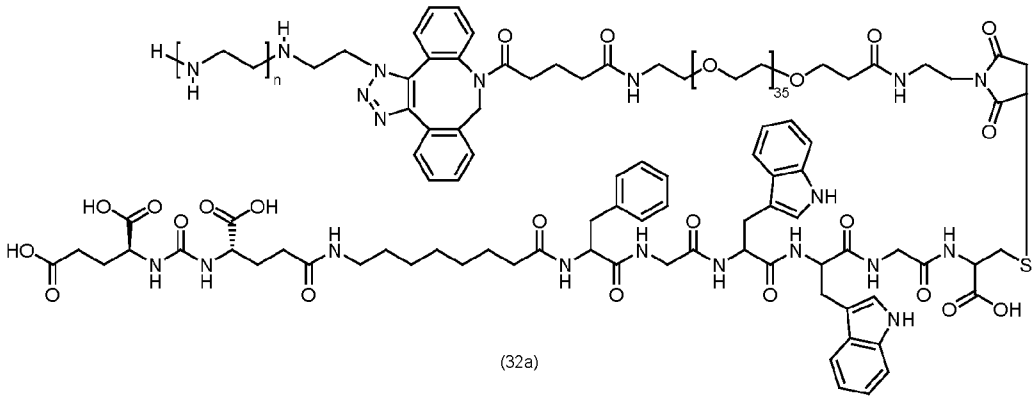
(28a)

;



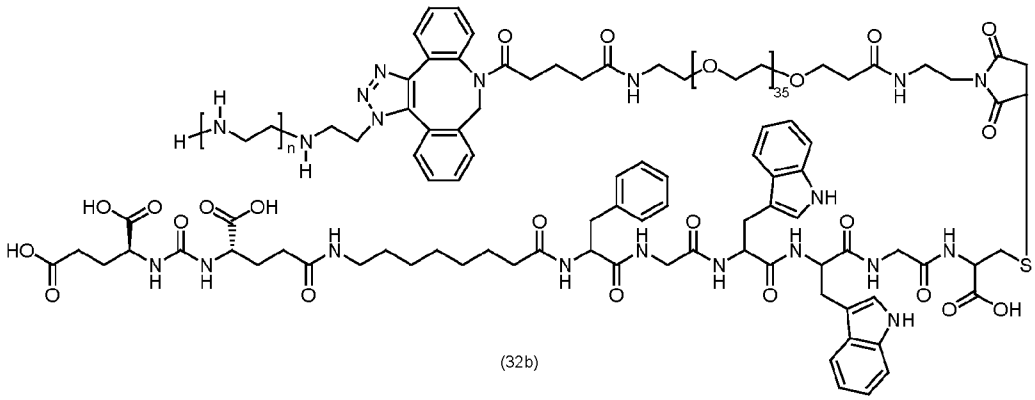
(28b)

;



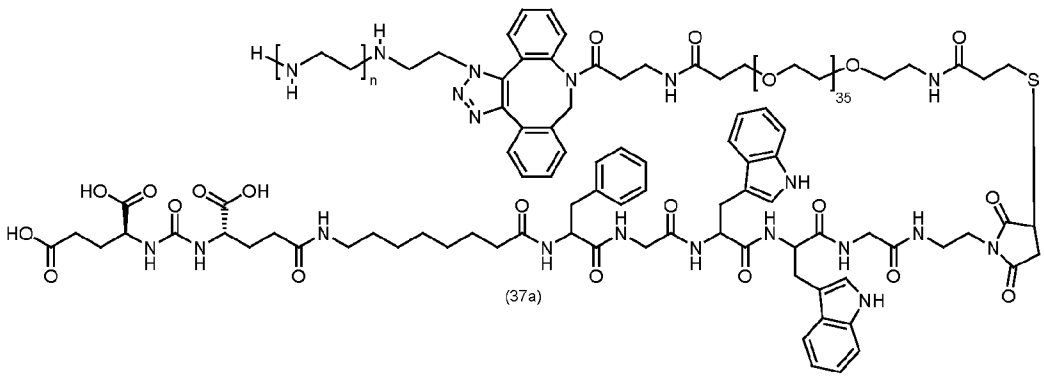
(32a)

;



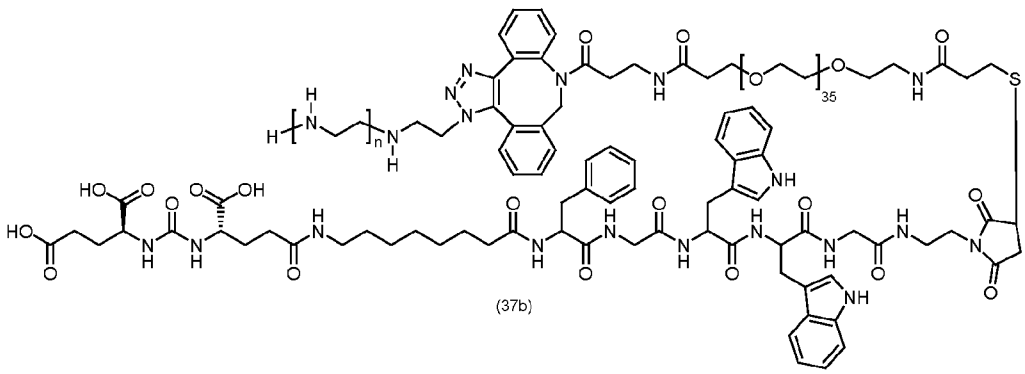
(32b)

;



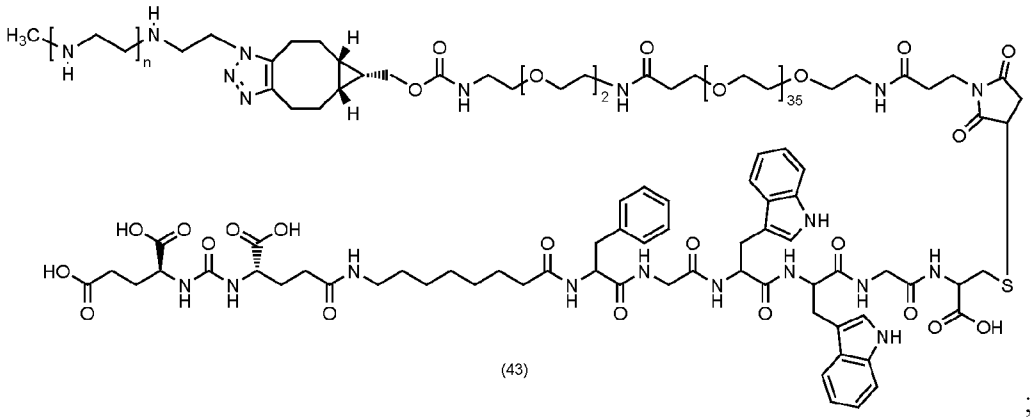
(37a)

;



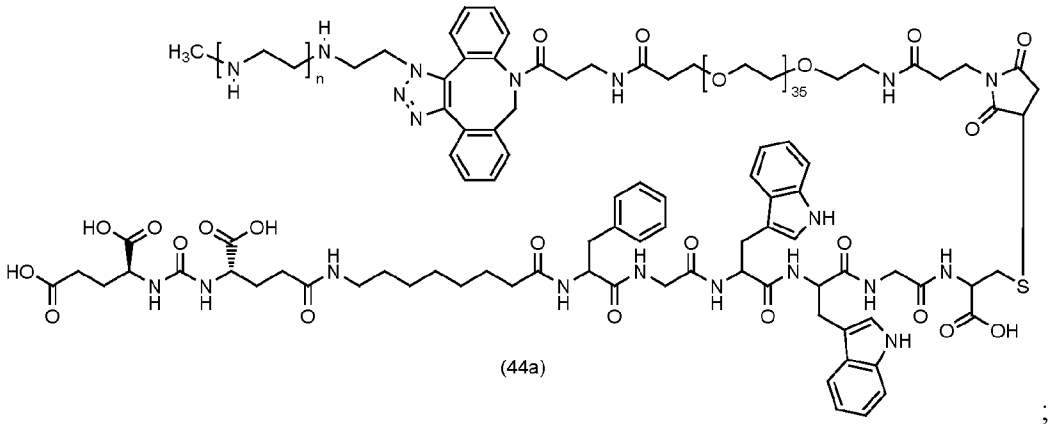
(37b)

;



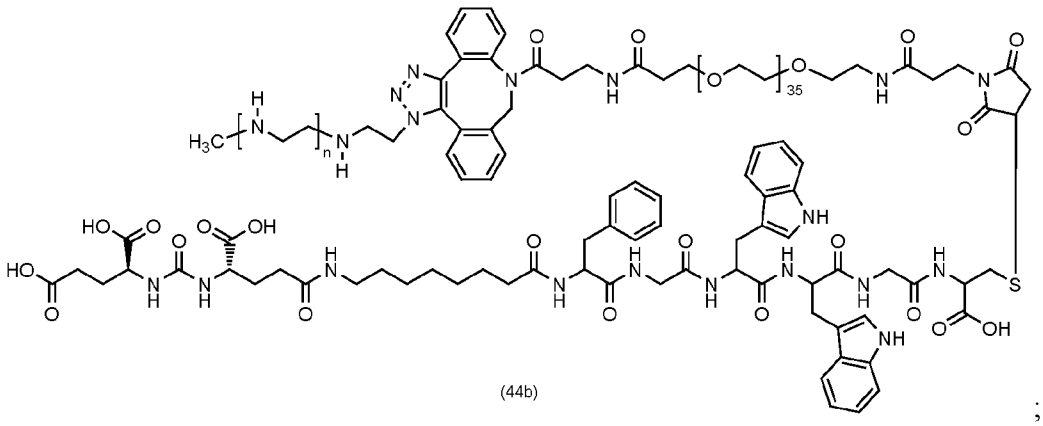
(43)

;



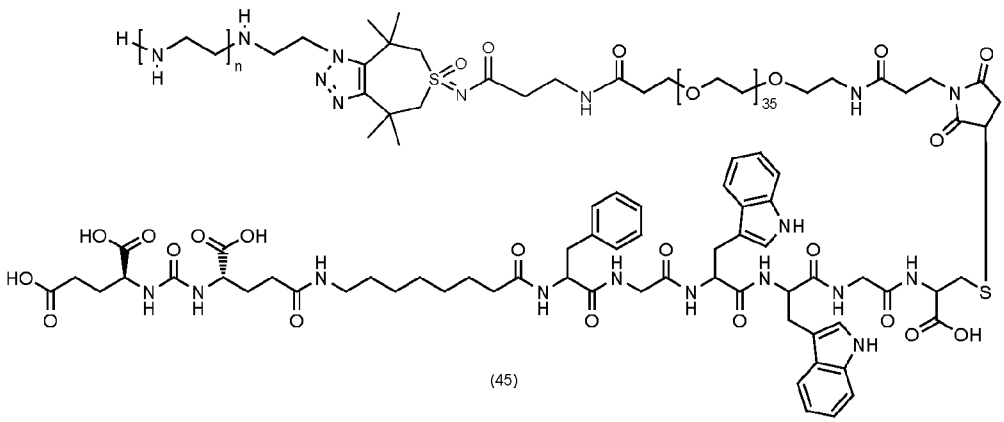
(44a)

;



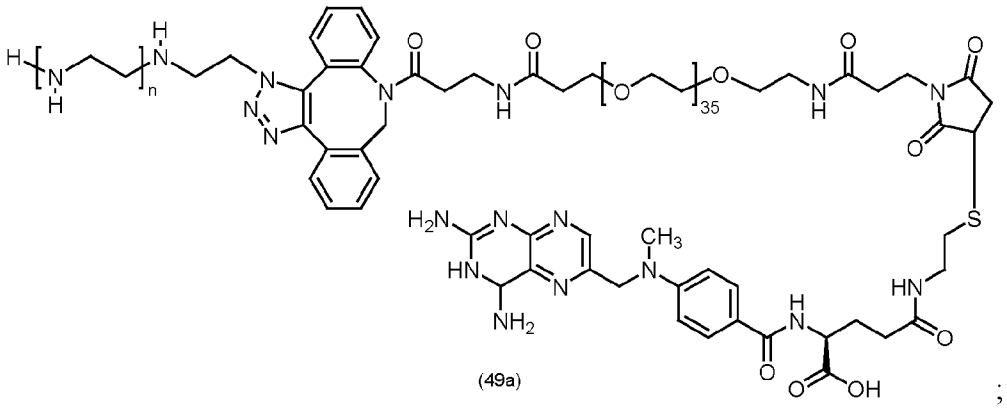
(44b)

;

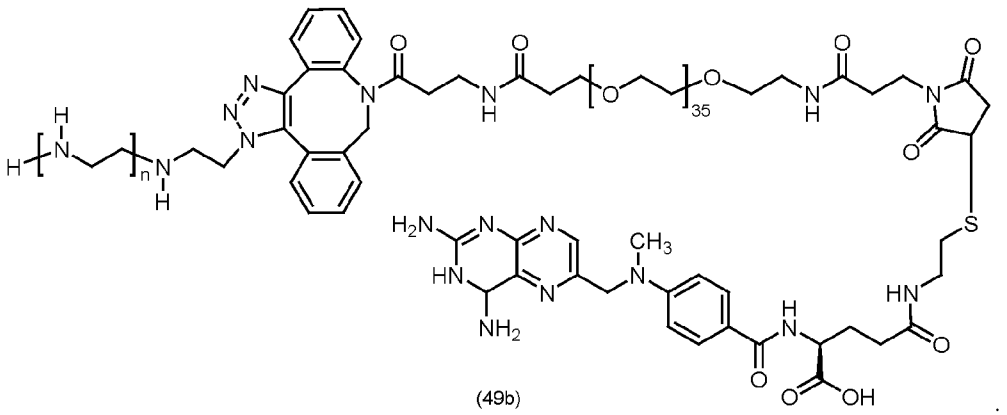


(45)

;



(49a) ;



(49b) .

33. The composition of any one of the preceding claims, wherein said composition further comprises a polyanion, preferably wherein said polyanion is a nucleic acid, wherein said polyanion is preferably non-covalently bound to said conjugate, and wherein said polyanion and said conjugate form a polyplex.

34. The composition of claim 33, wherein said polyanion is a nucleic acid, and wherein said nucleic acid is a dsRNA or a ssRNA.

35. The composition of claim 34, wherein said nucleic acid is a dsRNA.

36. The composition of claim 35, wherein said dsRNA is polyinosinic:polycytidylic acid (poly(IC)).

37. The composition of claim 34, wherein said nucleic acid is a ssRNA.

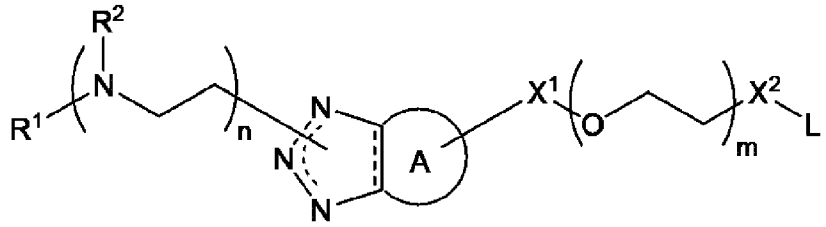
38. The composition of claim 37, wherein said ssRNA is a mRNA.

39. The composition of claim 33, wherein said polyanion is a nucleic acid, and wherein said nucleic acid is a DNA.

40. The composition of claim 39, wherein said DNA is a plasmid DNA.

41. A polyplex of a conjugate as defined in any one of the preceding claims and a polyanion, wherein said polyanion is preferably non-covalently bound to said conjugate, and wherein preferably the polyanion is a nucleic acid.

42. A polyplex comprising a conjugate of Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof, and a polyanion, preferably a nucleic acid, wherein said polyanion, preferably said nucleic acid is preferably non-covalently bound to said conjugate:



Formula I

wherein:

===== is a single bond or a double bond;

n is any integer between 1 and 1500;

m is a discrete number of repeating units m of 25 to 100, wherein preferably m is a discrete number of repeating units m of 25 to 60;

R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$;

R^2 is independently -H or an organic residue, wherein at least 80%, preferably wherein at least 90%, of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl

is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

L is a targeting fragment, wherein said targeting fragment is capable of binding to prostate specific membrane antigen (PSMA), and wherein preferably said targeting fragment is capable of binding to a cell expressing PSMA.

43. The polyplex of claim 41 or claim 42, wherein said polyanion is a nucleic acid, wherein said nucleic acis is an RNA.

44. The polyplex of claim 43, wherein said RNA is a dsRNA or a ssRNA.

45. The polyplex of claim 43, wherein said RNA is a dsRNA.

46. The polyplex of claim 45, wherein said dsRNA is polyinosinic:polycytidylic acid (poly(IC)).

47. The polyplex of claim 43, wherein said RNA is a ssRNA.

48. The polyplex of claim 47, wherein said ssRNA is a mRNA.

49. The polyplex of claim 41 or claim 42, wherein said polyanion is a nucleic acid, and wherein said nucleic acid is a DNA.

50. The polyplex of claim 49, wherein said DNA is a plasmid DNA.

51. A pharmaceutical composition comprising a composition of any one of the claims 1 to 40 or a polyplex of any one of the claims 41 to 50, and optionally one or more pharmaceutically acceptable excipient(s) and/or carrier(s).

52. A composition of any one of the claims 1 to 40 or a polyplex of any one of the claims 41 to 50 or a pharmaceutical composition according to claim 51, for use in the treatment of a

cancer, preferably a cancer characterized by cells that overexpress prostate-specific membrane antigen (PSMA).

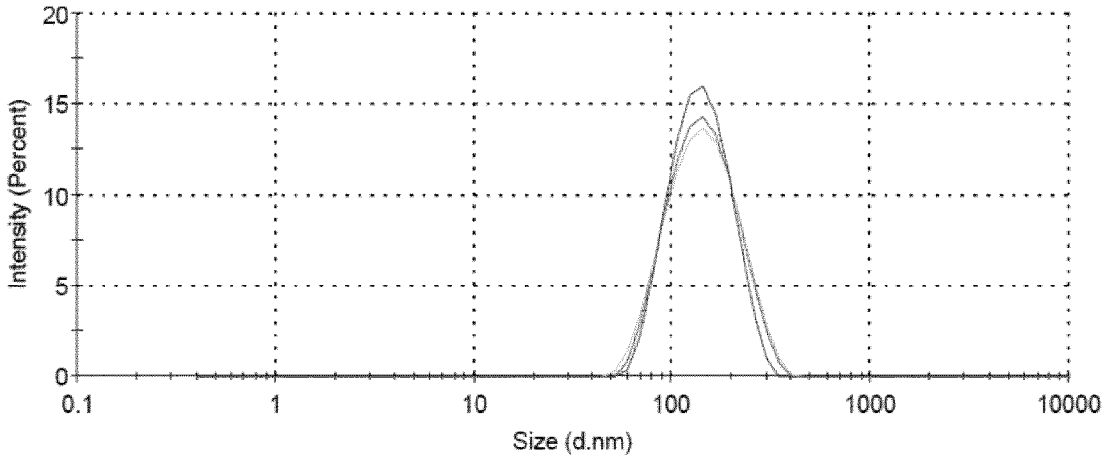

Intensity (Percent)
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)

FIG 1

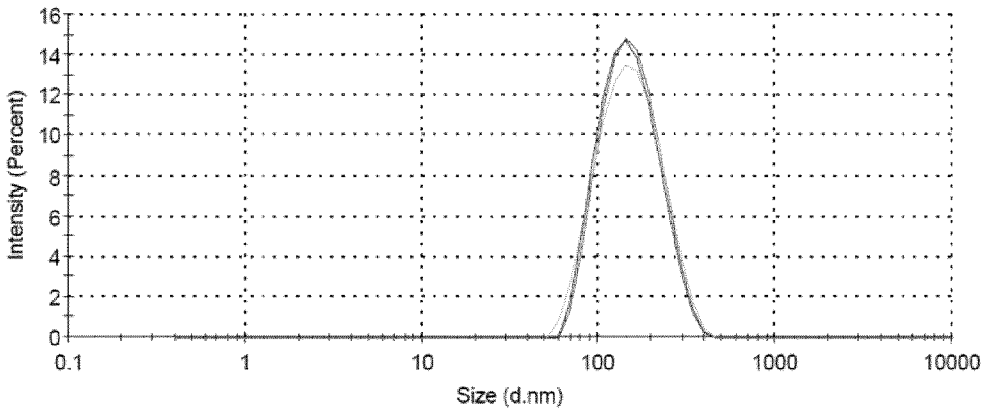

Intensity (Percent)
16
14
12
10
8
6
4
2
0
0.1
1
10
100
1000
10000
Size (d.nm)

FIG 2

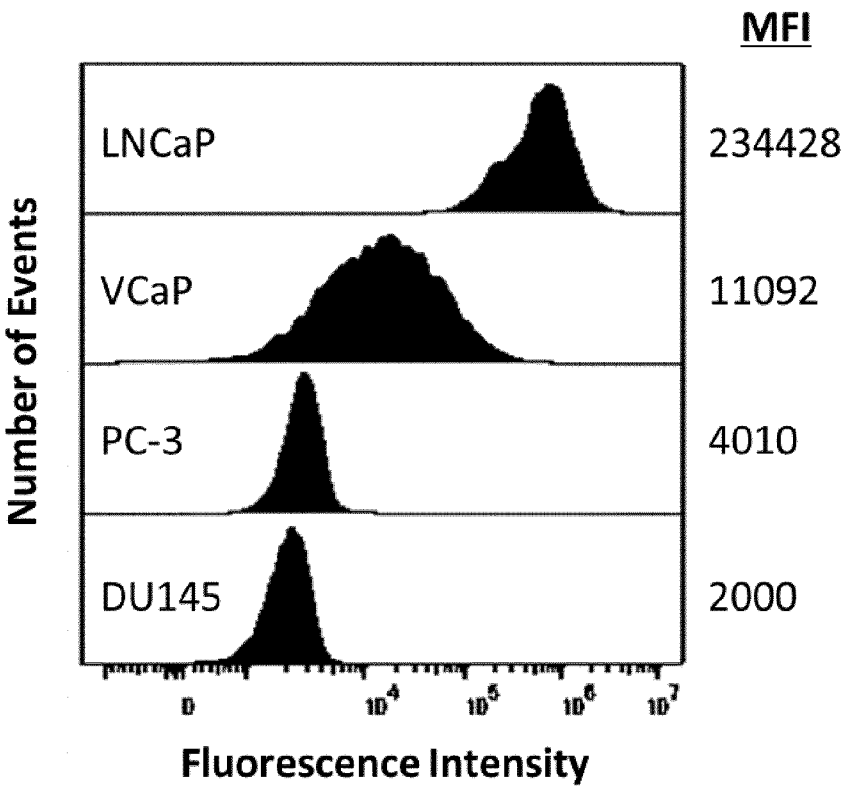

MFI
LNCaP
234428
VCaP
11092
PC-3
4010
DU145
2000
Number of Events
0
10^4
10^5
10^6
10^7
Fluorescence Intensity

FIG 3

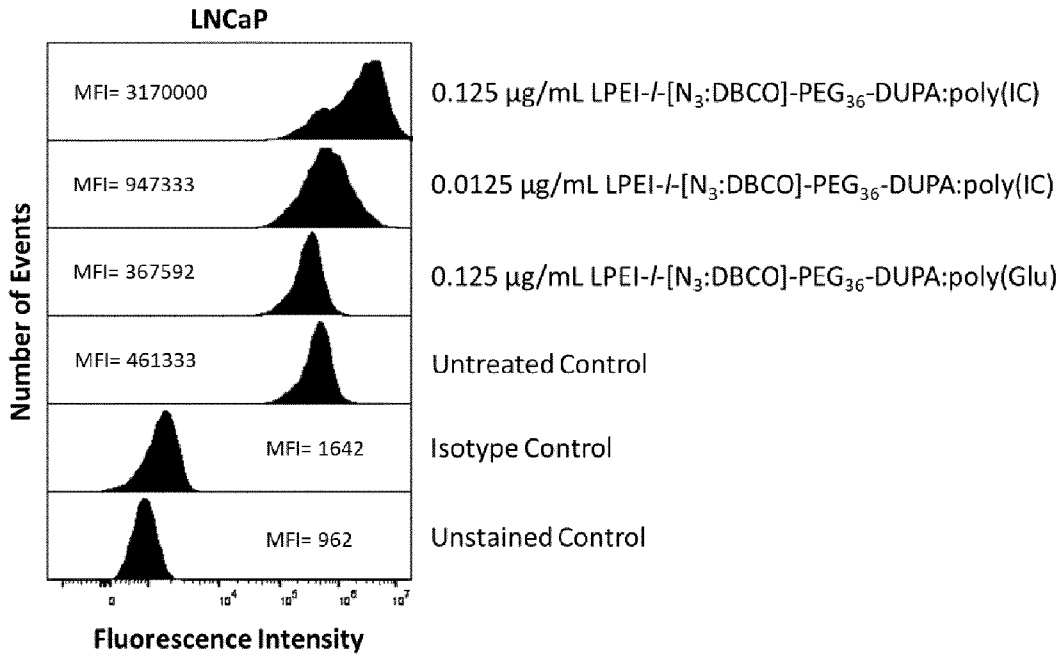

LNCaP
MFI= 3170000
MFI= 947333
MFI= 367592
MFI= 461333
MFI= 1642
MFI= 962
Number of Events
Fluorescence Intensity
0.125 µg/mL LPEI-/-[N3:DBCO]-PEG36-DUPA:poly(IC)
0.0125 µg/mL LPEI-/-[N3:DBCO]-PEG36-DUPA:poly(IC)
0.125 µg/mL LPEI-/-[N3:DBCO]-PEG36-DUPA:poly(Glu)
Untreated Control
Isotype Control
Unstained Control

FIG 4A

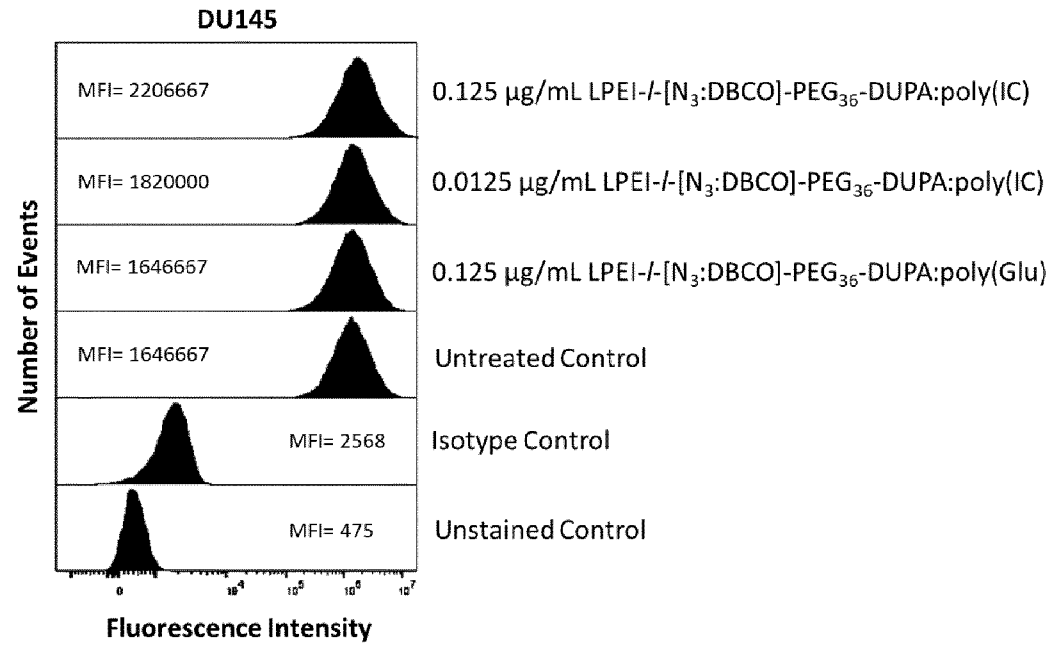

DU145
MFI= 2206667
MFI= 1820000
MFI= 1646667
MFI= 1646667
MFI= 2568
MFI= 475
Number of Events
Fluorescence Intensity
0.125 µg/mL LPEI-/-[N3:DBCO]-PEG36-DUPA:poly(IC)
0.0125 µg/mL LPEI-/-[N3:DBCO]-PEG36-DUPA:poly(IC)
0.125 µg/mL LPEI-/-[N3:DBCO]-PEG36-DUPA:poly(Glu)
Untreated Control
Isotype Control
Unstained Control

FIG 4B

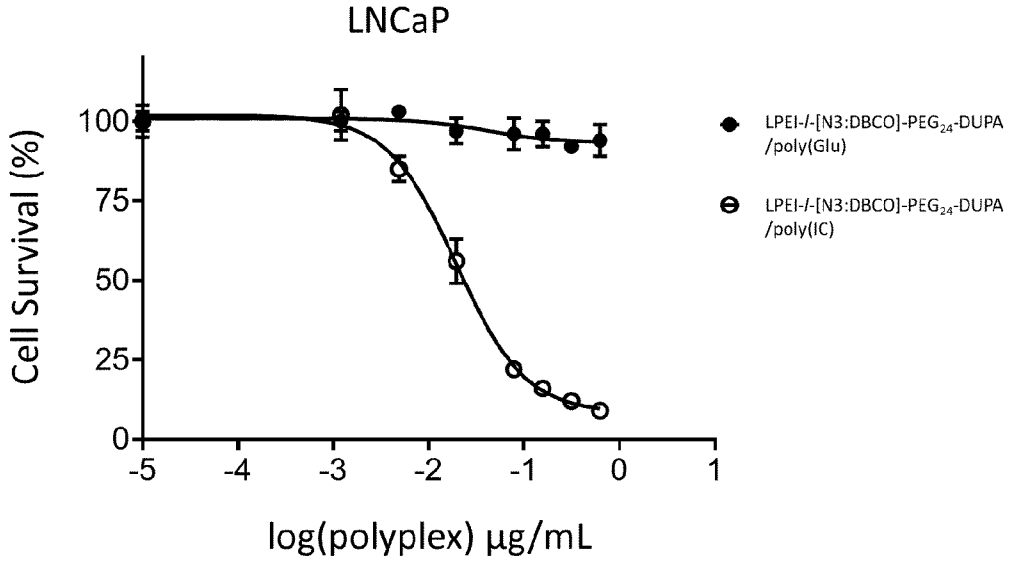

LNCaP
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI-/-[N3:DBCO]-PEG24-DUPA /poly(Glu)
LPEI-/-[N3:DBCO]-PEG24-DUPA /poly(IC)

FIG 5A

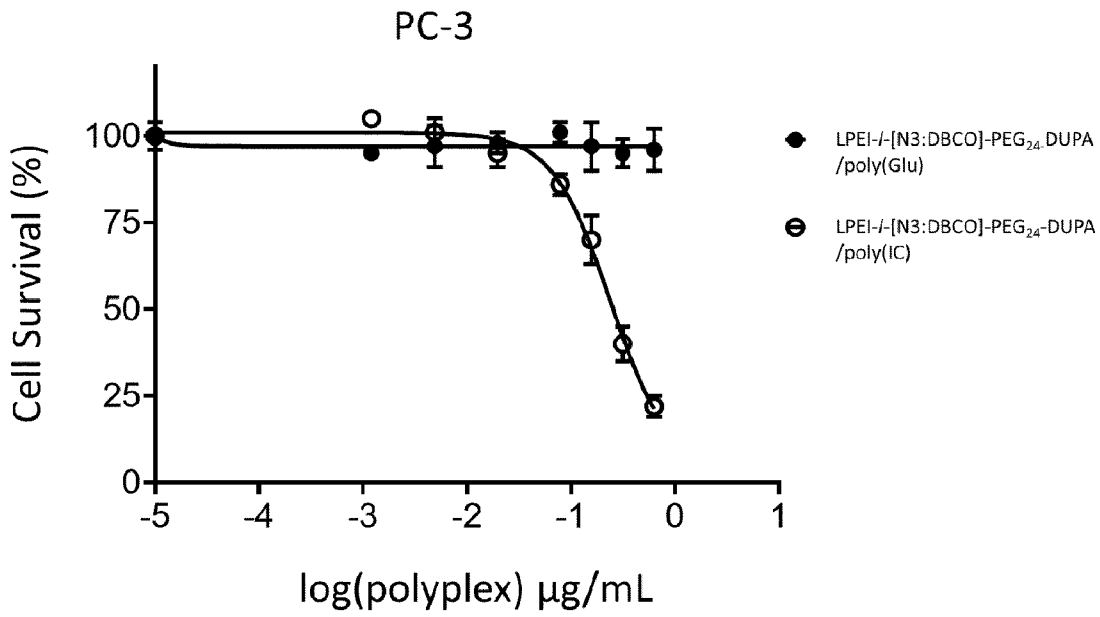

PC-3
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI-/-[N3:DBCO]-PEG24-DUPA /poly(Glu)
LPEI-/-[N3:DBCO]-PEG24-DUPA /poly(IC)

FIG 5B

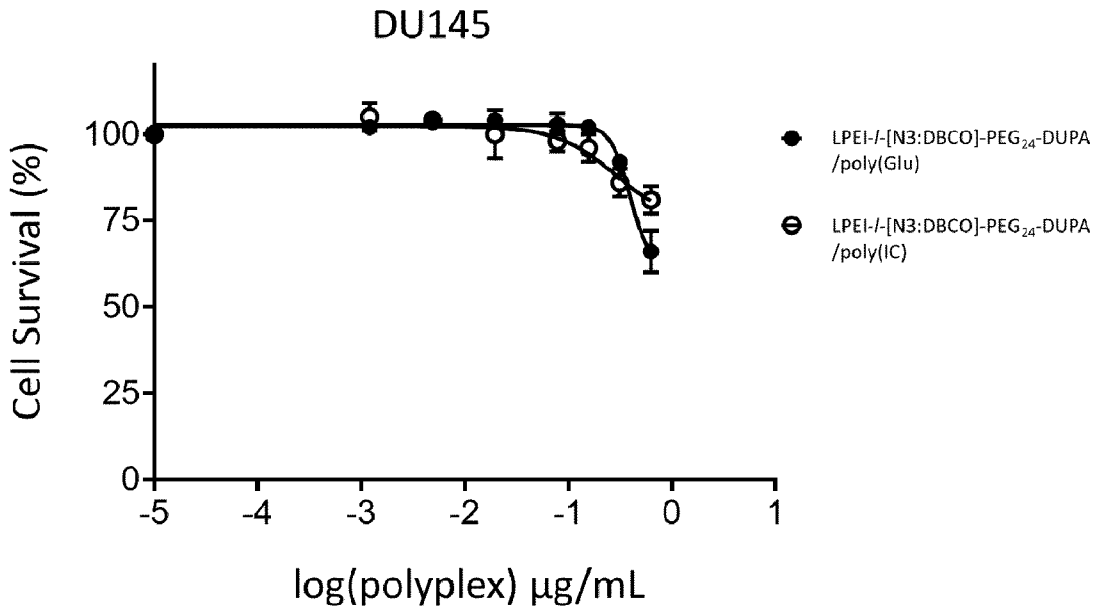

DU145
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI-/-[N3:DBCO]-PEG24-DUPA /poly(Glu)
LPEI-/-[N3:DBCO]-PEG24-DUPA /poly(IC)

FIG 5C

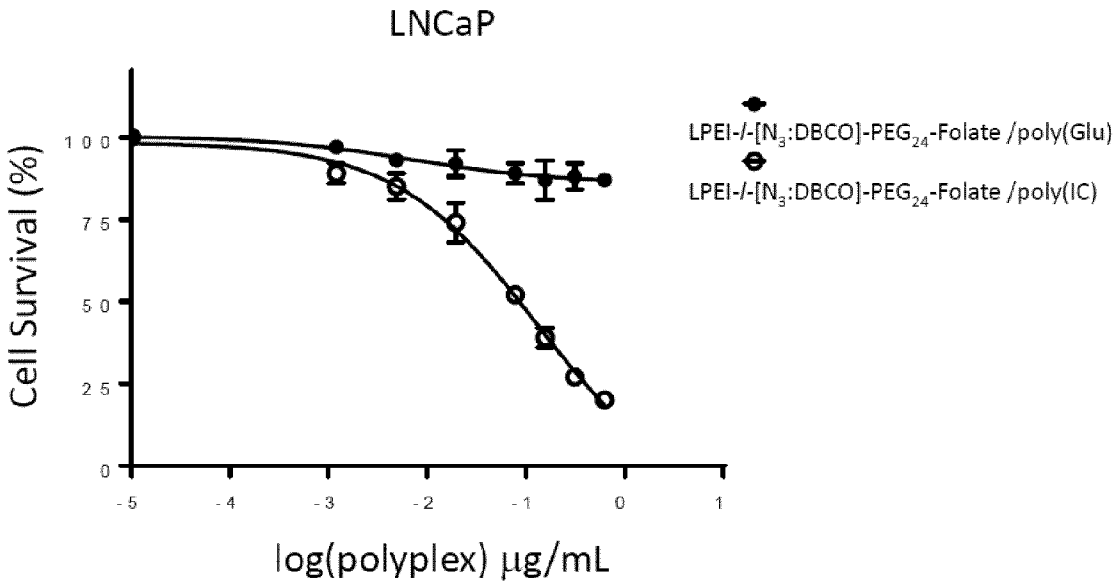

LNCaP
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI-/-[N3:DBCO]-PEG24-Folate /poly(Glu)
LPEI-/-[N3:DBCO]-PEG24-Folate /poly(IC)

FIG 5D

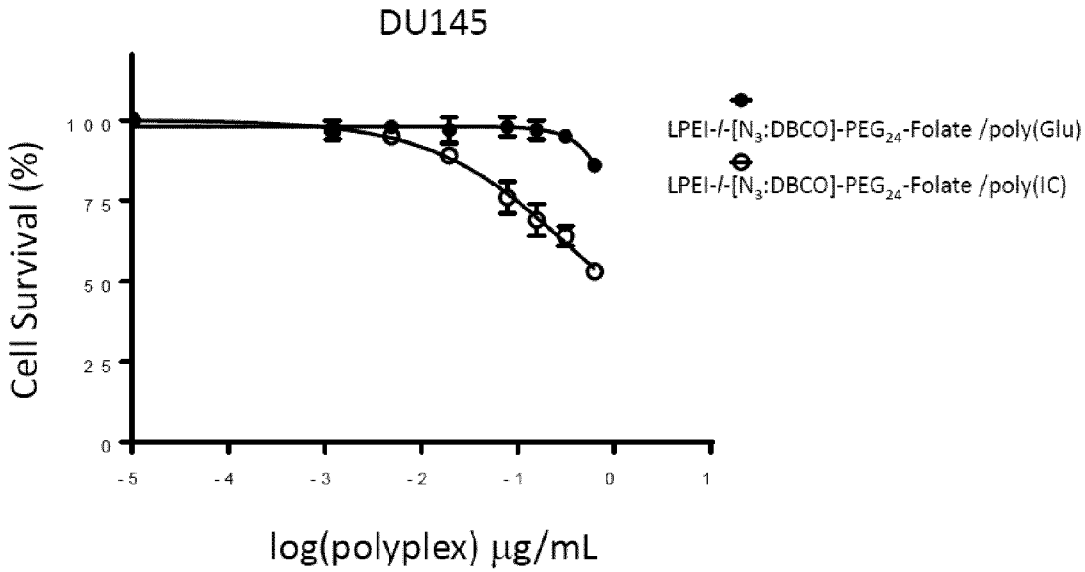

DU145
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI-/-[N3:DBCO]-PEG24-Folate /poly(Glu)
LPEI-/-[N3:DBCO]-PEG24-Folate /poly(IC)

FIG 5E

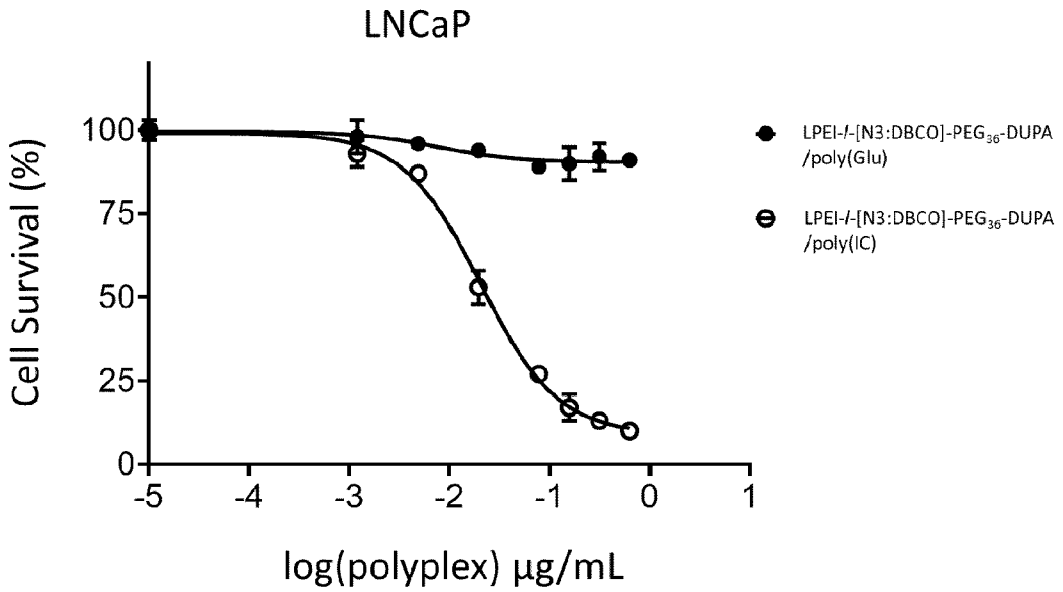

LNCaP
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI-/-[N3:DBCO]-PEG36-DUPA /poly(Glu)
LPEI-/-[N3:DBCO]-PEG36-DUPA /poly(IC)

FIG 6A

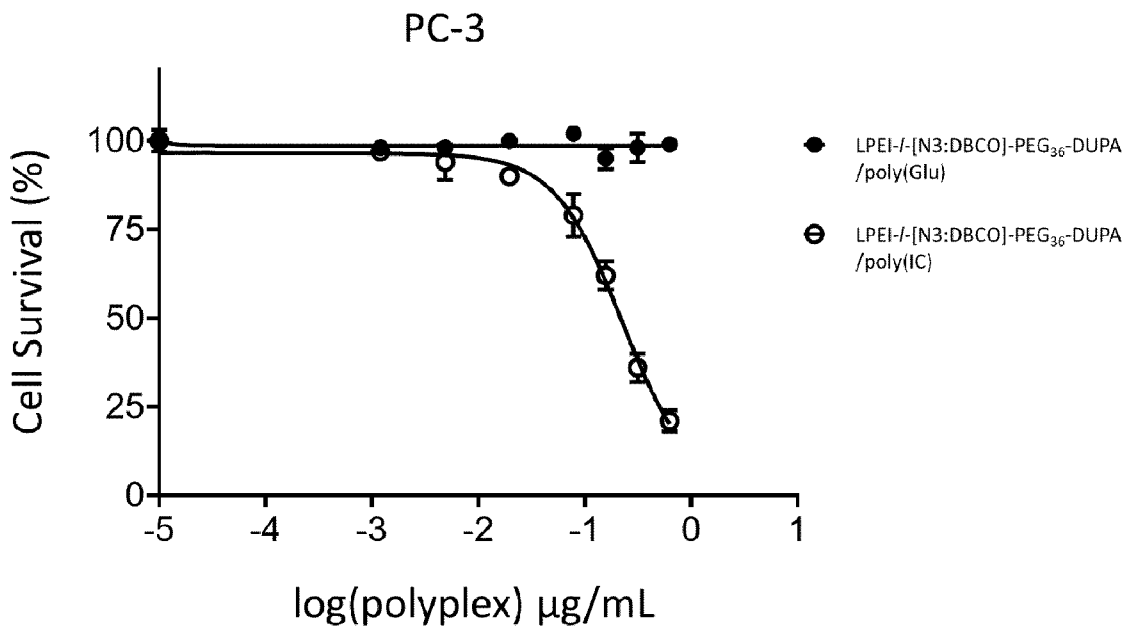

PC-3
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI-/-[N3:DBCO]-PEG36-DUPA /poly(Glu)
LPEI-/-[N3:DBCO]-PEG36-DUPA /poly(IC)

FIG 6B

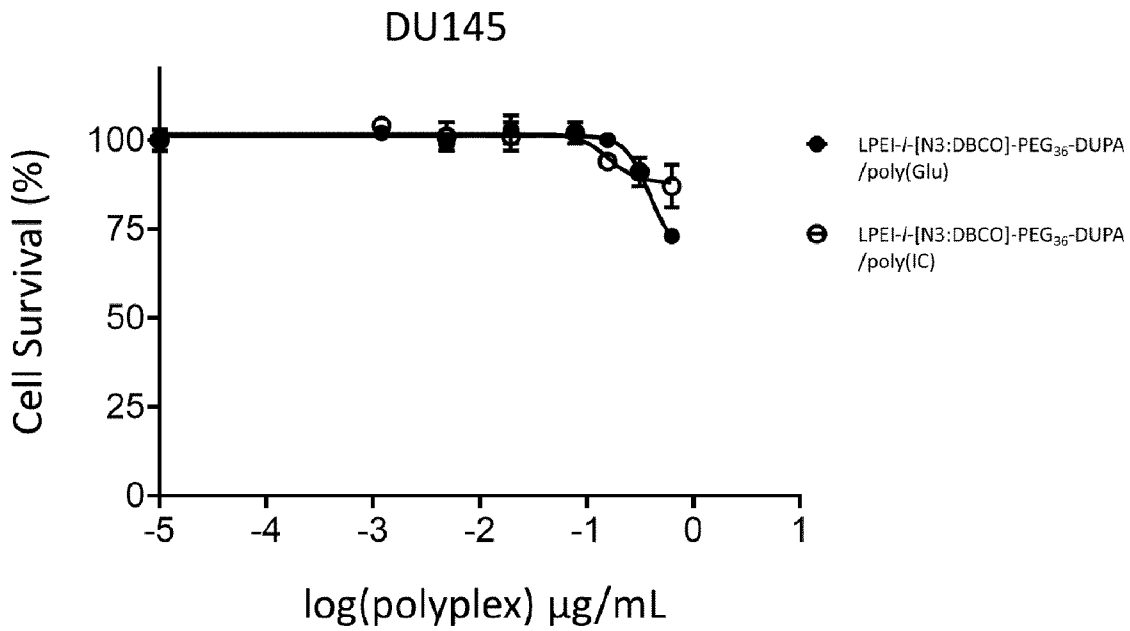

DU145
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI-/-[N3:DBCO]-PEG36-DUPA /poly(Glu)
LPEI-/-[N3:DBCO]-PEG36-DUPA /poly(IC)

FIG 6C

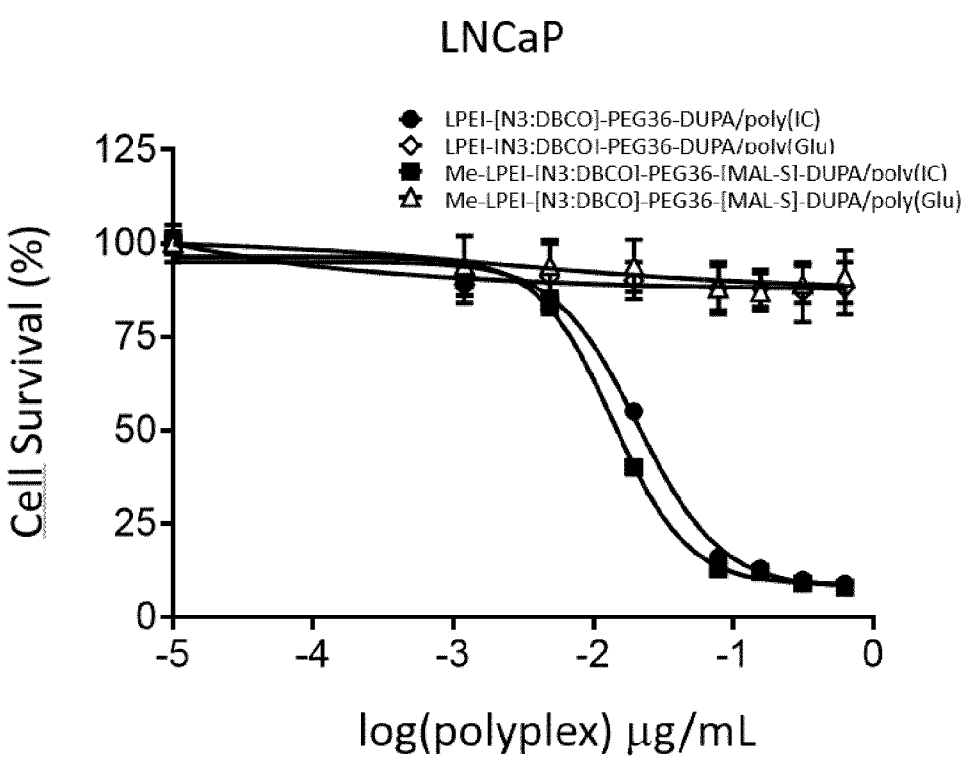

LNCaP
LPEI-[N3:DBCO]-PEG36-DUPA/poly(IC)
LPEI-[N3:DBCO]-PEG36-DUPA/poly(Glu)
Me-LPEI-[N3:DBCO]-PEG36-[MAL-S]-DUPA/poly(IC)
Me-LPEI-[N3:DBCO]-PEG36-[MAL-S]-DUPA/poly(Glu)
Cell Survival (%)
125
100
75
50
25
0
-5
-4
-3
-2
-1
0
log(polyplex) µg/mL

FIG 7

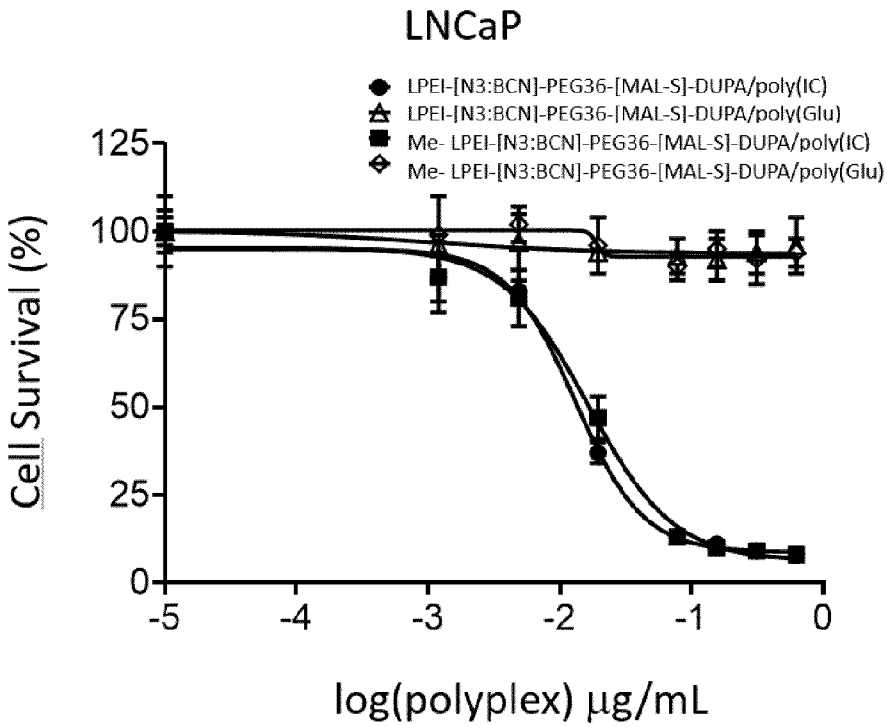

LNCaP
LPEI-[N3:BCN]-PEG36-[MAL-S]-DUPA/poly(IC)
LPEI-[N3:BCN]-PEG36-[MAL-S]-DUPA/poly(Glu)
Me- LPEI-[N3:BCN]-PEG36-[MAL-S]-DUPA/poly(IC)
Me- LPEI-[N3:BCN]-PEG36-[MAL-S]-DUPA/poly(Glu)
Cell Survival (%)
125
100
75
50
25
0
-5
-4
-3
-2
-1
0
log(polyplex) µg/mL

FIG 8

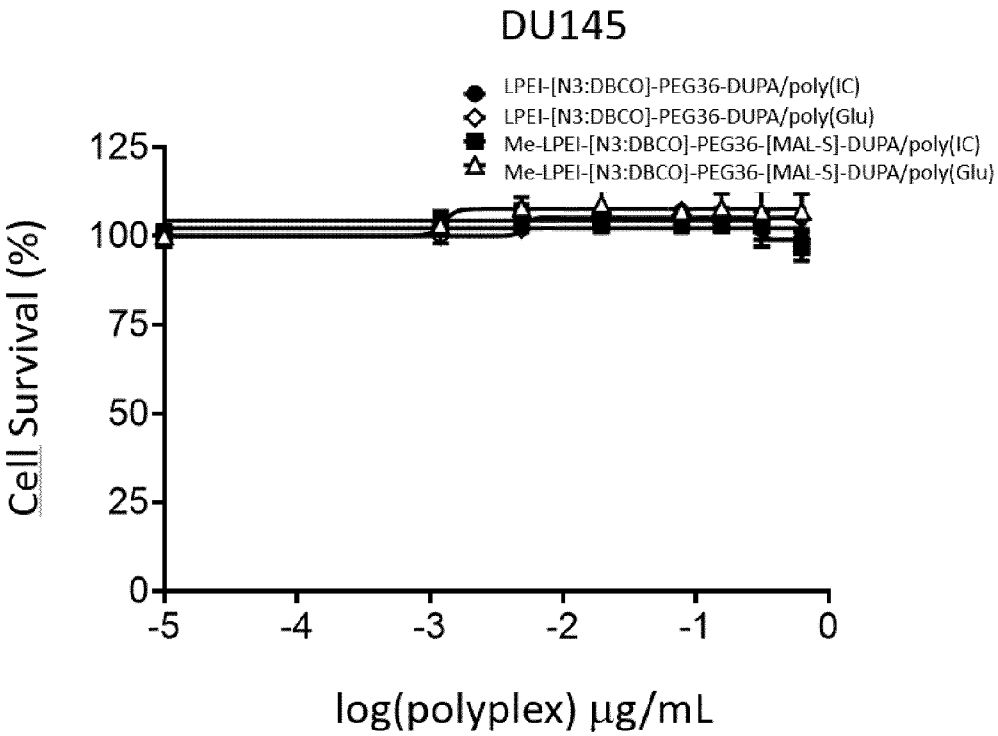

DU145
LPEI-[N3:DBCO]-PEG36-DUPA/poly(IC)
LPEI-[N3:DBCO]-PEG36-DUPA/poly(Glu)
Me-LPEI-[N3:DBCO]-PEG36-[MAL-S]-DUPA/poly(IC)
Me-LPEI-[N3:DBCO]-PEG36-[MAL-S]-DUPA/poly(Glu)
Cell Survival (%)
125
100
75
50
25
0
-5
-4
-3
-2
-1
0
log(polyplex) µg/mL

FIG 9

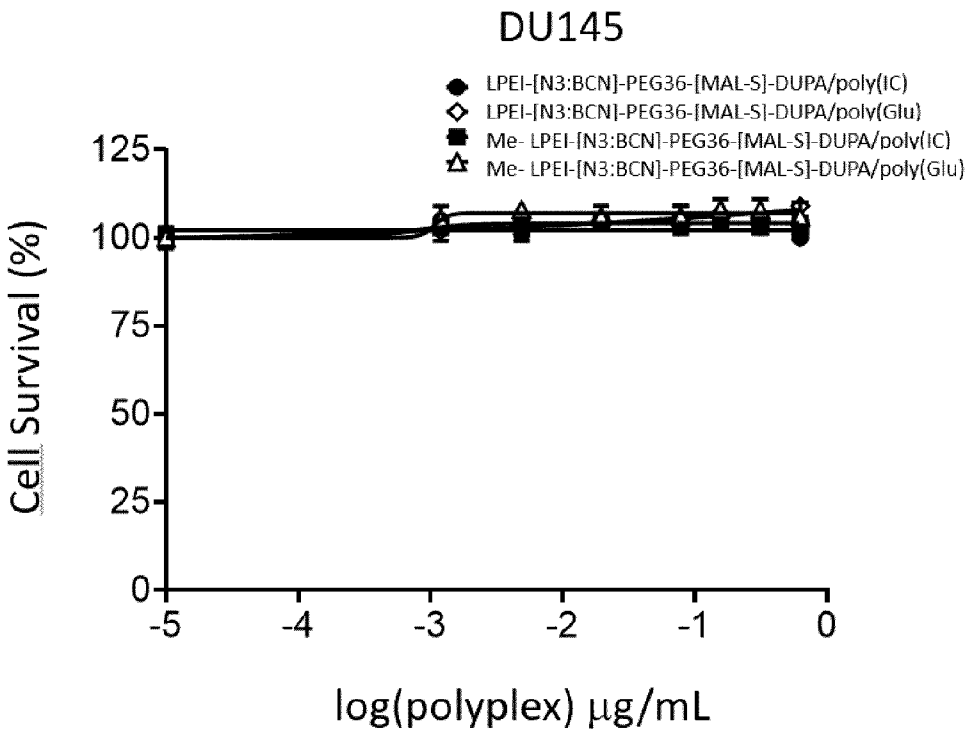

DU145
LPEI-[N3:BCN]-PEG36-[MAL-S]-DUPA/poly(IC)
LPEI-[N3:BCN]-PEG36-[MAL-S]-DUPA/poly(Glu)
Me- LPEI-[N3:BCN]-PEG36-[MAL-S]-DUPA/poly(IC)
Me- LPEI-[N3:BCN]-PEG36-[MAL-S]-DUPA/poly(Glu)
Cell Survival (%)
125
100
75
50
25
0
-5
-4
-3
-2
-1
0
log(polyplex) µg/mL

FIG 10

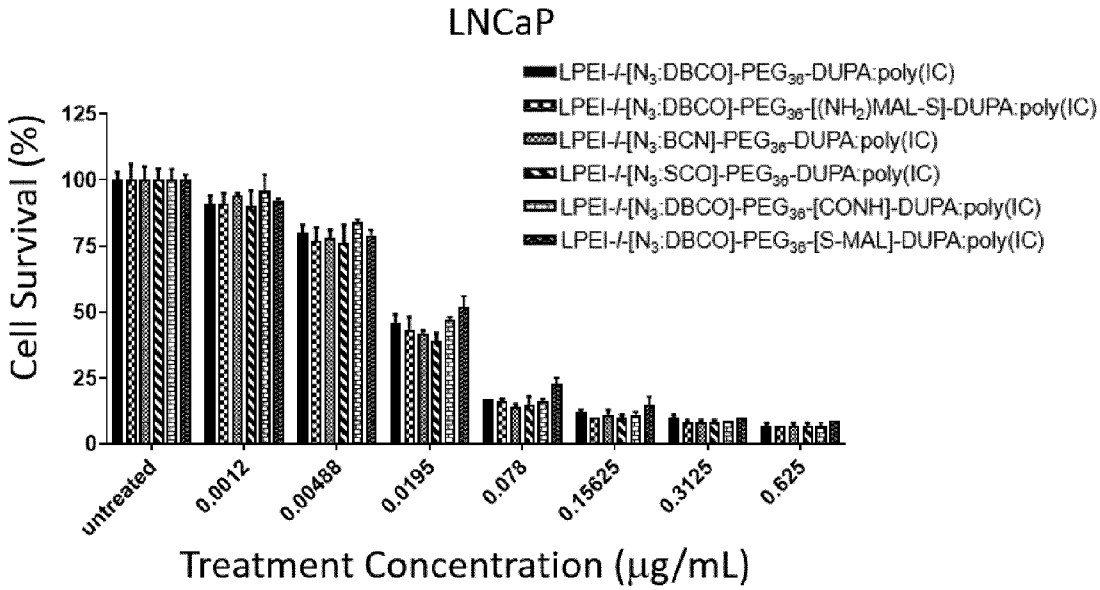

LNCaP
LPEI-/-[N3:DBCO]-PEG36-DUPA:poly(IC)
LPEI-/-[N3:DBCO]-PEG36-[(NH2)MAL-S]-DUPA:poly(IC)
LPEI-/-[N3:BCN]-PEG36-DUPA:poly(IC)
LPEI-/-[N3:SCO]-PEG36-DUPA:poly(IC)
LPEI-/-[N3:DBCO]-PEG36-[CONH]-DUPA:poly(IC)
LPEI-/-[N3:DBCO]-PEG36-[S-MAL]-DUPA:poly(IC)
Cell Survival (%)
125
100
75
50
25
0
untreated
0.0012
0.00488
0.0195
0.078
0.15625
0.3125
0.625
Treatment Concentration (µg/mL)

FIG 11

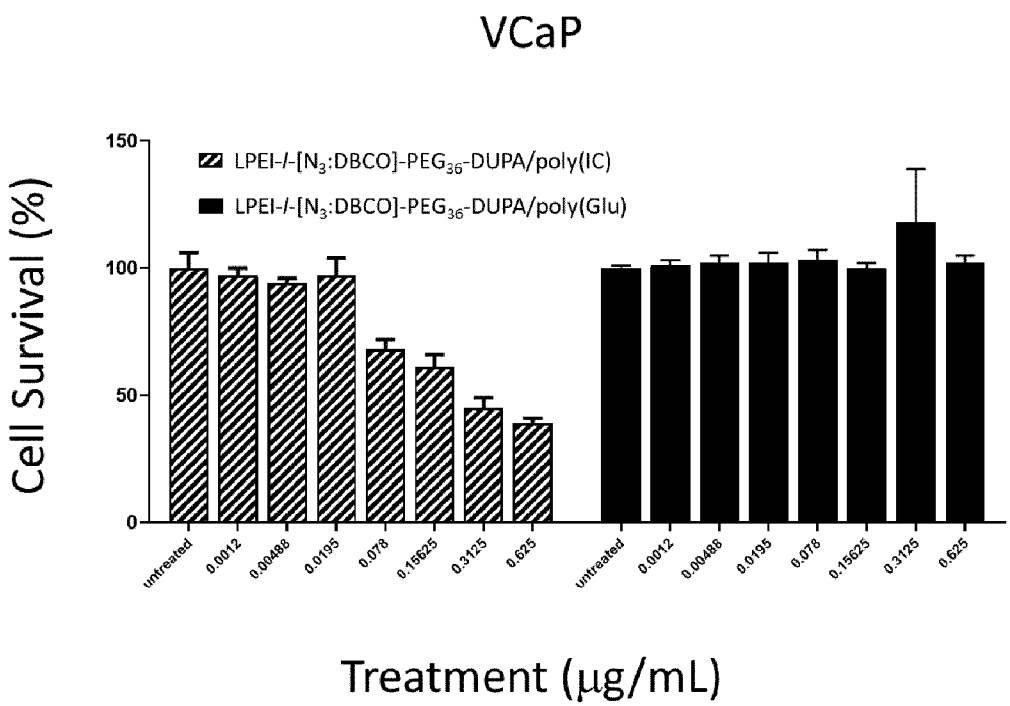

VCaP
LPEI-/-[N3:DBCO]-PEG36-DUPA/poly(IC)
LPEI-/-[N3:DBCO]-PEG36-DUPA/poly(Glu)
Cell Survival (%)
150
100
50
0
untreated
0.0012
0.00488
0.0195
0.078
0.15625
0.3125
0.625
Treatment (µg/mL)

FIG 12

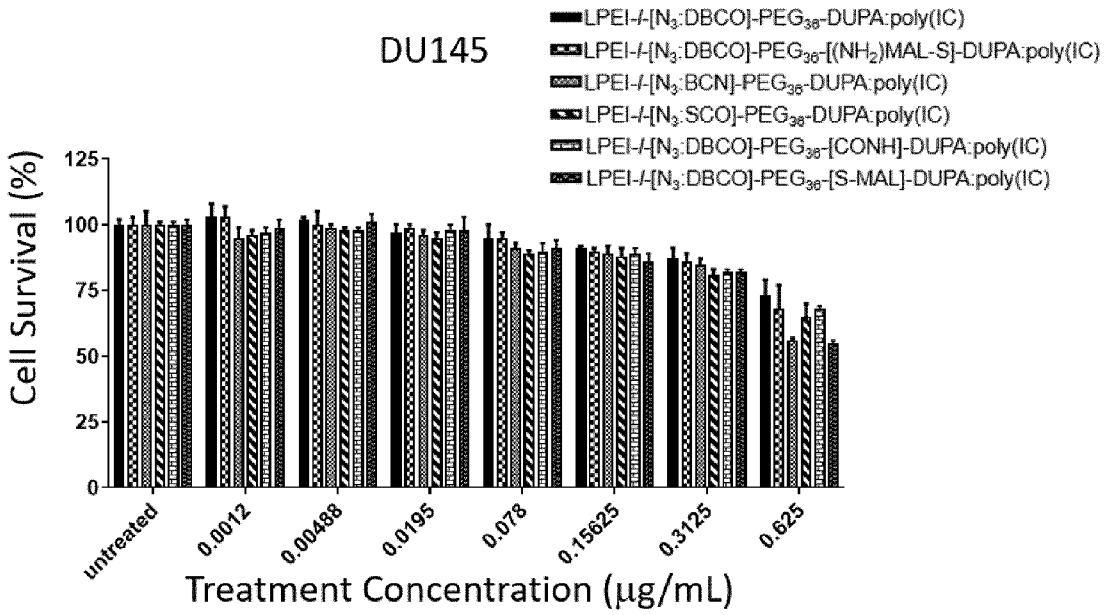

DU145
LPEI-/-[N3:DBCO]-PEG36-DUPA:poly(IC)
LPEI-/-[N3:DBCO]-PEG36-[(NH2)MAL-S]-DUPA:poly(IC)
LPEI-/-[N3:BCN]-PEG36-DUPA:poly(IC)
LPEI-/-[N3:SCO]-PEG36-DUPA:poly(IC)
LPEI-/-[N3:DBCO]-PEG36-[CONH]-DUPA:poly(IC)
LPEI-/-[N3:DBCO]-PEG36-[S-MAL]-DUPA:poly(IC)
Cell Survival (%)
125
100
75
50
25
0
untreated
0.0012
0.00488
0.0195
0.078
0.15625
0.3125
0.625
Treatment Concentration (µg/mL)

FIG 13

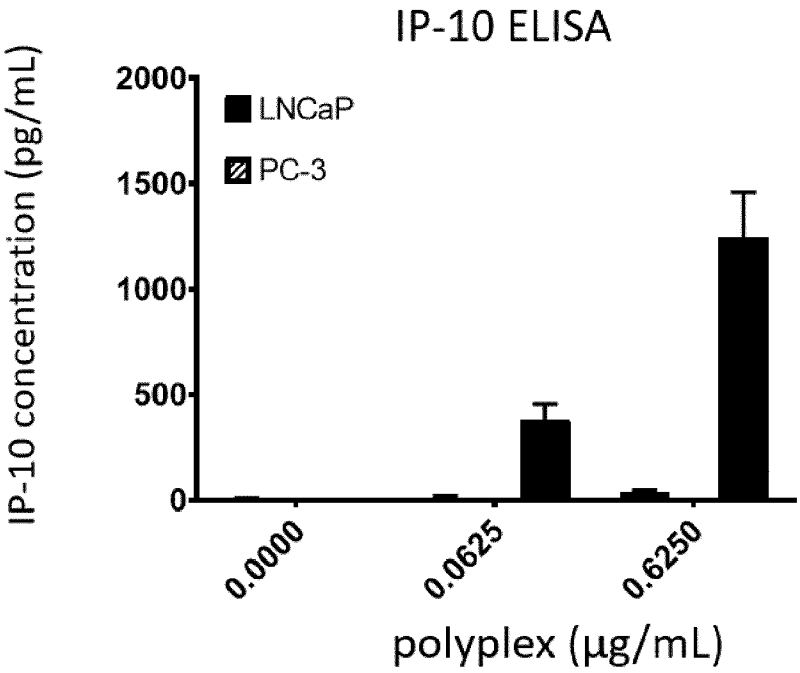


FIG 14A

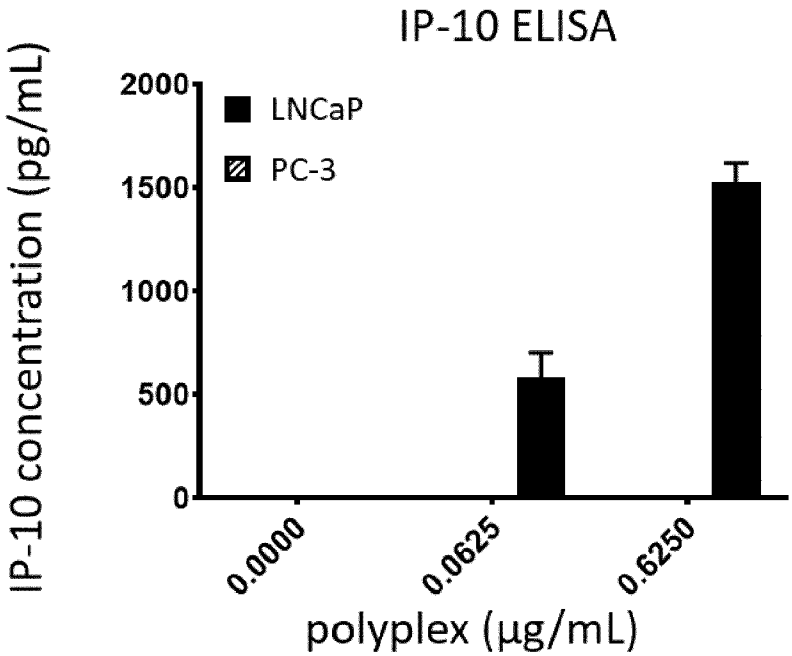


FIG 14B

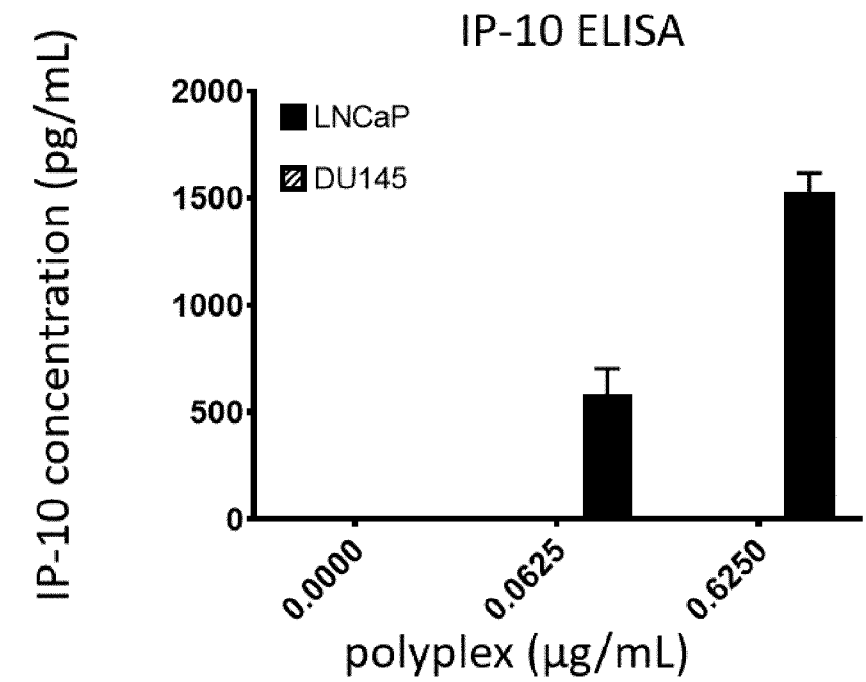


FIG 14C

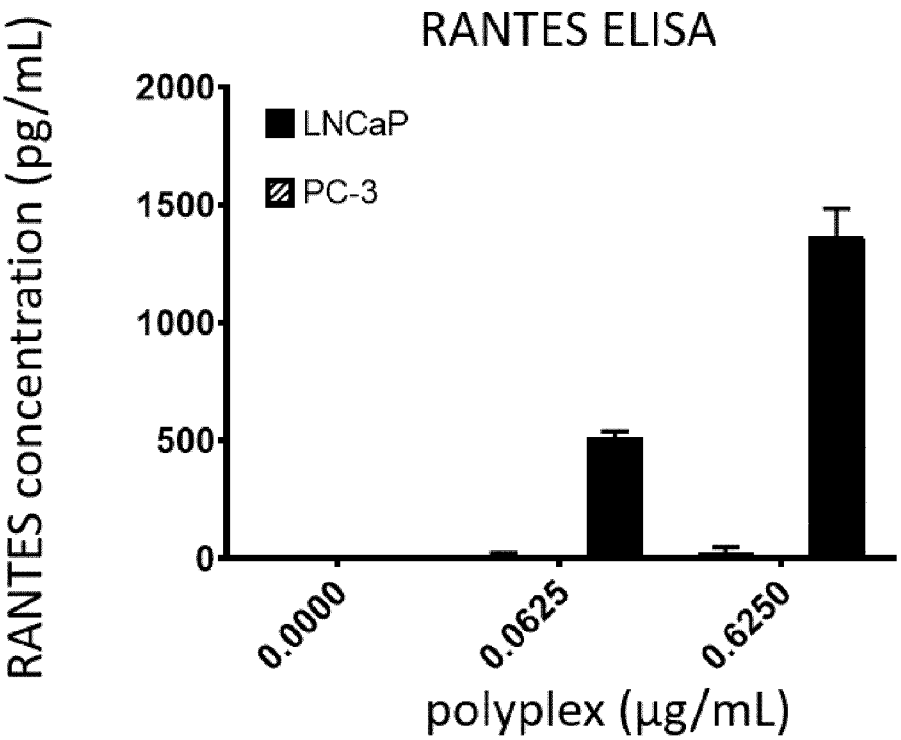


FIG 15A

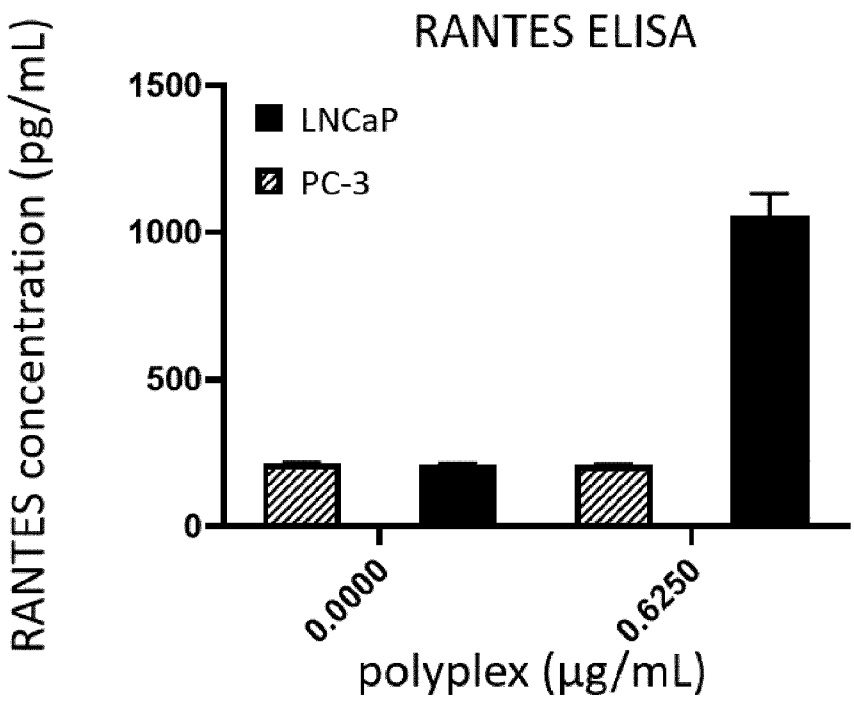


FIG 15B

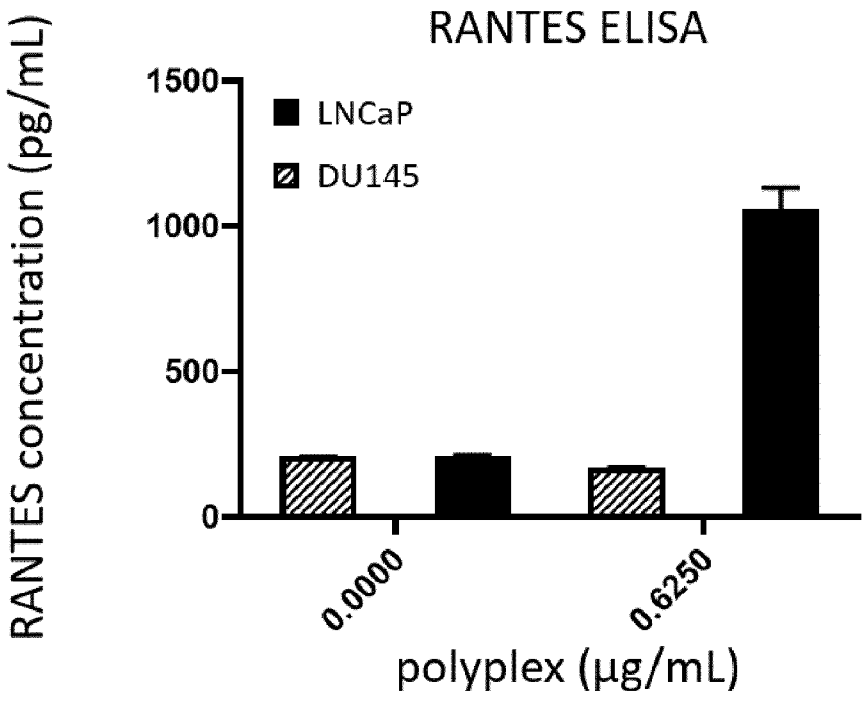


FIG 15C

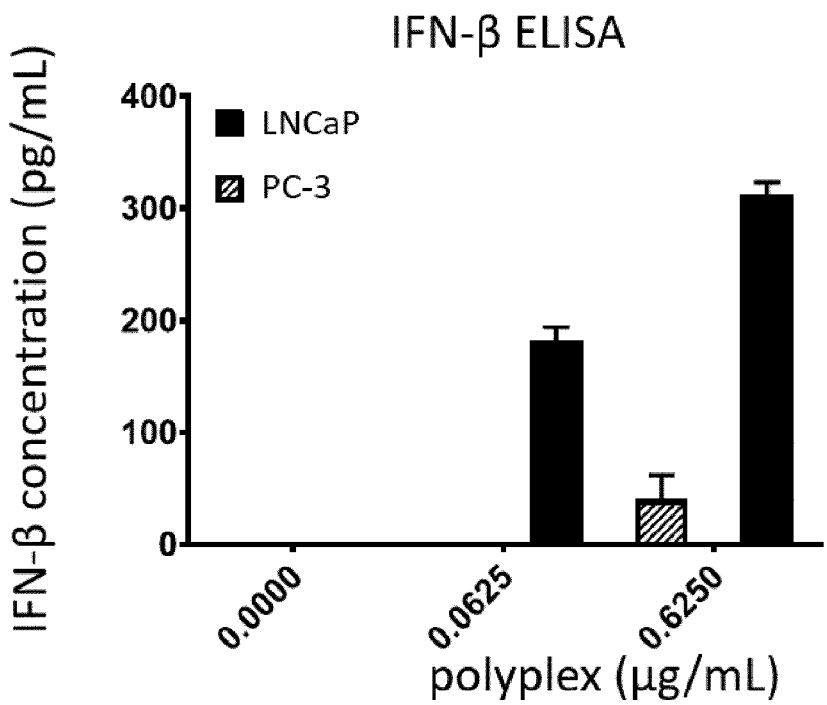


FIG 16A

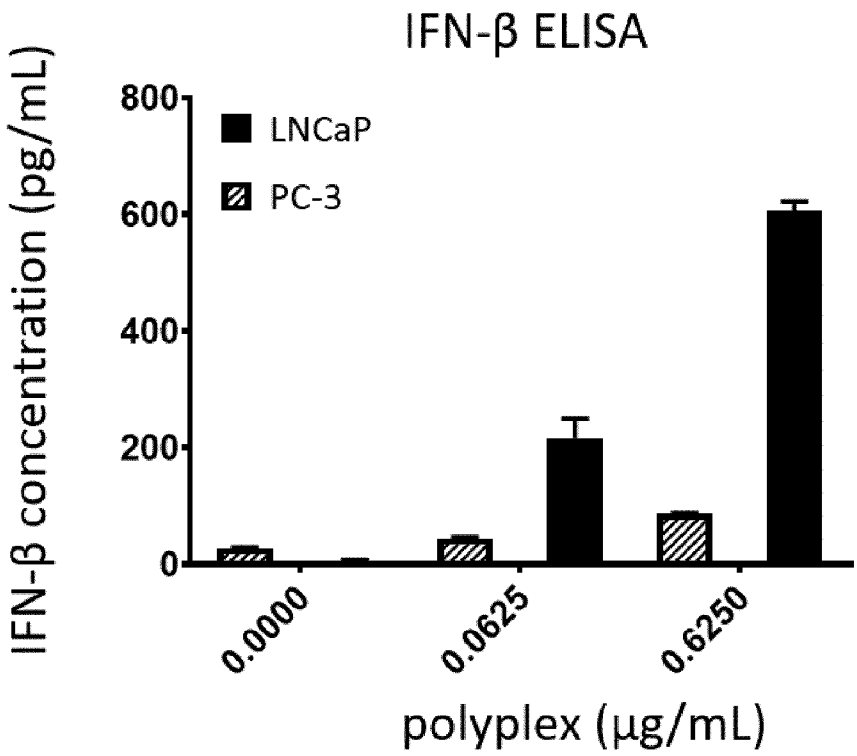


FIG 16B

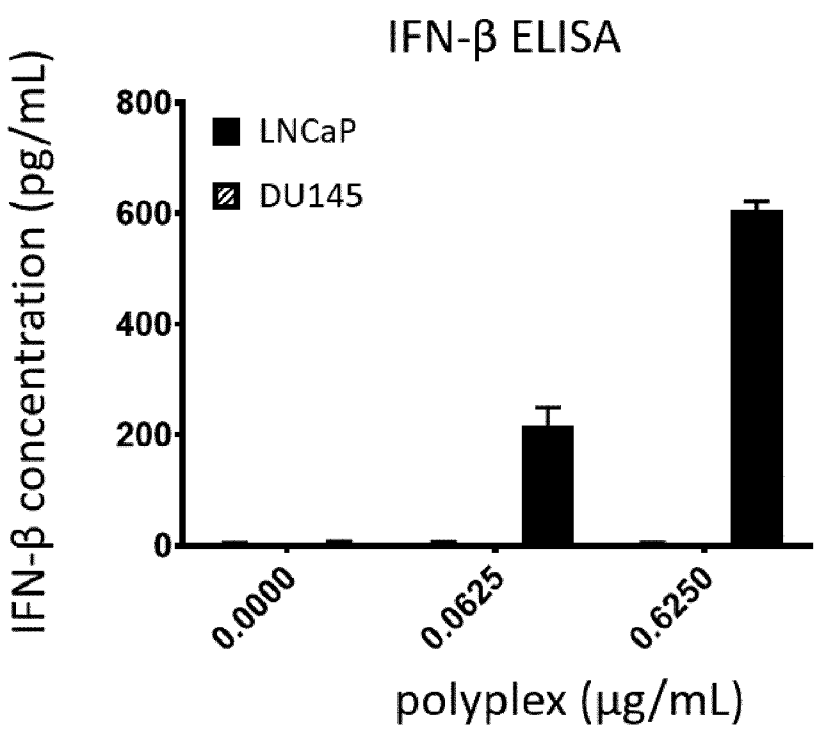

IFN-β ELISA
IFN-β concentration (pg/mL)
800
600
400
200
0
LNCaP
DU145
0.0000
0.0625
0.6250
polyplex (µg/mL)

FIG 16C

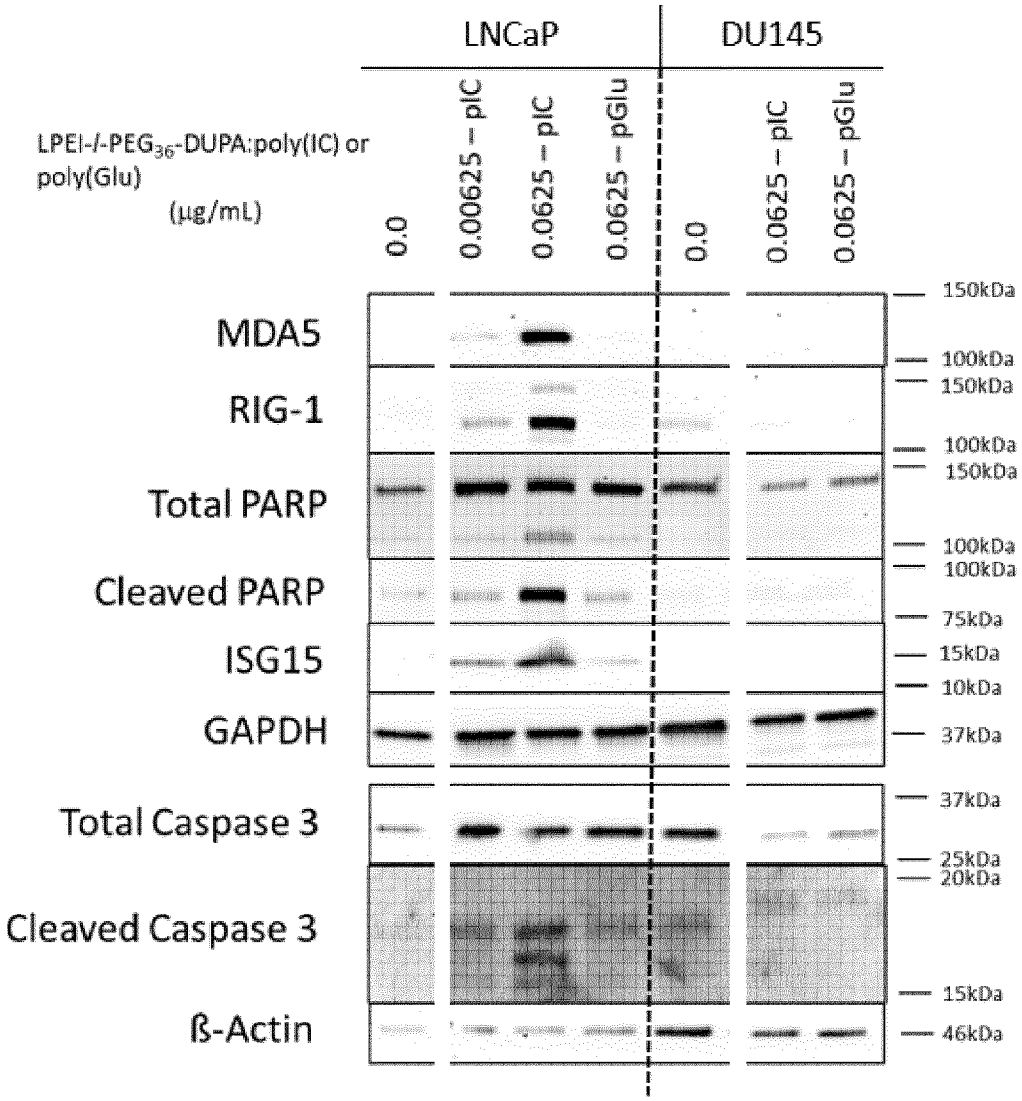

LNCaP
DU145
LPEI-/-PEG36-DUPA:poly(IC) or poly(Glu)
(µg/mL)
0.0
0.00625 – pIC
0.0625 – pIC
0.0625 – pGlu
0,0
0.0625 – pIC
0.0625 – pGlu
MDA5
RIG-1
Total PARP
Cleaved PARP
ISG15
GAPDH
Total Caspase 3
Cleaved Caspase 3
ß-Actin
150kDa
100kDa
150kDa
100kDa
150kDa
100kDa
100kDa
75kDa
15kDa
10kDa
37kDa
37kDa
25kDa
20kDa
15kDa
46kDa

FIG 17

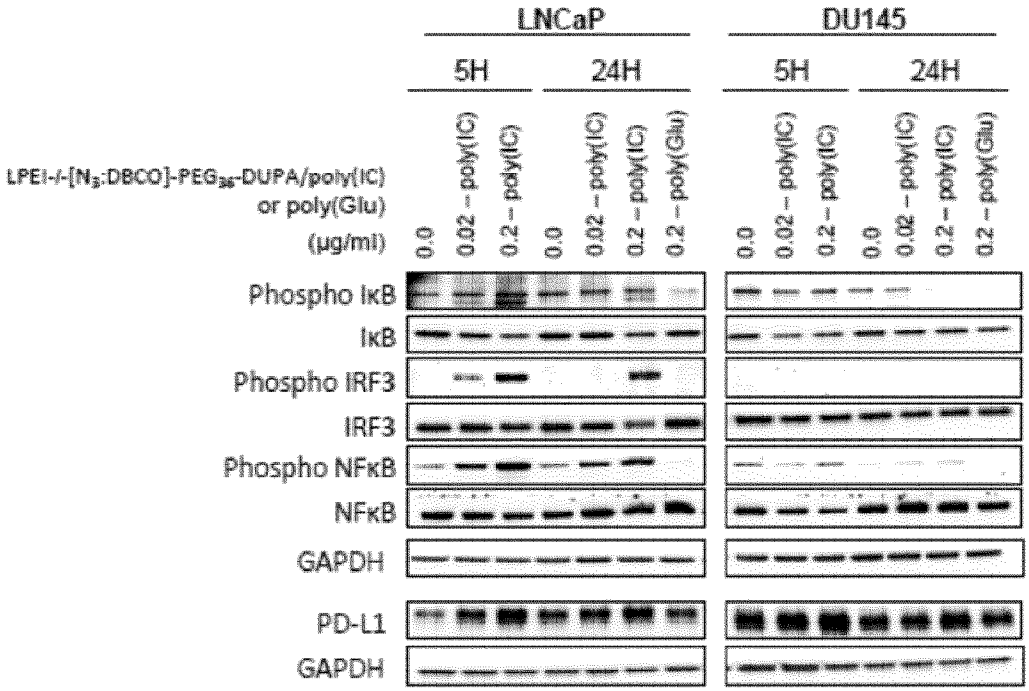

LNCaP
DU145
5H
24H
5H
24H
LPEI-f-[N3:DBCO]-PEG35-DUPA/poly(IC) or poly(Glu) (μg/ml)
0.0
0.02 – poly(IC)
0.2 – poly(IC)
0.0
0.02 – poly(IC)
0.2 – poly(IC)
0.2 – poly(Glu)
0.0
0.02 – poly(IC)
0.2 – poly(IC)
0.0
0.02 – poly(IC)
0.2 – poly(IC)
0.2 – poly(Glu)
Phospho IκB
IκB
Phospho IRF3
IRF3
Phospho NFκB
NFκB
GAPDH
PD-L1
GAPDH

FIG 18

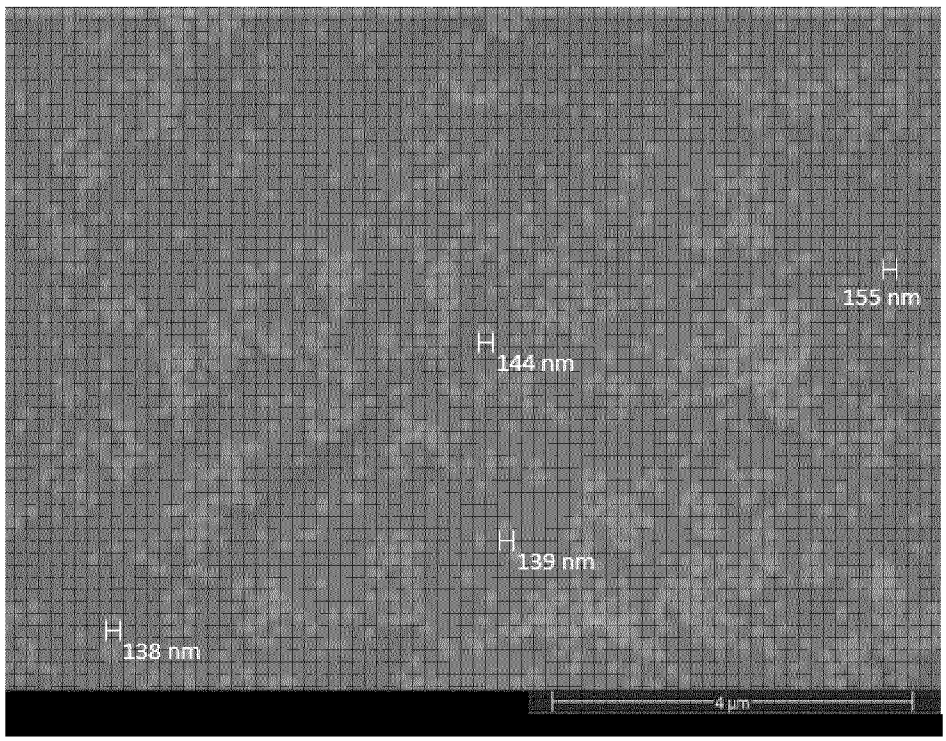

155 nm
144 nm
139 nm
138 nm
4 μm

FIG 19

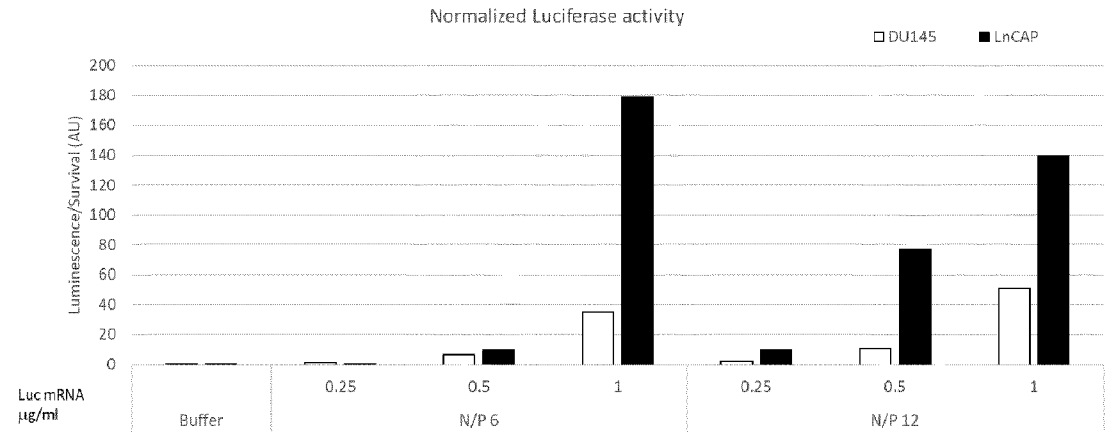


FIG 20

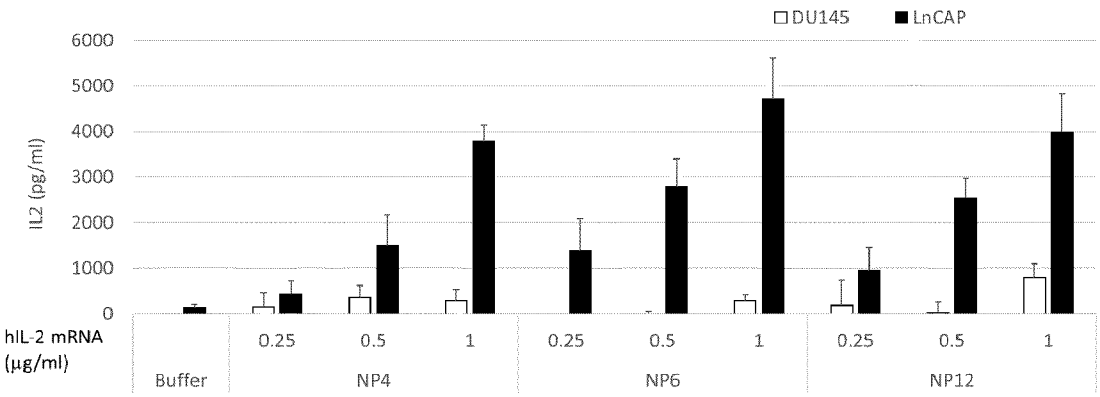


FIG 21

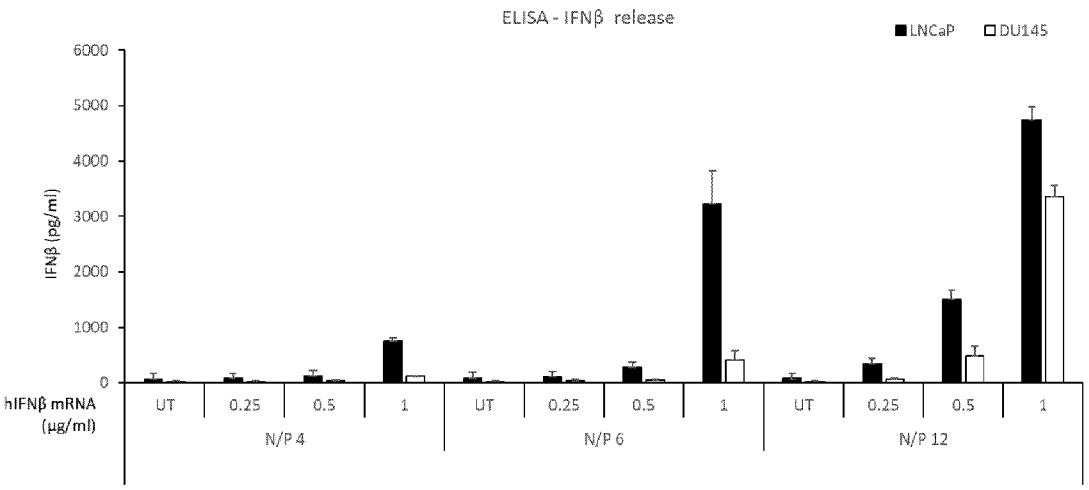


FIG 22

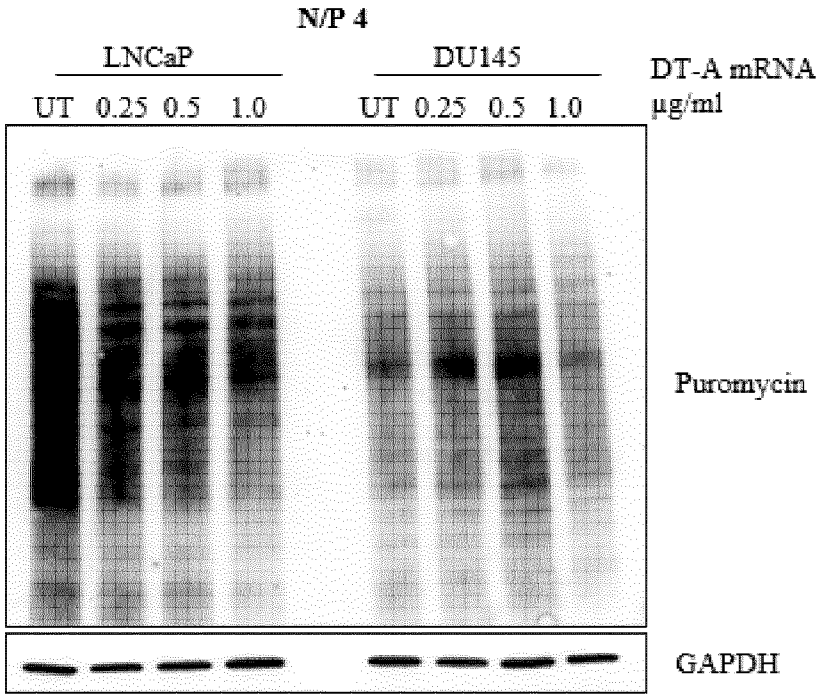

N/P 4
LNCaP
DU145
DT-A mRNA
µg/ml
UT 0.25 0.5 1.0
UT 0.25 0.5 1.0
Puromycin
GAPDH

FIG 23

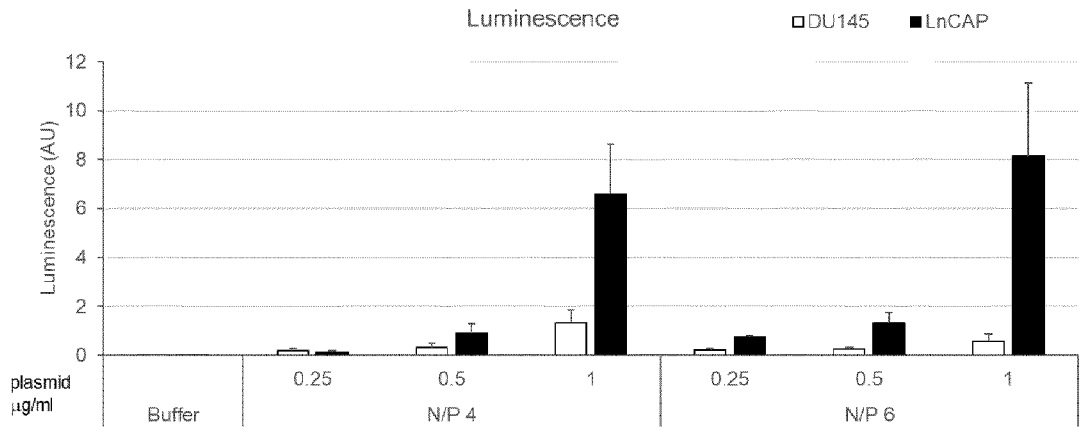

Luminescence
DU145
LnCAP
Luminescence (AU)
12
10
8
6
4
2
0
plasmid µg/ml
Buffer
0.25
0.5
1
0.25
0.5
1
N/P 4
N/P 6

FIG 24

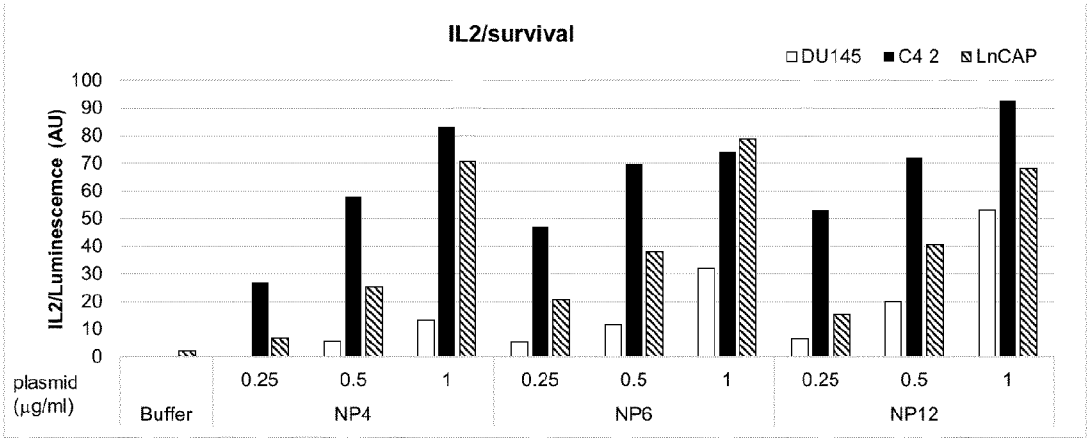

IL2/survival
DU145
C4 2
LnCAP
IL2/Luminescence (AU)
100
90
80
70
60
50
40
30
20
10
0
plasmid (µg/ml)
Buffer
0.25
0.5
1
0.25
0.5
1
0.25
0.5
1
NP4
NP6
NP12

FIG 25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/080997

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/080997

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/60 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Anonymous: "PEI-PEGs Archives - NSP-Functional Polymers & Copolymers", , 1 May 2021 (2021-05-01), pages 1-4, XP055912895, Retrieved from the Internet: URL:https://www.nanosoftpolymers.com/product-category/pei-pegs/ [retrieved on 2022-04-14] abstract	1,2
Y	WO 2020/201568 A1 (TARGIMMUNE THERAPEUTICS AG [CH]) 8 October 2020 (2020-10-08) page 54 - page 55 page 1	1-52

-/--

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 January 2024	01/02/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Langer, Miren

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2019/063705 A1 (TARGIMMUNE THERAPEUTICS AG [CH]) 4 April 2019 (2019-04-04) cited in the application claims 1-8	1-52
Y	WO 2015/173824 A1 (ALEX LEVITZKI MAN AND HOLDINGS LTD [IL]) 19 November 2015 (2015-11-19) cited in the application page 29; example 10	1-52
Y	JEFF HENISE ET AL: "Biodegradable Tetra-PEG Hydrogels as Carriers for a Releasable Drug Delivery System", BIOCONJUGATE CHEMISTRY, vol. 26, no. 2, 13 January 2015 (2015-01-13), pages 270-278, XP055637653, US ISSN: 1043-1802, DOI: 10.1021/bc5005476 figure 4	1-52
Y	BARKER KAROLYN ET AL: "Biodegradable DNA-enabled poly(ethylene glycol) hydrogels prepared by copper-free click chemistry", JOURNAL OF BIOMATERIALS SCIENCE. POLYMER EDITION. , vol. 27, no. 1 6 November 2015 (2015-11-06), pages 22-39, XP055777173, NL ISSN: 0920-5063, DOI: 10.1080/09205063.2015.1103590 Retrieved from the Internet: URL:https://www.tandfonline.com/doi/pdf/10.1080/09205063.2015.1103590?needAccess=true abstract page 27 - page 28	1-52
Y	ENRIQUE LALLANA ET AL: "Click Chemistry for Drug Delivery Nanosystems", PHARMACEUTICAL RESEARCH, KLUWER ACADEMIC PUBLISHERS-PLENUM PUBLISHERS, NL, vol. 29, no. 1, 13 September 2011 (2011-09-13), pages 1-34, XP019993267, ISSN: 1573-904X, DOI: 10.1007/S11095-011-0568-5 figure 5	1-52

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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