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(71) Applicant: **TARGIMMUNE THERAPEUTICS AG** [CH/CH]; Hochbergerstrasse 60c, 4057 Basel (CH).

(72) Inventors: **KITAS, Eric**; Eichbergweg 4, 4147 Aesch (CH). **ZIGLER, Maya**; Hirzbodenweg 17, 4052 Basel (CH). **POMBO-VILLAR, Esteban**; Höhenweg 63, 4102 Binningen (CH).

(74) Agent: **SPERRLE, Martin**; Latscha Schöllhorn Partner AG Grellingerstrasse 60, 4052 Basel (CH).

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(54) **Title:** TARGETED 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 preferably further conjugated to a targeting fragment 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.

TARGETED 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 delivers 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 supramolecular 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 supramolecular polyplexes can be taken up by cells *in vivo* where they can deliver the nucleic acid sequences intracellularly. Accordingly, supramolecular polyplexes comprising cationic polymers and nucleic acids can be used as vectors for therapy.

Despite their promise, technical challenges have arisen related to forming homogenous 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 or co-polymerized with 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 generally 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 with random inclusion of PEG fragments that are characterized on the basis of average PEG inclusion density. In such conjugates, PEG fragments, be it one or a multiple number, are bonded orthogonally to the LPEI fragment with generally 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 supramolecular polyplex. Accordingly, there is a need for homogenous LPEI-PEG conjugates with well-defined chemical structures.

SUMMARY OF THE INVENTION

The present invention provides conjugates comprising LPEI and 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 one of multiple nucleophiles of an LPEI backbone fragment. The discrete linkages not only ensure consistent and predictable ratios of LPEI to PEG fragments, but further ensure defined linear instead of random branched conjugates. Thus, the LPEI fragment is bonded in a linear end-to-end fashion to a single PEG fragment. The chemoselective bonding of the LPEI fragments to the 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 PEG fragments takes place by means of a [3+2] cycloaddition between an azide and an alkyne or alkene. Alternatively, said chemoselective bonding is by means of a thiol-ene reaction between a thiol and an alkene. When the chemoselective bond is between an azide and an alkyne or alkene, the resulting linkage is a 1,2,3-triazole (when an alkyne is coupled) or a 4,5-dihydro-1H-[1,2,3]triazole (when an alkene is coupled). When the chemoselective bond is between a thiol and an alkene, the resulting linkage is a thioether.

For the preferred conjugates of the present invention, the PEG fragment is further selectively linked with a targeting fragment to target a particular cell type so to target and facilitate the uptake of the inventive compositions, conjugates and/or polyplexes in said particular cell type. Thus, preferred embodiments comprise one or more (e.g., one) targeting fragment(s) such as hEGF, HER2 ligand, DUPA or folate or the like specifically connected to the LPEI-PEG diconjugates forming LPEI-PEG-Targeting fragment triconjugates, and capable of targeting the corresponding receptors such hEGFR, HER2, PSMA or folate 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 advantageously and surprisingly, the inventors have found that the resulting preferred conjugates and polyplexes in accordance with the present invention having a significant reduced heterogeneity due to the defined chemoselective bonding of the LPEI fragments to the PEG fragments, and thus a significant reduced number of potentially biologically active conjugates and polyplexes, not only form polyplexes of suitable size, 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.

Thus, in one aspect, the present invention provides a composition comprising a conjugate, wherein said conjugate comprises: a linear polyethyleneimine (LPEI) fragment comprising an alpha terminus and an omega terminus; a polyethylene glycol (PEG) fragment, preferably a linear polyethylene glycol (PEG) fragment, comprising a first terminal end and a second terminal end; wherein the omega terminus of the LPEI fragment is connected by a covalent linking moiety to the first terminal end of the PEG fragment; wherein said covalent linking moiety is not an amide; preferably wherein the alpha terminus of the LPEI fragment is bonded to a methyl group or a hydrogen atom, further preferably wherein the alpha terminus of the LPEI fragment is bonded to hydrogen atom; and preferably wherein the second terminal end of the PEG fragment is bonded to a targeting fragment.

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 the alpha terminus of said polyethyleneimine fragment is an initiation residue; wherein the omega terminus of the polyethyleneimine fragment is connected by a covalent linking moiety to the first terminal end of the polyethylene glycol fragment; wherein said covalent linking moiety is not a single bond and is not an amide; and wherein preferably the second terminal end of the polyethylene glycol fragment is capable of reacting, preferably wherein said second terminal end is capable of binding to a targeting fragment.

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 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 covalent linking group -Z-X^1-, wherein -Z- is not a single bond and -Z- is not an amide; wherein -X^1- is a divalent covalent linking moiety;

wherein the second terminal end of the polyethylene glycol fragment is capable of binding, preferably said polyethylene glycol fragment binds, to a targeting fragment. In a preferred embodiment of this aspect, 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:

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 any integer between 1 and 100;

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 preferably said targeting fragment is capable of binding to a cell, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, 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 any integer between 1 and 100;

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

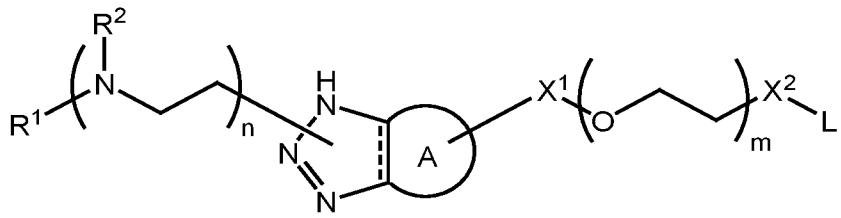
R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said $-(NR^2-CH_2-CH_2)_n-$ is H;

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 preferably said targeting fragment is capable of binding to a cell, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor.

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 any integer between 1 and 200;

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;

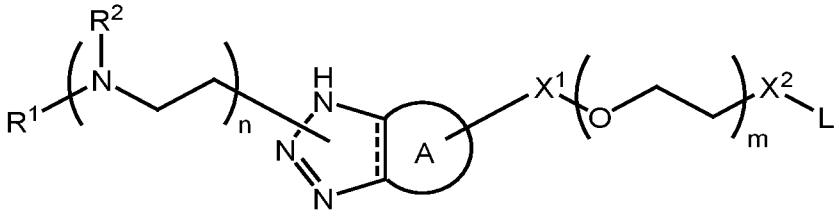
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor.

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 any integer between 1 and 200;

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;

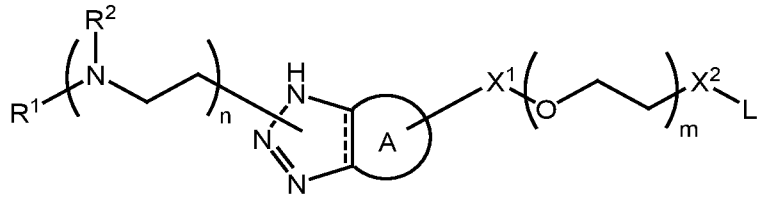
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor.

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 any integer between 1 and 200, preferably m is any integer between 1 and 100;

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 $-OSO_3H$;

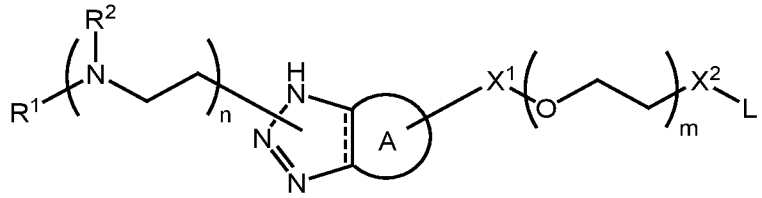
X^1 is a linking moiety of the formula $-(Y^1)_p-$, wherein p is an integer between 1 and 20, and each occurrence of Y^1 is independently selected from a chemical bond, $-CR^{11}R^{12}-$, -C(O)-, -O-, -S-, $-NR^{13}-$, an amino acid residue, a divalent phenyl moiety, a divalent heterocycle moiety, and 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 preferably capable of binding to a cell, 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:

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

n is any integer between 1 and 1500;

m is any integer between 1 and 200, preferably m is any integer between 1 and 100;

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 preferably capable of binding to a cell.

In a further aspect, the present invention provides a method of synthesizing a composition comprising a conjugate 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 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.

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.

In another aspect, the present invention provides a method of treating a disease or disorder such as a 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 XXX surprisingly not only maintain, but even increase their antitumor activity as 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 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 1A is a DLS back scatter plot taken in triplicate of a LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) polyplex measuring size distribution in HBG buffer at pH 7.2, 0.125 mg/mL, N/P ratio of 2.4. The z-average diameter was 306 nm with a polydispersity index (PDI) of 0.35.

FIG 1B is a DLS back scatter plot taken in triplicate of a LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) polyplex measuring size distribution in HBG buffer at pH 7.2, 0.125 mg/mL, N/P ratio of 4.0. The z-average diameter was 116 nm with a polydispersity index (PDI) of 0.08.

FIG 1C is a DLS back scatter plot taken in triplicate of a LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) polyplex measuring size distribution in HBG buffer at pH 7.2, 0.125 mg/mL, N/P ratio of 5.6. The z-average diameter was 107 nm with a polydispersity index (PDI) of 0.109.

FIG 2 is a DLS back scatter plot taken in triplicate of a LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu) polyplex measuring size distribution and ζ-potential in HBG buffer at pH 7.2, 0.1 mg/mL, 1 mL volume, N/P ratio of 4. The z-average diameter was 121 nm with a polydispersity index (PDI) of 0.087. The ζ-potential was 28.7 mV.

FIG 3 is a DLS back scatter plot taken in triplicate of a LPEI-*l*-[N_3:DBCO]-PEG_{23}-OMe:poly(IC) polyplex measuring size distribution and ζ-potential in 50 mM acetate buffer, 5% glucose at pH 4.3, 0.1875 mg/mL, 1 mL volume, N/P ratio of 4. The z-average diameter was 107 nm with a polydispersity index (PDI) of 0.139. The ζ-potential was 31.4 mV.

FIG 4 is a DLS back scatter plot taken in triplicate of a LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC) polyplex measuring size distribution and ζ-potential in 50 mM acetate buffer, 5% glucose at pH 4.3, 0.1875 mg/mL, 1 mL volume, N/P ratio of 4. The z-average diameter was 156 nm with a polydispersity index (PDI) of 0.144. The ζ-potential was 38.3 mV.

FIG 5 is a DLS back scatter plot taken in triplicate of a LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) polyplex measuring distribution and ζ-potential at 0.1875 mg/mL, 1 mL volume, N/P 4. The z-average diameter was 120 nm with a polydispersity index (PDI) of 0.125. The ζ-potential was 31.1 mV.

FIG 6A is a plot of cell survival in MCF7 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF: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 A431 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.FIG 7A is a plot of cell survival in MCF7 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-

hEGF:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.FIG 7B is a plot of cell survival in A431 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 8A is a plot of cell survival in MCF7 cells as a function of treatment with LPEI-*l*--[N_3:DBCO]-PEG_{12}-hEGF:poly(IC) and LPEI-*l*--[N_3:DBCO]-PEG_{12}-hEGF:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 8B is a plot of cell survival in A431 cells as a function of treatment with LPEI-*l*--[N_3:DBCO]-PEG_{12}-hEGF:poly(IC) and LPEI-*l*--[N_3:DBCO]-PEG_{12}-hEGF:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 9A is a plot of cell survival in MCF7 cells as a function of treatment with LPEI-*l*--[N_3:DBCO]-PEG_4-hEGF:poly(IC) and LPEI-*l*--[N_3:DBCO]-PEG_4-hEGF:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 9B is a plot of cell survival in A431 cells as a function of treatment with LPEI-*l*--[N_3:DBCO]-PEG_4-hEGF:poly(IC) and LPEI-*l*--[N_3:DBCO]-PEG_4-hEGF:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 10A is a plot of cell survival in MCF7 cells as a function of treatment with non-targeted polyplexes LPEI-*l*--[N_3:DBCO]-PEG_{23}-OMe:poly(IC) and LPEI-*l*--[N_3:DBCO]-PEG_{23}-OMe:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 10B is a plot of cell survival in A431 cells as a function of treatment with non-targeted polyplexes LPEI-*l*-PEG_{23}-OMe:poly(IC) and LPEI-*l*-PEG_{23}-OMe:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 11A 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 11B 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 11C 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 12A 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 12B 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 12C 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 13 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}-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.

FIG 14 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}-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.

FIG 15 is a plot of cell survival in SKOV3 and MCF7 cells treated with LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC) polyplexes at various concentrations.

FIG 16A is a plot of cell survival in MCF7 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 16B is a plot of cell survival in SKBR3 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 16C is a plot of cell survival in BT474 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(Glu). The X axis indicates the log of concentration of poly(IC) or poly(Glu) delivered.

FIG 17 is a plot of IP-10 secretion in A431 and MCF7 cells as a function of treatment with LPEI-*l*-PEG_{24}-hEGF:poly(IC) polyplexes at 0.125, 0.25, 0.5, and 1 μg/mL.

FIG 18 is a Western Blot imaging analysis showing qualitative levels of EGFR phosphorylation as a function of treatment with full serum, starved serum, LPEI-*l*-PEG_{24}-EGF, LPEI-*l*-PEG_{24}-hEGF:poly(IC), and hEGF.

FIG 19A 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 19B 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 19C 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 20A 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 20B 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 20C 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 21A is a plot of IFN-ß secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) concentration in LNCaP cells and PC-3 cells.

FIG 21B is a plot of IFN-ß secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration in LNCaP cells and PC-3 cells.

FIG 21C is a plot of IFN-ß secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration in LNCaP cells and DU145 cells.

FIG. 22 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.

FIG 23 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 24A is a plot of luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] compared to the control delivery vehicle Messenger MAX. The luminescence was measured at N/P ratios of 4, 6 and

12, and at concentrations from 0.125 to 1.0 mg/mL of LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] and lipofectamine messenger MAX at 24 hours after treatment.

FIG 24B is a plot of luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] compared to the control delivery vehicle jetPEI. The luminescence was measured at N/P ratios of 4, 6 and 12, and at concentrations from 0.125 to 1.0 mg/mL of LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] and jetPEI at 24 hours after treatment.

FIG 24C is a plot of the ratio of luminescence (AU) between Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] with Messenger MAX as a comparison delivery vehicle. The luminescence was measured at N/P ratios of 4, 6 and 12, and at concentrations from 0.125 to 1.0 mg/mL of LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] and lipofectamine messenger MAX at 24 hours after treatment, and the ratio was calculated by dividing the luminescence signal from RencaEGFR M1 H cells by the luminescence signal from Renca parental cells.

FIG 24D is a plot of the ratio of luminescence (AU) between Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] with jetPEI as a comparison delivery vehicle. The luminescence was measured at N/P ratios of 4, 6 and 12, and at concentrations from 0.125 to 1.0 mg/mL of LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] and jetPEI at 24 hours after treatment, and the ratio was calculated by dividing the luminescence signal from RencaEGFR M1 H cells by the luminescence signal from Renca parental cells.

FIG 24E is a plot of percent survival in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] compared to the control delivery vehicle Messenger MAX. The percent survival was measured at N/P ratios of 4, 6 and 12, and at concentrations from 0.125 to 1.0 mg/mL of LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] and Messenger MAX at 24 hours after treatment.

FIG 25A shows relative luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] at 24 h after treatment at an N/P of 4.

FIG 25B shows relative luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] at 24 h after treatment at an N/P of 6.

FIG 25C shows relative luminescence (AU) in Renca parenteral cells and Renca EGFR

M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] at 48 h after treatment at an N/P of 4.

FIG 25D shows relative luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] at 48 h after treatment at an N/P of 6.

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 is 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 preferably comprise an LPEI fragment, a PEG fragment, and a targeting fragment. In preferred embodiments, 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 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 “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 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 polypetides 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 $(AA)_a$ being 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 refers 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,

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. l. 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 piece.

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 (LPEI) fragment comprising an alpha terminus and an omega terminus; a polyethylene glycol (PEG) fragment, preferably a linear polyethylene glycol (PEG) fragment, comprising a first terminal end and a second terminal end; wherein the omega terminus of the LPEI fragment is connected by a covalent linking moiety to the first terminal end of the PEG fragment; wherein said covalent linking moiety is not an amide; preferably wherein the alpha terminus of the LPEI fragment is bonded to a methyl group or a hydrogen atom, further preferably wherein the alpha terminus of the LPEI fragment is bonded to hydrogen atom; and preferably wherein the alpha terminus of the PEG fragment is bonded to a targeting fragment.

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 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 covalent linking group $-Z-X^1-$,wherein -Z- is not a single bond and -Z- is not an amide; wherein $-X^1-$ is a divalent covalent linking moiety; wherein the second terminal end of the polyethylene glycol fragment is capable of binding, preferably said polyethylene glycol fragment binds, to a targeting fragment. In a preferred embodiment of this aspect, said composition consists of said conjugate. In a preferred embodiment, linear polyethyleneimine fragment is of the formula $R^1\text{-}(NR^2\text{-}CH_2\text{-}CH_2)_n\text{-}$, n is any integer between 1 and 1500. In a further preferred embodiment, said $R^1\text{-}(NR^2\text{-}CH_2\text{-}CH_2)_n$-moiety is a disperse polymeric moiety with between about 115 and about

1150 repeating units n and a dispersity of about 5 or less, preferably between about 280 and about 700 repeating units n with a dispersity of about 3 or less, and further preferably between about 350 and about 630 repeating units n with a dispersity of about 2 or less, and wherein preferably R^1 is -H or $-CH_3$.

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: 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 any integer between 1 and 100; 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 preferably capable of binding to a cell 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 any integer between 1 and 100; 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 preferably capable of binding to a cell.

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: 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 any integer between 1 and 100; 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 preferably said targeting fragment is capable of binding to a cell,

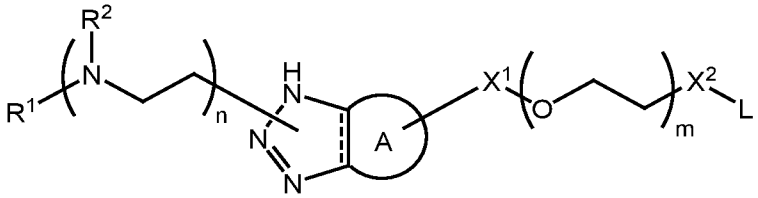
and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor, and

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 any integer between 1 and 100; 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 preferably said targeting fragment is capable of binding to a cell, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor.

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:

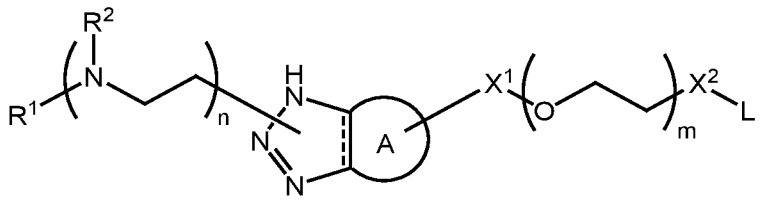


Formula I

wherein: ===== is a single bond or a double bond; n is any integer between 1 and 1500; m is any integer between 1 and 200, preferably m is any integer between 1 and 100; 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 preferably capable of binding to a cell, 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:

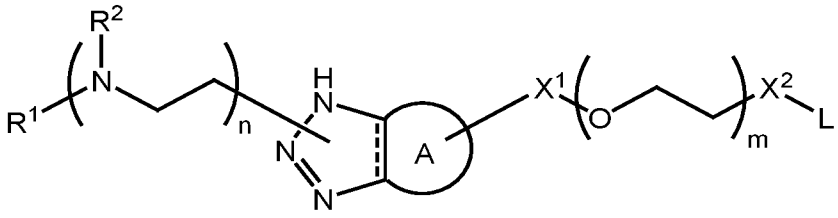


Formula I

wherein: ═══ is a single bond or a double bond; n is any integer between 1 and 1500; m is any integer between 1 and 200, preferably m is any integer between 1 and 100; 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 preferably capable of binding to a cell. 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 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 any integer between 1 and 200;

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

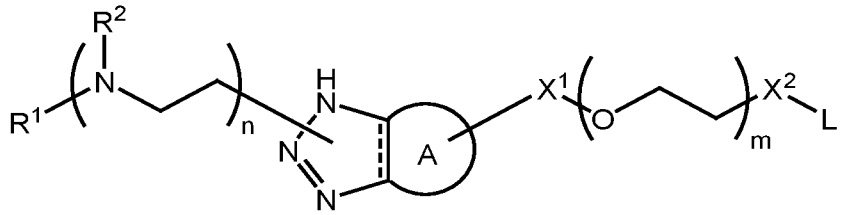
independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are attached, can combine to form one or more fused C_6-C_{10} aryl, C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is 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, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. 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 any integer between 1 and 200;

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 $-(NR^2\text{-}CH_2\text{-}CH_2)_n-$ 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, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$.

In some embodiments, the 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, preferably wherein the covalent linking moiety produces a linear end-to-end linkage between the LPEI fragment and the PEG fragment. 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 comprised in the composition are comprised by said conjugate, as preferably determined by UV spectroscopy or mass sspectrometry. 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, succinimide, or 7- to 8-membered heterocycloalkenyl, wherein the heterocycloalkyl or heterocycloalkenyl does not comprise heteroatoms other than N, O and S, and wherein each cyclooctene, heterocycloalkyl or heterocycloalkenyl is optionally substituted at any position with one or more R^{A1}.

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

In some embodiments, Ring A is cyclooctene, succinimide, 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, succinimide, 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, succinimide, 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, succinimide, 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, succinimide, 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 hEGF, one of skill in the art will recognize other suitable electrophilic functional groups 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.

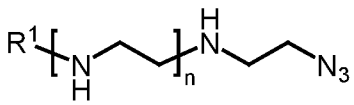
LPEI Fragment

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 initator 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 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-

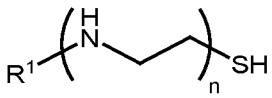
CH_2]– and is substituted at the omega terminus with an azide group which can be coupled to an alkyne or alkene substituent on a PEG fragment. In some preferred embodiments, the alpha terminus of the LPEI fragment can be substituted with a hydrogen atom or a C_1-C_6 alkyl, preferably a hydrogen or C_1-C_4 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:



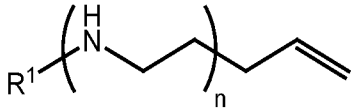
wherein R^1 can be any suitable initiation residue, preferably a hydrogen or C_1-C_6 alkyl, preferably hydrogen or C_1-C_4 alkyl, more preferably hydrogen or methyl, most preferably a hydrogen.

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 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 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 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 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 präffix "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]–. 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 PEG fragment comprises the alkene or alkyne functional group. For example, in some preferred embodiments, 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 below) which can be coupled to the azide group of a corresponding LPEI fragment.

In some preferred embodiments, the 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., hEGF, HER2, folate, or DUPA) (e.g., via a linking moiety "X^2" as discussed below); in some preferred embodiments, there is no additional substitution present on the PEG fragment.

The PEG fragment can comprise a range of lengths (i.e., repeating units represented by the variable "m"). In other embodiments, the PEG fragment can comprise a discrete number of repeating -O-CH_2-CH_2- units and is not defined in terms of an average chain length. In a preferred embodiment, said said -(O-CH_2-CH_2)$_m$- is a disperse polymeric moiety. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprises, preferably consists of, a discrete number of repeating units m. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprises, preferably consists of, a discrete number of contiguous repeating units m.

In some preferred embodiments, the PEG fragment is a disperse polymeric moiety comprising between about 1 and about 200 repeating units, preferably between about 1 and about 200 repeating units. In some preferred embodiments, the PEG fragment can comprise between 1 and 100 repeating units (i.e., -O-CH_2-CH_2-). Preferably the PEG fragments of the present invention comprise between about 1 and about 100 repeating units, between about 1 and about 90 repeating units, between about 1 and about 80 repeating units, between about 1 and about 70 repeating units, between about 1 and about 60 repeating units, between about 1 and about 50 repeating units, between about 1 and about 50 repeating units, between about 1 and about 40 repeating units, between about 1 and about 30 repeating units, or between about 1 and about 20 repeating units. In some other preferred embodiments, the PEG fragments comprise a discrete number of repeating units m, preferably 12 repeating units or 24 repeating units. In some embodiment, said polyethylene glycol fragment is a disperse polymeric moiety with between about 2 and about 80 repeating units and a dispersity of about 2.0 or less, preferably of about 1.8 or less, further of about 1.5 or less; preferably between about 2 and about 70 repeating units with a dispersity of about 1.8 or less, preferably of about 1.5 or less; more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5 or less. In some embodiment, said -(O-CH_2-CH_2)$_m$-moiety is a disperse polymeric moiety with between about 2 and about 80 repeating units and a dispersity of about 2.0 or less, preferably between about 2 and about 70 repeating units with a dispersity of about 1.8 or less; more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5 or less.

In a preferred embodiment, said polyethylene glycol fragment PEG fragment comprises, preferably consists of, a discrete number of repeating units m, preferably of 12 or 24 repeating

units. In a preferred embodiment, said m (of said -(O-CH_2-CH_2)$_m$-moiety) comprises, preferably consists of, a discrete number of repeating units m, preferably of 12 or 24 repeating units.

In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 2 to 100, preferably of a discrete number of repeating units m of 4 to 60. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 4 to 60, preferably of a discrete number of repeating units m of 10 to 60. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 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 repeating units m of 4, 8, 12, 16, 20, 24, 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 4. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 12. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of repeating units m of 24. 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 contiguous repeating units m of 2 to 100, preferably of a discrete number of contiguous repeating units m of 4 to 60. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 4 to 60, preferably of a discrete number of contiguous repeating units m of 10 to 60. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 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 4, 8, 12, 16, 20, 24, 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 4. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 12. In a preferred embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 24. In a preferred

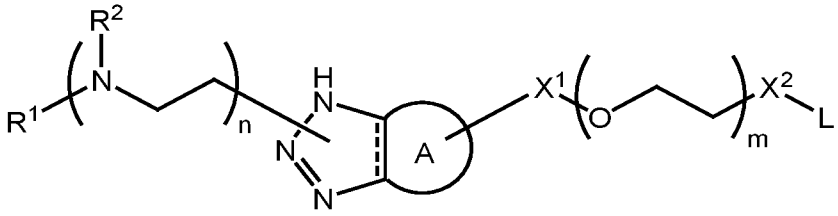
embodiment, the PEG fragment comprise, preferably consist of, a discrete number of contiguous repeating units m of 36.

In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety of Formula I* or Formula I comprise, preferably consist of, a discrete number of repeating units m of 2 to 100, preferably of a discrete number of repeating units m of 4 to 60. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of repeating units m of 4 to 60, preferably of a discrete number of repeating units m of 10 to 60. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of repeating units m of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 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 comprise, preferably consist of, a discrete number of repeating units m of 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, or 60. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of repeating units m of 4. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of repeating units m of 12. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of repeating units m of 24. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of repeating units m of 36.

In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety of Formula I* or Formula I comprise, preferably consist of, a discrete number of contiguous repeating units m of 2 to 100, preferably of a discrete number of contiguous repeating units m of 4 to 60. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of contiguous repeating units m of 4 to 60, preferably of a discrete number of contiguous repeating units m of 10 to 60. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of contiguous repeating units m of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 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 comprise, preferably consist of, a discrete number of contiguous repeating units m of 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, or 60. In a preferred embodiment said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of contiguous repeating units m of 4. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of contiguous

repeating units m of 12. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of contiguous repeating units m of 24. In a preferred embodiment, said -(O-CH_2-CH_2)$_m$-moiety comprise, preferably consist of, a discrete number of contiguous repeating units m of 36.

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 2 to 100, preferably of a discrete number of repeating units m of 4 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;

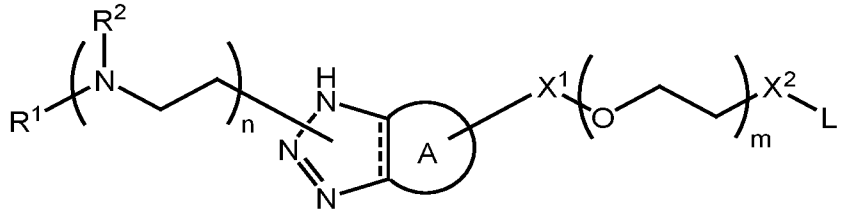
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. 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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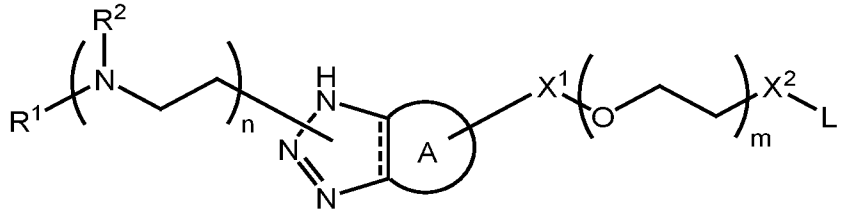
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 preferably said targeting fragment is capable of binding to a cell, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. 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 of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



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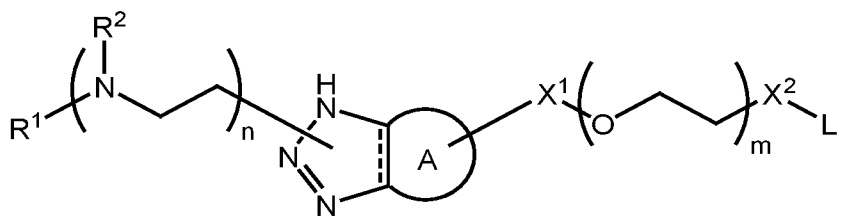
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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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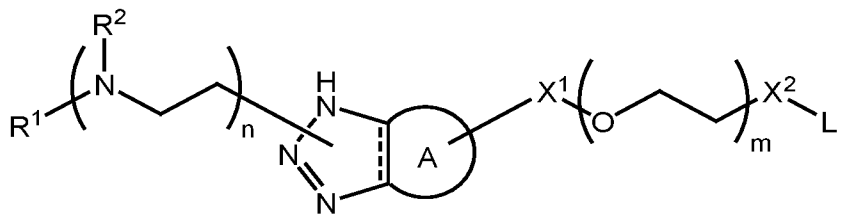
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 preferably said targeting fragment is capable of binding to a cell, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. 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 of the Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof:



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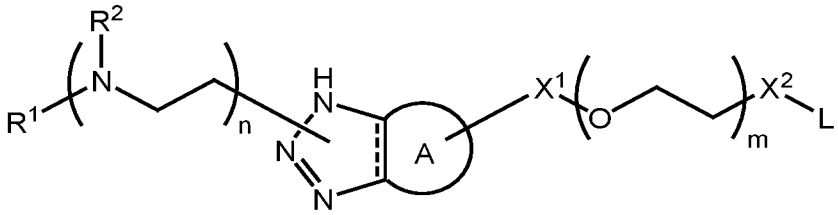
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L is a targeting fragment, wherein preferably said targeting fragment is capable of binding to a cell, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. In a preferred embodiment, said R^1 is -H. 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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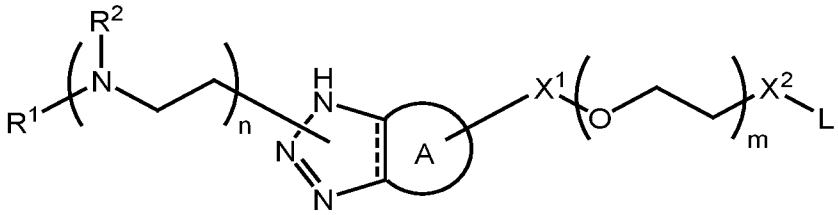
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X^1 is a divalent covalent linking moiety;

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, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. 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 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 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 $-(NR^2\text{-}CH_2\text{-}CH_2)_n-$ 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

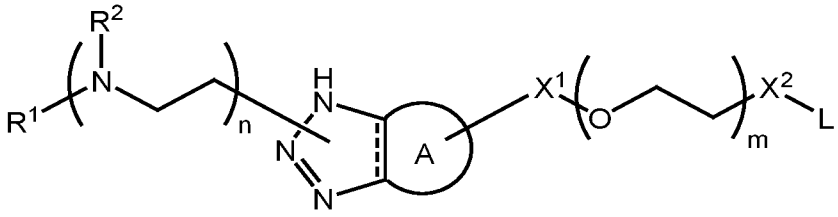
is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

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, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. 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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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, and wherein further preferably said targeting fragment is capable of binding

to a cell surface receptor. 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 an PEG fragment present (i) as a disperse polymeric moiety, wherein m is between about 2 and about 80 and a dispersity of about 2 or less, preferably between about 2 and about 70 with a dispersity of about 1.8 or less; more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or (ii) as a discrete number of repeating units m, wherein preferably discrete number of repeating units m are 12 or 24 repeating units.

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, 12 repeating units. 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, preferably consisting of, 24 repeating units.

Targeting Fragment

The inventive conjugates comprise a targeting fragment which allows to direct the inventive conjugate 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”, as used herein refers to a protein, 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 selected from a growth factor receptor, an extracellular matrix protein, a cytokine receptor, a hormone receptor, a glycosylphosphatidylinositol (GPI) anchored membrane protein, a carbohydrate-binding integral membrane protein, a lectin, an ion channel, a G-protein coupled receptor, and an enzyme-linked receptor such as a tyrosine kinase-coupled receptor.

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 selected from a growth factor receptor, an extracellular matrix protein, a cytokine receptor, a hormone receptor, a glycosylphosphatidylinositol (GPI) anchored membrane protein, a carbohydrate-binding integral membrane protein a lectin, an ion channel, a G-protein coupled receptor, and an enzyme-linked receptor such as a tyrosine kinase-coupled receptor. In a preferred embodiment, said cell surface receptor is a growth factor receptor. In a preferred embodiment, said cell surface receptor is an extracellular matrix protein. In a preferred embodiment, said cell surface receptor is a cytokine receptor. In a preferred embodiment, said cell surface receptor is a hormone receptor. In a preferred embodiment, said cell surface receptor is a glycosylphosphatidylinositol (GPI) anchored membrane protein. In a preferred embodiment, said cell surface receptor is a carbohydrate-binding integral membrane protein. In a preferred embodiment, said cell surface receptor is a lectin. In a preferred embodiment, said cell surface receptor is an ion channel. In a preferred embodiment, said cell surface receptor is an enzyme-linked receptor, wherein preferably said enzyme-linked receptor is a tyrosine kinase-coupled receptor.

In a preferred embodiment, said cell surface receptor is selected from an epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), prostate surface membrane antigen (PSMA), an insulin-like growth factor 1 receptor (IGF1R), a vascular endothelial growth factor receptor (VEGFR), a platelet-derived growth factor receptor (PDGFR) and a fibroblast growth factor receptor (FGFR). In a preferred embodiment, said cell surface receptor is an epidermal growth factor receptor (EGFR). In a preferred embodiment, said cell surface receptor is a human epidermal growth factor receptor 2 (HER2). In a preferred embodiment, said cell surface receptor is a prostate surface membrane antigen (PSMA). In a preferred embodiment, said cell surface receptor is an insulin-like growth factor 1 receptor (IGF1R). In a preferred embodiment, said cell surface receptor is a vascular

endothelial growth factor receptor (VEGFR). In a preferred embodiment, said cell surface receptor is a platelet-derived growth factor receptor (PDGFR). In a preferred embodiment, said cell surface receptor is a fibroblast growth factor receptor (FGFR).

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

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

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

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 strong 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, in analogy as exemplified in Example 23. 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 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, in analogy as exemplified in Example 23.

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 westernblot 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 vs 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 polyIC) 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, cell titerglow 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. lt 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 growth factor or a functional fragment thereof, preferably hEGF), a hormone or a functional fragment thereof, preferably insulin, 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 growth factor or a fragment, preferably a functional fragment, thereof (e.g., hEGF); 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, preferably hEGF, 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 some embodiments, said targeting fragment L is selected from hEGF; an anti-HER2 peptide, preferably an anti-HER2 antibody or affibody; DUPA; a folate receptor-targeting fragment, folic acid; a somatostatin receptor-targeting fragment, preferably somatostatin and/or octreotide; an integrin-targeting fragment, preferably an arginine-glycine-aspartic acid (RGD)-containing fragment; a low pH insertion peptide; an asialoglycoprotein receptor-targeting fragment, preferably asialoorosomucoid; an insulin-receptor targeting fragment, preferably insulin; a mannose-6-phosphate receptor targeting fragment, preferably mannose-6-phosphate; a mannose-receptor targeting fragment, preferably mannose; a Sialyl Lewis^x antigen targeting fragments, preferably E-selectin; a sigma-2 receptor agonist, preferably N,N-dimethyltryptamine (DMT), sphingolipid-derived amine, and/or steroid, more preferably progesterone; a p32-targeting ligand, preferably anti-p32 antibody or p32-binding LyP-1 tumor-homing peptide; a Trop-2 targeting fragment, preferably an anti-Trop-2 antibody and/or antibody fragment; insulin-like growth factor 1; vascular endothelial growth factor; platelet-derived growth factor; and fibroblast growth factor.

In some embodiments, said targeting fragment L is selected from a targeting fragment derived from hEGF; an anti-HER2 peptide, preferably an anti-HER2 antibody or affibody; DUPA; folic acid; a somatostatin receptor-targeting fragment, preferably somatostatin and/or octreotide; an integrin-targeting fragment, preferably an arginine-glycine-aspartic acid (RGD)-containing fragment; a low pH insertion peptide; asialoglycoprotein receptor-targeting fragment, , preferably asialoorosomucoid; an insulin-receptor targeting fragment, preferably insulin; a mannose-6-phosphate receptor targeting fragment, preferably mannose-6-phosphate; a mannose-receptor targeting fragment, preferably mannose; a Sialyl Lewis^x antigen targeting

fragments, preferably E-selectin; a sigma-2 receptor agonist, preferably N,N-dimethyltryptamine (DMT), sphingolipid-derived amine, and/or steroid, more preferably progesterone; a p32-targeting ligand, preferably anti-p32 antibody or p32-binding LyP-1 tumor-homing peptide; a Trop-2 targeting fragment, preferably an anti-Trop-2 antibody and/or antibody fragment; insulin-like growth factor 1; vascular endothelial growth factor; platelet-derived growth factor; and fibroblast growth factor.

In a preferred embodiment, said targeting fragment is selected from an EGFR targeting fragment; a PSMA targeting fragment; an anti-HER2 peptide, preferably an anti-HER2 antibody or affibody; folic acid; a somatostatin receptor-targeting fragment, preferably somatostatin and/or octreotide; an integrin-targeting fragment, preferably an arginine-glycine-aspartic acid (RGD)-containing fragment; a low pH insertion peptide; asialoglycoprotein receptor-targeting fragment, preferably asialoorosomucoid; an insulin-receptor targeting fragment, preferably insulin; a mannose-6-phosphate receptor targeting fragment, preferably mannose-6-phosphate; a mannose-receptor targeting fragment, preferably mannose; a Sialyl Lewis^x antigen targeting fragments, preferably E-selectin; a sigma-2 receptor agonist, preferably N,N-dimethyltryptamine (DMT), sphingolipid-derived amine, and/or steroid, more preferably progesterone; a p32-targeting ligand, preferably anti-p32 antibody or p32-binding LyP-1 tumor-homing peptide; a Trop-2 targeting fragment, preferably an anti-Trop-2 antibody and/or antibody fragment; insulin-like growth factor 1; vascular endothelial growth factor; platelet-derived growth factor; and fibroblast growth factor.

In a preferred embodiment, the targeting fragment is an epidermal growth factor such as human epidermal growth factor (hEGF), wherein typically and preferably said coupling to the rest of said conjugate is effected via an amino group of said hEGF. The hEGF can be selectively taken up by cells that have increased expression (e.g., overexpression) of human epidermal growth factor receptor (EGFR).

In a preferred embodiment, said targeting fragment is capable of binding to epidermal growth factor receptor (EGFR), which is also named herein as EGFR targeting fragment.

EGFR is a transmembrane glycoprotein that is a member of the protein kinase superfamily and a receptor for members of the epidermal growth factor family. EGFR is a cell surface protein that binds to epidermal growth factor, thus inducing receptor dimerization and tyrosine autophosphorylation leading to cell proliferation. In a preferred embodiment, said EGFR targeting fragment is capable of binding to epitopes on the extracellular domain of EGFR.

In a preferred embodiment, said targeting fragment is capable of binding to a cell EGFR expressing. In a preferred embodiment, said targeting fragment is capable of binding to a cell overexpressing EGFR. In one embodiment, said cell overexpressing EGFR means that the level of EGFR expressed in said cell of a certain tissue is elevated in comparison to the level of EGFR as measured in a normal healthy cell of the same type of tissue under analogous conditions. In one embodiment, said cell overexpressing EGFR refers to an increase in the level of EGFR 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 EGFR relates to expression of EGFR that is at least 10-fold, further preferably at least 20-fold, as compared to the expression of EGFR in a normal cell or in a normal tissue.

In a preferred embodiment, said targeting fragment is capable of binding to a cell expressing or overexpressing EGFR. For example, EGFR is overexpressed in neoplastic tissue and cancer types, such as glioma and carcinoma or cancer of epithelial origin, including of head and neck, thyroid, breast, ovarian, colon, gastric colorectal, stomach small intestine, cervix, bladder, lung, nasopharyngeal and esophageal tissue, such as squamous cells (e.g., Gan et al., J Cell Mol Med. 2009 Sep; 13(9b): 3993–4001; Aratani et al., Anticancer Research June 2017, 37 (6) 3129-3135), in particular in glioma, non-small-cell-lung-carcinoma, breast cancer, glioblastoma, squamous cell carcinoma, e.g. head and neck squamous cell carcinoma, small intestinal, colorectal cancer, adenocarcinoma, ovary cancer, bladder cancer or prostate cancer, and metastases thereof.

EGFR expression and overexpression are detected preferably using a monoclonal antibody targeting EGFR, e.g. by immunohistochemical methods (as e.g. described in Kriegs et al., Nature, 2019, 9:13564; Prenzel et al., Endocr Relat Cancer 8, 11-31, 2001). A cut-off of 5% or more EGFR positive cells can be used to define EGFR expression in different types of tissues or cells. Thus, cells or tissue with <5% positive cells can be considered to be negative.

In a preferred embodiment, said targeting fragment is capable of specifically binding to EGFR. Typically, specific binding refers to a binding affinity or dissociation constant K_D of the targeting fragment in the range of between about 1 x 10^{-3} M and about 1 x 10^{-12} M. In preferred embodiment, said targeting fragment is capable of specifically binding to EGFR, 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. In a preferred embodiment, said targeting fragment is capable of specifically binding to EGFR, wherein typically and preferably said affinity or specific binding is measured by the dissociation constant (K_D) and said 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. To detect binding or the complex or measure affinity, molecules can be analyzed using a competition binding assay, typically and preferably such as Biacore 3000 instrument (Biacore Inc., Piscataway NJ; as described, for example, in Wei-Ting Kuo et al., PLoS One. 2015, 10(2): e0116610 or in US2017224620A1). Preferably, binding results in formation of a complex between the EGFR targeting fragment and EGFR, wherein the binding or complex can be detected.

In a preferred embodiment, said targeting fragment is an EGFR antibody, an EGFR affibody, an EGFR aptamer, an EGFR targeting peptide or an EGFR targeting tyrosine kinase inhibitor. In a preferred embodiment, said EGFR targeting fragment is an EGFR antibody, an EGFR affibody, an EGFR aptamer, an EGFR targeting peptide or an EGFR targeting tyrosine kinase inhibitor.

In a preferred embodiment, said targeting fragment is an EGFR targeting peptide. An EGFR targeting peptide refers, typically and preferably, to peptide ligands of EGFR. Such peptide ligands are known to the skilled person and have been described, for example in US2017224620A1 and by Gent et al., 2018, Pharmaceutics 2018, 10, 2 (the disclosures of which are incorporated herein by reference in its entirety). EGFR targeting peptides have low immunogenic potential and show good penetration into solid tumor tissues.

In a preferred embodiment, said EGFR targeting peptide has a molecular weight of about 1000 g/mol to about 2000 g/mol, preferably of about 1100 g/mol to about 1900g/mol, further preferably of about 1200 g/mol to about 1800 g/mol, and again more preferably of about 1300 g/mol to about 1700 g/mol.

In a preferred embodiment, the EGFR targeting peptide comprises, or preferably consists of, the sequence YHWYGYTPQNVI (GE11) (SEQ ID NO: 9). In a preferred embodiment, said targeting fragment comprises, or preferably consists of, the sequence YHWYGYTPQNVI (GE11) (SEQ ID NO: 9). GE-11 has excellent affinity towards EGFR and shows also binding specificity for EGFR (kd = 22 nM) (Ruoslahtiet al., Adv. Mater. 2012, 24, 3747–3756; Li et al., J. Res. Commun. 2005, 19, 1978–1985). GE11 moves from EGFR after the addition of the physiologic ligand EGF, demonstrating both its selective binding to EGFR and its receptor affinity. GE11 has been reported to have a high potential to accelerate

nanoparticle endocytosis due to an alternative EGFR-dependent actin-driven pathway. (Mickeler et al., Nano Lett. 2012, 12, 3417–3423; Song et al., FASEB J. 2009, 23, 1396–1404) It has been showed that the EGFR level on the surface of cancer cells remains constant after treatment with GE11 polyplexes, indicating an EGFR recycling process with a prolonged receptivity of the cells for circulating GE11 polyplexes.

In a preferred embodiment, said EGFR targeting fragment comprises, or preferably consists of, GE11 (SEQ ID NO: 9), in particular, in use for treating solid tumors characterized by EGFR-overexpressing cells. The inventive conjugate and polyplexes comprising, or preferably consisting, GE11 as the targeting fragment are believed to be stable polyplexes ensuring that the polyanion and nucleic acid payload is not released before the polyplex has reached its target cell.

In a preferred embodiment, said targeting fragment is an EGFR antibody. An EGFR antibody refers to an antibody that binds to EGFR. In a preferred embodiment, said EGFR antibody is a human. In a preferred embodiment, said EGFR antibody is a humanized EGFR antibody. In a preferred embodiment, said EGFR antibody is a monoclonal human. In a preferred embodiment, said EGFR antibody is a humanized EGFR antibody. In a preferred embodiment, said EGFR antibody is a monoclonal fully human EGFR antibody. In another preferred embodiment, the EGFR antibody is a scFv or Fab fragment.

EGFR antibodies are known to the skilled person and have been described for example in WO2008/105773 and in WO2017/185662 (the disclosure of which is incorporated herein by reference in its entirety) and include Bevacizumab, Panitumumab, Cetuximab, Tomuzotuximab, Futuximab, Zatuximab, Modotuximab, Imgatuzumab, Zalutumumab, Matuzumab, Necitumumab, Nimotuzumab, CEVIAvax EGF, clones EGFR, L8A4, E6.2, TH190DS, Pep2, Pep3, LR-DM1, P1X, YC088, ratML66, FM329, TGM10-1, F4, 2F8, 15H8, TAB-301MZ-S(P), mAb528, 2224, E7.6.3, C225, CBL155, MR1, MR1, L211C, N5-4, TH83DS, L2-12B, 15H8, 12Do3, 7A7, 42C11 (MOB-1078z), PABL-080, HPAB-2204LY-S(P), VHH205, ABT-806, , Tab-271MZ, Hu225, LA22, Fab fragment DL11, Fab fragment DX 1-6, VHH104, OA-cb6, 07D06, Fab fragment HPAB-0419-FY-F(E), Fab fragment TAB-285MZ-F(E), Fab fragment TAB-293MZ-F(E), Fab fragment HPAB-0136-YJ-F(E), FGF-R2, EG-19-11, Fab fragment pSEX81-63, DX 1-4, scFv fragment DX 1-6, EG-26-11, EG-26-11, DX1-4, TAB-326MZ, scFv fragment 528, scFv fragment LA1, scFv fragment 07D06, single domain antibody VHH139, scFv fragment EG-19-11, single domain Antibody VHH134, single domain Antibody 9G8, ABT-414, AMG-595, and IMGN-289. One of ordinary skill in the art

will appreciate that any antibody that recognizes and/or specifically binds to EGFR may be used in accordance with the present invention.

In a preferred embodiment, said targeting fragment is an EGFR inhibitor. An EGFR inhibitor refers to targeting fragment that block cell-surface localization and signaling of the EGFR, such as oligosaccharyltransferase inhibitors like nerve growth inhibitor-1; or EGFR kinase inhibitors, such as afatinib, erlotinib, osimertinib and gefitinib. EGFR inhibitors are known to the skilled person and have been described for example in WO2018078076 and in US2017224620A1 (the disclosure of which is incorporated herein by reference in its entirety).

In a preferred embodiment, said targeting fragment is an EGFR aptamer. Preferred EGFR targeting aptamers include, but are not limited to those disclosed in Na Li et al. (PLoS One. 2011; 6(6): e20299), Deng-LiangWang et al. (Biochemical and Biophysical Res Com, 453(4), 2014, pp 681-685), Min Woo Kim et al. (Theranostics 2019; 9(3):837-852), Akihiro Eguchi et al. (JACS Au 2021, 1, 5, 578-585) or Yingpan Song et al. (RSC Adv., 2020, 10, 28355–28364), the disclosures of which are incorporated herein by reference in its entirety.

The term EGFR aptamer includes also EGFR aptamer derivatives and/or functional fragments of EGFR aptamer. In some embodiments, in the EGFR aptamer derivatives fewer than 30, 25, 20, 15, 10, 5, 4, 3, 2, or 1 nucleic acid is substituted relative to the corresponding EGFR aptamer. In some embodiments, the sequences of the EGFR aptamer derivatives are at least 80%, preferably 85%, more preferably 90%, again more preferably 95%, most preferably 99% identical with the corresponding EGFR aptamer.

In a preferred embodiment, said targeting fragment is an EGFR affibody. Preferred EGFR affibodies include, but are not limited to ZEGFR:1907, ZEGFR:2377 or ZEGFR:03115 (available from Affibody Medical AB) or the dimeric form of these affibodies. In a preferred embodiment said EGFR affibody has the sequence of SEQ ID NO: 8.

In a preferred embodiment, said targeting fragment is the EGFR ligand epidermal growth factor (EGF). Thus, in a preferred said targeting fragment is epidermal growth factor (EGF). In a preferred embodiment, said targeting fragment is human EGF (hEGF), mouse EGF (mEGF), rat EGF, or guinea pig EGF. In a very preferred embodiment, said targeting fragment is human EGF (hEGF). In a very preferred embodiment, said targeting fragment comprises, preferably consists of, the sequence of SEQ ID NO: 7.

In some embodiments, EGF is modified, e.g., by deleting or exchanging one or more amino acids or truncation of EGF. Modified and/or truncated EGF molecules are for example disclosed in WO2019023295A1. EGF has many residues conserved across rat, mouse, guinea

pig and human species (Savage et al., J. Biol. Chem.., 247: 7612-7621, 1973; Carpenter and Cohen, Ann. Rev. Biochem., 48: 193-316, 1979; Simpson et al., Eur J Biochem, 153:629-37, 1985). In particular, six cysteine residues at positions 6, 14, 20, 31, 33, and 42 are conserved as they form three disulfide bridges to provide conserved tertiary protein structure. Also conserved across all four species are residues as positions 7, 9, 11, 12, 13, 15, 18, 21, 24, 29, 32, 34, 36, 37, 39, 41, 46, and 47. Many of these residues may be expected to facilitate or provide key binding interactions with the corresponding EGFR. It has been described that both the full length human EGF (53 residues) and a truncated form (48 residues), which results from trypsin cleavage, retain strong binding affinity and activation of the EGFR (Calnan et al., 47(5):622-7, 2000; Gregory, Regul Pept, 22:217-26, 1988). Mutagenesis studies have been reported for various residues to correlate the effect of replacement of specific residues on binding of EGF to the EGFR or activation of the EGFR (Campion et al., Biochemistry, 29, 9988-9993, 1990; Engler et al., J. Biol. Chem., 267:2274-2281, 1992; Tadaki and Niyogi. J. Biol. Chem., 268: 10114-10119, 1993). An x-ray crystal structure of EGF bound to EGFR has been solved which shows key binding interactions and also identifies residues not directly involved in binding (Ogiso et al., Cell, Vol. 110, 775-787, 2002).

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:

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 any integer between 1 and 100;

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 comprises, or preferably consists of, the sequence YHWYGYTPQNVI (GE11) (SEQ ID NO: 9), 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 any integer between 1 and 100;

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

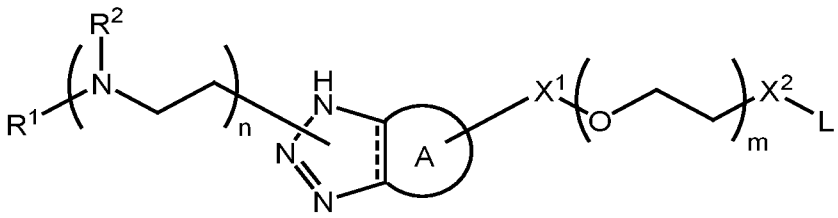
R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said -(NR^2-CH_2-CH_2)$_n$– is H;

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 comprises, or preferably consists of, the sequence YHWYGYTPQNVI (GE11) (SEQ ID NO: 9).

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 any integer between 1 and 200;

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,

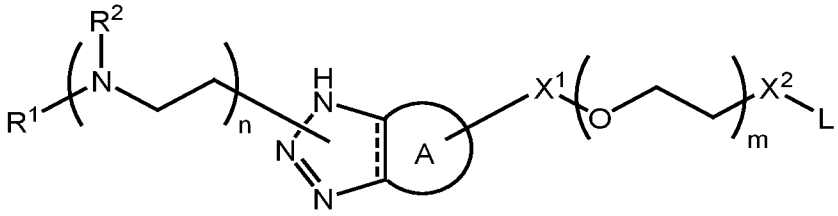
C_5-C_6 heteroaryl, or C_3-C_6 cycloalkyl rings, wherein each fused aryl, heteroaryl, or cycloalkyl is optionally substituted with one or more R^{A2}; R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

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 comprises, or preferably consists of, the sequence YHWYGYTPQNVI (GE11) (SEQ ID NO: 9).

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 any integer between 1 and 200;

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;

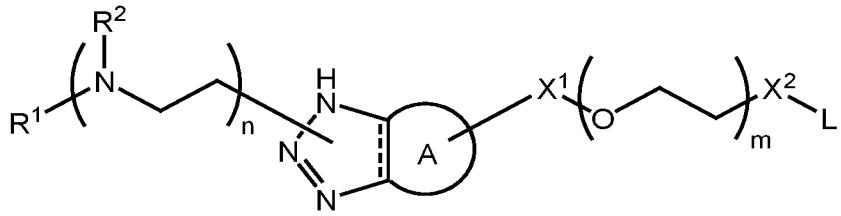
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 comprises, or preferably consists of, the sequence YHWYGYTPQNVI (GE11) (SEQ ID NO: 9).

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 2 to 100, preferably of a discrete number of repeating units m of 4 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;

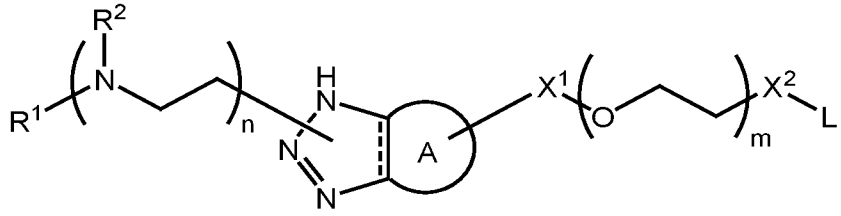
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 EGFR targeting fragment, wherein preferably said EGFR targeting fragment is capable of specifically binding to a cell expressing, preferably overexpressing, EGFR.

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 contiguous repeating units m of 2 to 100, preferably of a discrete number of contiguous repeating units m of 4 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;

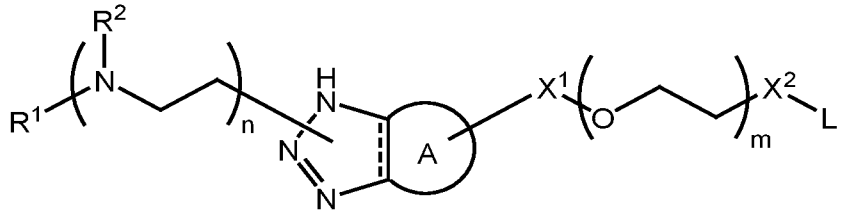
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 EGFR targeting fragment, wherein preferably said EGFR targeting fragment is capable of specifically binding to a cell expressing, preferably overexpressing, EGFR.

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 2 to 100, preferably of a discrete number of repeating units m of 4 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;

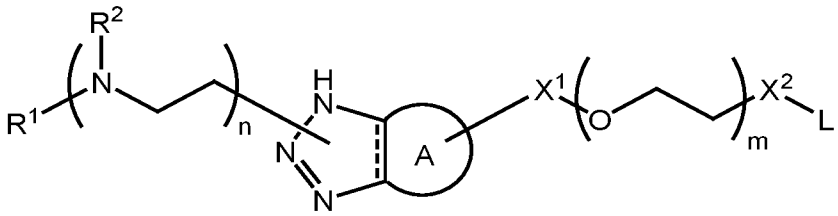
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor.

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 2 to 100, preferably of a discrete number of contiguous repeating units m of 4 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, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor.

In a preferred embodiment, said targeting fragment is capable of binding to prostate surface membrane antigen (PSMA), which is also named herein as PSMA targeting fragment.

PSMA is a multifunctional transmembrane protein that functions as a glutamate carboxypeptidase and also demonstrates rapid, ligand-induced internalization and recycling (Liu H, et al., 1998, Cancer Res 58:4055–4060). 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). 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, preferably said PSMA targeting fragment, is capable of binding to a cell expressing PSMA. In a preferred embodiment, said targeting fragment, preferably said PSMA targeting fragment, is capable of binding to a cell overexpressing PSMA. For example, 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). PSMA is expressed and overexpressed also in other tumor types (Mhawech-Fauceglia et al., Histopathology 2007, 50:472–483; Israeli RS et al, Cancer Res 1994, 54:1807-1811; Chang SS et al, Cancer Res 1999, 59:3192-198).

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, 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 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 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 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. 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; 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 (Lupold et al., 2002, Cancer Res., 62:4029; and Chu et al., 2006, Nuc. Acid Res., 34: e73), derivatives thereof, and/or functional fragments thereof. 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.

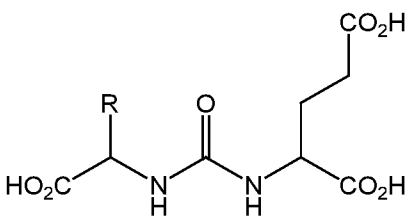
In preferred embodiments, said small molecule PSMA targeting fragments are urea-based inhibitors (herein also called urea-based peptidase inhibitors), more preferably small urea-based inhibitors, such as disclosed in Kularatne et al., Mol Pharmaceutics 2009, 6, 780; Kularatne et al., Mol. Pharmaceutics 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.

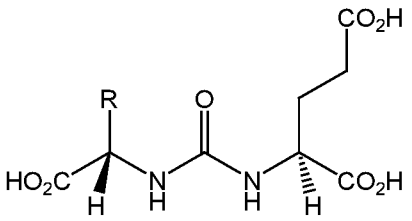
The term “urea based PSMA peptidase inhibitor” relate to a PSMA peptidase inhibitor comprising an urea group. The term “dipeptide urea based PSMA peptidase inhibitor” relate to

PSMA peptidase inhibitor comprising an 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***:



1

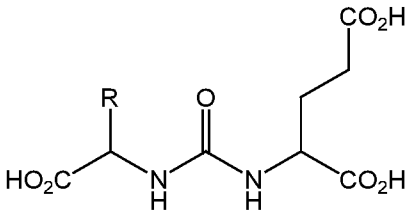


1*

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 group serve as the point of covalent attachment to the X^2 linking moiety and the PEG fragment respectively, 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 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 serve as the point of covalent attachment to the X^2 linking

moiety and the PEG fragment respectively.

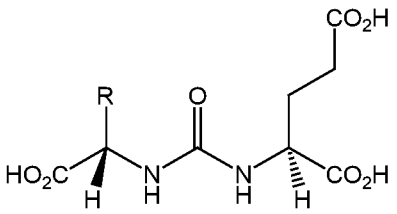
In a preferred embodiment, said targeting fragment is a glutamate-urea moiety of formula **1**:



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 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 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 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 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 any integer between 1 and 200, preferably m is any integer between 1 and 100; 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 surface 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 any integer between 1 and 200, preferably m is any integer between 1 and 100; 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 surface 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 any integer between 1 and 200, preferably m is any integer between 1 and 100; 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 any integer between 1 and 100; 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 surface 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*);

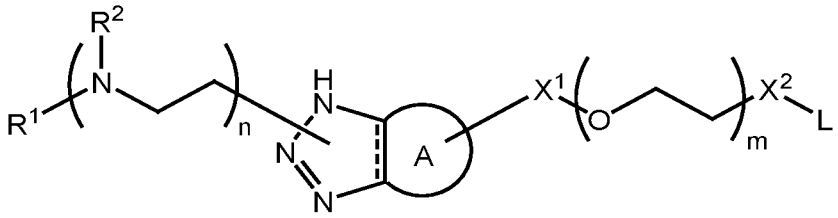
wherein n is any integer between 1 and 1500; m is any integer between 1 and 200, preferably m is any integer between 1 and 100; R^1 is an initiation residue, wherein preferably R^1 is -H or $-CH_3$; R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90% of said R^2 in said $-(NR^2-CH_2-CH_2)_n$–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 surface 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 any integer between 1 and 100; 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 any integer between 1 and 200;

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};

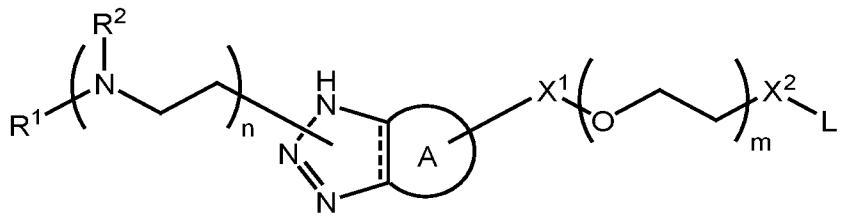
R^{A2} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, halogen -SO_3H, or -OSO_3H;

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 2 to 100, preferably of a discrete number of repeating units m of 4 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;

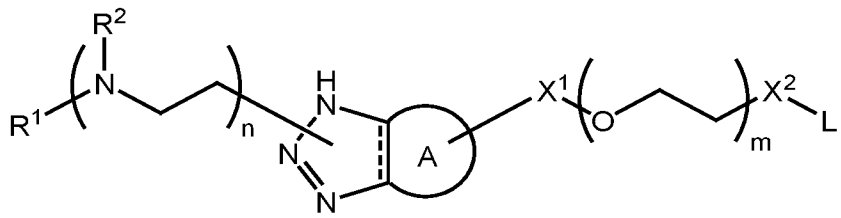
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 2 to 100, preferably of a discrete number of repeating units m of 4 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;

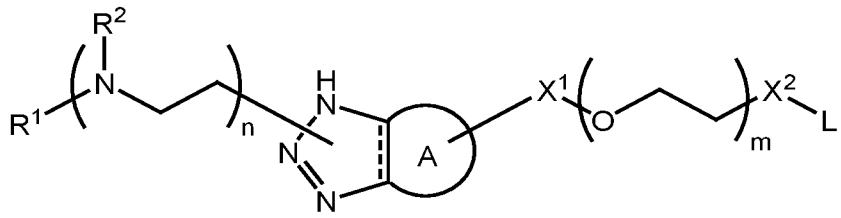
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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;

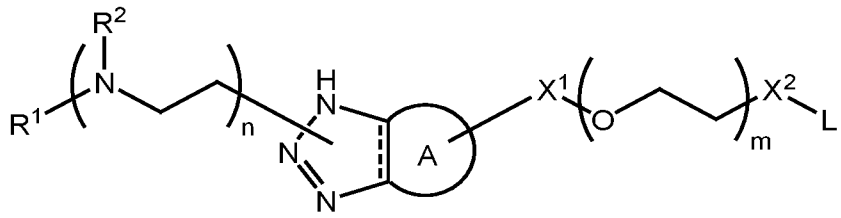
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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;

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 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;

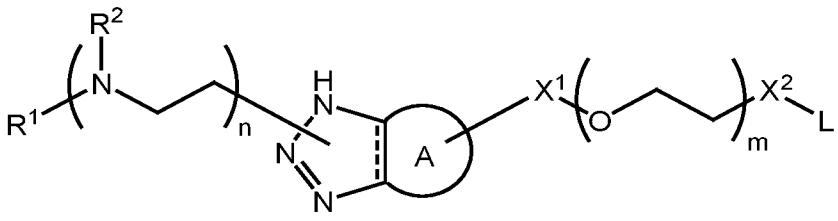
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 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 contiguous repeating units m of 2 to 100, preferably of a discrete number of contiguous repeating units m of 4 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;

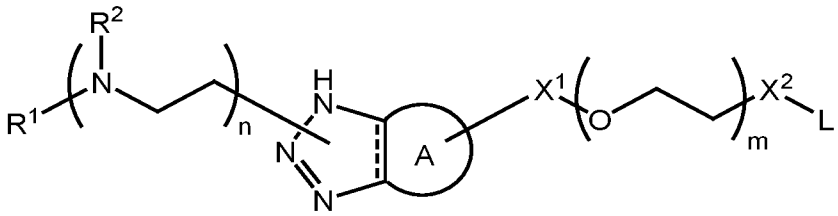
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 contiguous repeating units m of 2 to 100, preferably of a discrete number of contiguous repeating units m of 4 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;

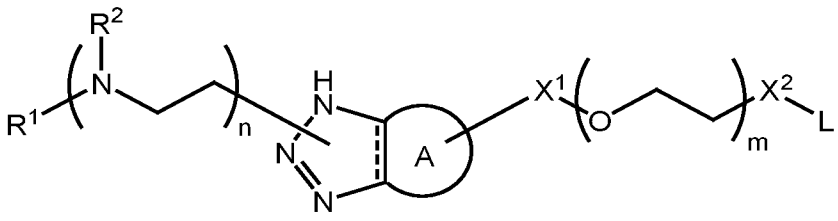
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 contiguous 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;

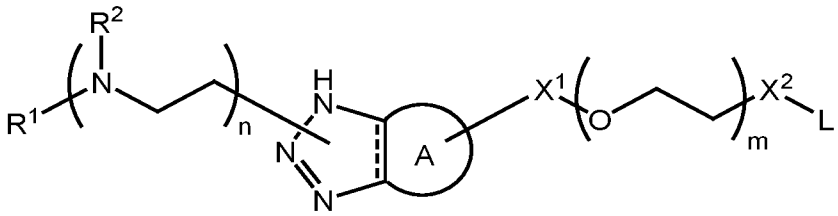
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 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 contiguous 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;

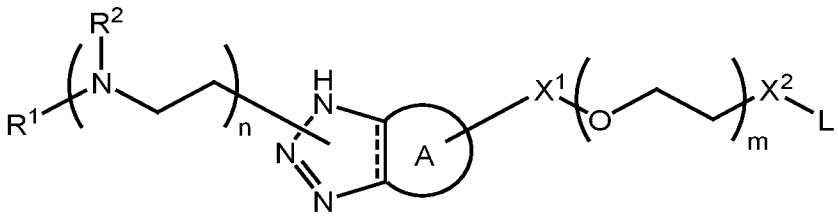
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 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, preferably consisting of, 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 2 to 100, preferably of a discrete number of contiguous repeating units m of 4 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;

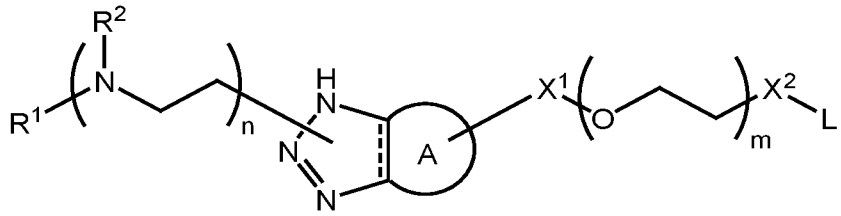
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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 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***.

In another aspect, the present invention provides a composition comprising, preferably consisting of, 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 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 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 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).

In a preferred embodiment, said targeting fragment is capable of binding to an asialoglycoprotein receptor (ASGPr), which is also named herein as ASGPr targeting fragment. Thus, in some embodiments said targeting fragment is an ASGPr targeting fragment. Asialoglycoprotein receptors (ASGPr) are carbohydrate binding proteins (i.e., lectins) which bind asialoglycoprotein and glycoproteins, preferably galactose-terminal glycoproteins and preferably branched galactose-terminal glycoproteins. Preferably said ASGPr targeting

fragment is capable of binding to epitopes on the extracellular domain of ASGPr.

Preferably, said ASGPr targeting fragment is capable of binding to a cell expressing ASGPr. In a preferred embodiment, said targeting fragment is capable of binding to a cell overexpressing ASGPr, preferably a hepatocyte. In a preferred embodiment, said targeting fragment is capable of binding to a cell ASGPr expressing. In a preferred embodiment, said targeting fragment is capable of binding to a cell overexpressing ASGPr. In one embodiment, said cell overexpressing ASGPr means that the level of ASGPr expressed in said cell of a certain tissue is elevated in comparison to the level of ASGPr as measured in a normal healthy cell of the same type of tissue under analogous conditions. In one embodiment, said cell overexpressing ASGPr refers to an increase in the level of ASGPr 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 ASGPr relates to expression of ASGPr that is at least 5-fold, preferably at least 10-fold, further preferably at least 20-fold, as compared to the expression of ASGPr in a normal cell or in a normal tissue. For example, ASGPr is overexpressed in liver cells, preferably hepatocytes, and liver cancer cells. In preferred embodiments, the ASGPr targeting fragment is capable of binding to a liver cell, preferably a hepatocyte or cancerous liver cell and metastases thereof.

Preferably said ASGPr targeting fragment is capable of specifically binding to ASGPr. Typically, specific binding refers to a binding affinity or dissociation constant (KD) of the targeting fragment between about 1 x 10^{-3} M and about 1 x 10^{-12} M. To detect binding of the complex or measure affinity, molecules can be analyzed using a competition binding assay, such as with a Biacore 3000 instrument (see, e.g., Kuo et al., PLoS One, 2015; 10(2): e01166610). Preferably said ASGPr targeting fragment is capable of specifically binding to ASGPr with a binding affinity equal to or greater than that of galactose.

In a preferred embodiment, said ASGPr targeting fragments include small molecules or small molecule ligand, peptides, proteins, more preferably ASGPr antibodies, ASGPr affibodies, ASGPr aptamers, ASGPr targeting peptides, lactose, galactose, N-acetylgalactosamine (GalNAc), galactosamine, N-formylgalactosamine, N-acetyl-galactosamine, N-propionylgalactosamine, N-n-butanoylgalactosamine, and N-iso-butanoylgalactosamine, and combinations thereof (Iobst, S. T. and Drickamer, K. J.B.C. 1996, 271, 6686). In some embodiments, ASGPr targeting fragments are monomeric (i.e., having a single galactosamine). In some embodiments, ASGPr targeting fragments are multimeric (i.e., having multiple galactosamines).

In a preferred embodiment, the ASGPr targeting fragment is a galactose cluster. A galactose cluster is understood as a molecule having two to four terminal galactose derivatives. As used herein, the term galactose derivative includes both galactose and derivatives of galactose having affinity for the asialoglycoprotein receptor equal to or greater than that of galactose. Preferably the galactose derivative is selected from galactose, galactosamine, N-formylgalactosamine, N-acetylgalactosamine, N-propionyl-galactosamine, N-n-butanoylgalactosamine, and N-iso-butanoylgalactosamine. Preferably the galactose derivative is an N-acetyl-galactosamine (GalNAc).

In preferred embodiments, a galactose cluster contains three galactose derivatives each linked to a central branch point, preferably wherein each terminal galactose derivative is attached to the remainder of the galactose cluster through its C-1 carbon. In preferred embodiments, the galactose derivative is linked to the branch point via linkers or spacers, preferably flexible hydrophilic spacers, more preferably PEG spacers and yet more preferably PEG3 spacers.

In preferred embodiments, a galactose cluster has three terminal galactosamines or galactosamine derivatives each having affinity for the ASGPr (i.e., is a tri-antennary galactose derivative cluster). In some embodiments the galactose cluster comprises tri-antennary galactose, tri-valent galactose and galactose trimer. Preferably the galactose cluster has three terminal N-acetyl-galactosamines.

In another preferred embodiment, the targeting fragment is folic acid, wherein typically and preferably said coupling to the rest of said conjugate is effected via the terminal carboxyl group of said folic acid. In some preferred embodiments, the targeting fragment can be folate. Without wishing to be bound by theory, folate can be selectively taken up in cells that have increased expression (e.g., overexpression) of folate receptor.

In further preferred embodiments the targeting fragment are HER2 targeting ligands, which in some embodiments can be selectively taken up in cells that have increased expression (e.g., overexpression) of HER2.

In some embodiments, the targeting fragment can be a somatostatin receptor-targeting fragment. Without wishing to be bound by theory, the somatostatin receptor-targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of somatostatin receptors such as somatostatin receptor 2 (SSTR2).

In some embodiments, the targeting fragment can be an integrin-targeting fragment such as arginine-glycine-aspartic acid (RGD)-containing ligands (e.g., cyclic RGD ligands).

Without wishing to be bound by theory, the integrin-targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of integrins (e.g., RGD integrins such as $\alpha_v\beta_6$ integrin or $\alpha_v\beta_8$ integrin).

In some embodiments, the targeting fragment can be a low pH insertion peptides (pHLIP). Without wising to be bound by theory, the low pH insertion peptide can be selectively taken up by cells that exist in a low pH microenvironment. In some embodiments, the targeting fragment can be an asialoglycoprotein receptor-targeting fragment such as asialoorosomucoid. Without wising to be bound by theory, the asialoglycoprotein receptor-targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of asialoglycoprotein receptors. In some embodiments, the targeting fragment can be an insulin-receptor targeting fragment such as insulin. Without wishing to be bound by theory, the insulin-receptor targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of insulin receptors. In some embodiments, targeting fragment can be a mannose-6-phosphate receptor targeting fragment such as mannose-6-phosphate. Without wishing to be bound by theory, the mannose-6-phosphate receptor targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of mannose-6-phosphate receptors (e.g., monocytes). In some embodiments, the targeting fragment can be a mannose receptor-targeting fragment such as mannose. Without wishing to be bound by theory, the mannose-receptor-targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of mannose receptors. In some embodiments, the targeting fragment can be a Sialyl Lewis^x antigen targeting fragments such as E-selectin. Without wishing to be bound by theory, the Sialyl Lewis^x antigen-targeting fragments can be selectively taken up by cells that have increased expression (e.g., overexpression) of glycosides such as Sialyl Lewis^x antigens. In some embodiments, the targeting fragment can be N-acetyllactosamine targeting fragment. Without wishing to be bound by theory, the N-acetyllactosamine targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) N-acetyllactosamine. In some embodiments, the targeting fragment can be a galactose targeting fragment. Without wishing to be bound by theory, the galactose targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of galactose. In some embodiments, the targeting fragment can be a sigma-2 receptor agonist, such as N,N-dimethyltryptamine (DMT), a sphingolipid-derived amine, and/or a steroid (e.g., progesterone). Without wishing to be bound by theory, the sigma-2 receptor agonist can be selectively taken up by cells that have increased expression (e.g.,

overexpression) of sigma-2 receptors. In some embodiments, the targeting fragment can be a p32-targeting ligand such as anti-p32 antibody or p32-binding LyP-1 tumor-homing peptide. Without wising to be bound by theory, the p32-targeting ligand can be selectively taken up by cells that have increased expression (e.g., overexpression) of the mitochondrial protein p32. In some embodiments, the targeting fragment can be a Trop-2 targeting fragment such as an anti-Trop-2 antibody and/or antibody fragment. Without wishing to be bound by theory, the Trop-2 targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of Trop-2. In some embodiments, the targeting fragment is an insulin-like growth factor 1 receptor-targeting fragment, such as insulin-like growth factor 1. Without wishing to be bound by theory, the insulin-like growth factor 1 receptor-targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of insulin-like growth factor 1 receptor. In some embodiments, the targeting fragment can be a VEGF receptor-targeting fragment such as VEGF. Without wishing to be bound by theory, the VEGF receptor-targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of VEGF receptor. In some embodiments, the targeting fragment can be a platelet-derived growth factor receptor-targeting fragment such as platelet-derived growth factor. Without wishing to be bound by theory, the platelet-derived growth factor receptor-targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of platelet-derived growth factor receptor. In some embodiments, the targeting fragment can be a fibroblast growth factor receptor-targeting fragment such as fibroblast growth factor. Without wishing to be bound by theory, the fibroblast growth factor receptor-targeting fragment can be selectively taken up by cells that have increased expression (e.g., overexpression) of fibroblast growth factor receptor.

Coupling of PEG Fragment to Targeting fragment

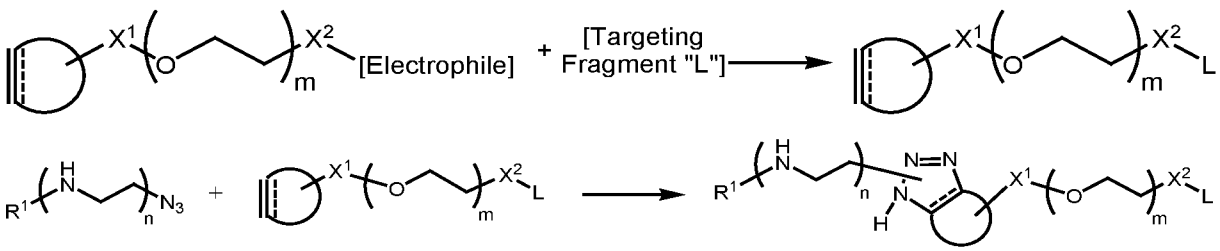
In some embodiments, the second terminal end of the PEG fragment is functionalized with a linking group (i.e., X^2) that links the PEG fragment to a targeting fragment. Typically, the linking moiety X^2 comprises a reactive group for coupling to an appropriate, i.e. complementary reactive group on the 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.

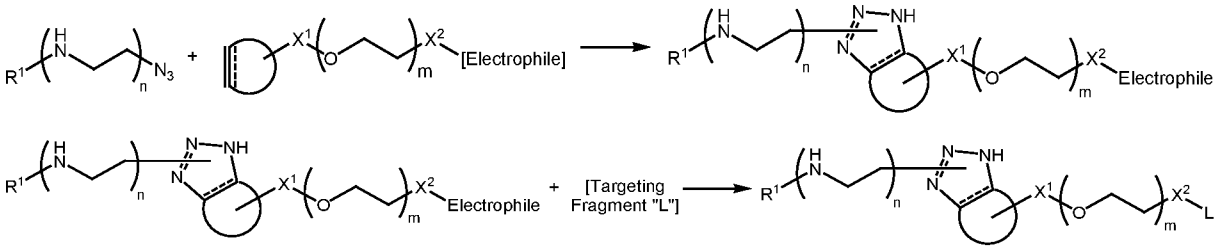
Scheme 1. Exemplary coupling difunctional PEG to targeting fragment followed by LPEI



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.

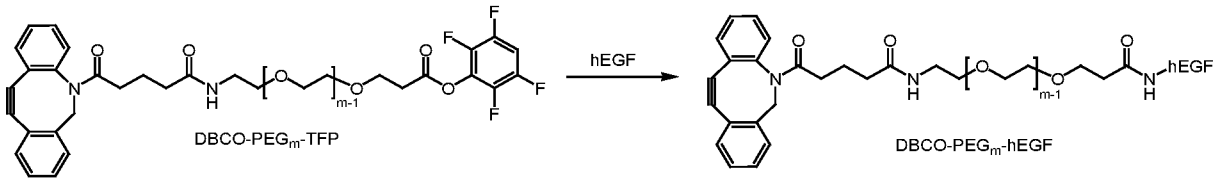
Scheme 2. Exemplary coupling difunctional PEG to LPEI followed by targeting fragment.



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.

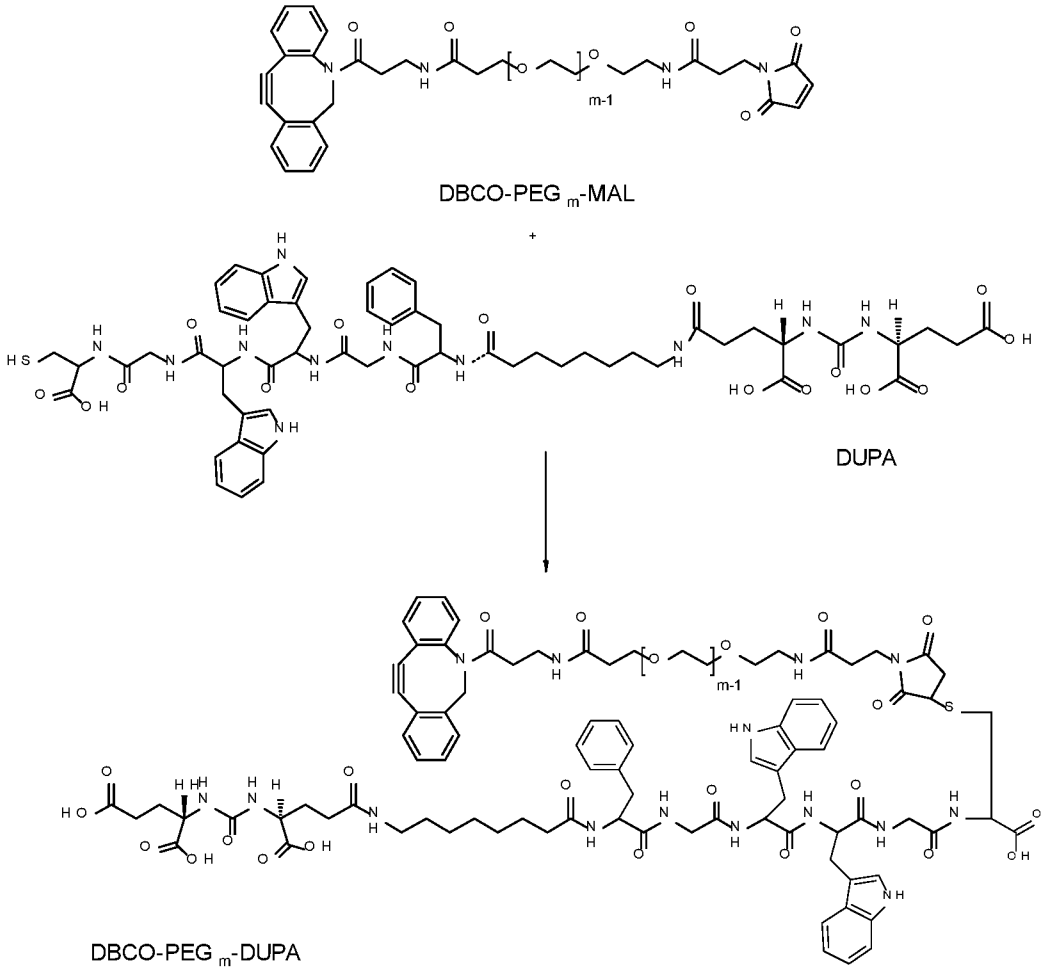
Scheme 3. Exemplary coupling of bifunctional PEG to hEGF.



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 with hEGF in solution results

in a nucleophilic substitution via a nucleophilic amine of hEGF to produce an hEGF-modified PEG. The DBCO group can be used in subsequent reactions for coupling to an LPEI fragment. The variable m represents the number of repeating PEG units as described herein.

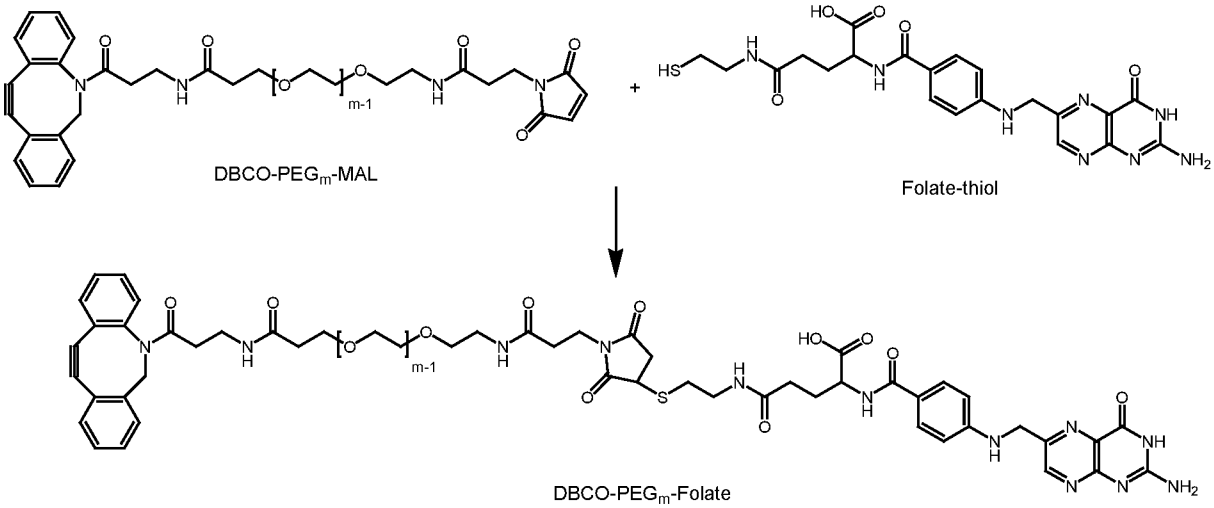
Scheme 4. Exemplary coupling of bifunctional PEG to DUPA.



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 the number of repeating PEG units as described herein.

Scheme 5. Exemplary coupling of bifunctional PEG to folate.



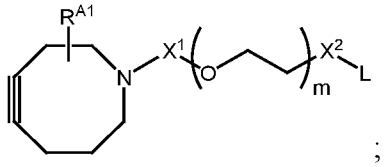
As shown above in Scheme 5, PEG can be modified to include an electrophilic maleimide (MAL) group. The maleimide-substituted PEG can be coupled to nucleophilic partner such as a folate residue which itself is modified to contain a nucleophilic group (e.g., thiol). Treatment of the MAL-modified PEG in 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 the number of repeating PEG units as described herein.

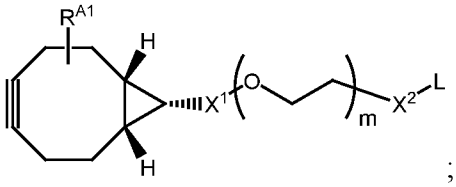
Coupling of PEG Fragment to LPEI Fragment

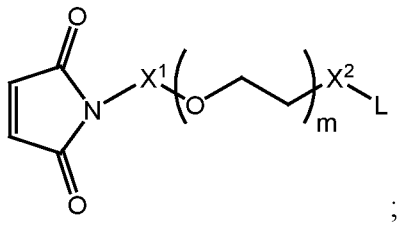
Before or after coupling the bi-functionalized PEG fragment to a targeting fragment, the bi-functionalized PEG fragment can be coupled to an LPEI fragment. In preferred embodiments, the bi-functionalized PEG fragment is coupled to LPEI using cycloaddition chemistry, e.g., a 1,3-dipolar cycloaddition 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.

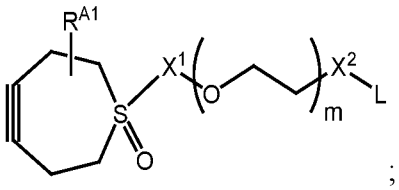
One of skill in the art will appreciate that any suitable alkene or alkyne groups can be used to react with an azide group to couple the LPEI fragment to the PEG fragment. In some preferred embodiments, incorporation of alkene or alkyne groups into ring systems introduces strain into the ring systems. 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

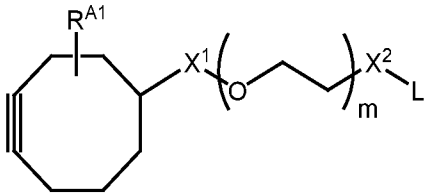
an alkyne group, or eight-membered rings that include a *trans* alkene group. For example, suitable alkyne groups such as cyclooctyne (OCT), monofluorinated cyclooctyne (MOFO), difluorocycloalkyne (DIFO), dibenzocyclooctynol (DIBO), dibenzoazacyclooctyne (DIBAC), bicyclononyne (BCN), biarylazacyclooctynone (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:

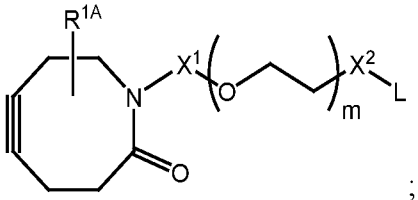

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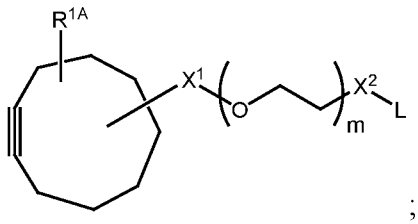

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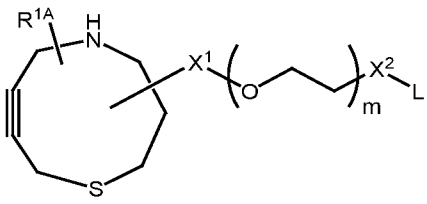

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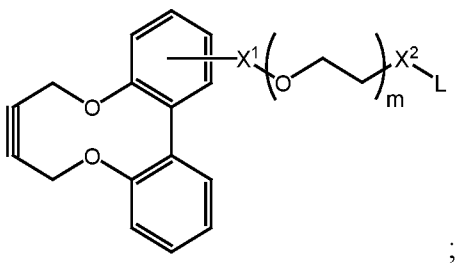

;


;


;


;


; or


;

wherein the variables X^1, X^2, R^{1A}, 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.

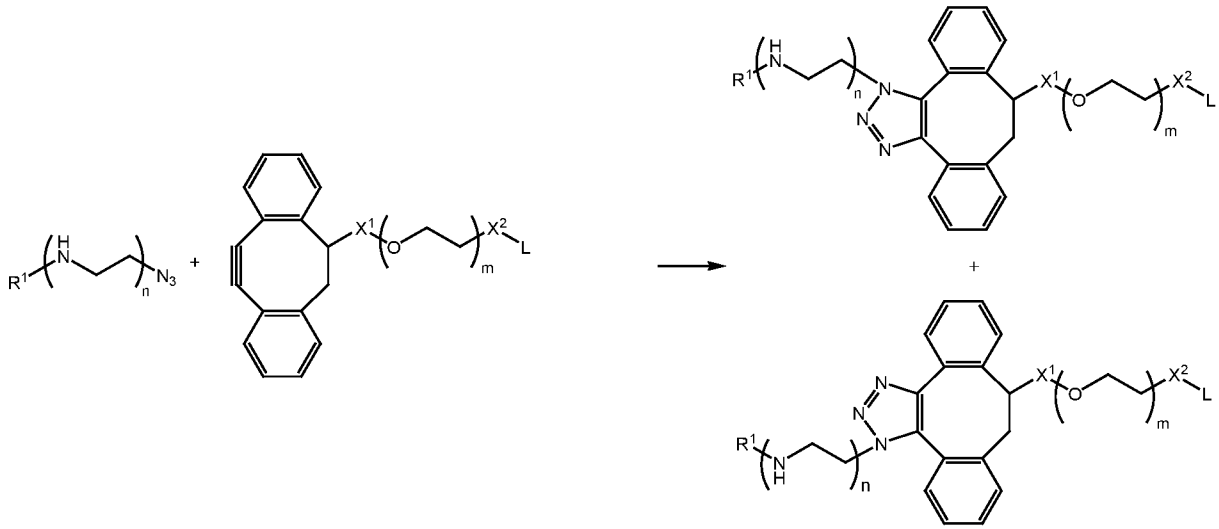
In some preferred embodiments, the [3+2] azide-alkyne cycloaddition reaction takes place at a pH of 5 or below, preferably 4 or below. As set forth below in the Comparative Example, no reaction occurred when a PEG fragment modified with an activated alkyne was treated with a non-azide containing LPEI 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.

Thus, 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.

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,

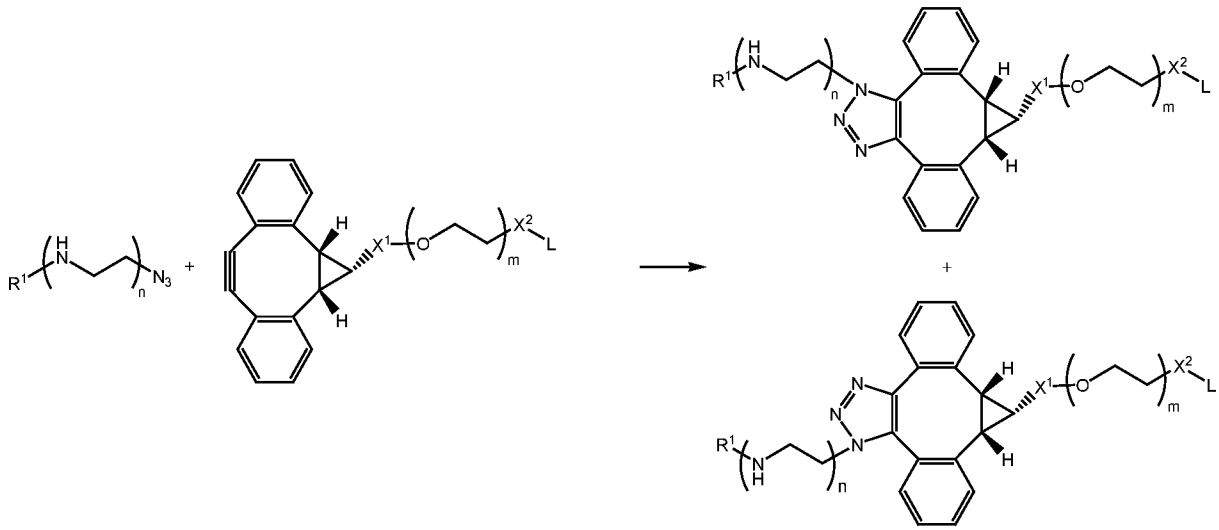
wherein said reaction occurs at a pH below about 5, preferably 4 or below, and wherein preferably said omega terminus of said linear polyethyleneimine fragment comprises an azide, and wherein said first terminal end of said polyethylene glycol fragment comprises an alkene or an alkyne, and wherein said 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.

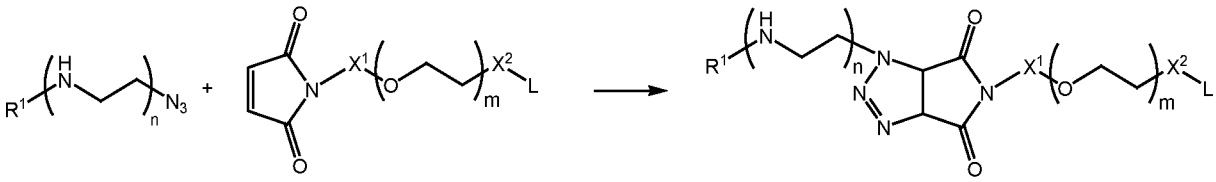
Scheme 7. Coupling of LPEI to Bicyclononyne (BCN)-modified PEG



As shown above in Scheme 7, in some embodiments PEG can be modified to include a

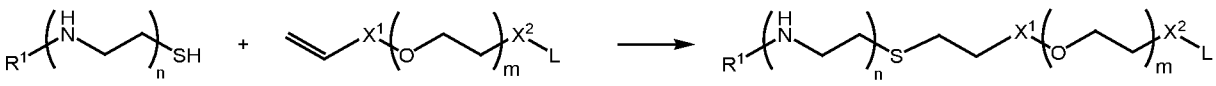
strained alkyne group such bicyclononyne (BCN). Treatment of the BCN-modified PEG in solution with an azide-modified LPEI results in a [3+2] cycloaddition of the azide to the alkyne of BCN to produce a 1, 2, 3 triazole. One of skill in the art will appreciate that the reaction shown above in Scheme 7 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.

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.

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.

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.

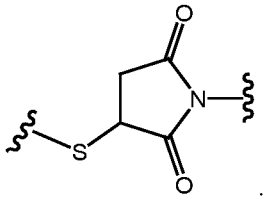
In one aspect, X^1 is a linking moiety of the formula $–(Y^1)_p–$, wherein p is an integer between 1 and 20, and each occurrence of Y^1 is independently selected from a chemical bond, $-CR^{11}R^{12}-$, -C(O)-, -O-, -S-, $-NR^{13}-$, an amino acid residue, a divalent phenyl moiety, a divalent carbocyle 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.

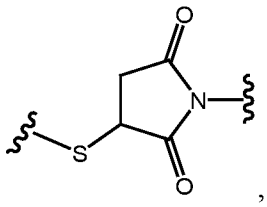
In some embodiments, when Y^1 is an amino acid residue, it can be oriented in any direction, i.e., -C(O)-CHR-NH- or -NH-CHR-C(O)-, wherein "R" represents the side-chain of a naturally occurring amino acid.

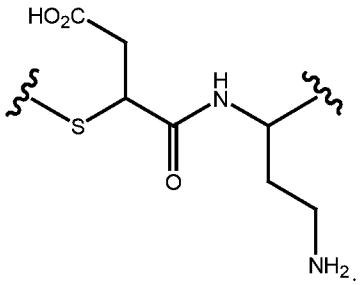
In some embodiments, the divalent heteroaryl moiety is a divalent heteroaryl group comprising one or more heteroatoms selected from O, N, S, and P, preferably one or two atoms selected from O and N. In some embodiments, the divalent heteroaryl moiety is a divalent furan, pyrrole, imidazole, pyrazole, 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}.

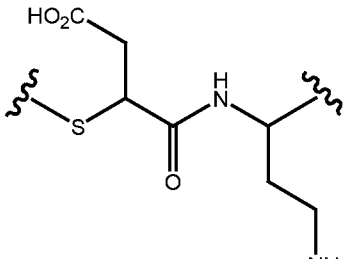
In the embodiments below for X^1, unless otherwise specified, a wavy line indicates a bond in any direction, i.e., to a PEG fragment or to the divalent covalent linking moiety (e.g., "Z" or Ring A).

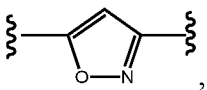
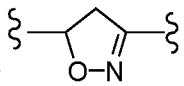
In some embodiments, the divalent heterocycle moiety is a divalent heterocycle group comprising one or more heteroatoms selected from O, N, S, and P, preferably one or two atoms selected from O and N. In some embodiments, the divalent heterocycle moiety is a divalent tetrahydrofuran, pyrrolidine, piperidine, or 4,5-Dihydro-isoxazole, each optionally substituted with one or more R^{14}. In some preferred embodiments, the divalent heterocycle moiety is a

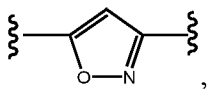
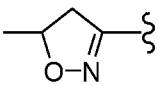
succinimide. In some preferred embodiments, two Y^1 can combine to form a linking moiety or partial linking moiety of the formula .

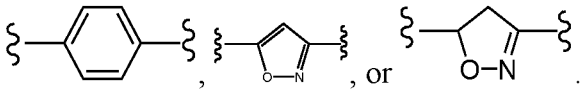
In a further preferred embodiment, two Y^1 can combine to form a linking moiety or partial linking moiety of the formula , wherein the wavy line next to the sulfur represents the direction of connectivity towards the targeting fragment.

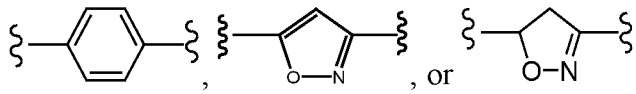
In a further preferred embodiment, Y^1 can comprise a linking moiety or partial linking moiety of the formula: .

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.

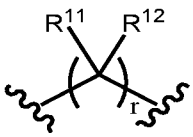
In some embodiments, X^1 is a linking moiety of the formula $–(Y^1)_p–$, wherein p is an integer between 1 and 8, and each occurrence of Y^1 is independently selected from a chemical bond, $-CHR^{11}-$, -C(O)-, -O-, -S-, -NH-, $-C_6H_4-$, , or .

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 .

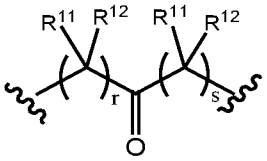
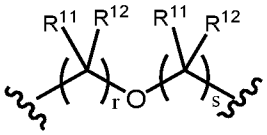
In some embodiments, X^1 is a linking moiety of the formula $–(Y^1)_p–$, wherein p is an integer between 1 and 8, and each occurrence of Y^1 is independently selected from a chemical bond, -CH_2-, -C(O)-, -O-, -S-, -NH-, .

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-, , wherein Y^1 is only -NH- when it is adjacent to a -C(O)- group to form a carbamate or amide.

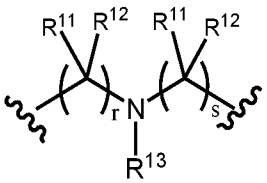
In some embodiments, X^1 is

, 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.

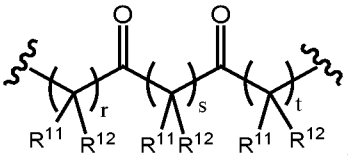
In some embodiments, X^1 is

, 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 $–[OCH_2$-$CH_2]_m–$.

In some embodiments, X^1 is

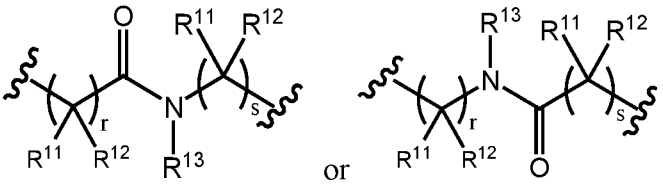
, wherein s and t are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 7; and wherein R^{11}, R^{12}, and R^{13} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more 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 $–[OCH_2$-$CH_2]_m–$.

In some embodiments, X^1 is



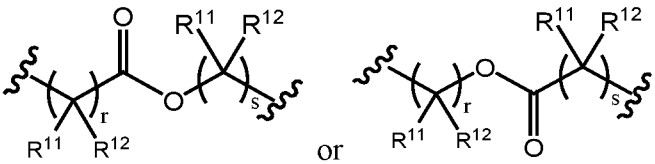
, wherein r is an integer between 0 and 3, preferably between 1 and 3, more preferably between 1 and 2; s and t are each independently an integer between 0 and 2, preferably 0 and 1; wherein the sum of r, s, and t is less than or equal to 6; and wherein R^{11} and R^{12} are independently -H or C_1-C_6 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 –$[OCH_2$-$CH_2]_m$–.

In some embodiments, X^1 is



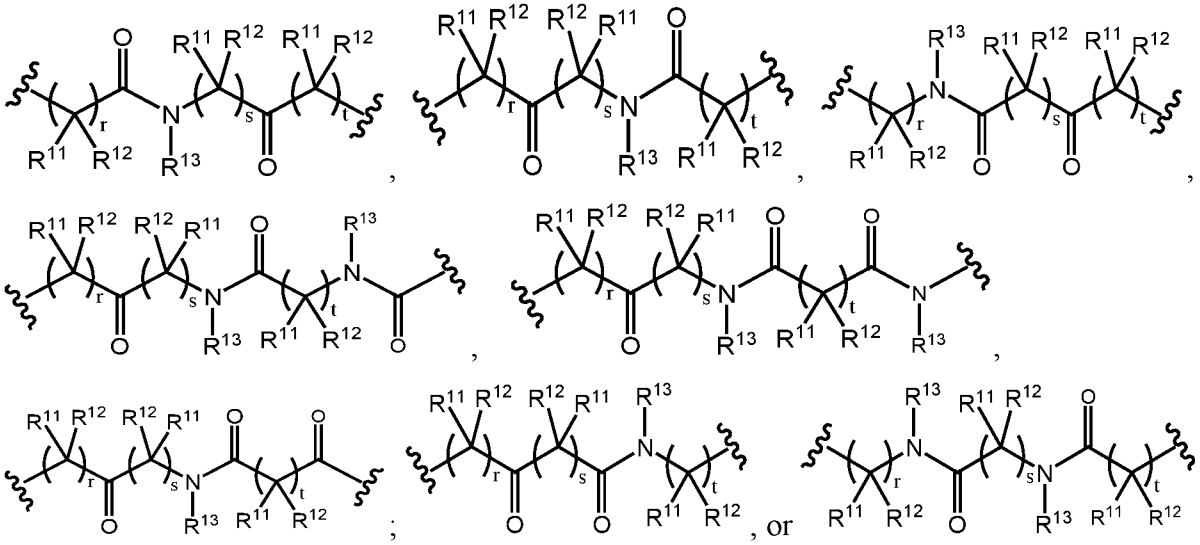
, wherein r and s are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 6; and wherein R^{11}, R^{12} and R^{13} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more 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 –$[OCH_2$-$CH_2]_m$–.

In some embodiments, X^1 is



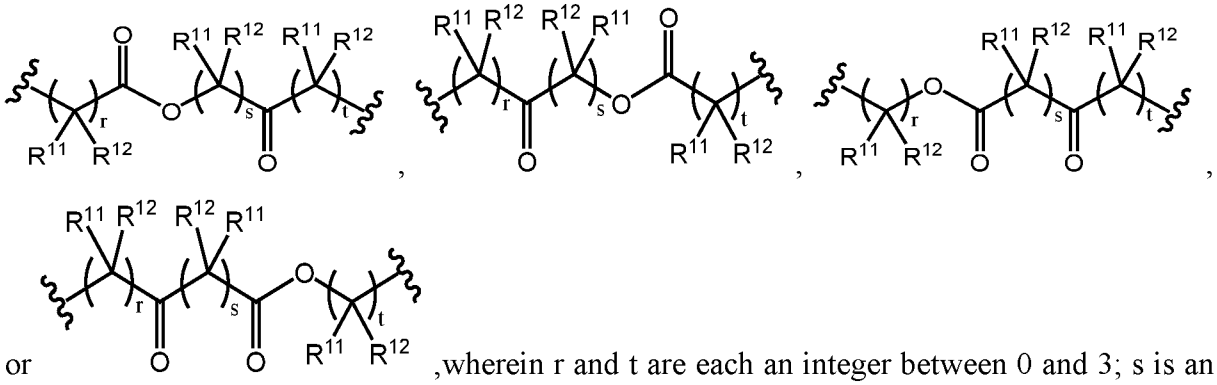
, wherein r and s are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 6; and wherein R^{11}, R^{12} and R^{13} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more 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 –$[OCH_2$-$CH_2]_m$–.

In some embodiments, X^1 is



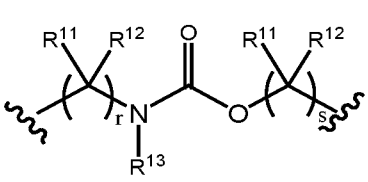
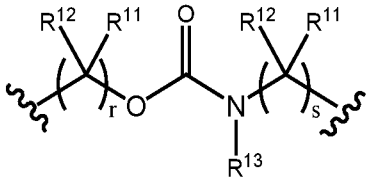
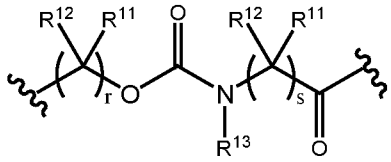
wherein r and t are each an integer between 0 and 3 and s is an integer between 0 and 3; preferably wherein r is 0, s is 2 or 3, and t is 2; wherein the sum of r, s and t is less than or equal to 5; and wherein R^{11}, R^{12} and R^{13} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 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 $–[OCH_2\text{-}CH_2]_m–$.

In some embodiments, X^1 is



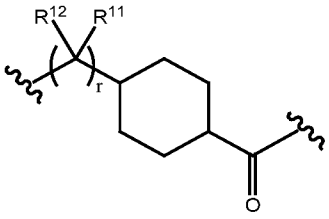
wherein r and t are each an integer between 0 and 3; s is an integer between 0 and 3; wherein the sum or r, s and t is less than or equal to 5; and wherein R^{11} and R^{12} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to 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 $–[OCH_2\text{-}CH_2]_m–$.

In some embodiments, X^1 is

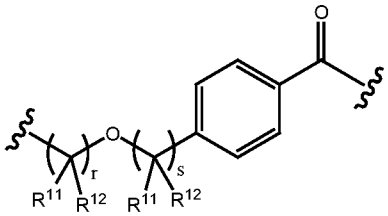


, or

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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 –$[OCH_2$-$CH_2]_m$–.

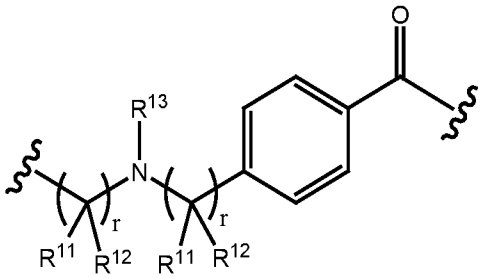
In some embodiments, X^1 is


, 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 –$[OCH_2$-$CH_2]_m$–.

In some embodiments, X^1 is

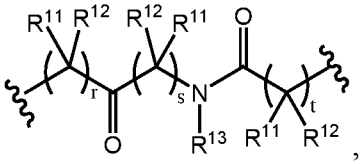
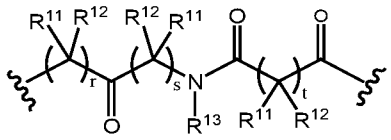
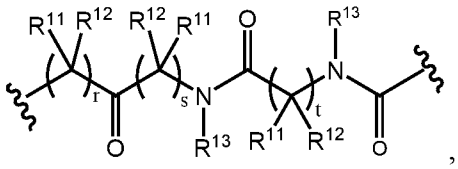
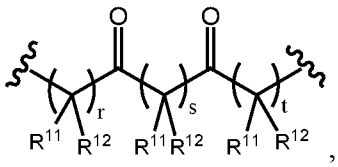
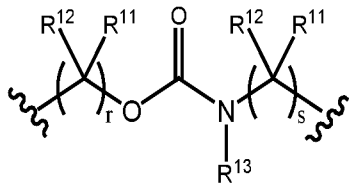
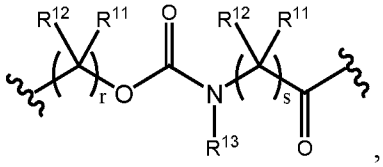
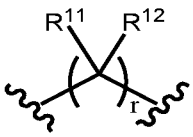
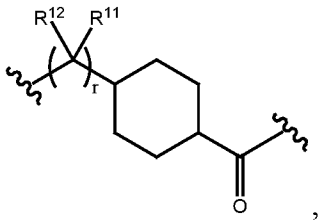
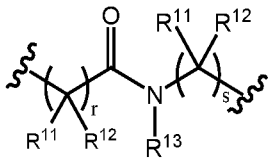
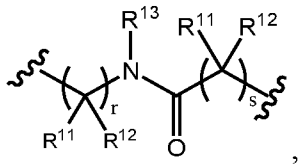
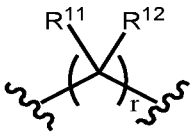
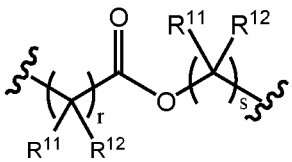
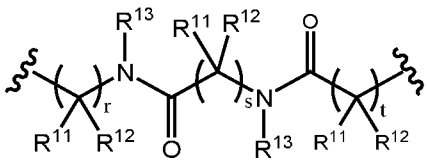
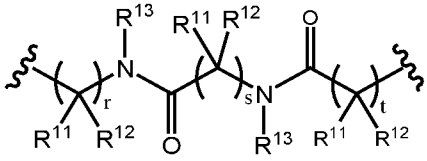

, wherein r and s are each independently an integer between 0 and 4, preferably between 0 and 2, more preferably between 1 and 2; wherein the sum of r and s is less than or equal to 5; and wherein R^{11}, and R^{12} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -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 –$[OCH_2$-$CH_2]_m$–.

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 $–[OCH_2\text{-}CH_2]_m–$.

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


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, or

; wherein:

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

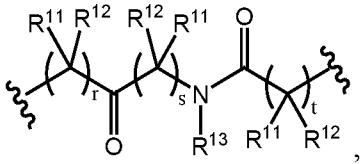
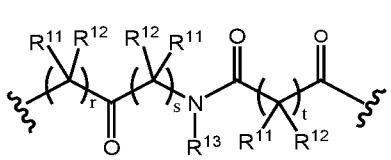
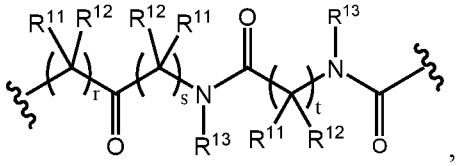
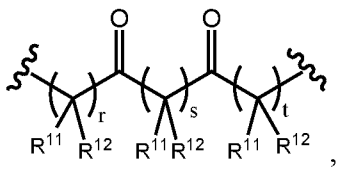
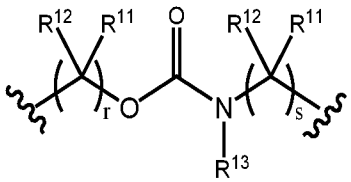
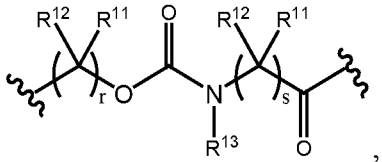
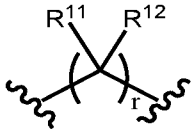
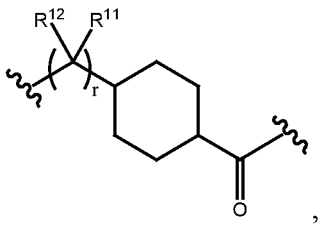
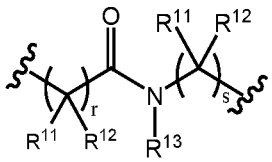
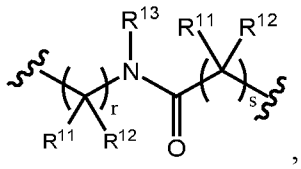
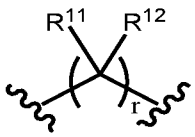
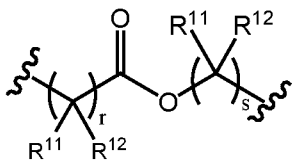
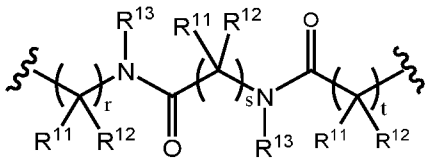
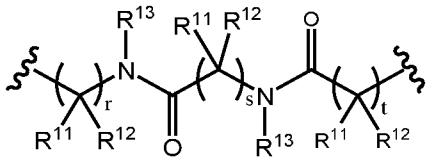
s is independently, at each occurrence, 0-6, preferably 0, 2, 4;

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-CH_2]$_m$–.

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


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; wherein:

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

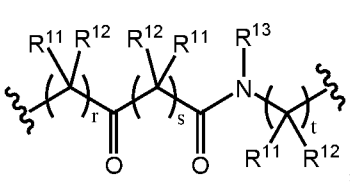
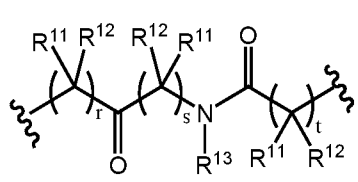
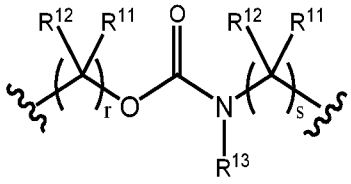
s is independently, at each occurrence, 0-6, preferably 0, 2, 4;

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]$_m$–.

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;

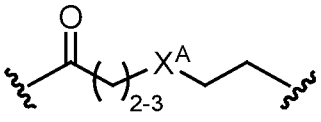
s is independently, at each occurrence, 0-6, preferably 0, 2, 3, or 4; more preferably 2 or 3;

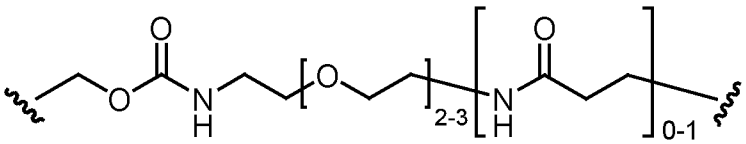
t is independently, at each occurrence, 0-6, preferably 0, 1, 2, 4; more preferably 2;

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

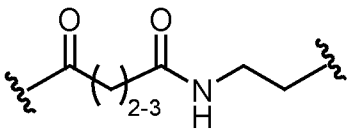
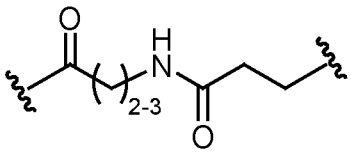
R^{13} is -H. Preferably the wavy line nearest to the integer "r" is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line nearest to the integer "s" or "t" group is a bond to the PEG fragment –$[OCH_2$-$CH_2]_m$–.

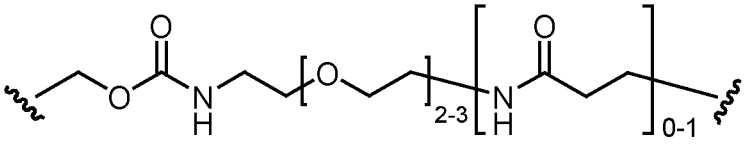
In some preferred embodiments, X^1 is selected from:


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

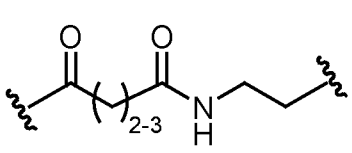
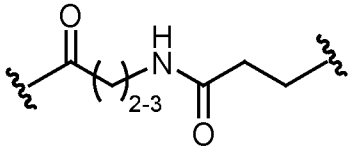
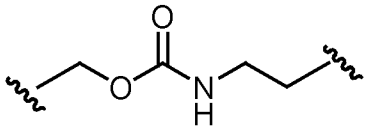
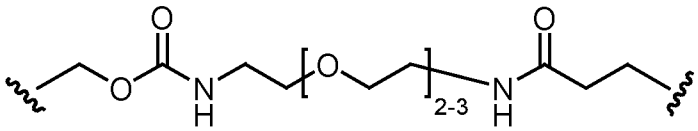

. Preferably the wavy line on the left side is a bond to the divalent covalent linking moiety (e.g., "Z" or Ring A) and the wavy line on the right side is a bond to the PEG fragment –$[OCH_2$-$CH_2]_m$–.

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

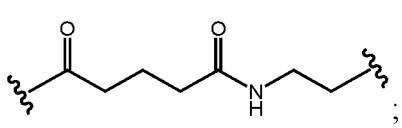
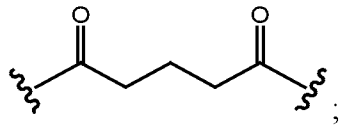
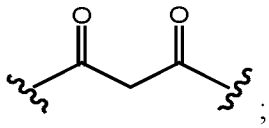
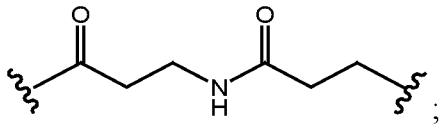
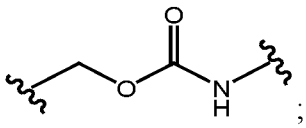
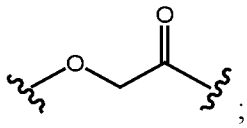
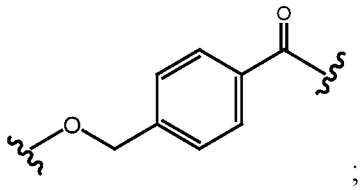
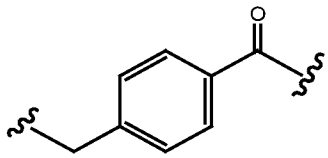
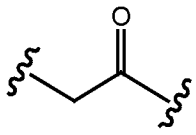
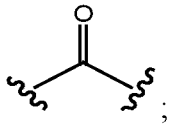
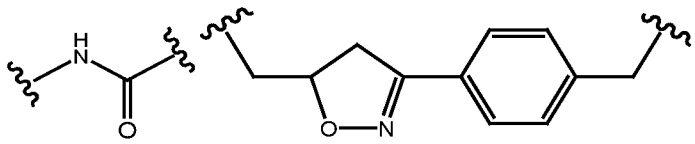
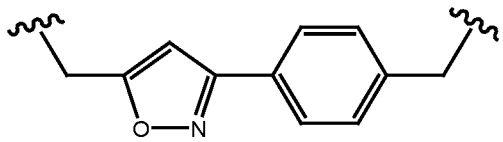

;

; 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 –$[OCH_2$-$CH_2]_m$–.

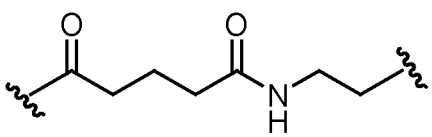
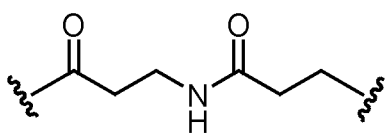
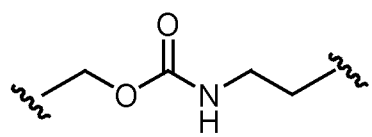
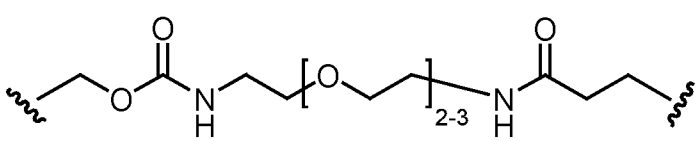
In some preferred embodiments, X^1 is selected from:


;

;

, 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 $–[OCH_2\text{-}CH_2]_m–$.

In some embodiments, X^1 is selected from:


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,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 $–[OCH_2\text{-}CH_2]_m–$.

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 $–[OCH_2\text{-}CH_2]_m–$.

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.

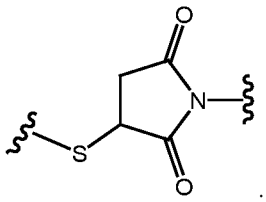
In some embodiments, R^{21}, R^{22} and R^{23} are each independently, at each occurrence, -H, $-CO_2H$, or C_1-C_6 alkyl. In some embodiments, R^{21}, R^{22} and R^{23} are each, independently -H or C_1-C_4 alkyl, preferably C_1-C_2 alkyl.

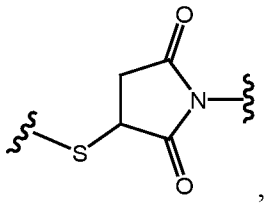
In some embodiments, R^{21}, R^{22}, R^{23}, and R^{24} are -H.

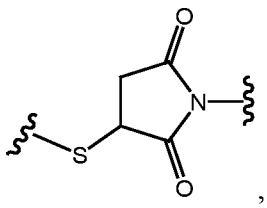
In some embodiments, R^{24} is independently -H, C_1-C_6 alkyl, or oxo.

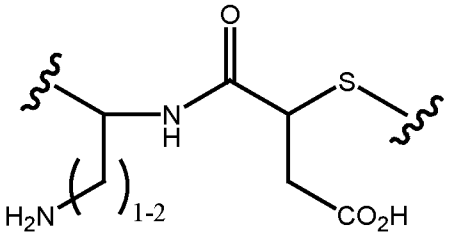
In some embodiments, the divalent heteroaryl moiety is a divalent heteroaryl group comprising one or more heteroatoms selected from O, N, S, and P, preferably one or two atoms selected from O and N. In some embodiments, the divalent heteroaryl moiety is a divalent furan, pyrrole, imidazole, pyrazole, 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}.

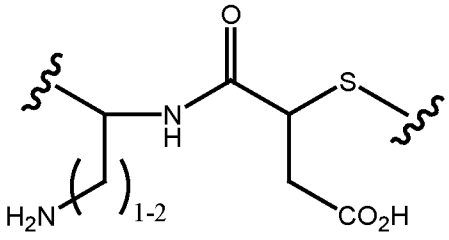
In the embodiments below for X^2, unless otherwise specified, a wavy line indicates a bond in any direction, i.e., to a PEG fragment (-[OCH_2CH_2]$_m$-) or to a targeting fragment (i.e., "L").

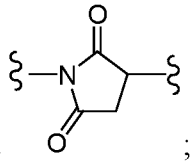
In some embodiments, the divalent heterocycle moiety is a divalent heterocycle group comprising one or more heteroatoms selected from O, N, S, and P, preferably one or two atoms selected from O and N. In some embodiments, the divalent heterocycle moiety is a divalent tetrahydrofuran, pyrrolidine, piperidine, 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) and the wavy line next to the nitrogen represents a bond to the the PEG fragment (–[OCH_2-CH_2]$_m$–).

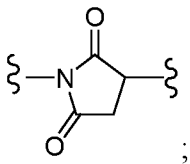
In a further preferred embodiment, two Y^2 can combine to form a linking moiety or partial linking moiety of the formula , wherein the wavy line next to the sulfur represents a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line next to nitrogen represents a bond to the targeting fragment (L).

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

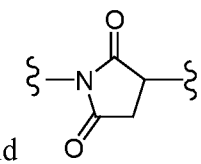
In a further preferred embodiment, Y^2 can comprise a linking moiety or partial linking moiety of the formula: , wherein the wavy line next to the sulfur represents the direction of connectivity towards the targeting fragment.

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

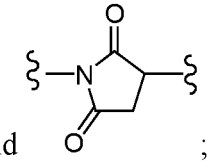
R^{21} and R^{22} are independently, at each occurrence, -H, $-CO_2H$, or C_1-C_6 alkyl, wherein each C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl.

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

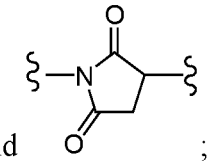
R^{21} is independently, at each occurrence, -H, $-CO_2H$, or C_1-C_4 alkyl (preferably C_1 alkyl), wherein each C_1-C_4 alkyl is optionally substituted with one or more C_6-C_{10} aryl or 5 to 8-membered heteroaryl.

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

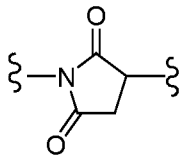
R^{21} is independently, at each occurrence, -H, $-CO_2H$, or C_1-C_4 alkyl (preferably C_1 alkyl), wherein each C_1-C_4 alkyl is optionally substituted with one or more C_6-C_{10} aryl or 5 to 8-membered heteroaryl.

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

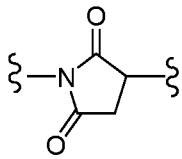
R^{21} is independently, at each occurrence, -H, $-CO_2H$, or C_1-C_3 alkyl (preferably C_1 alkyl), wherein each C_1-C_3 alkyl is optionally substituted with one or more phenyl or indole.

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.

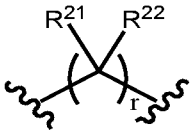
In some embodiments, X^2 is a linking moiety of the formula $–(Y^2)_q–$, wherein q is an integer between 1 and 40, and each occurrence of Y^2 is independently selected from a chemical bond, $-CHR^{21}-$, -NH-, -O-, -S-, -C(O)-, an amino acid residue, and  , wherein Y^2 is only -NH- when it is adjacent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

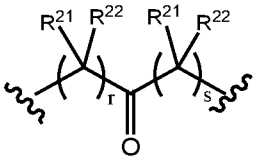
direction, i.e., -C(O)-CHR-NH- or -NH-CHR-C(O)-, wherein "R" represents the side-chain of a naturally occurring amino acid.

In some embodiments, X^2 is

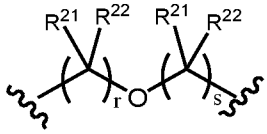


, wherein r is an integer between 1 and 8, preferably between 1 and 4, more preferably between 1 and 2; and wherein R^{21} and R^{22} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H.

In some embodiments, X^2 is

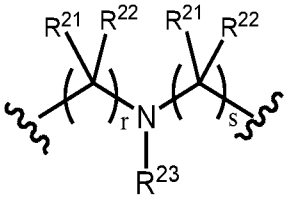


, or



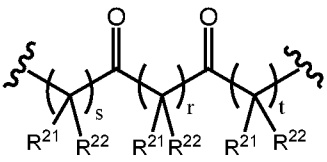
, wherein r and s are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 7; and wherein R^{21} and R^{22} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably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

In some embodiments, X^2 is



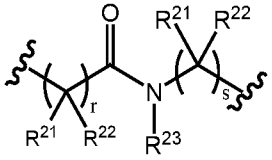
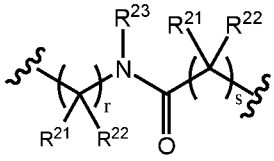
, wherein s and t are each independently an integer between 0 and 4, preferably between 1 and 3, more preferably between 1 and 2; and wherein the sum of r and s is less than or equal to 7; and wherein R^{21}, R^{22}, and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more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

In some embodiments, X^2 is

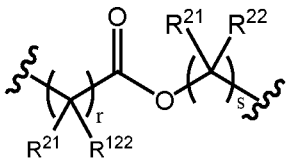
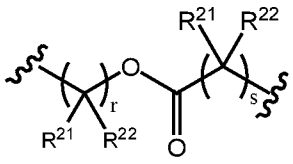


, wherein r is an integer between 0 and 3, preferably between 1 and 3, more preferably between 1 and 2; s and t are each independently an integer between 0 and 2, preferably 0 and 1; wherein the sum of r, s, and t is less than or equal to 6; and wherein R^{21} and R^{22} are independently -H or C_1-C_6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

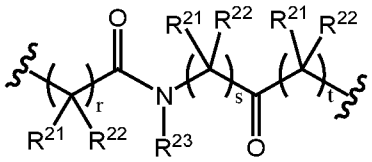
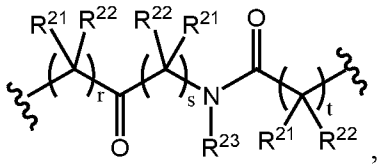
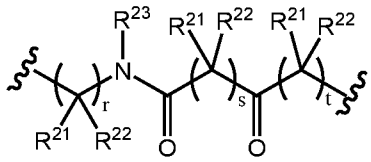
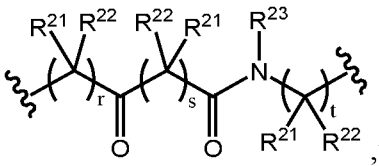
In some embodiments, X^2 is

 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]$_m$–) and the wavy line nearest to the integer "s" is a bond to the targeting fragment (L).

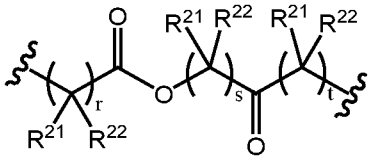
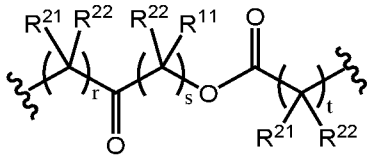
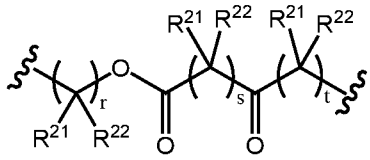
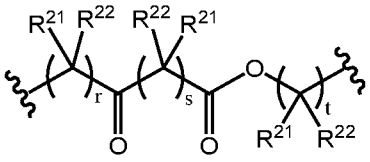
In some embodiments, X^2 is

 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]$_m$–) and the wavy line nearest to the integer "s" is a bond to the targeting fragment (L).

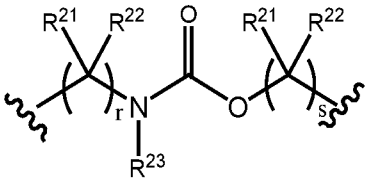
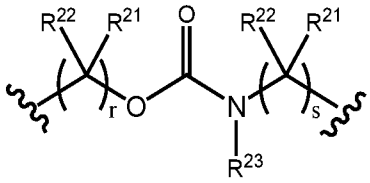
In some embodiments, X^2 is

, , , 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]$_m$–) and the wavy line nearest to the integer "t" is a bond to the targeting fragment (L).

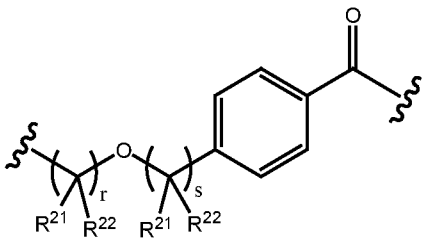
In some embodiments, X^2 is


,

,

, 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^{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]$_m$–) and the wavy line nearest to the integer "t" is a bond to the targeting fragment (L).

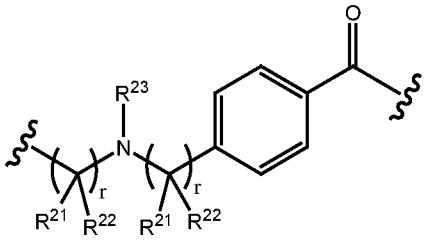
In some embodiments, X^2 is


, 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]$_m$–) and the wavy line nearest to the integer "s" is a bond to the targeting fragment (L).

In some embodiments, X^2 is

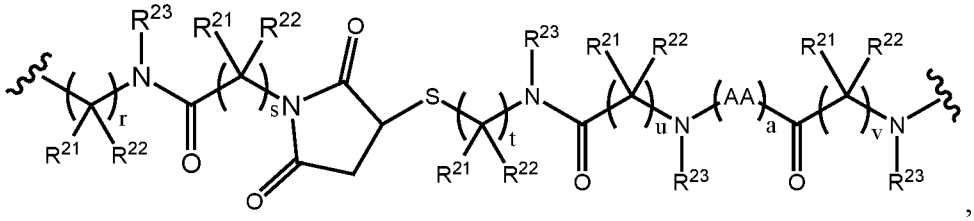

, wherein r and s are each independently an integer between 0 and 4, preferably between 0 and 2, more preferably between 1 and 2; wherein the sum of r and s is less than or equal to 5; and wherein R^{21}, and R^{22} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line nearest to the carbonyl group is a bond to the targeting fragment (L).

In some embodiments, X^2 is

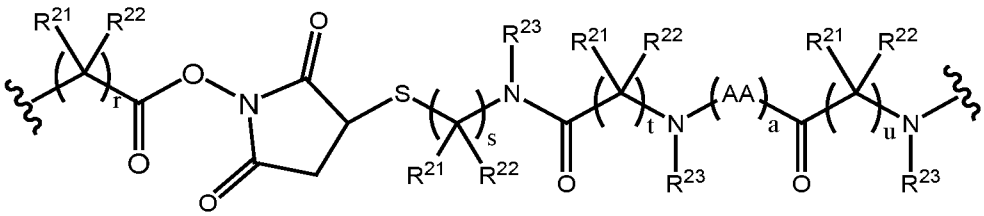


, wherein r and s are each independently an integer between 0 and 4, preferably between 0 and 2; wherein the sum of r and s is less than or equal to 5; and wherein R^{21}, R^{22} and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line nearest to the integer "r" is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line nearest to the carbonyl group is a bond to the targeting fragment (L).

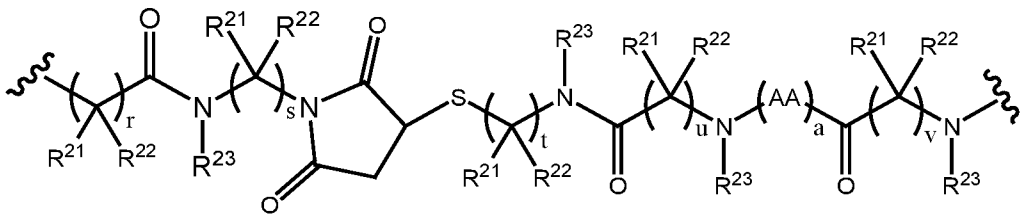
In some embodiments, X^2 is selected from:



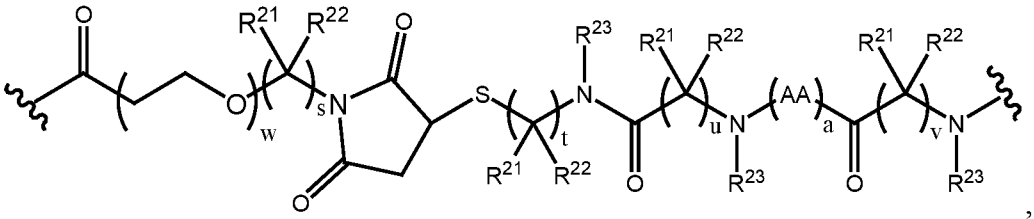
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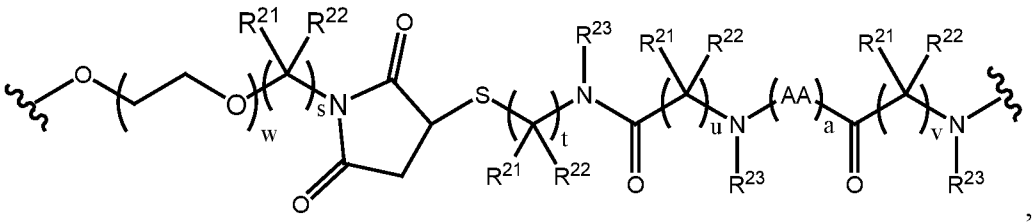
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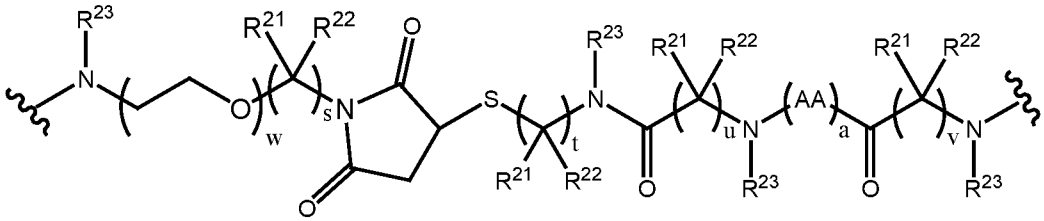
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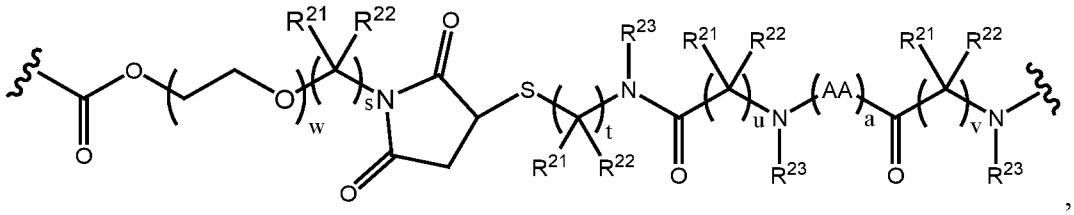


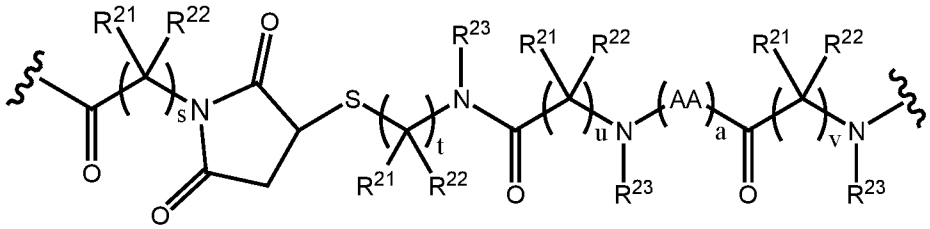
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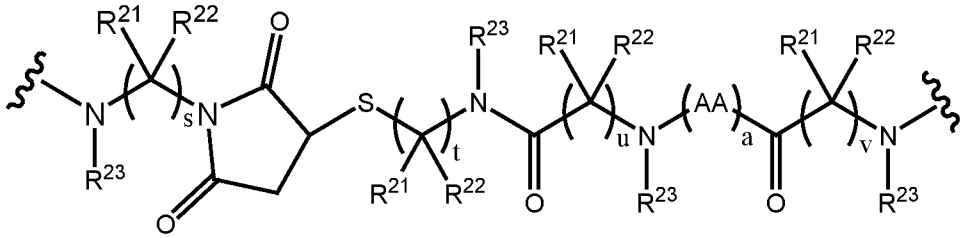


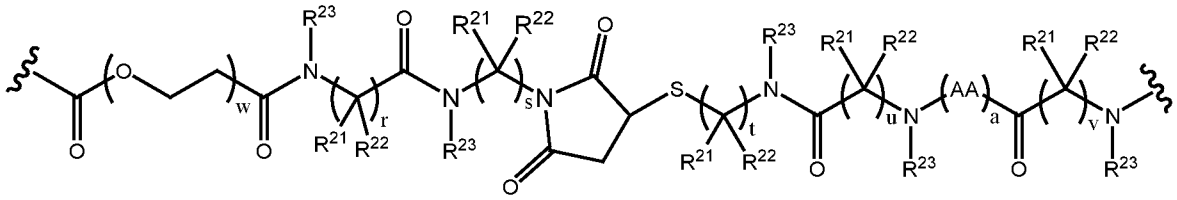
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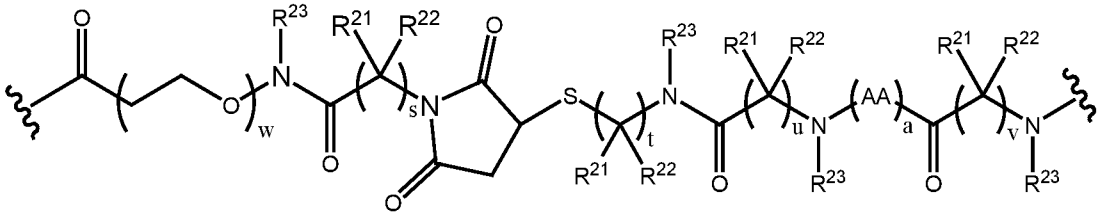

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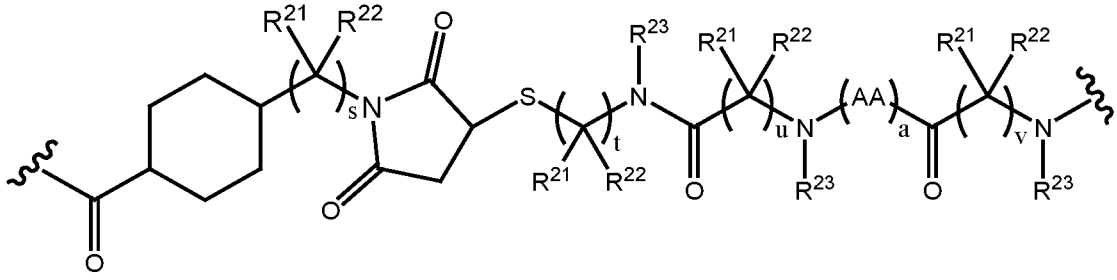

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, or


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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;

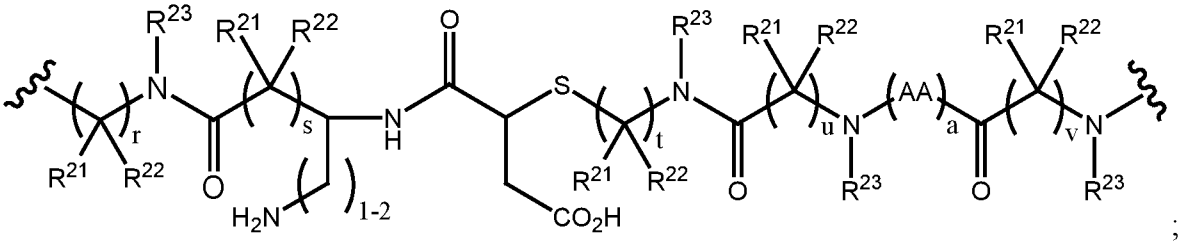
AA is an amino acid residue, preferably a naturally occurring amino acid residue; yet more preferably wherein AA is an an amino acid selected from Arg, His, Lys, Asp, Glu, Ser, Thr, Asn, Gln, Cys, Sec, Gly, Pro, Ala, Val, Ile, Leu, Met, Phe, Tyr, and Trp;

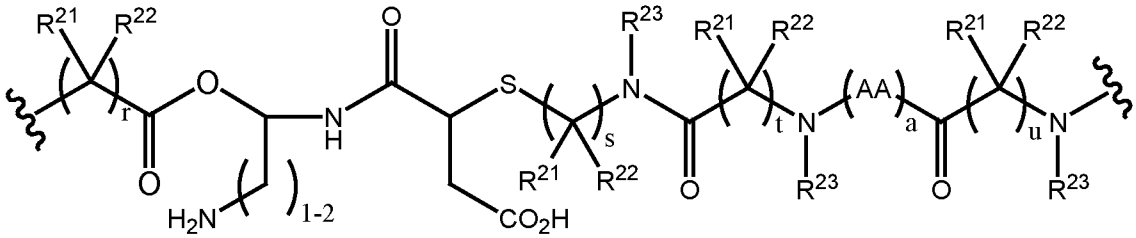
a is an integer between 0 and 10, preferably between 0 and 6; more preferably between 0 and 4;

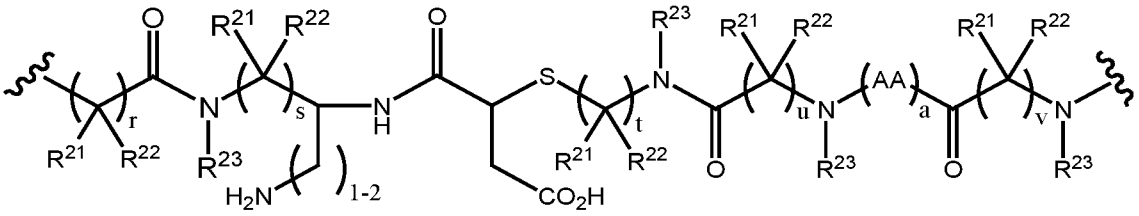
and wherein R^{21}, R^{22} and R^{23} are independently –H, C_1-C_6 alkyl or (-COOH), preferably –H, C_1-C_2 alkyl or (-COOH), more preferably –H or (-COOH). Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

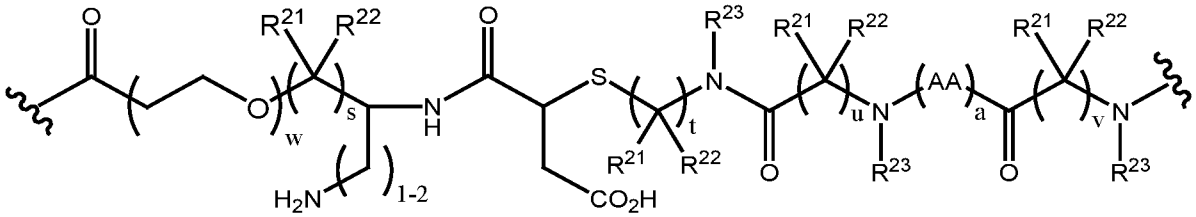
In some preferred embodiments, $(AA)_a$ comprises a tri-peptide selected from Trp-Trp-Gly or Trp-Gly-Phe. In some preferred embodiments, $(AA)_a$ is Trp-Trp-Gly-Phe (SEQ ID NO:2).

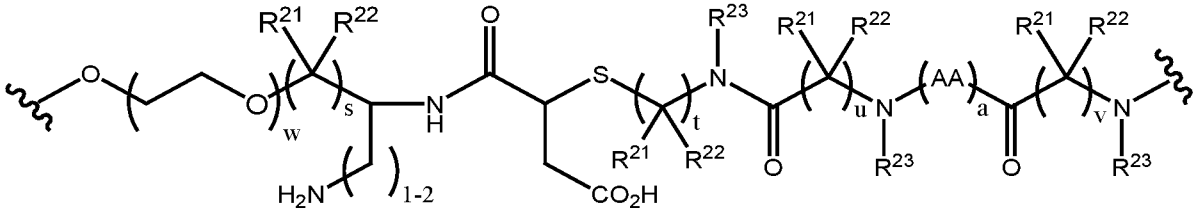
In some embodiments, X^2 is selected from:

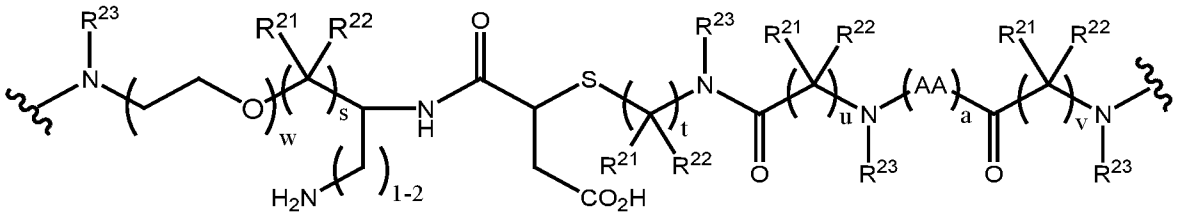

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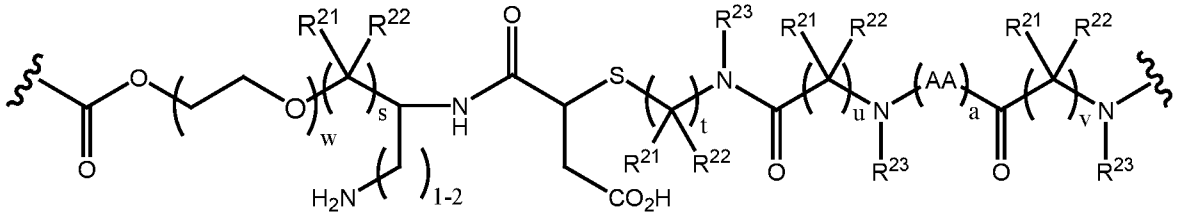

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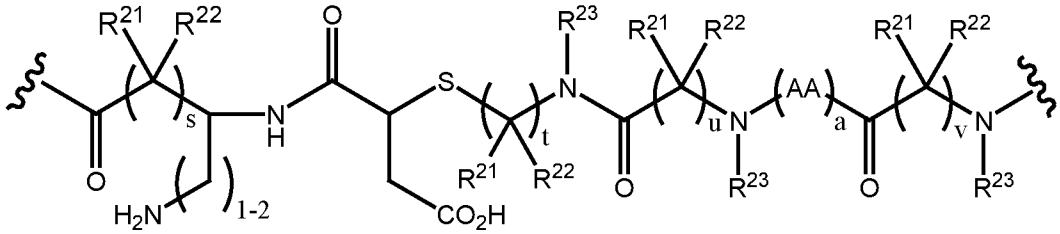

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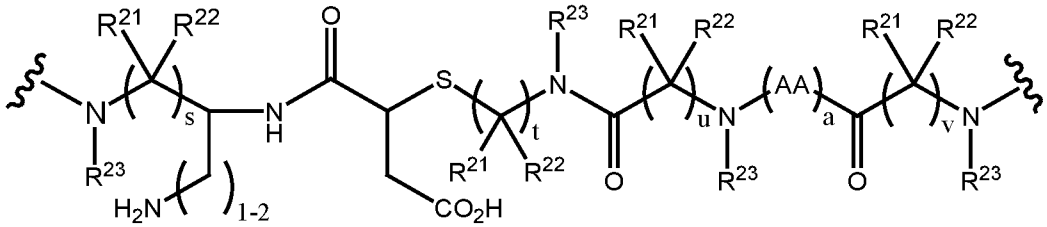

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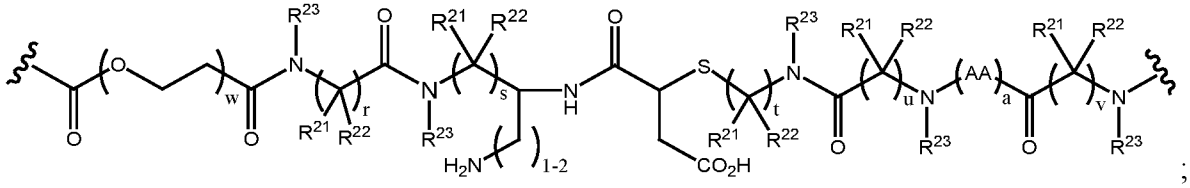

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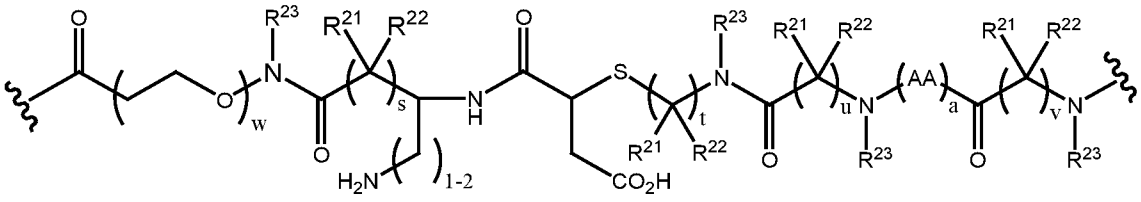

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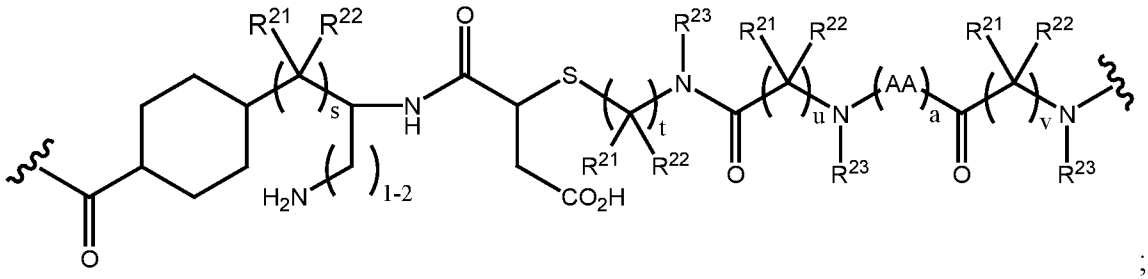

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; or



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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;

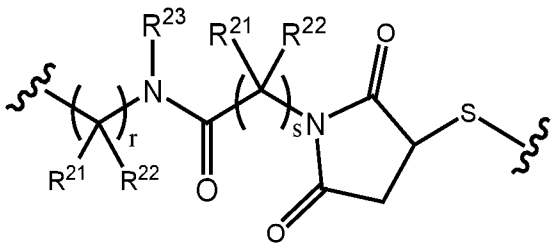
AA is an amino acid residue, preferably a naturally occurring amino acid residue; yet more preferably wherein AA is an an amino acid selected from Arg, His, Lys, Asp, Glu, Ser, Thr, Asn, Gln, Cys, Sec, Gly, Pro, Ala, Val, Ile, Leu, Met, Phe, Tyr, and Trp;

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

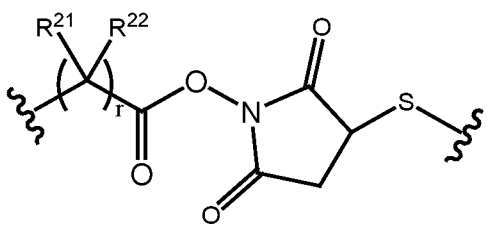
and wherein R^{21}, R^{22} and R^{23} are independently –H, C_1-C_6 alkyl or (-COOH), preferably –H, C_1-C_2 alkyl or (-COOH), more preferably –H or (-COOH). Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

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

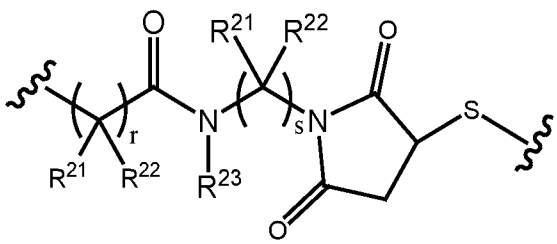
In some embodiments, X^2 is selected from:



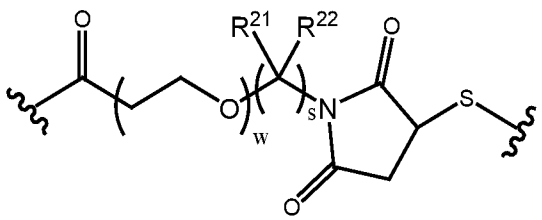
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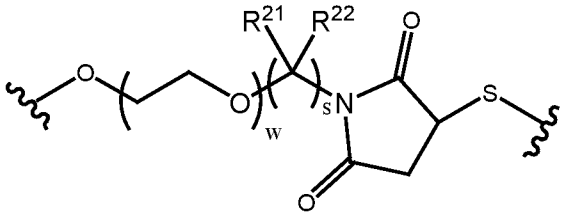
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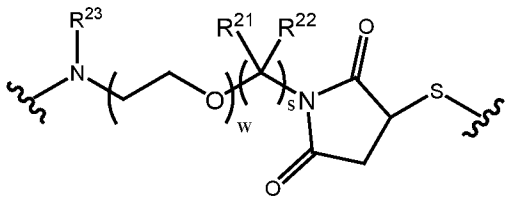


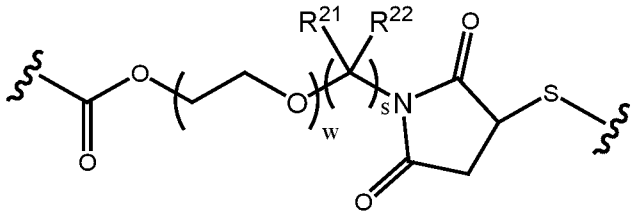
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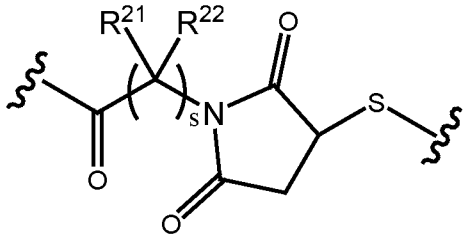


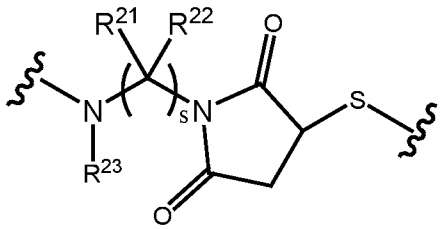
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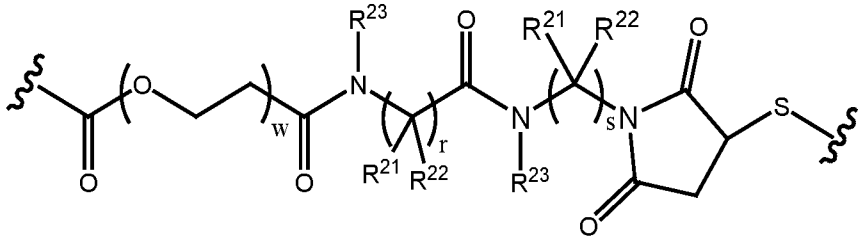

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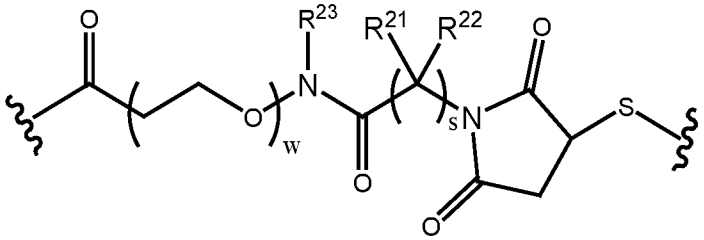

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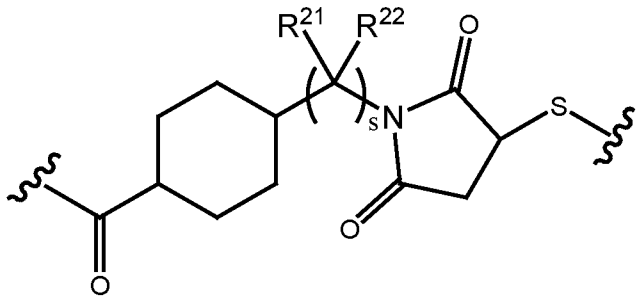

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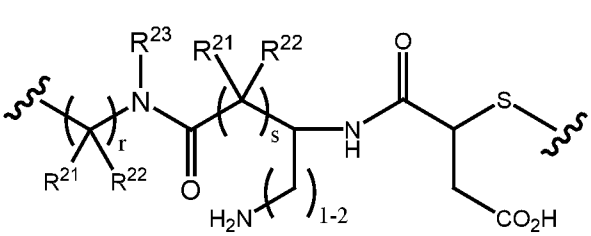

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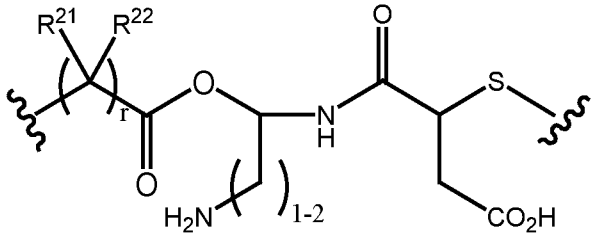

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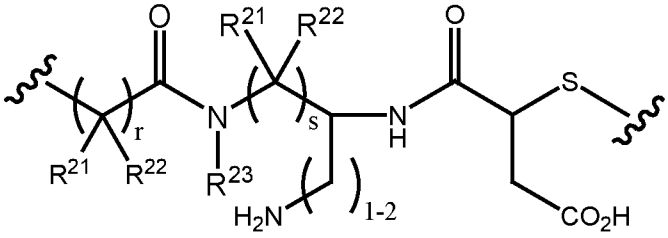
wherein r and s are each independently an integer between 0 and 4, preferably between 0 and 2; w is an integer between 0 and 10;

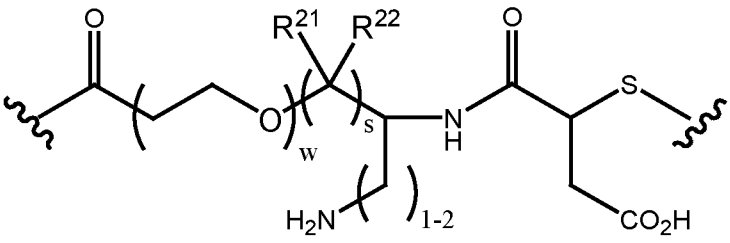
and wherein R^{21}, R^{22} and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line on the left side is a bond to the PEG fragment (–$[OCH_2$-$CH_2]_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

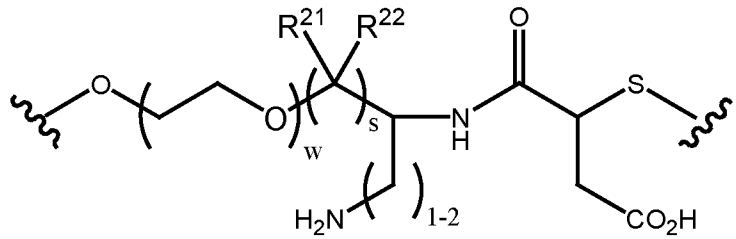
In some embodiments, X^2 is selected from:

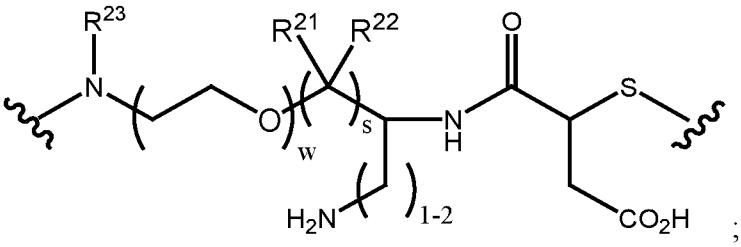

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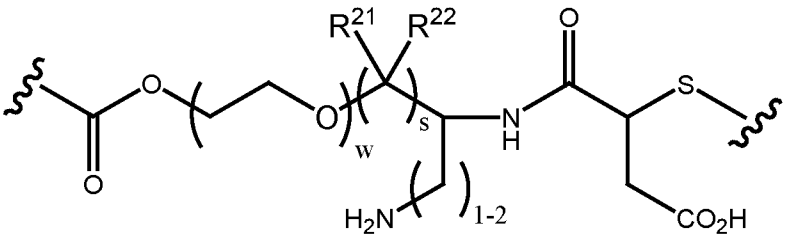

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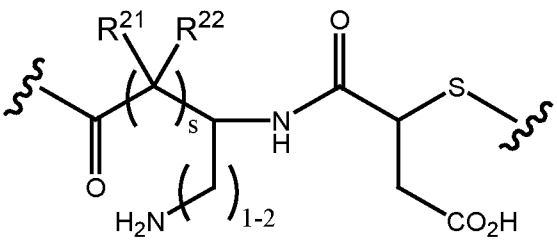

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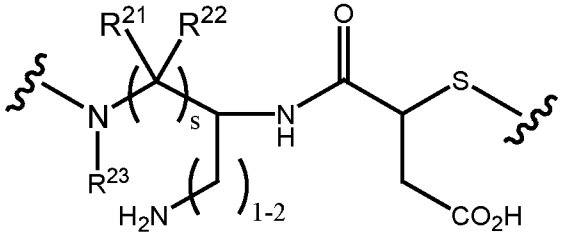

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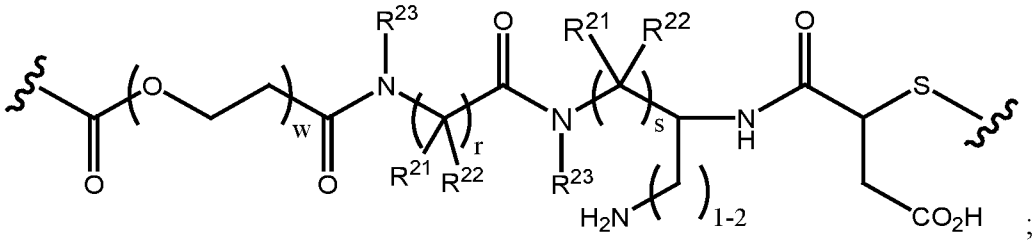

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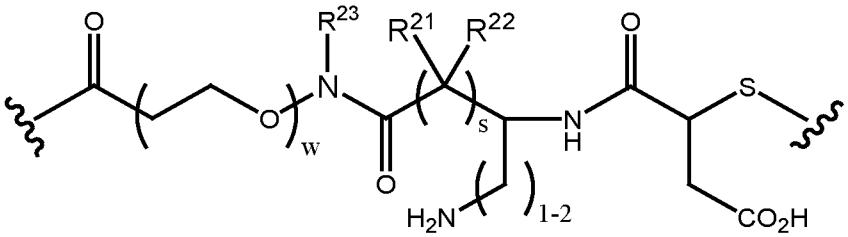

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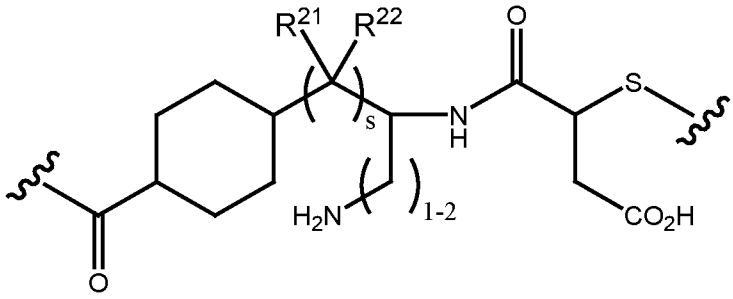
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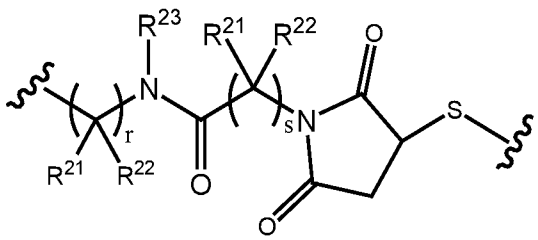


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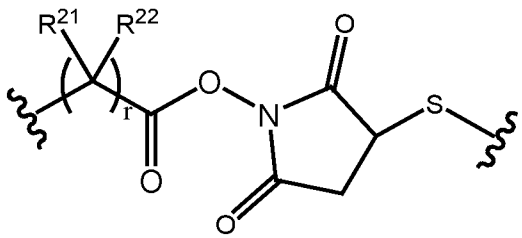
wherein r and s are each independently an integer between 0 and 4, preferably between 0 and 2; w is an integer between 0 and 10;

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 (–$[OCH_2$-$CH_2]_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

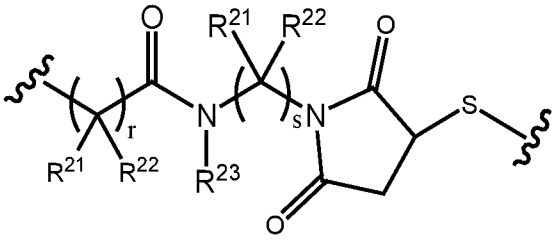
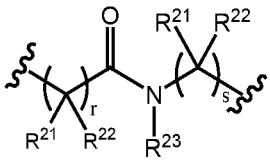
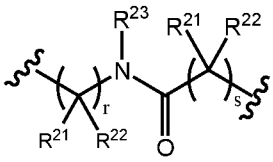
In some preferred embodiments, X^2 is selected from:

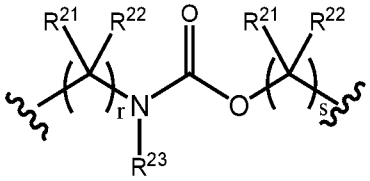
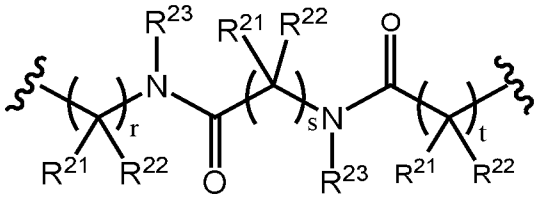


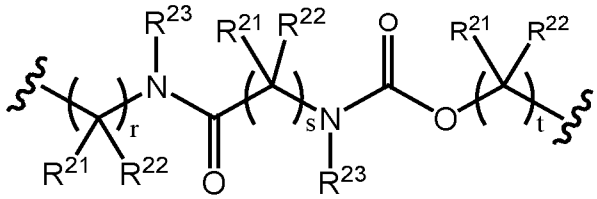
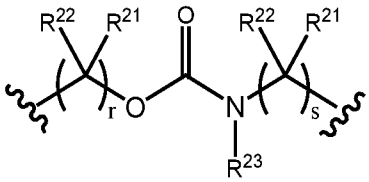
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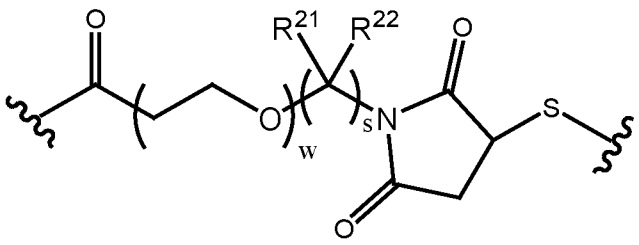


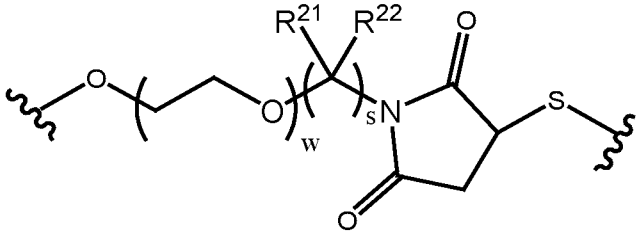
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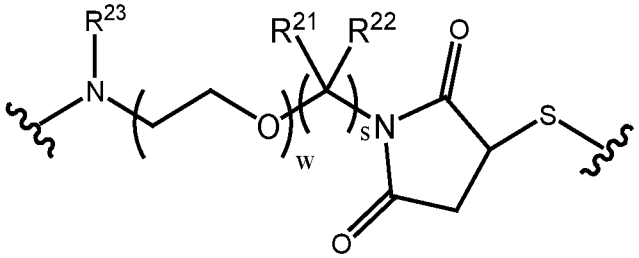

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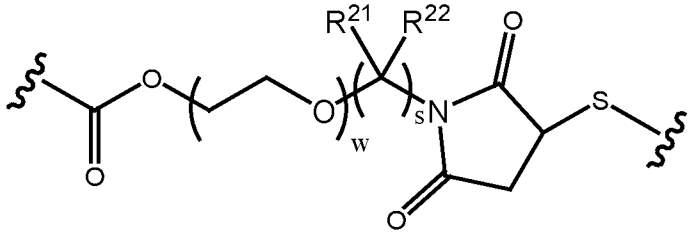

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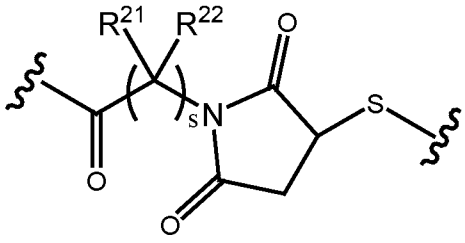

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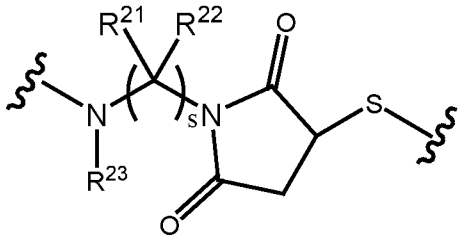

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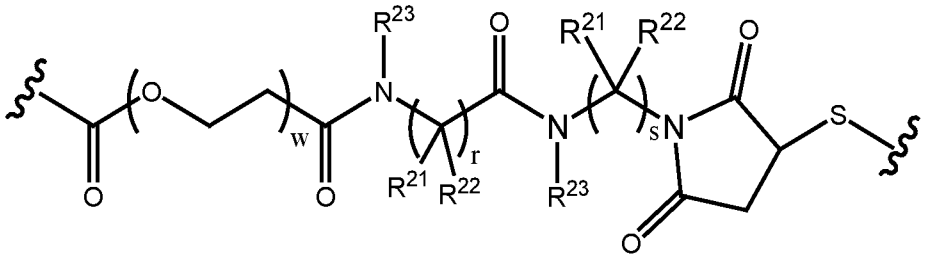

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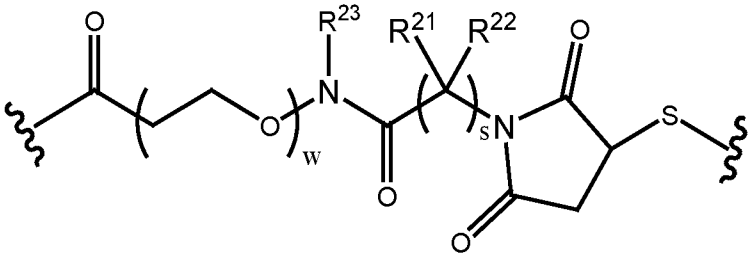

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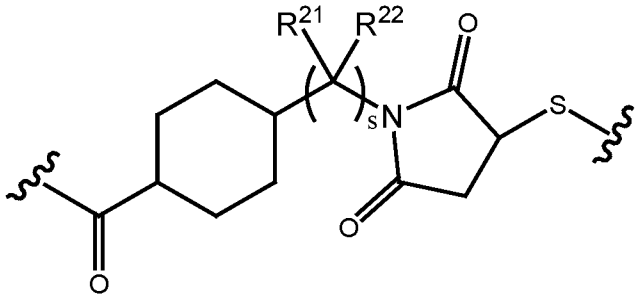

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wherein;

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;

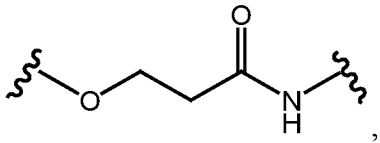
AA is an amino acid selected from Arg, His, Lys, Asp, Glu, Ser, Thr, Asn, Gln, Cys, Sec, Gly, Pro, Ala, Val, Ile, Leu, Met, Phe, Tyr, and Trp;

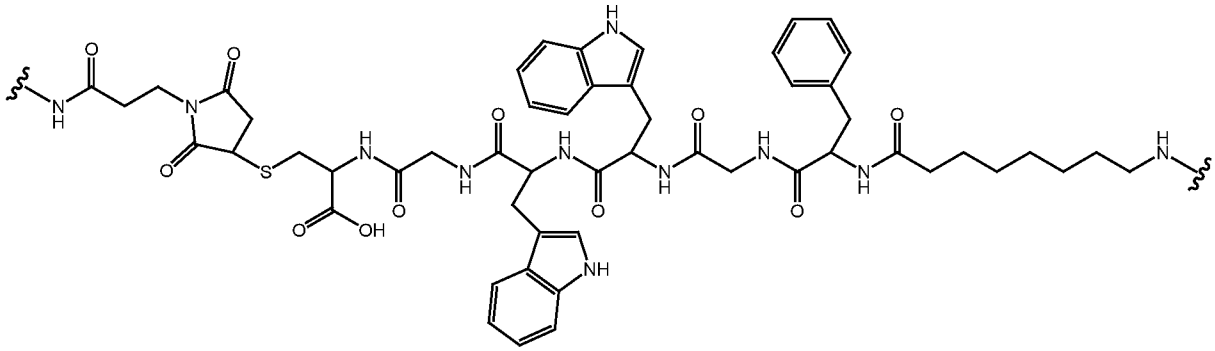
a is an integer between 0 and 10, preferably between 0 and 6; more preferably between 0 and 4;

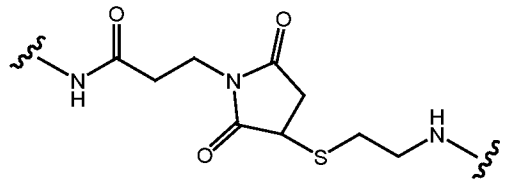
and wherein R^{21}, R^{22} and R^{23} are independently -H or C_1-C_6 alkyl, preferably -H or C_1-C_2 alkyl, more preferably -H. Preferably the wavy line on the left side is a bond to the PEG fragment (–$[OCH_2$-$CH_2]_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

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

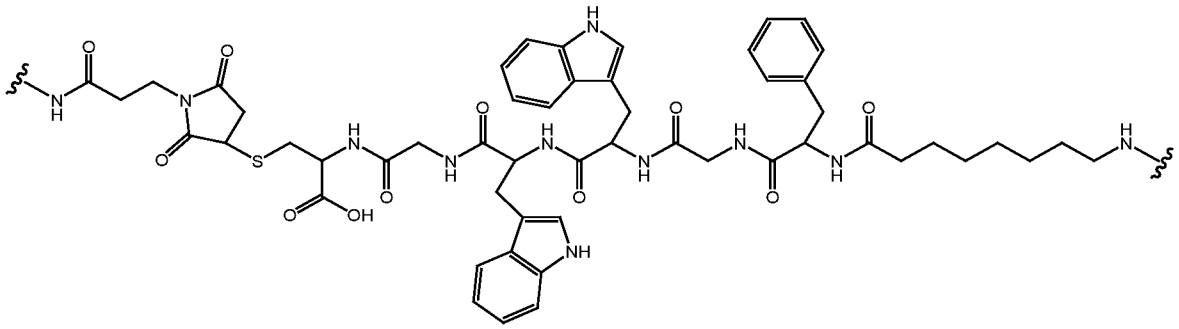
In some embodiments, X^2 comprises or alternatively is a urea, a carbamate, a carbonate, or an ester. In preferred embodiments, X^2 is selected from:


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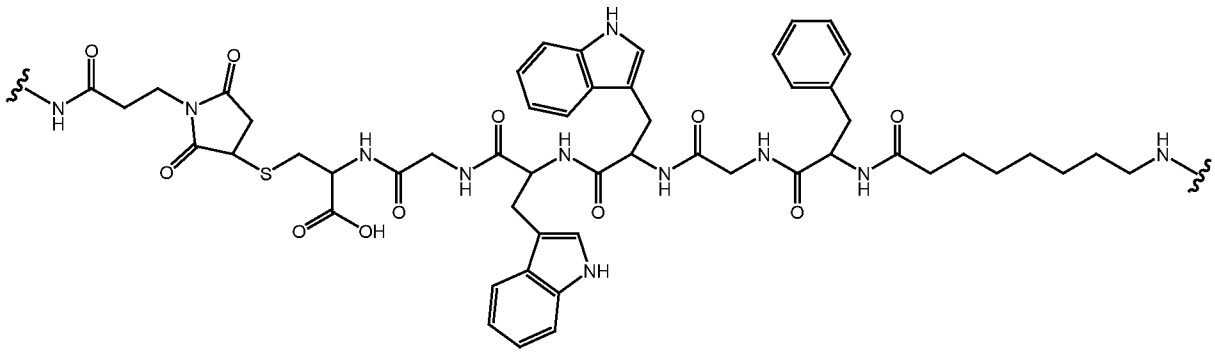

and . Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

In a preferred embodiment said X^2 is


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Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

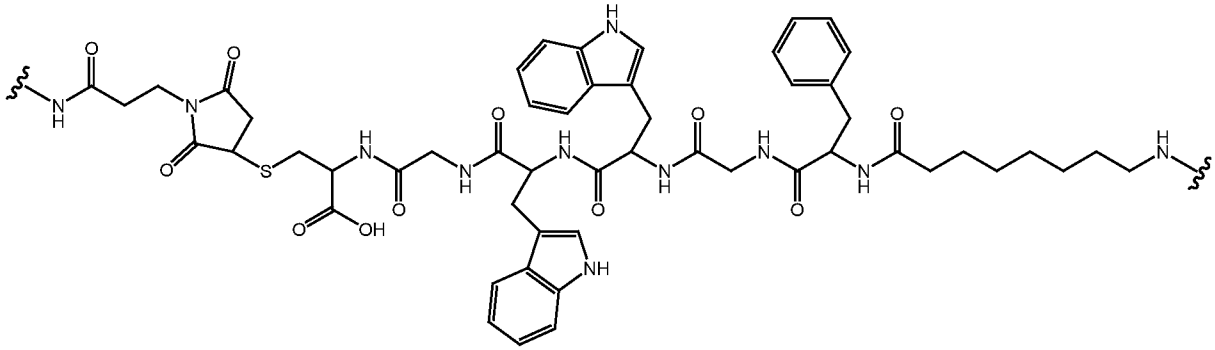
In a further preferred embodiment said X^2 is



and said L of said triconjugate is the DUPA residue (HOOC(CH_2)$_2$-CH(COOH)-NH-CO-NH-

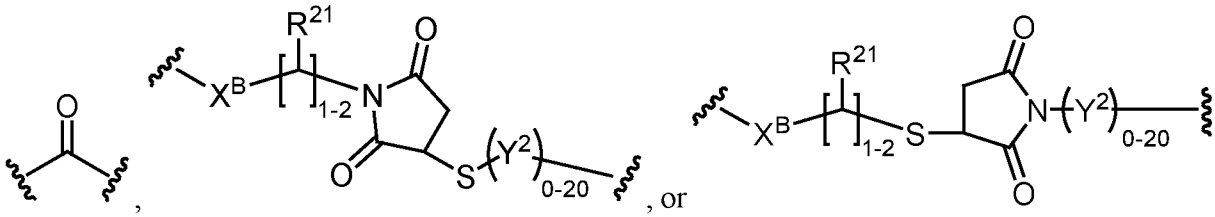
CH(COOH)-$(CH_2)_2$-CO-). Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line on the right side is a bond to the DUPA residue.

In a further preferred embodiment said X^2 is



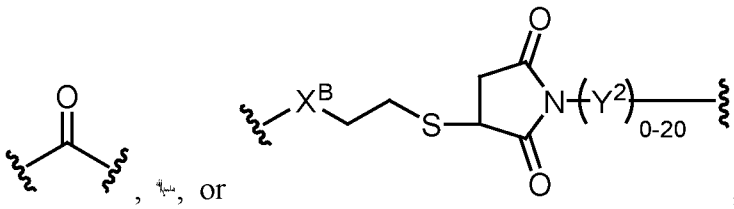
and said L of said triconjugate is the DUPA residue (HOOC$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-), wherein the terminus with the amide group of said X^2 is bonded to the PEG fragment (–[OCH_2-CH_2]$_m$–) and wherein the terminus with the amine functionality is bonded to the DUPA residue (HOOC$(CH_2)_2$-CH(COOH)-NH-CO-NH-CH(COOH)-$(CH_2)_2$-CO-).

In some embodiments, X^2 is selected from:



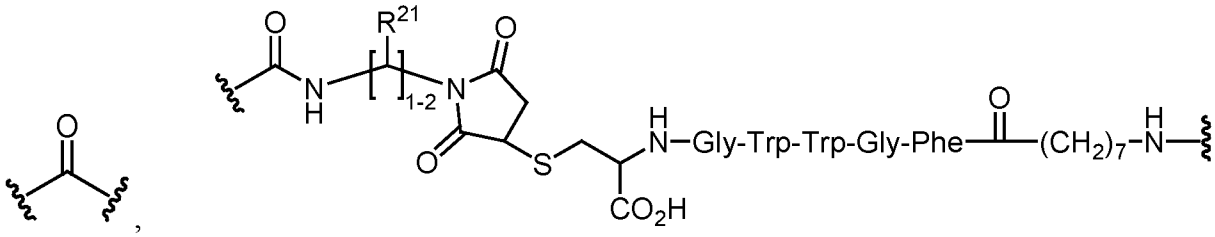
, wherein X^B is -C(O)NH- or -NH-C(O)-, and wherein Y^2 and R^{21} are as defined above. Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

In some embodiments, X^2 is selected from:

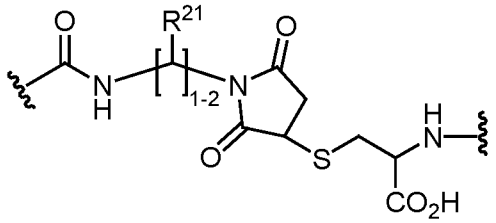
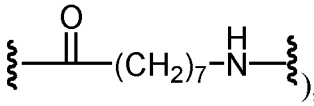


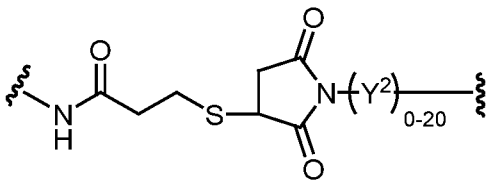
, wherein X^B is -C(O)NH- or -NH-C(O)-, and wherein Y^2 and R^{21} are as defined above. Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

In some embodiments, X^2 is selected from:



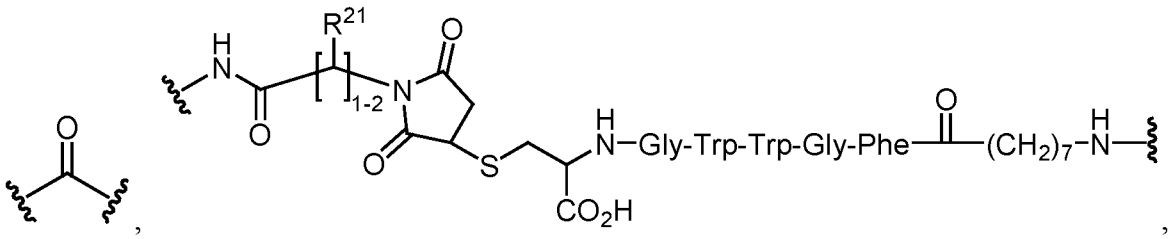
(SEQ ID NO. 10, wherein SEQ ID NO:10 is defined as W1-Gly-Trp-Trp-Gly-Phe-W2,

wherein W1 is  and W2 is ), or

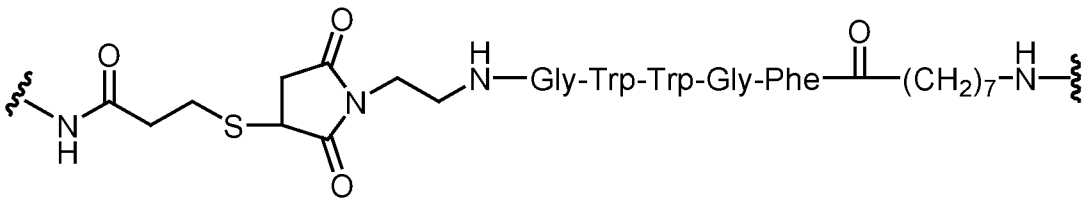


; wherein Y^2 and R^{21} are as defined above. Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

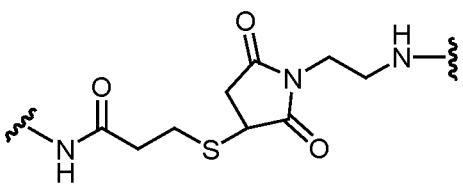
In some embodiments, X^2 is selected from:

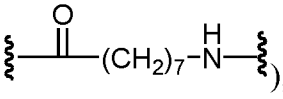


, or

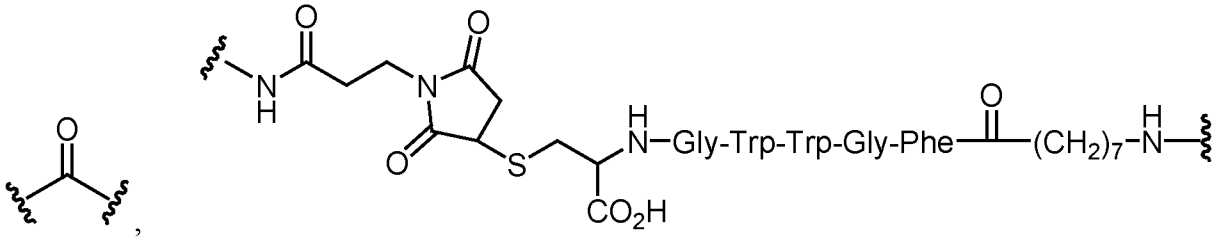


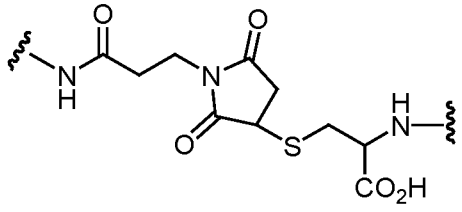
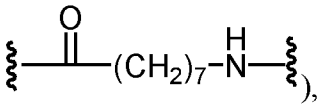
(SEQ ID NO. 14, wherein SEQ ID NO:14 is defined as W9-Gly-Trp-Trp-Gly-Phe-W10, wherein W9 is

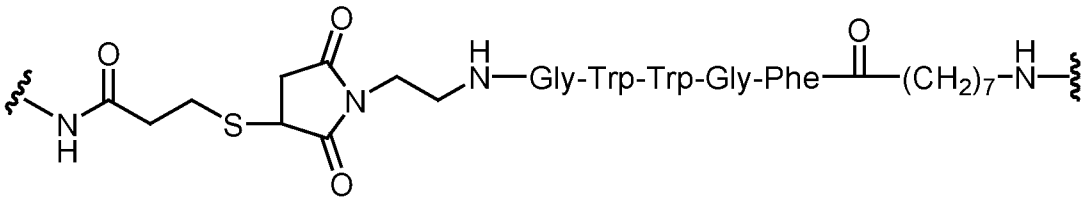


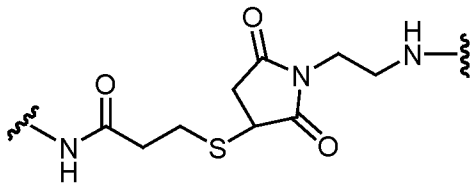
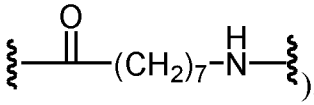
and W10 is ); wherein R^{21} is as defiend above; preferably R^{21} is -H or -CH_2-NH_2; more preferably -H. Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

In some embodiments, X^2 is selected from:

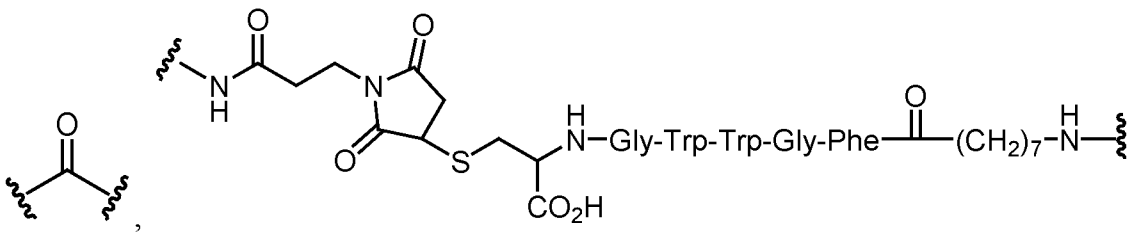


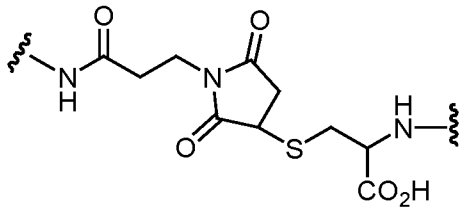
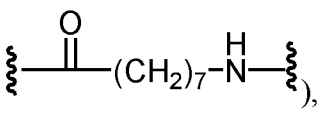
(SEQ ID No. 11, wherein SEQ ID NO:11 is defined as W3-Gly-Trp-Trp-Gly-Phe-W4, wherein W3 is  and W4 is ),

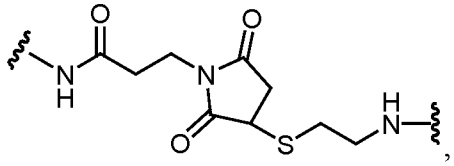
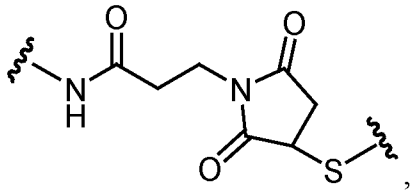


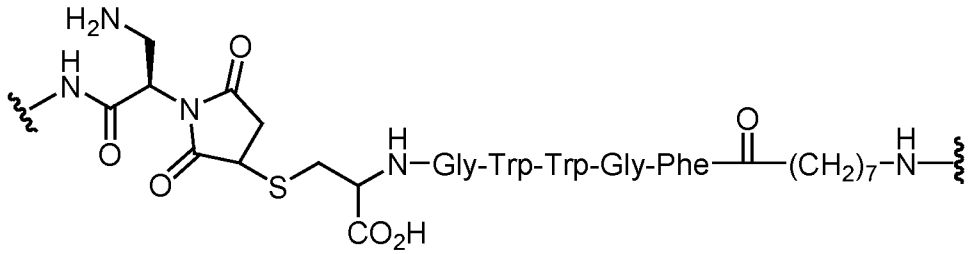
or (SEQ ID NO. 14, wherein SEQ ID NO:14 is defined as W9-Gly-Trp-Trp-Gly-Phe-W10, wherein W9 is  and W10 is ). Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

In some embodiments, X^2 is selected from:

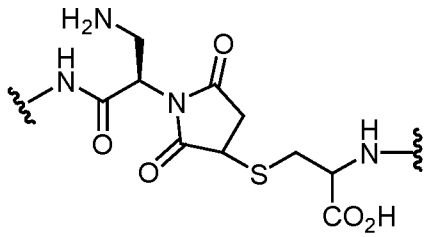
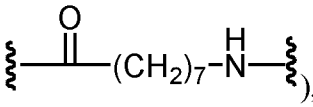


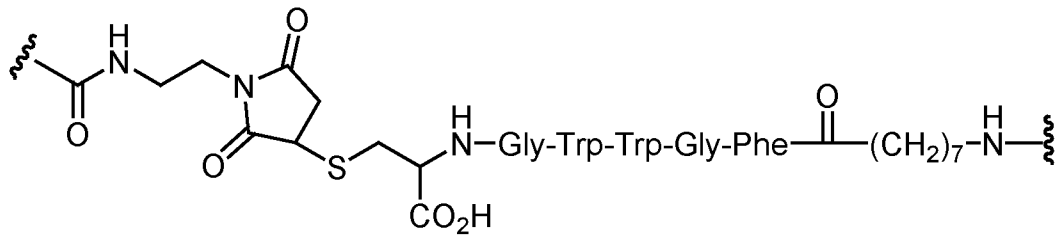
(SEQ ID No. 11, wherein SEQ ID NO:11 is defined as W3-Gly-Trp-Trp-Gly-Phe-W4, wherein W3 is  and W4 is ),


,

,

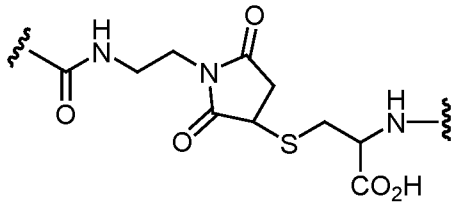
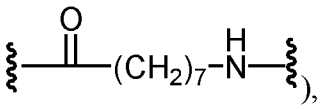


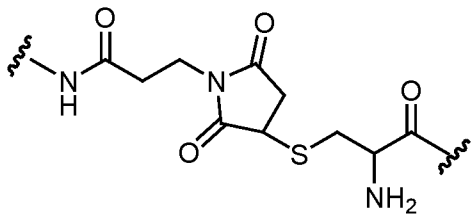
(SEQ ID No. 12, wherein SEQ ID NO:12 is defined as W5-Gly-Trp-Trp-Gly-Phe-W6, wherein W5 is

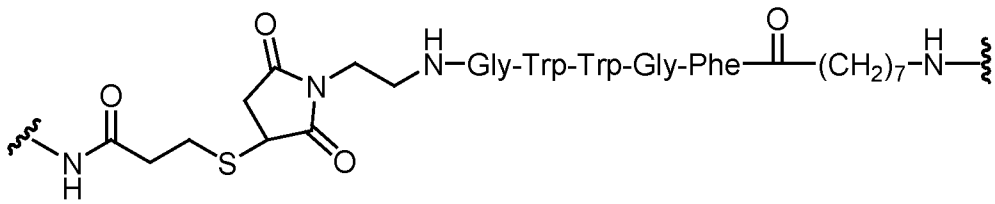

and W6 is

),



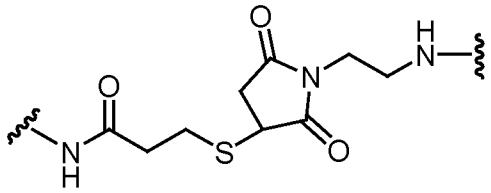
, (SEQ ID No. 13, wherein SEQ ID NO:13 is defined as W7-Gly-Trp-Trp-Gly-Phe-W8, wherein W7 is

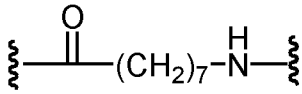
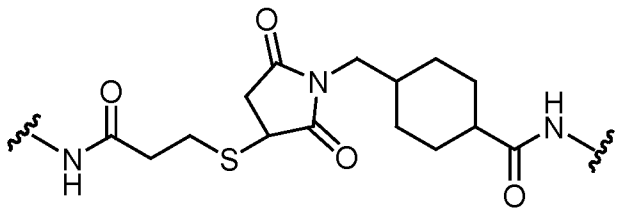

and W8 is

),


,

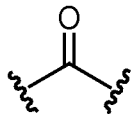


(SEQ ID NO. 14, wherein SEQ ID NO:14 is defined as W9-Gly-Trp-Trp-Gly-Phe-W10, wherein W9 is

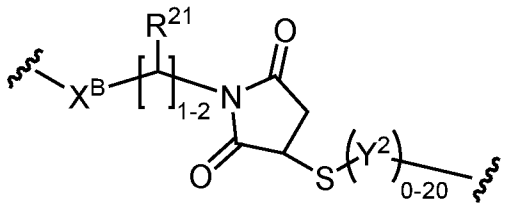


and W10 is , or . Preferably the wavy line on the left side is a bond to the PEG fragment (–[OCH_2-CH_2]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

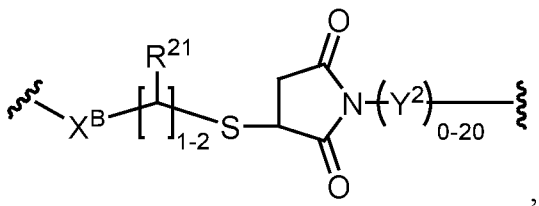
In some embodiments, X^2 is:

.

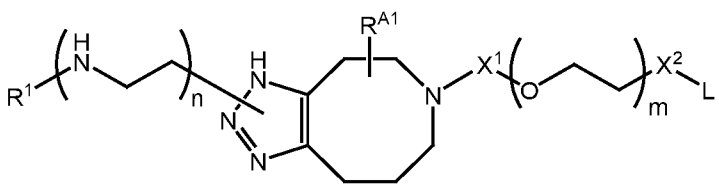
In some embodiments, X^2 is:

, 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]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

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]$_m$–) and the wavy line on the right side is a bond to the targeting fragment (L).

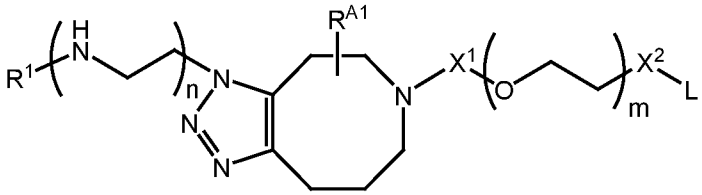
In some embodiments, the composition comprises a conjugate of the Formula IA:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

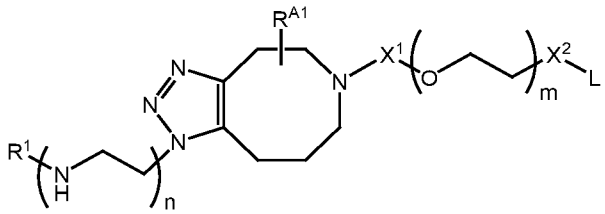
In some embodiments, the composition comprises a conjugate of the Formula IA-1:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

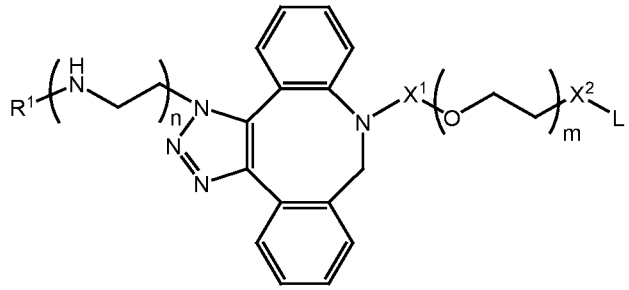
In some embodiments, the composition comprises a conjugate of the Formula IA-2:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

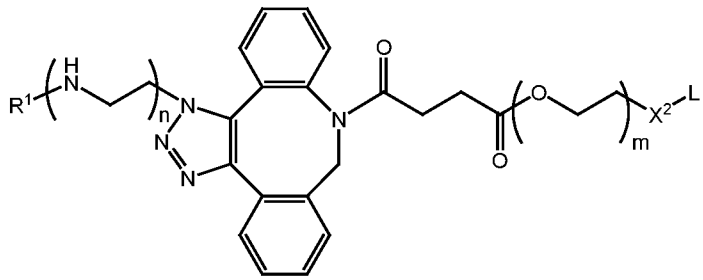
In some embodiments, the composition comprises a conjugate of the Formula IA-3:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

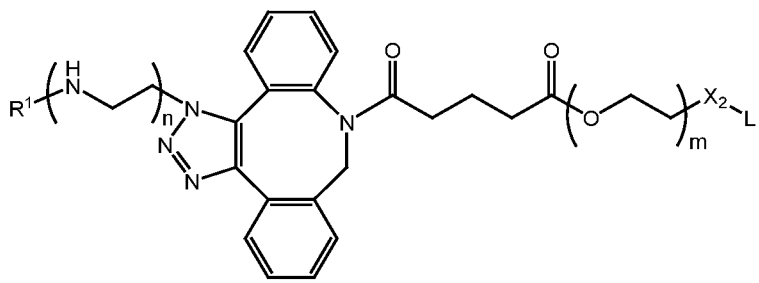
In some embodiments, the composition comprises a conjugate of the Formula IA-3a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

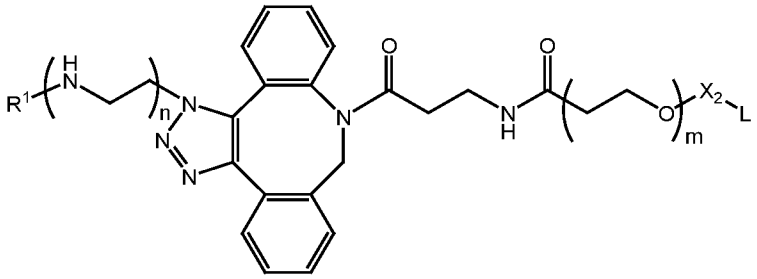
In some embodiments, the composition comprises a conjugate of the Formula IA-3b:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

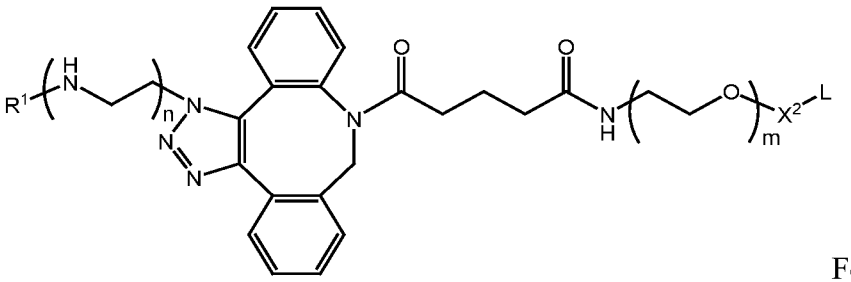
In some embodiments, the composition comprises a conjugate of the Formula IA-3c:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

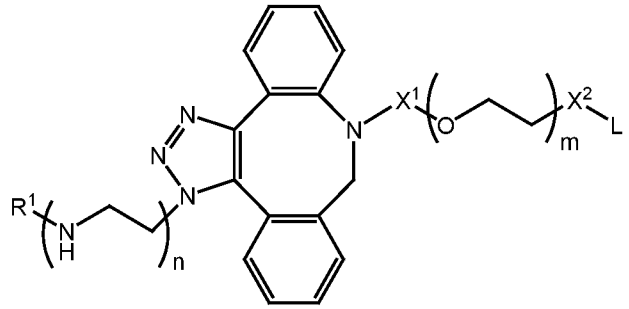
In some embodiments, the composition comprises a conjugate of the Formula IA-3d:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

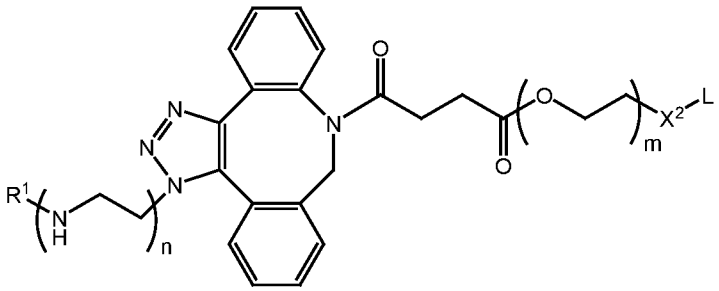
In some embodiments, the composition comprises a conjugate of the Formula IA-4:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

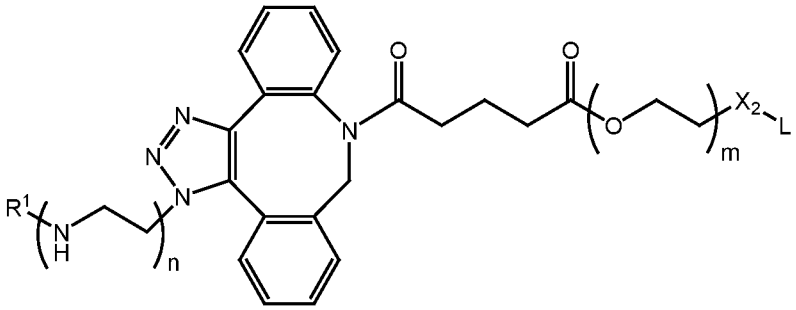
In some embodiments, the composition comprises a conjugate of the Formula IA-4a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

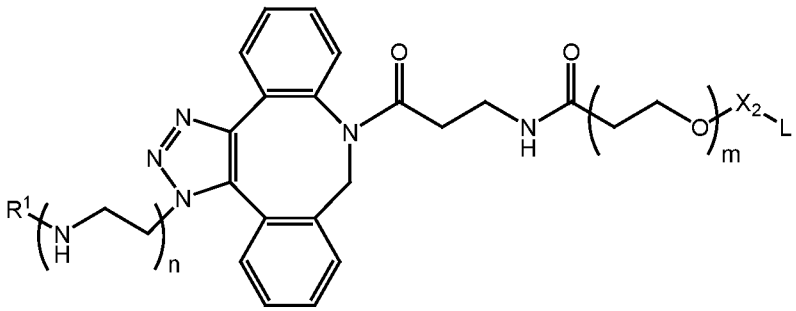
In some embodiments, the composition comprises a conjugate of the Formula IA-4b:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

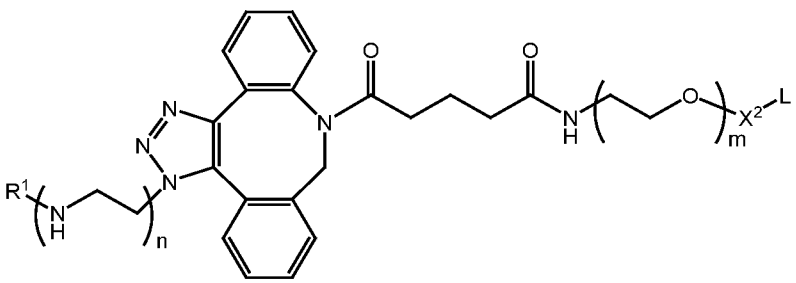
In some embodiments, the composition comprises a conjugate of the Formula IA-4c:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

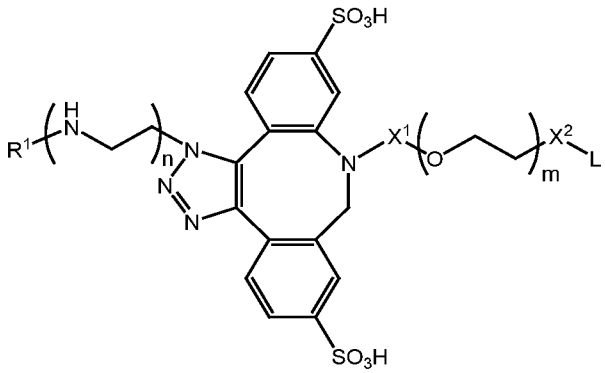
In some embodiments, the composition comprises a conjugate of the Formula IA-4d:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

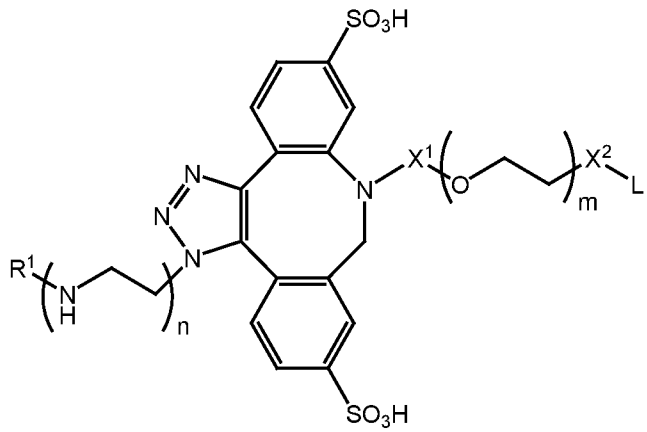
In some embodiments, the composition comprises a conjugate of the Formula IA-5:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

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

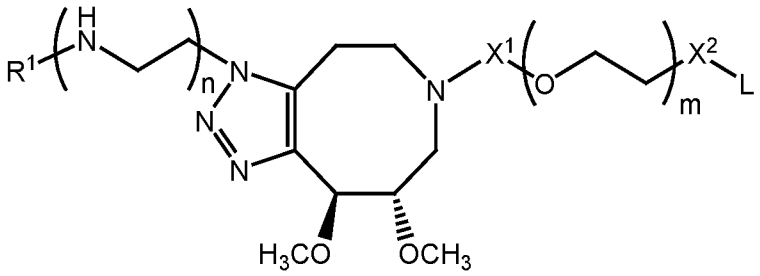


Formula IA-6,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

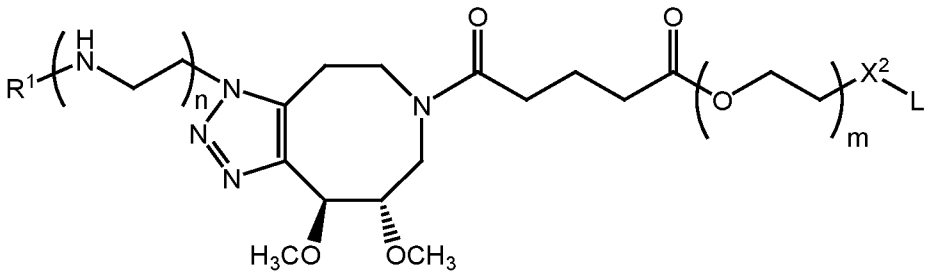
In some embodiments, the composition comprises a conjugate of the Formula IA-7:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

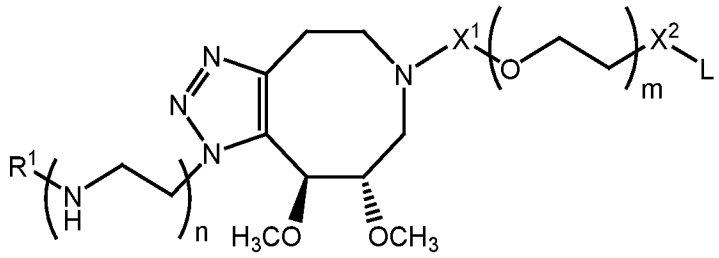
In some embodiments, the composition comprises a conjugate of the Formula IA-7a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

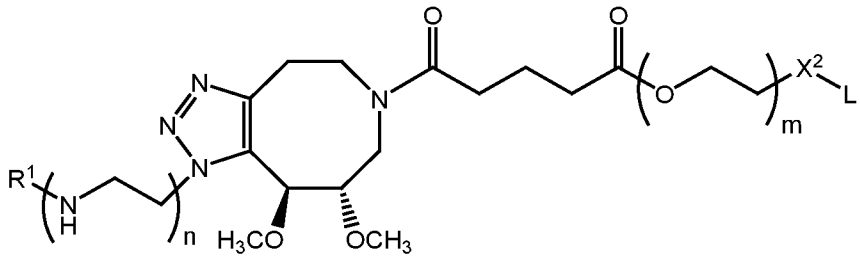
In some embodiments, the composition comprises a conjugate of the Formula IA-8:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

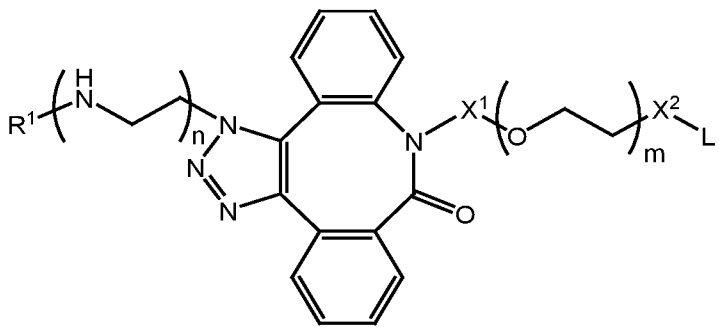
In some embodiments, the composition comprises a conjugate of the Formula IA-8a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

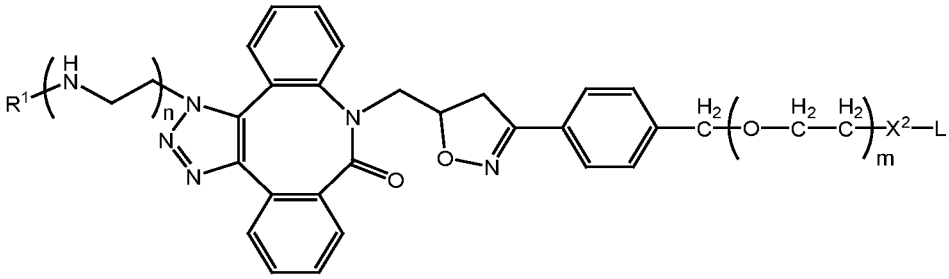
In some embodiments, the composition comprises a conjugate of the Formula IA-9:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

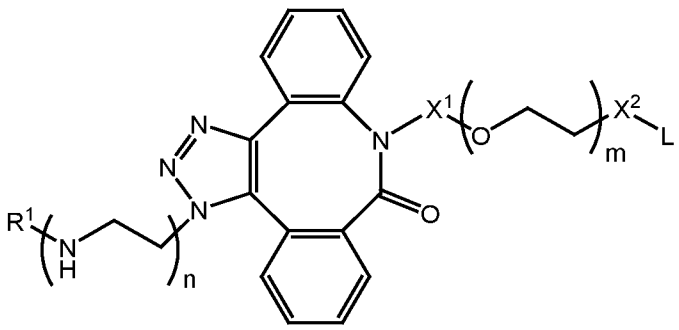
In some embodiments, the composition comprises a conjugate of the Formula IA-9a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

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

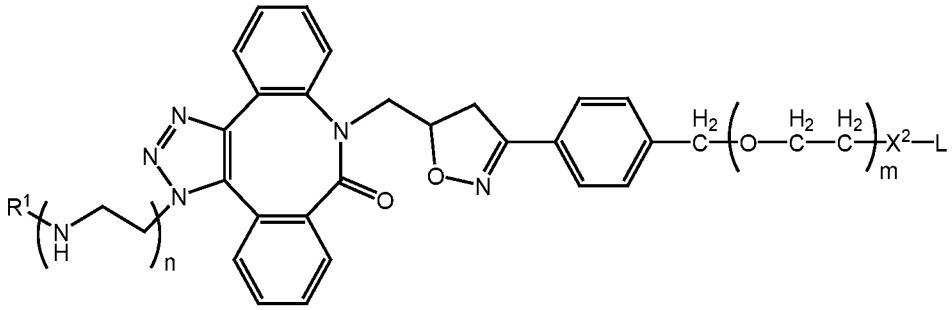


Formula IA-10,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more

preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

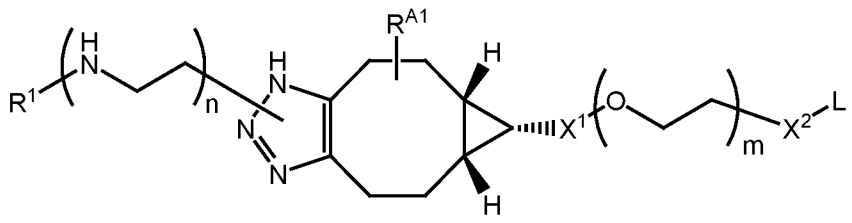
In some embodiments, the composition comprises a conjugate of the Formula IA-10a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

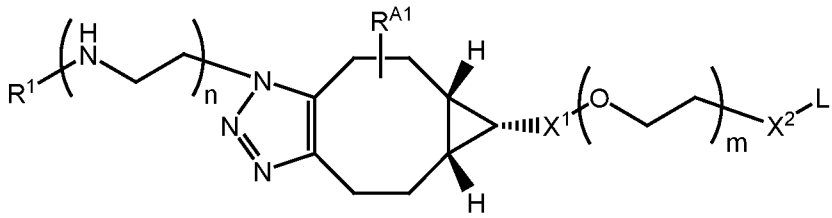
In some embodiments, the composition comprises a conjugate of the Formula IB:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

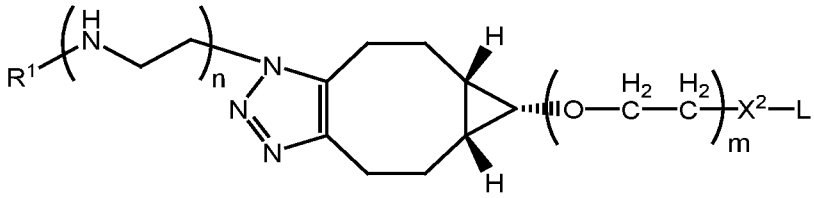
In some embodiments, the composition comprises a conjugate of the Formula IB-1:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

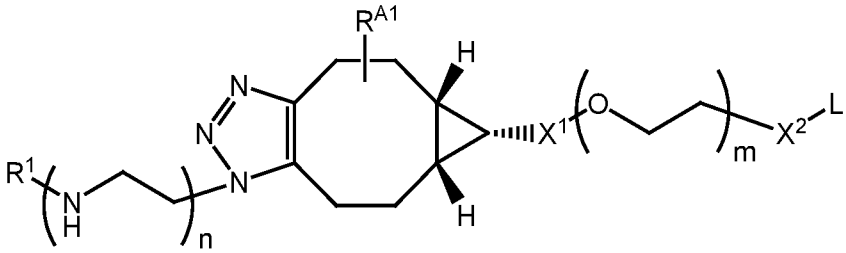
In some embodiments, the composition comprises a conjugate of the Formula IB-1a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

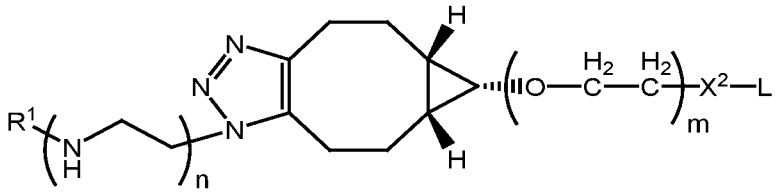
In some embodiments, the composition comprises a conjugate of the Formula IB-2:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

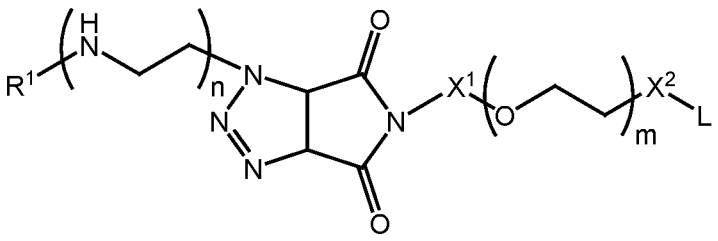
In some embodiments, the composition comprises a conjugate of the Formula IB-2a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

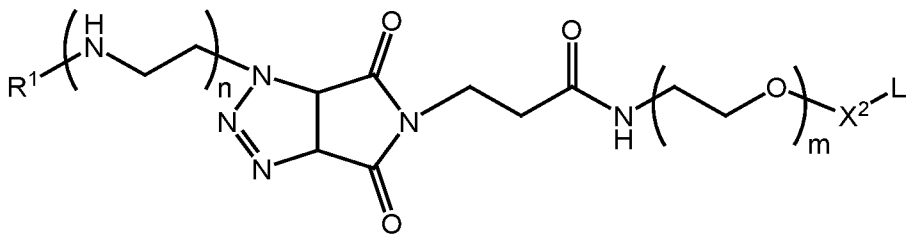
In some embodiments, the composition comprises a conjugate of the Formula IC:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

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

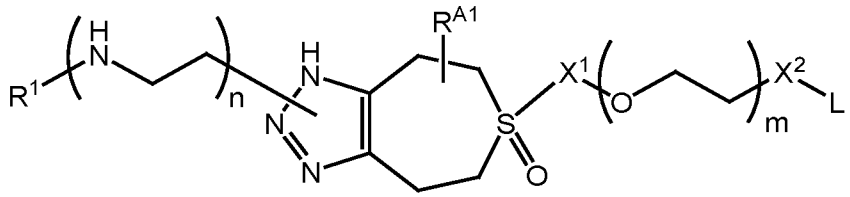


Formula IC-1,

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 between about 2 and about 80 and a dispersity of about 2 or less, more

preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

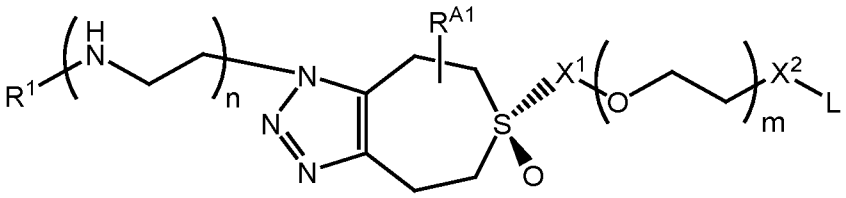
In some embodiments, the composition comprises a conjugate of the Formula ID:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

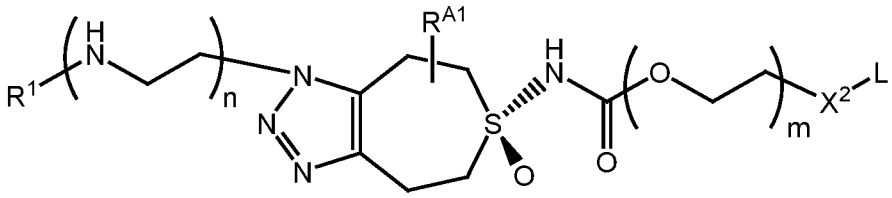
In some embodiments, the composition comprises a conjugate of the Formula ID-1:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

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

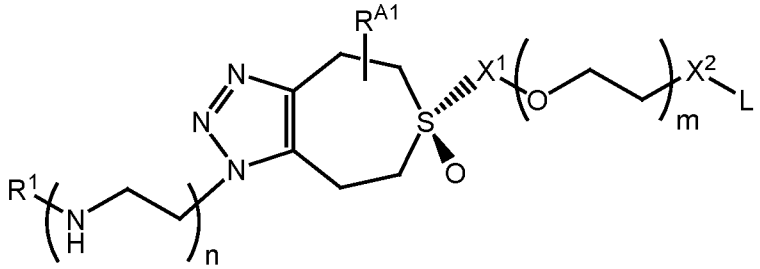


Formula ID-1a,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

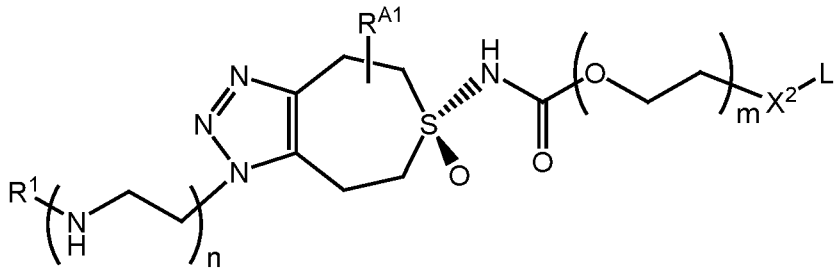
In some embodiments, the composition comprises a conjugate of the Formula ID-2:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

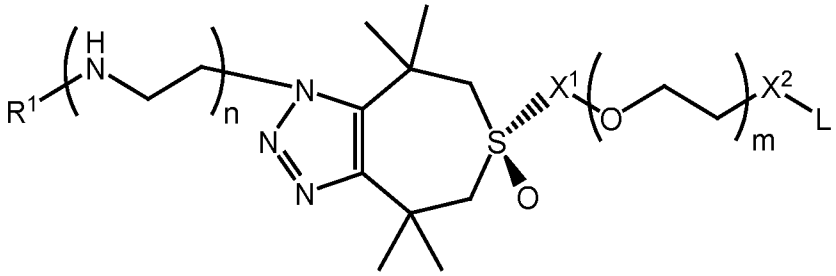
In some embodiments, the composition comprises a conjugate of the Formula ID-2a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

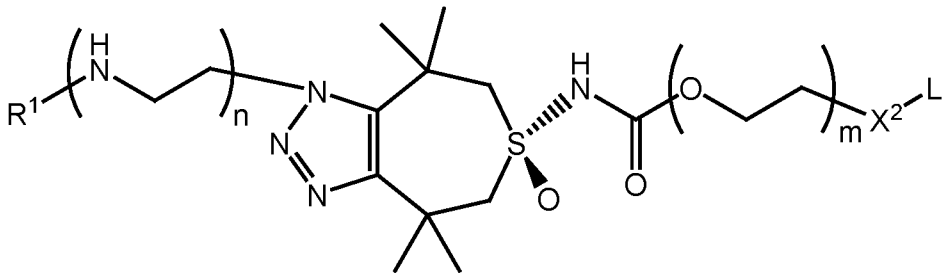
In some embodiments, the composition comprises a conjugate of the Formula ID-3:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

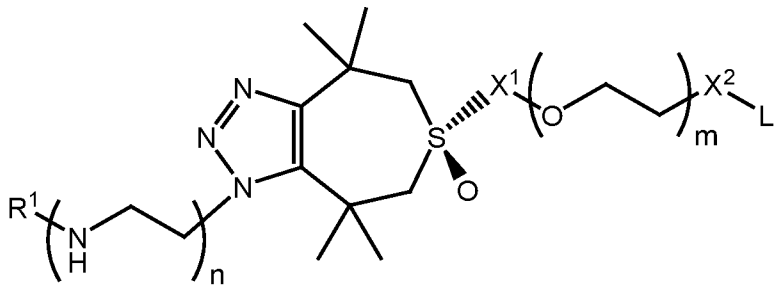
In some embodiments, the composition comprises a conjugate of the Formula ID-3a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

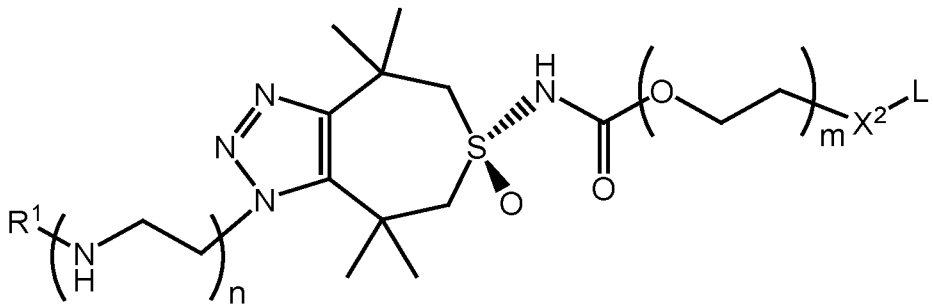
In some embodiments, the composition comprises a conjugate of the Formula ID-4:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

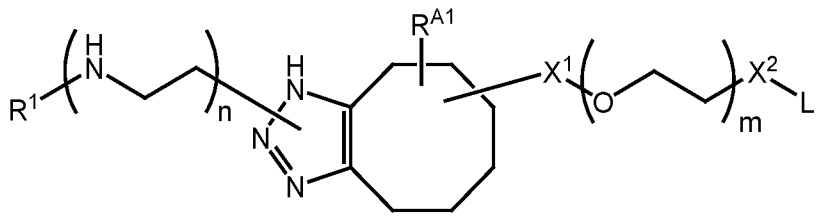
In some embodiments, the composition comprises a conjugate of the Formula ID-4a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

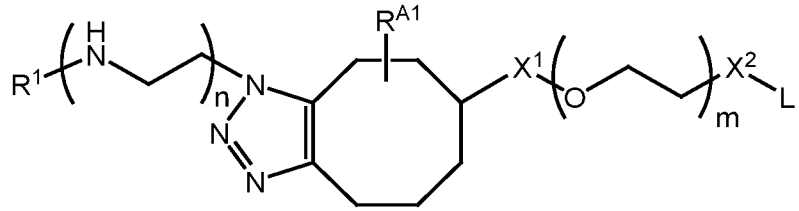
In some embodiments, the composition comprises a conjugate of the Formula IE:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

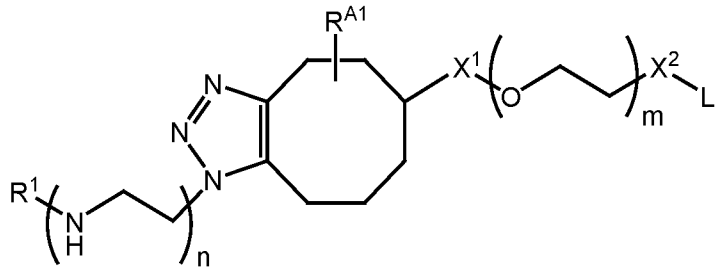
In some embodiments, the composition comprises a conjugate of the Formula IE-1:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

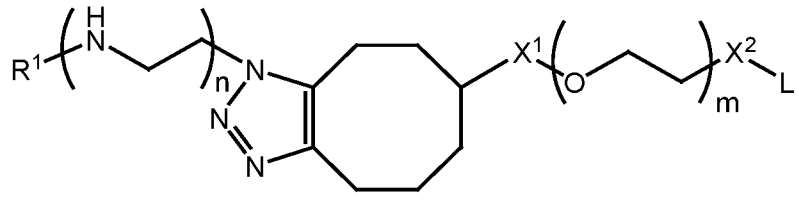
In some embodiments, the composition comprises a conjugate of the Formula IE-2:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

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

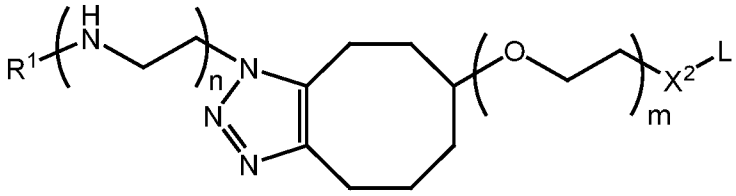


Formula IE-3,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

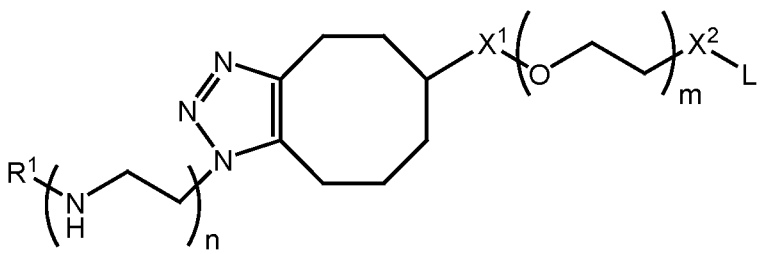
In some embodiments, the composition comprises a conjugate of the Formula IE-3a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

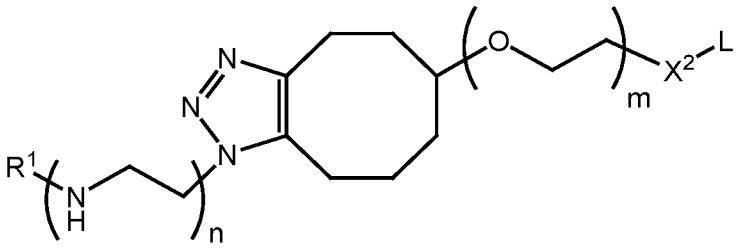
In some embodiments, the composition comprises a conjugate of the Formula IE-4:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

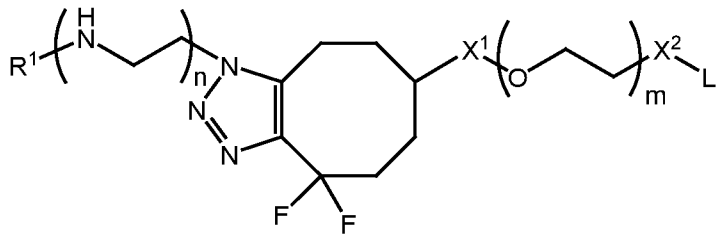
In some embodiments, the composition comprises a conjugate of the Formula IE-4a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

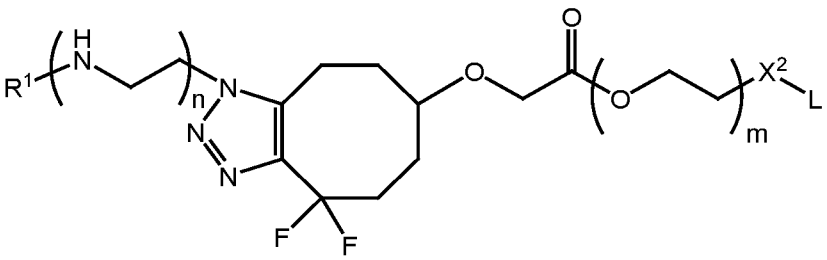
In some embodiments, the composition comprises a conjugate of the Formula IE-5:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

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

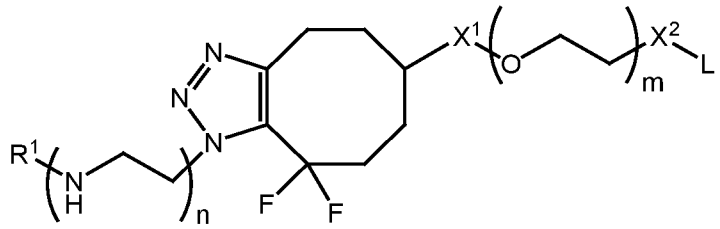


Formula IE-5a,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

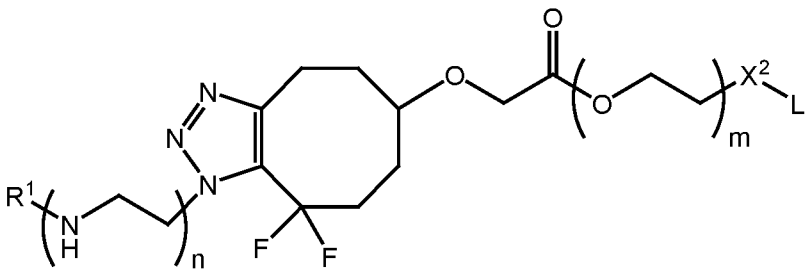
In some embodiments, the composition comprises a conjugate of the Formula IE-6:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

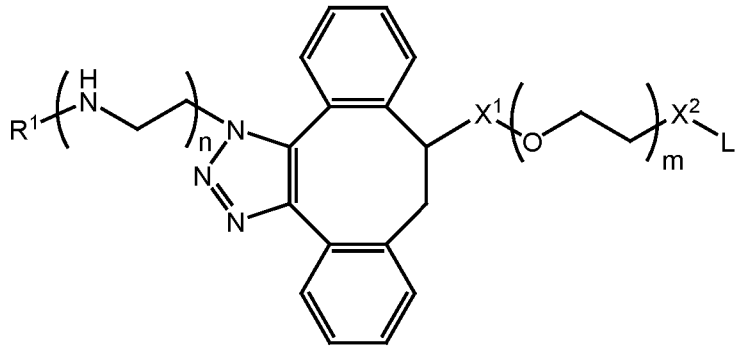
In some embodiments, the composition comprises a conjugate of the Formula IE-6a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

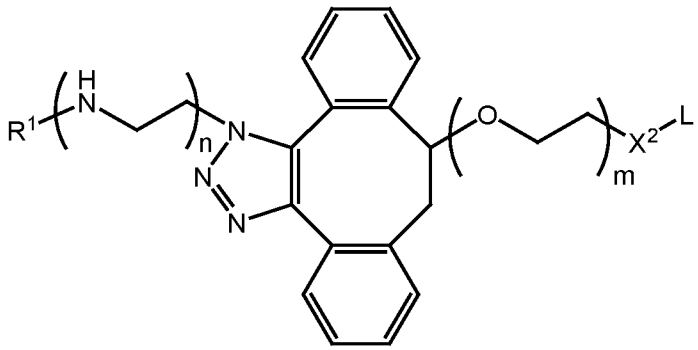
In some embodiments, the composition comprises a conjugate of the Formula IE-7:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

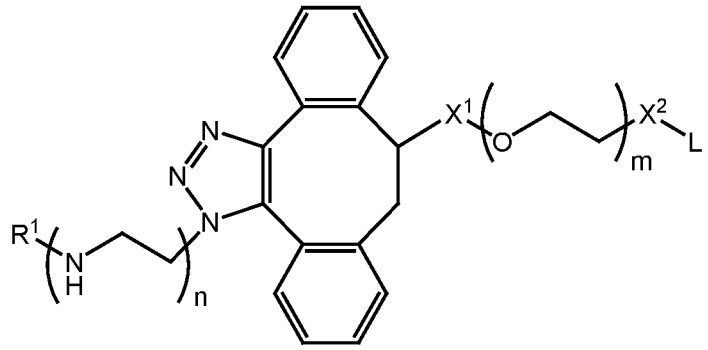
In some embodiments, the composition comprises a conjugate of the Formula IE-7a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

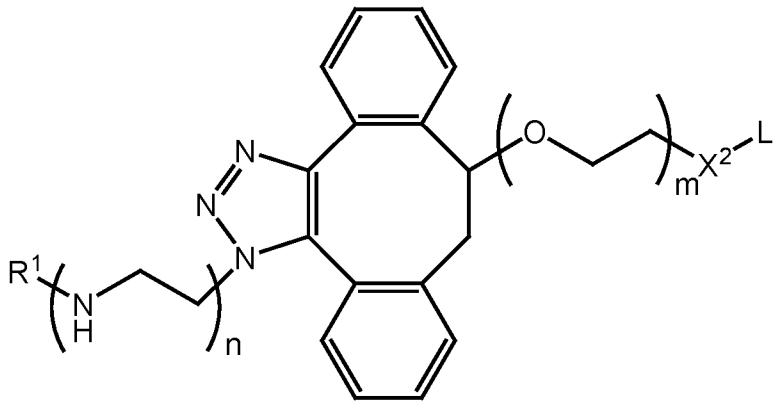
In some embodiments, the composition comprises a conjugate of the Formula IE-8:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

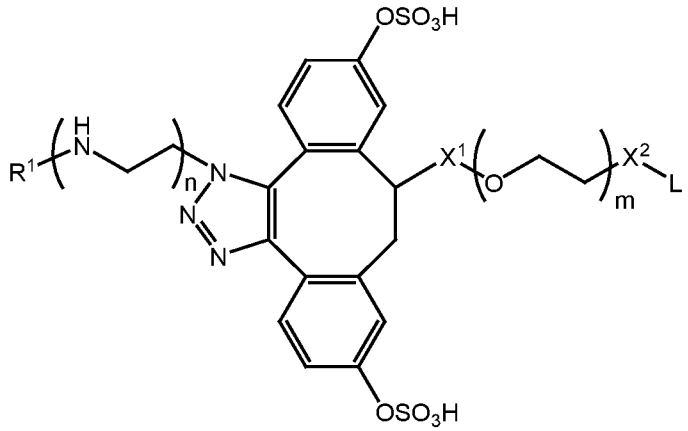
In some embodiments, the composition comprises a conjugate of the Formula IE-8a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

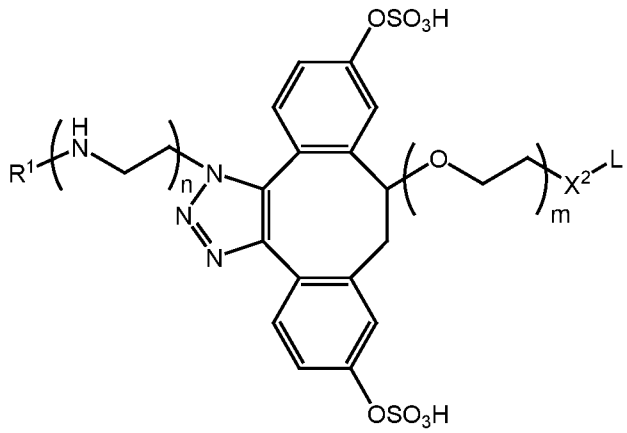
In some embodiments, the composition comprises a conjugate of the Formula IE-9:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

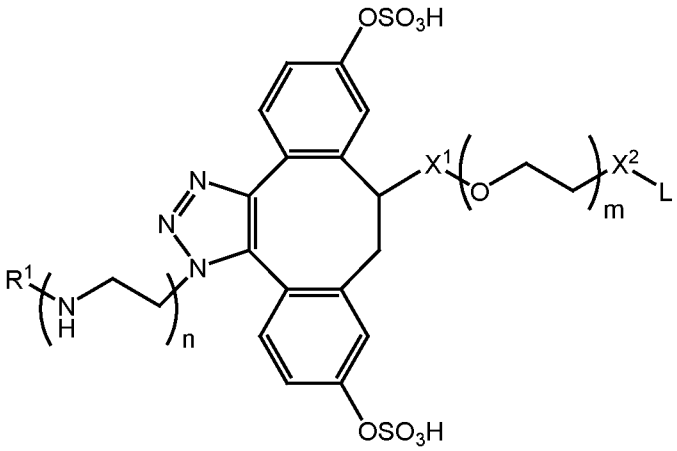
In some embodiments, the composition comprises a conjugate of the Formula IE-9a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

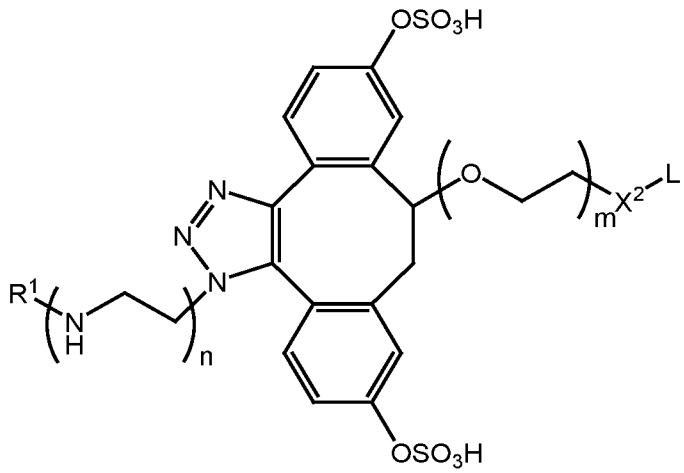
In some embodiments, the composition comprises a conjugate of the Formula IE-10:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

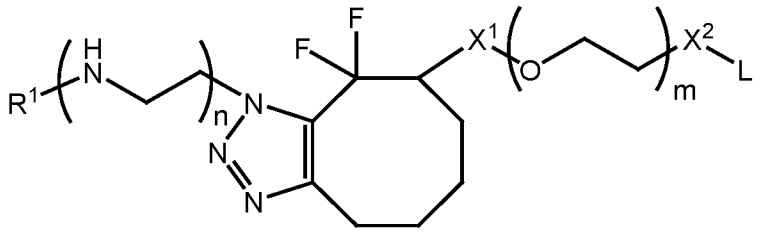
In some embodiments, the composition comprises a conjugate of the Formula IE-10a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

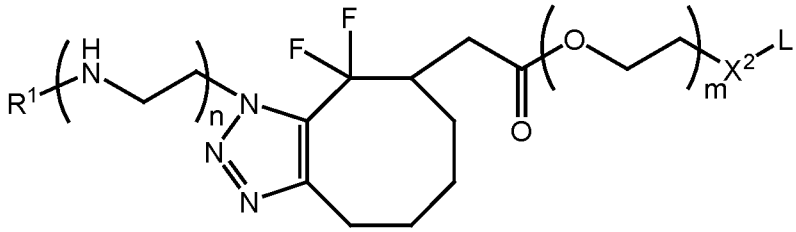
In some embodiments, the composition comprises a conjugate of the Formula IE-11:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

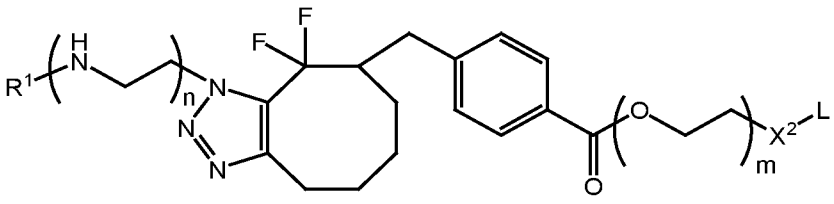
In some embodiments, the composition comprises a conjugate of the Formula IE-11a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

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

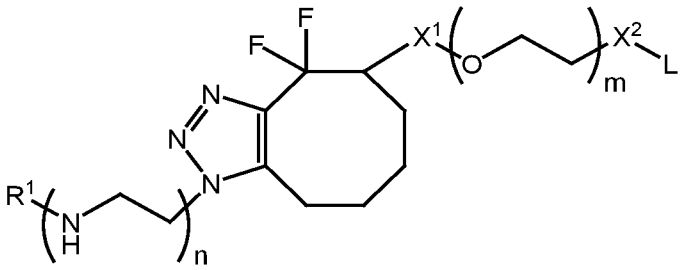


Formula IE-11b,

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 between about 2 and about 80 and a dispersity of about 2 or less, more

preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

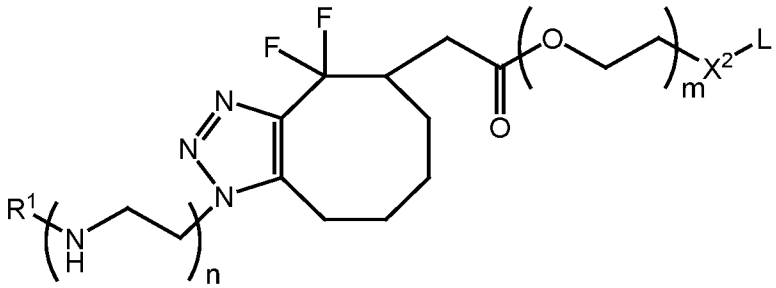
In some embodiments, the composition comprises a conjugate of the Formula IE-12:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

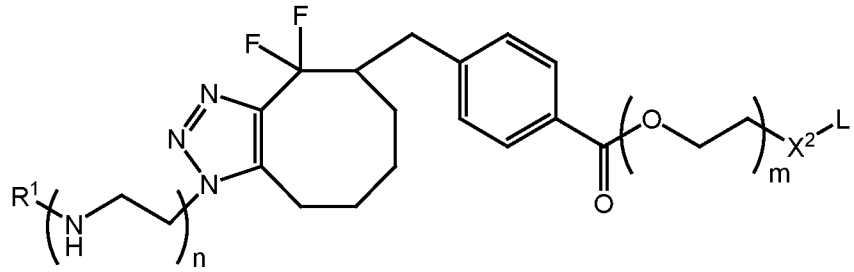
In some embodiments, the composition comprises a conjugate of the Formula IE-12a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

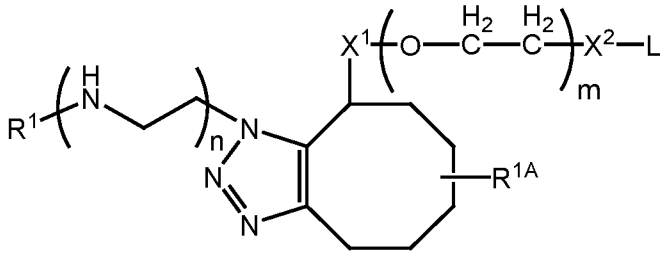
In some embodiments, the composition comprises a conjugate of the Formula IE-12b:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

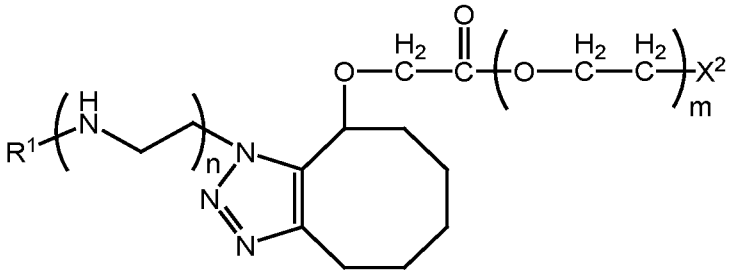
In some embodiments, the composition comprises a conjugate of the Formula IE-13:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

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

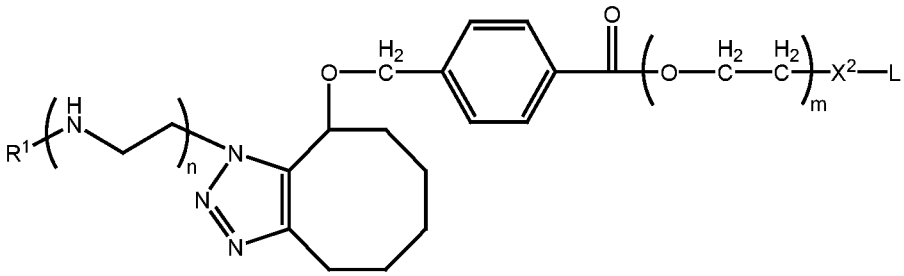


Formula IE-13a,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

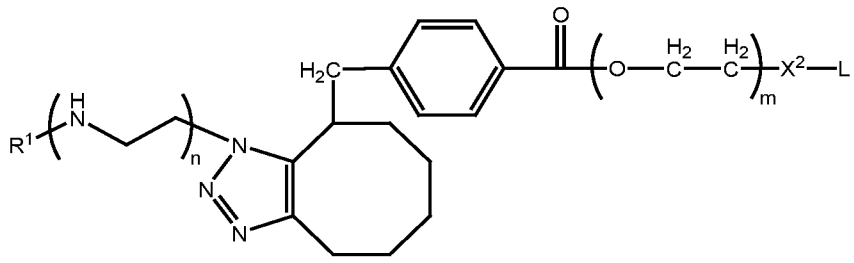
In some embodiments, the composition comprises a conjugate of the Formula IE-13b:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

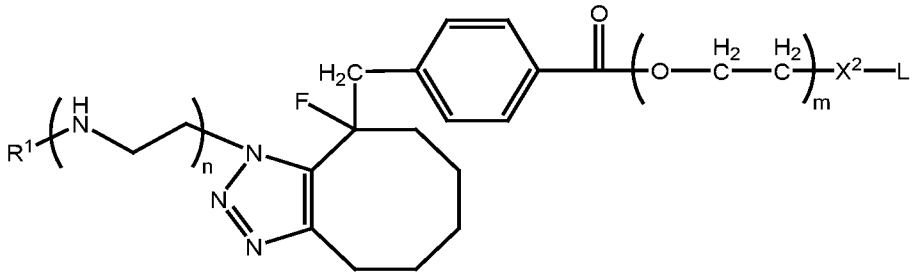
In some embodiments, the composition comprises a conjugate of the Formula IE-13c:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

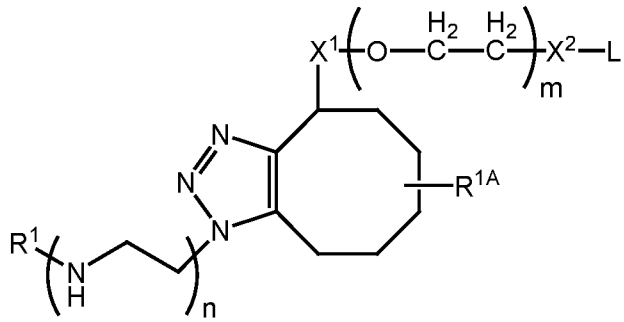
In some embodiments, the composition comprises a conjugate of the Formula IE-13d:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

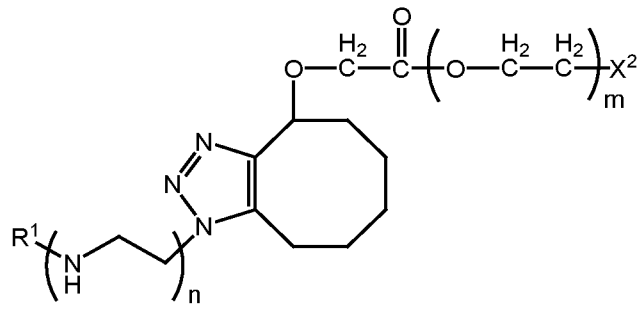
In some embodiments, the composition comprises a conjugate of the Formula IE-14:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

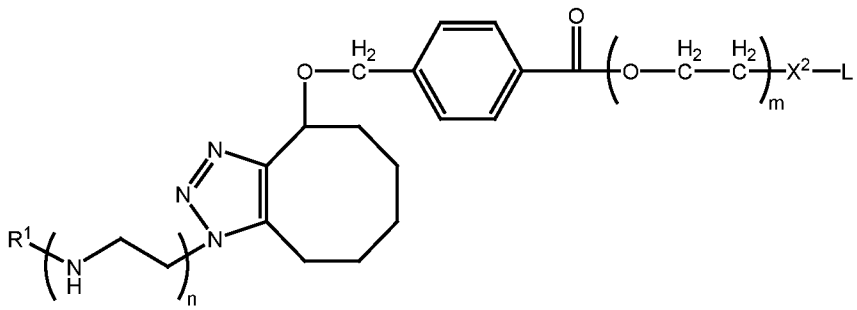
In some embodiments, the composition comprises a conjugate of the Formula IE-14a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

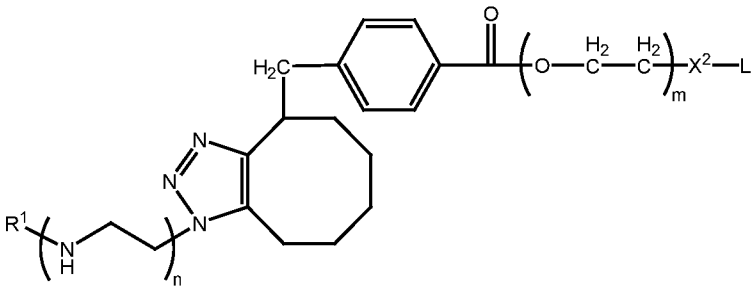
In some embodiments, the composition comprises a conjugate of the Formula IE-14b:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

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

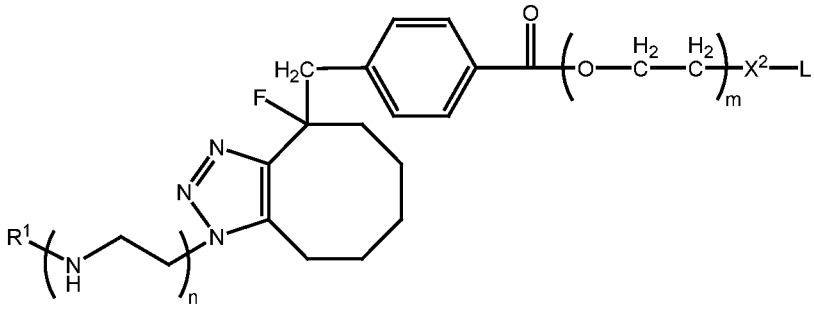


Formula IE-14c,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more

preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

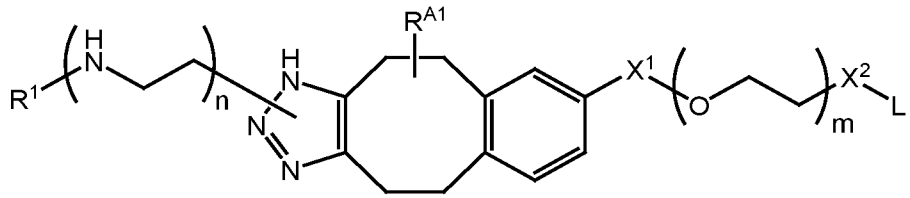
In some embodiments, the composition comprises a conjugate of the Formula IE-14d:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

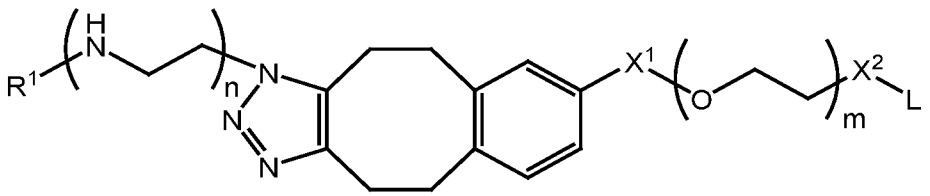
In some embodiments, the composition comprises a conjugate of the Formula IH:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

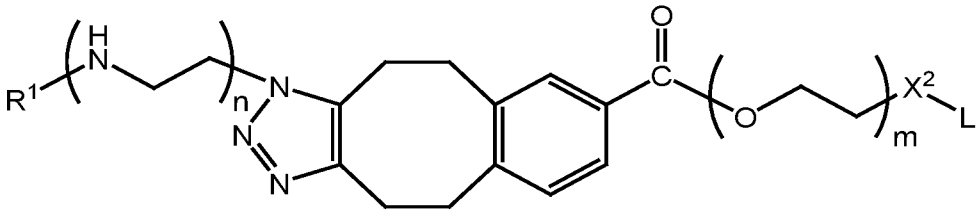
In some embodiments, the composition comprises a conjugate of the Formula IH-1:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

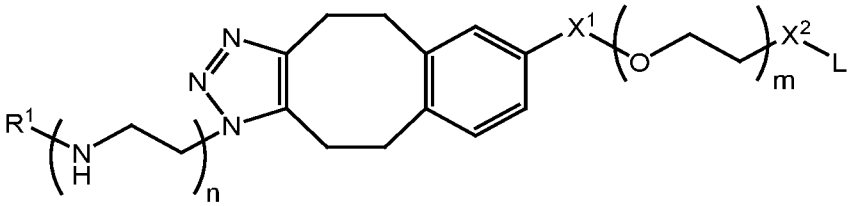
In some embodiments, the composition comprises a conjugate of the Formula IH-1a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

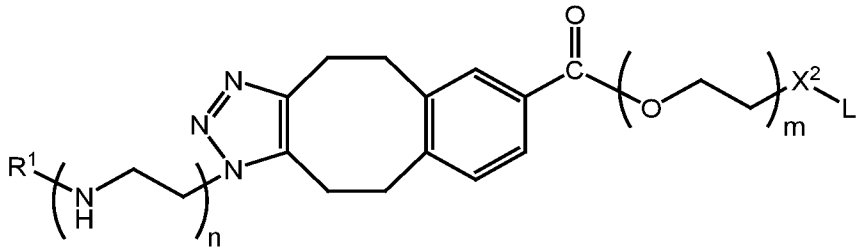
In some embodiments, the composition comprises a conjugate of the Formula IH-2:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

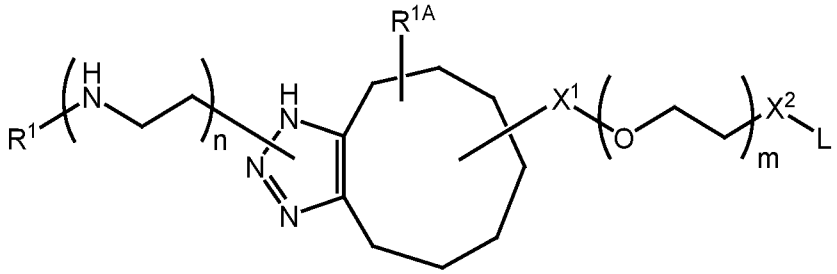
In some embodiments, the composition comprises a conjugate of the Formula IH-2a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

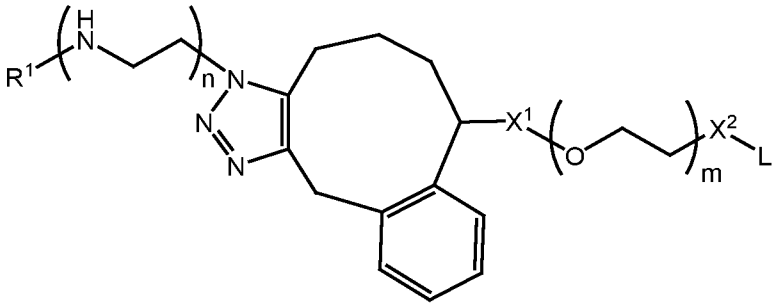
In some embodiments, the composition comprises a conjugate of the Formula IJ:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

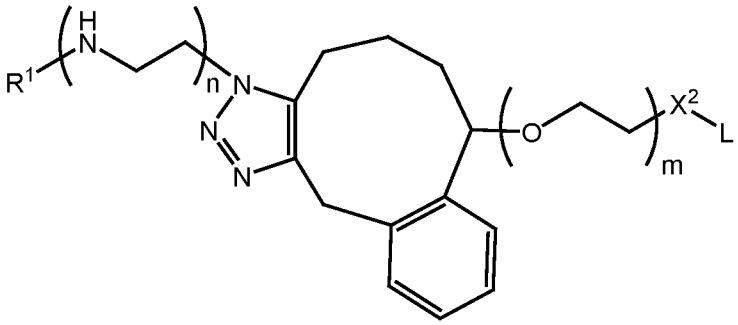
In some embodiments, the composition comprises a conjugate of the Formula IJ-1:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

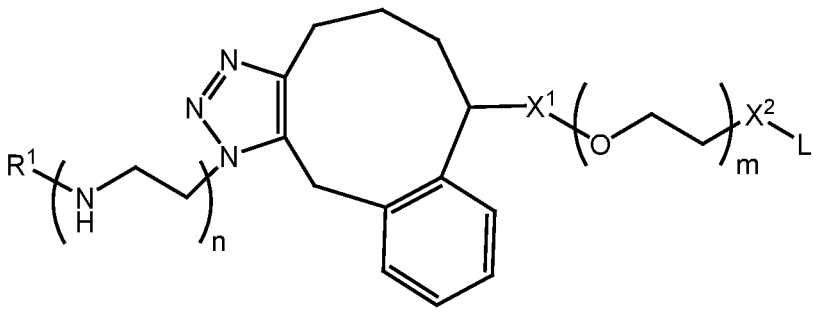
In some embodiments, the composition comprises a conjugate of the Formula IJ-1a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

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

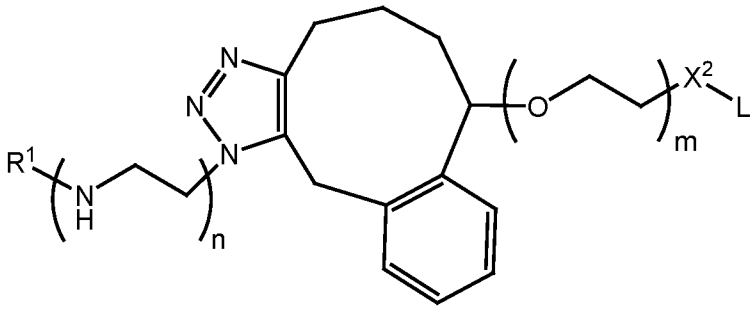


Formula IJ-2,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more

preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

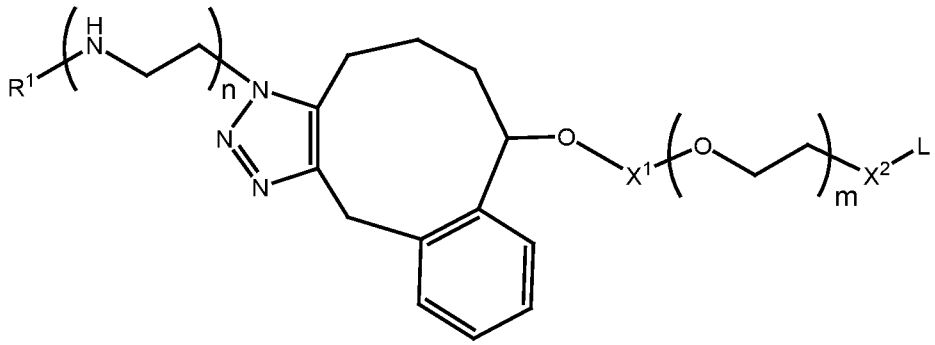
In some embodiments, the composition comprises a conjugate of the Formula IJ-2a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

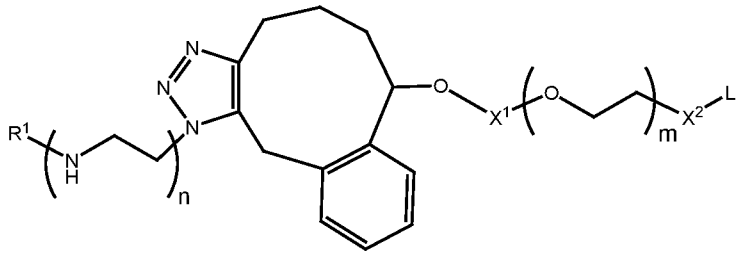
In some embodiments, the composition comprises a conjugate of the Formula IJ-3:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

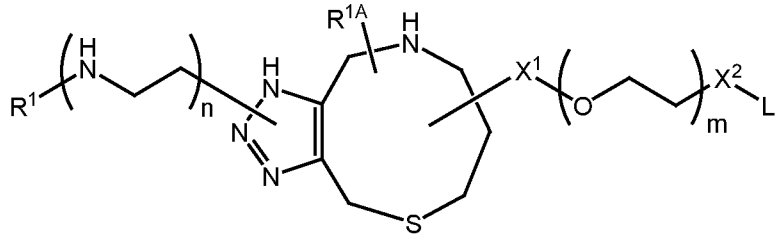
In some embodiments, the composition comprises a conjugate of the Formula IJ-4:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

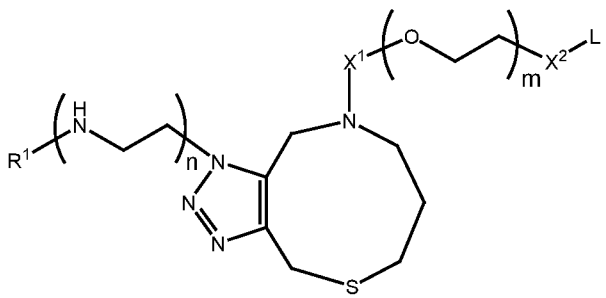
In some embodiments, the composition comprises a conjugate of the Formula IK:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

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

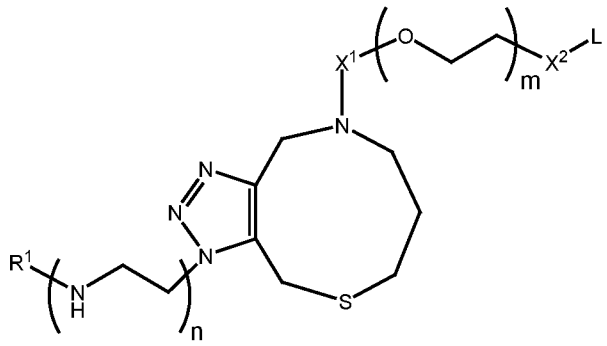


Formula IK-1,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

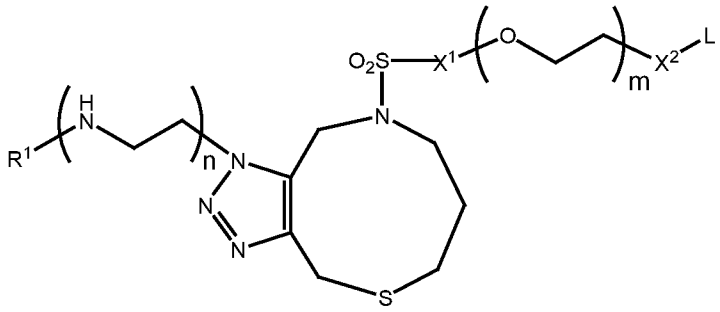
In some embodiments, the composition comprises a conjugate of the Formula IK-2:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

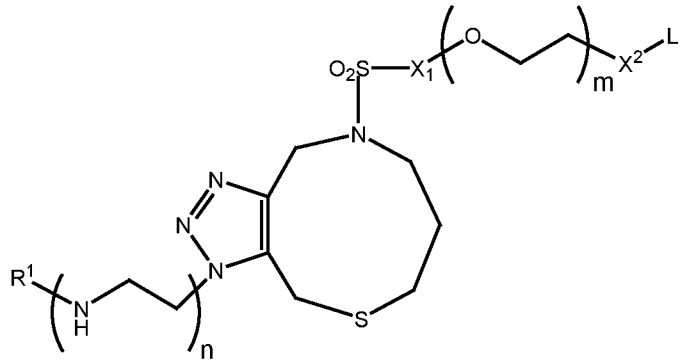
In some embodiments, the composition comprises a conjugate of the Formula IK-3:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

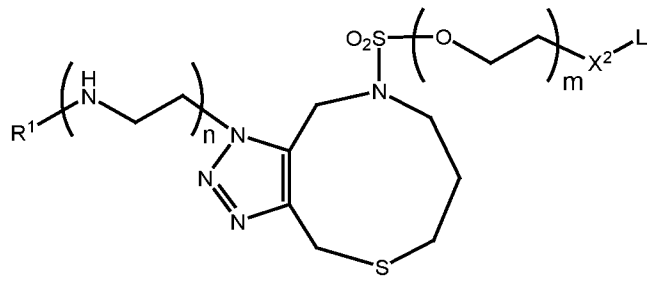
In some embodiments, the composition comprises a conjugate of the Formula IK-4:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

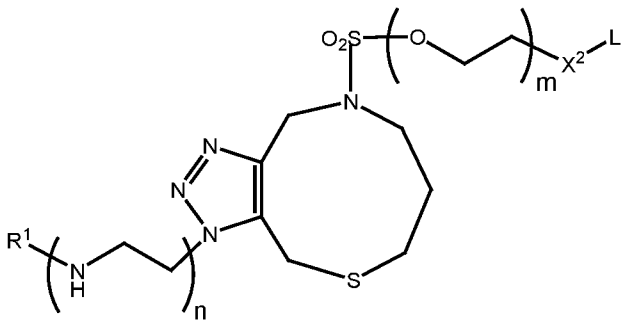
In some embodiments, the composition comprises a conjugate of the Formula IK-3a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

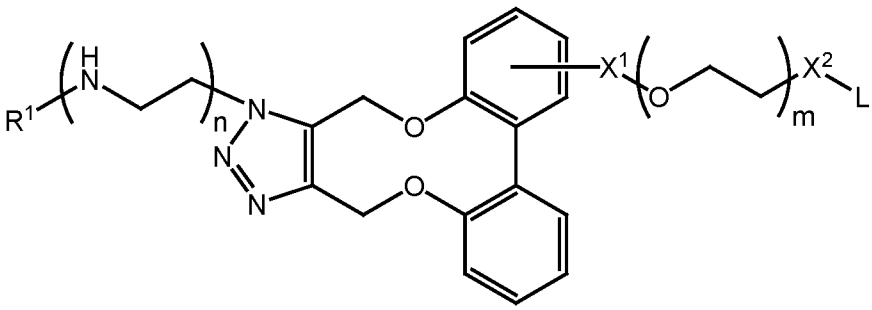
In some embodiments, the composition comprises a conjugate of the Formula IK-4a:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

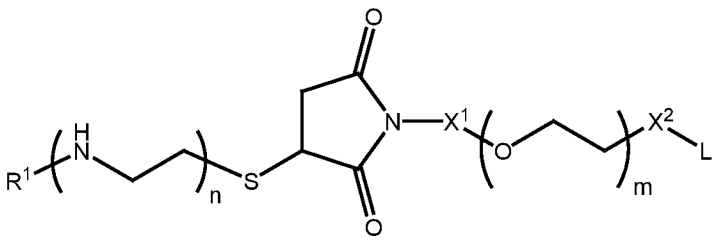
In some embodiments, the composition comprises a conjugate of the Formula IL:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

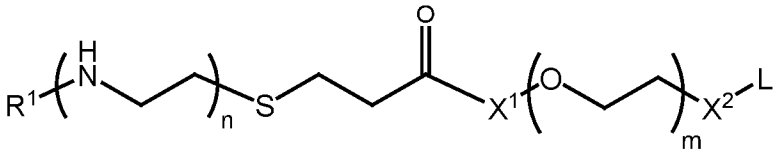
In some embodiments, the composition comprises a conjugate of the Formula IM:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

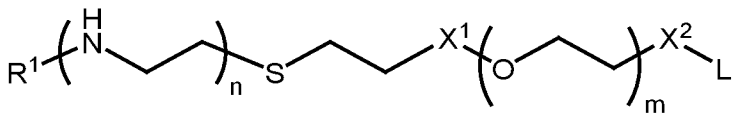
In some embodiments, the composition comprises a conjugate of the Formula IN:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

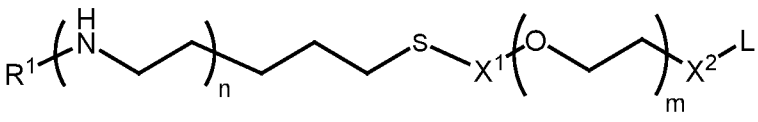
In some embodiments, the composition comprises a conjugate of the Formula IO:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

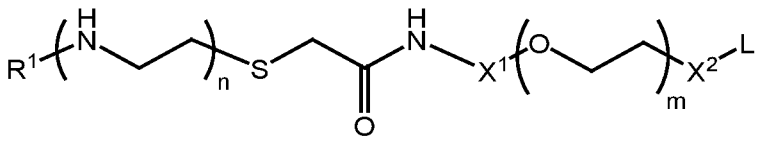
In some embodiments, the composition comprises a conjugate of the Formula IP:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

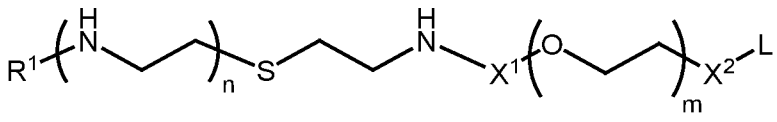
In some embodiments, the composition comprises a conjugate of the Formula IQ:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

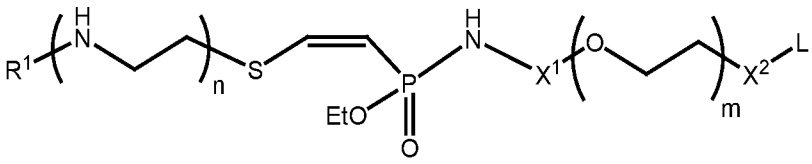
In some embodiments, the composition comprises a conjugate of the Formula IR:



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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

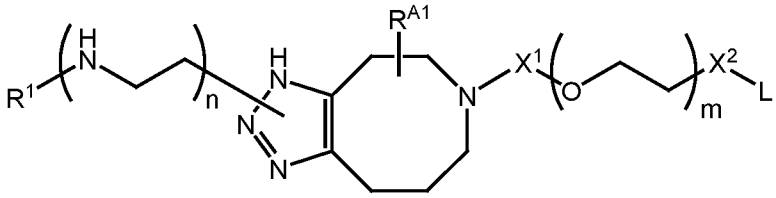
In some embodiments, the composition comprises a conjugate of the Formula IQ:



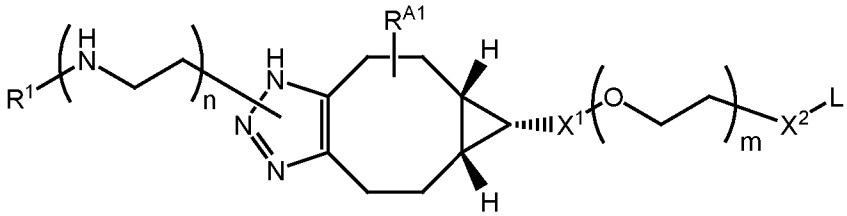
Formula IS,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

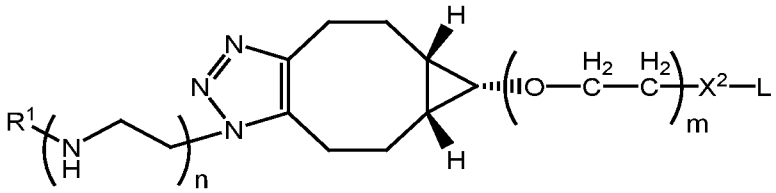
In a further preferred embodiment, said conjugate of Formula I is selected from:



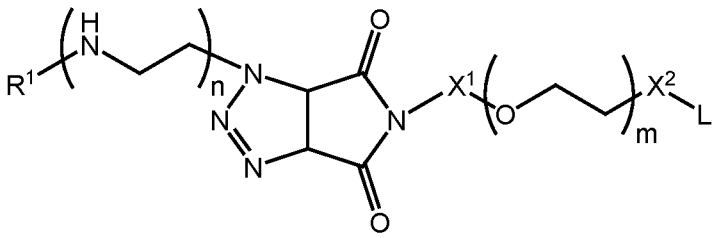
Formula IA,



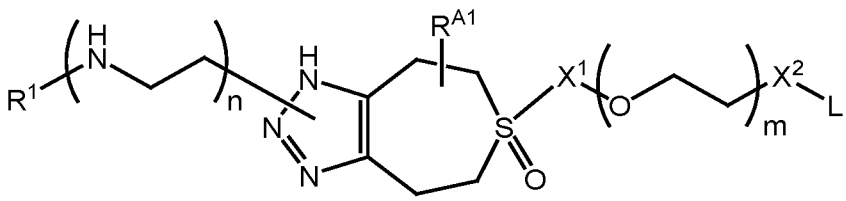
Formula IB,



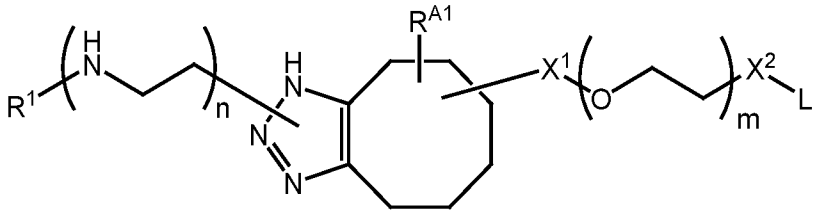
Formula IB-2a,



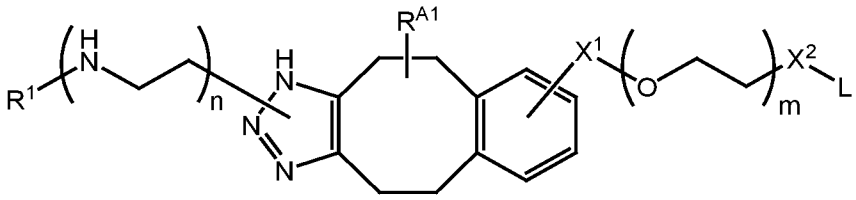
Formula IC,



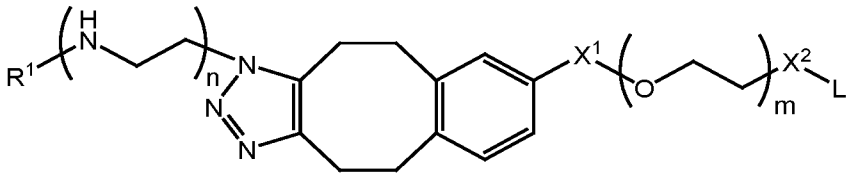
Formula ID,



Formula IE,



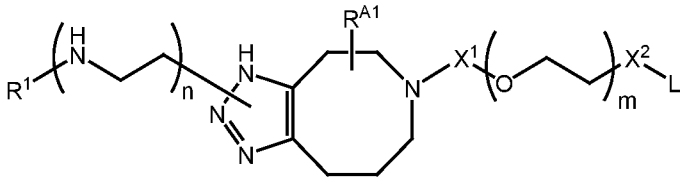
Formula IH,



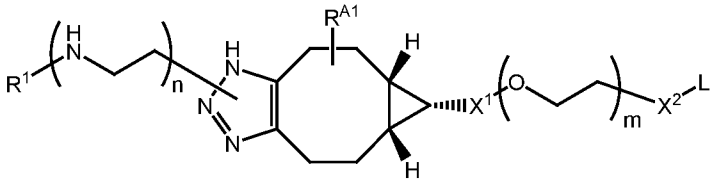
Formula IH-1,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

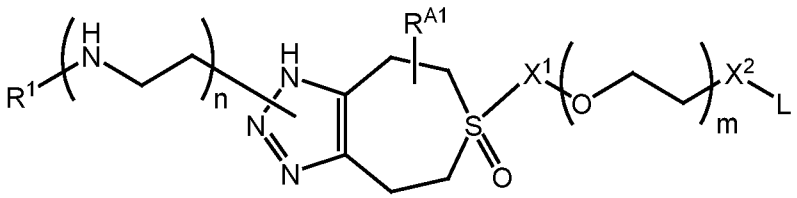
In some embodiments, said conjugate of Formula I is selected from:



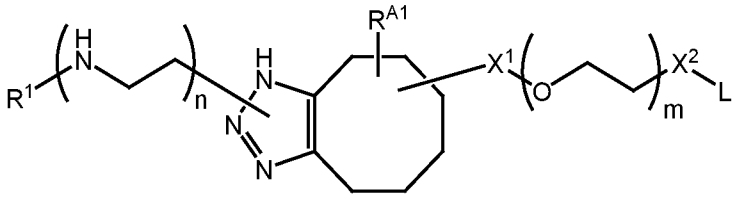
Formula IA,



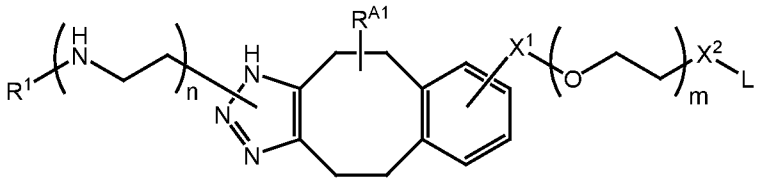
Formula IB,



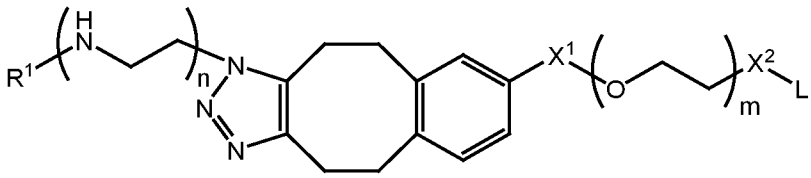
Formula ID,



Formula IE,



Formula IH, and

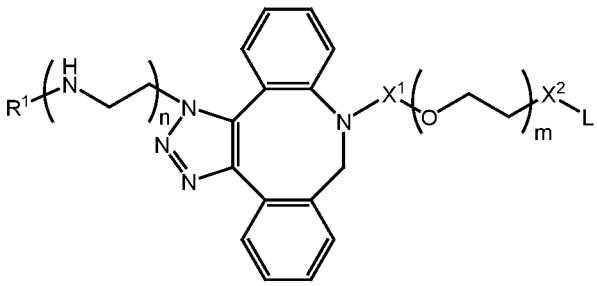


Formula IH-1,

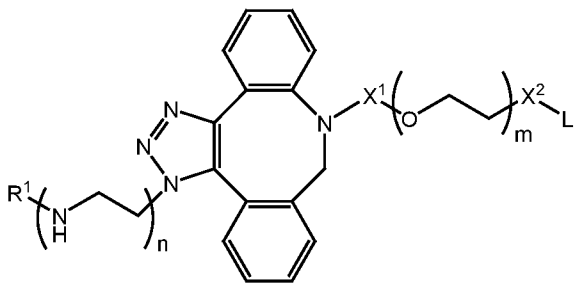
preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

In preferred embodiments, of any of Formulae IA, IB, IC, ID, IE, and/or IH, R^{1A} is -H.

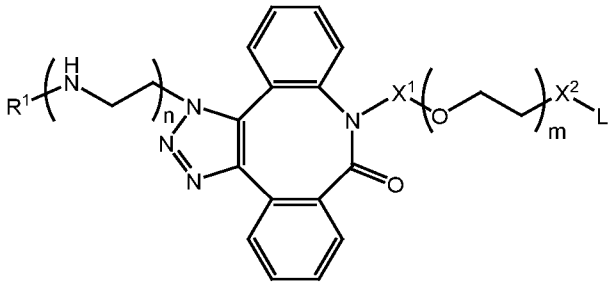
In another preferred embodiment, said conjugate of Formula I is selected from:



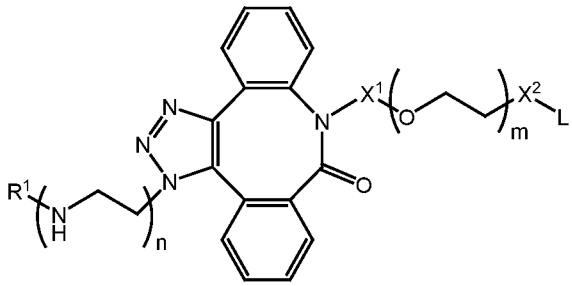
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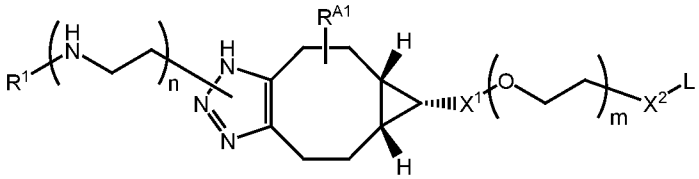
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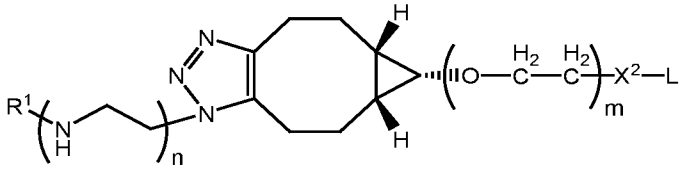
Formula IA-9,



Formula IA-10,



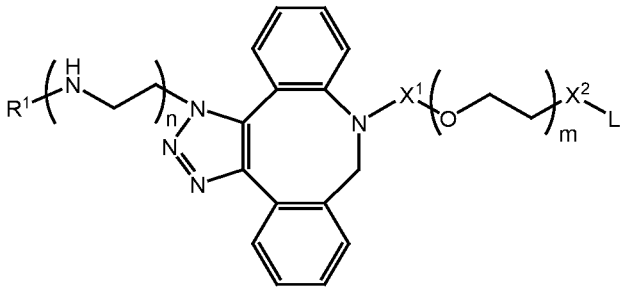
Formula IB,



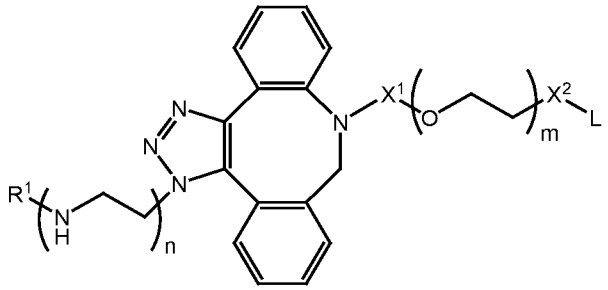
Formula IB-2a,

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 between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

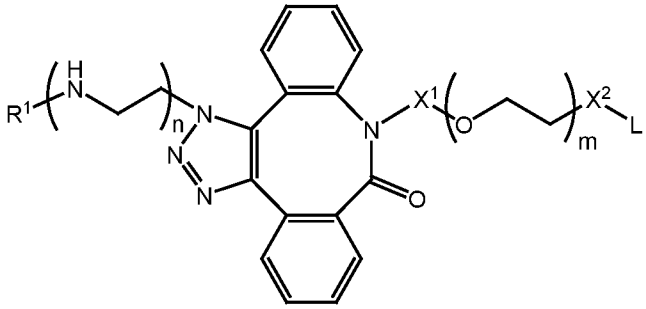
In some embodiments, said conjugate of Formula I is selected from:



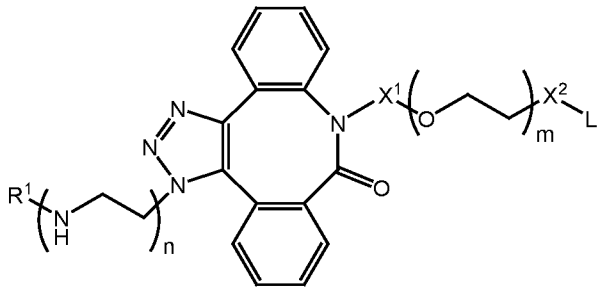
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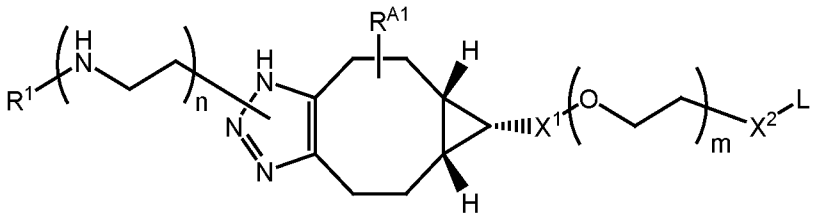
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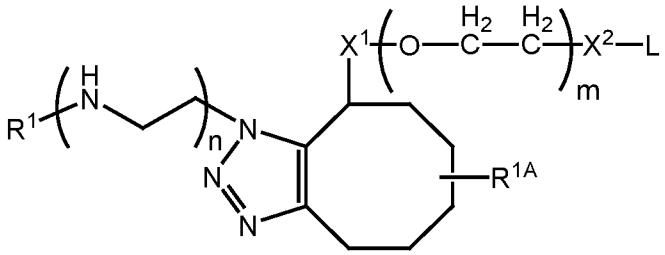
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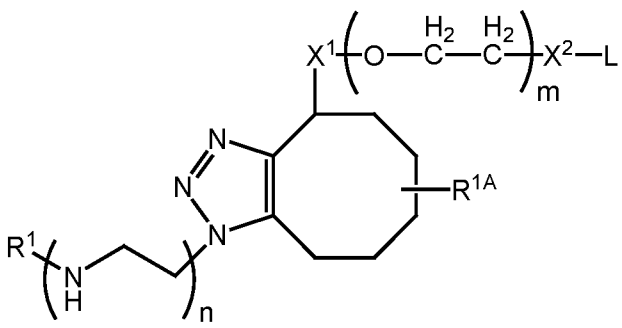
Formula IA-10,



Formula IB,



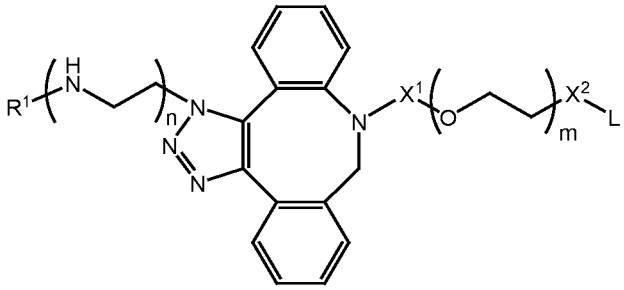
Formula IE-13, and



Formula IE-14,

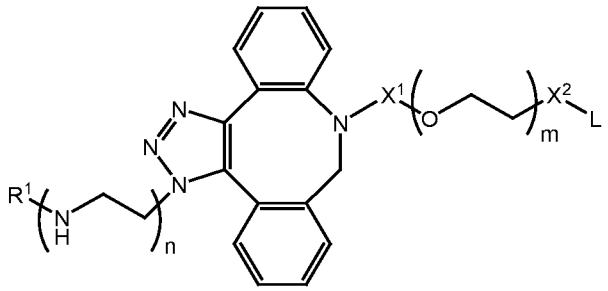
preferably wherein n is between about 280 and about 700 with a dispersity of about 3 or less, more preferably between about 350 and about 630 with a dispersity of about 2 or less, and again more preferably between about 400 and 580 with a dispersity about 1.2 or less, and preferably wherein m is between about 2 and about 80 and a dispersity of about 2 or less, more preferably between about 2 and about 70 with a dispersity of about 1.8 or less; again more preferably between about 2 and about 50 repeating units with a dispersity of about 1.5, or alternatively m is a discrete number of repeating units, preferably wherein m is 12 or 24.

In some embodiments, said conjugate of Formula I is selected from:



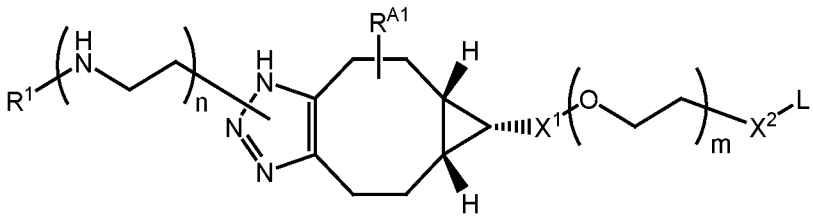
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and



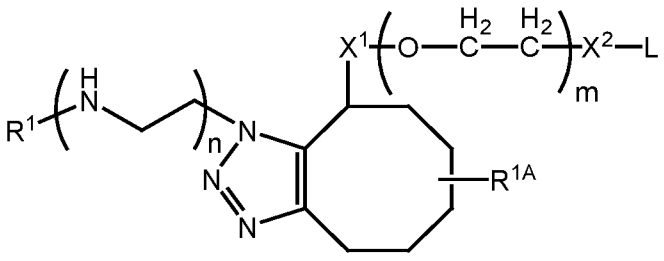
Formula IA-4.

In some embodiments, said conjugate of Formula I is selected from:

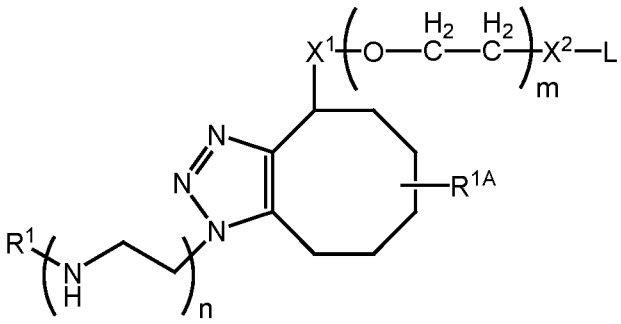


Formula IB.

In some embodiments, said conjugate of Formula I is selected from:

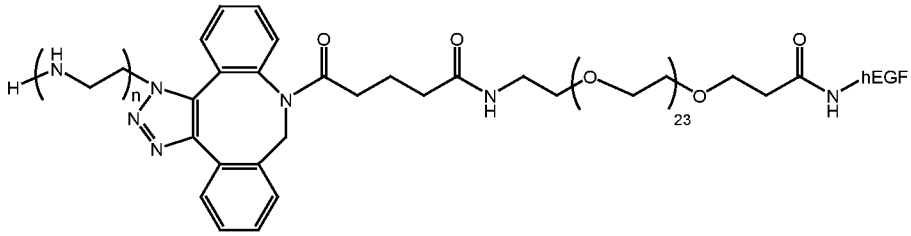


Formula IE-13, and



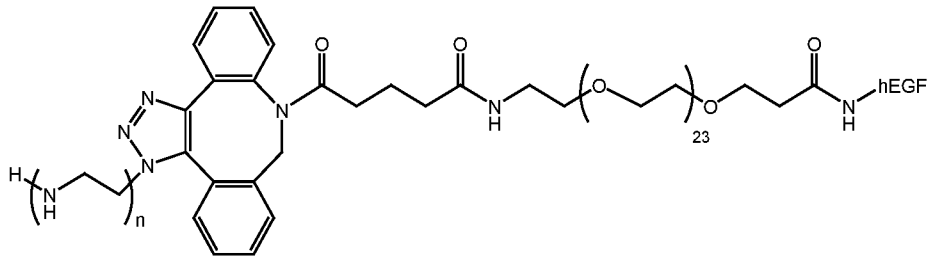
Formula IE-14.

In some embodiments, the composition comprises a conjugate of the formula:



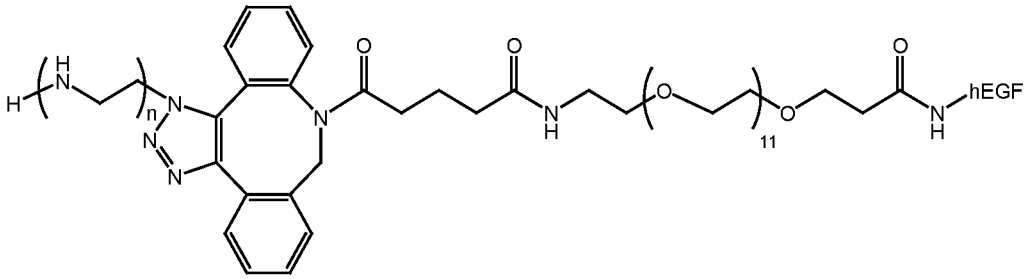
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



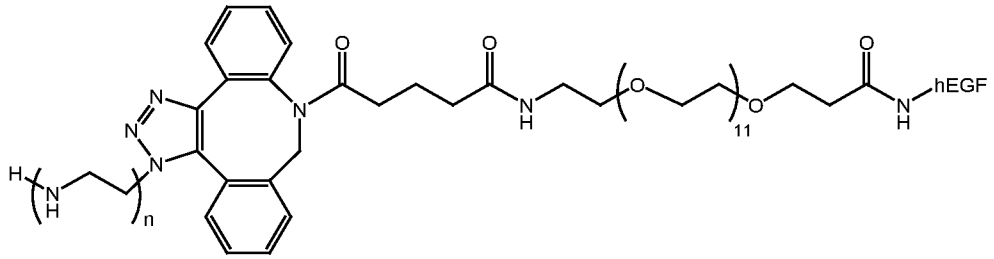
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



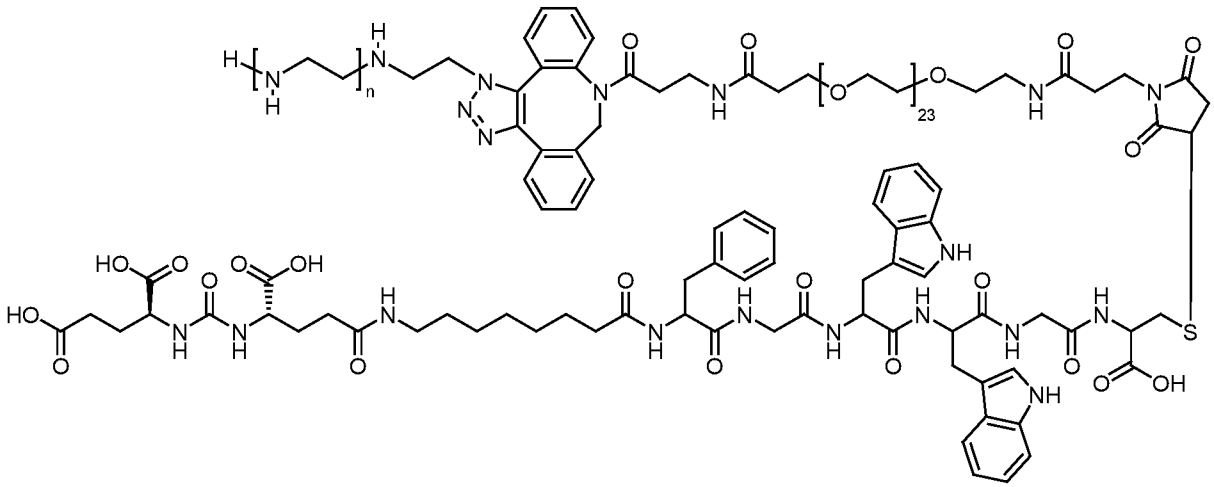
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



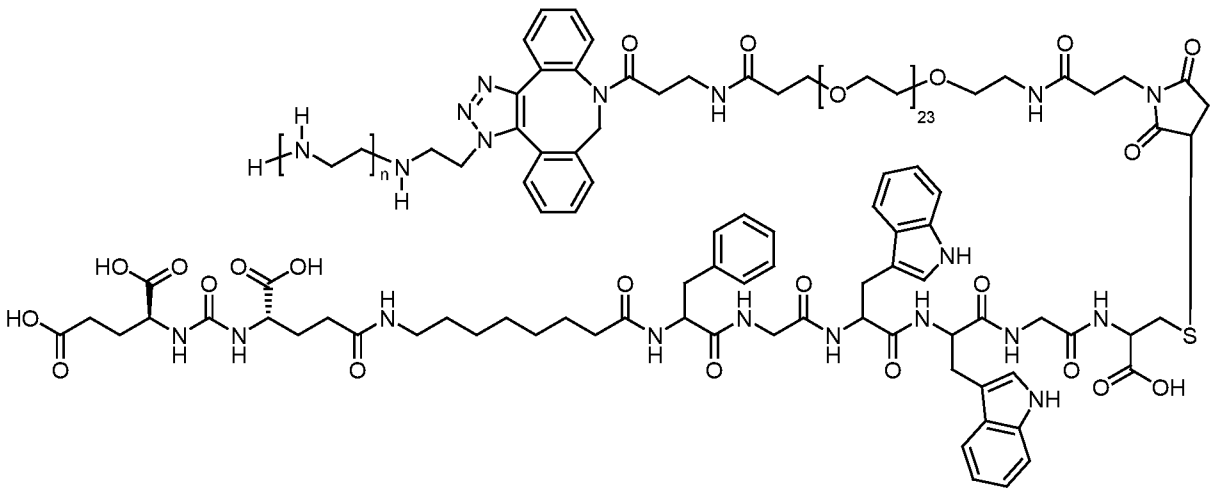
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



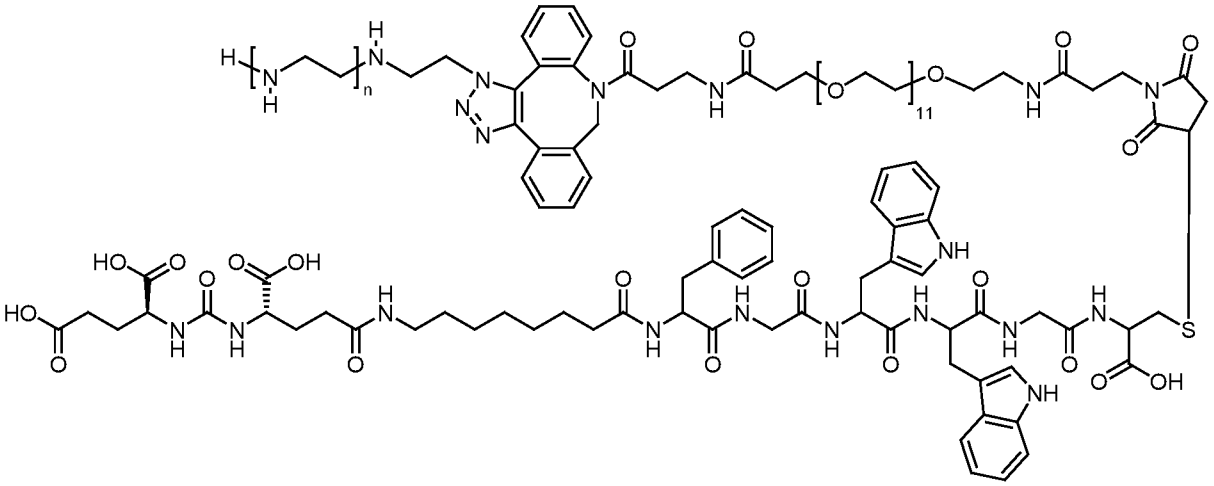
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



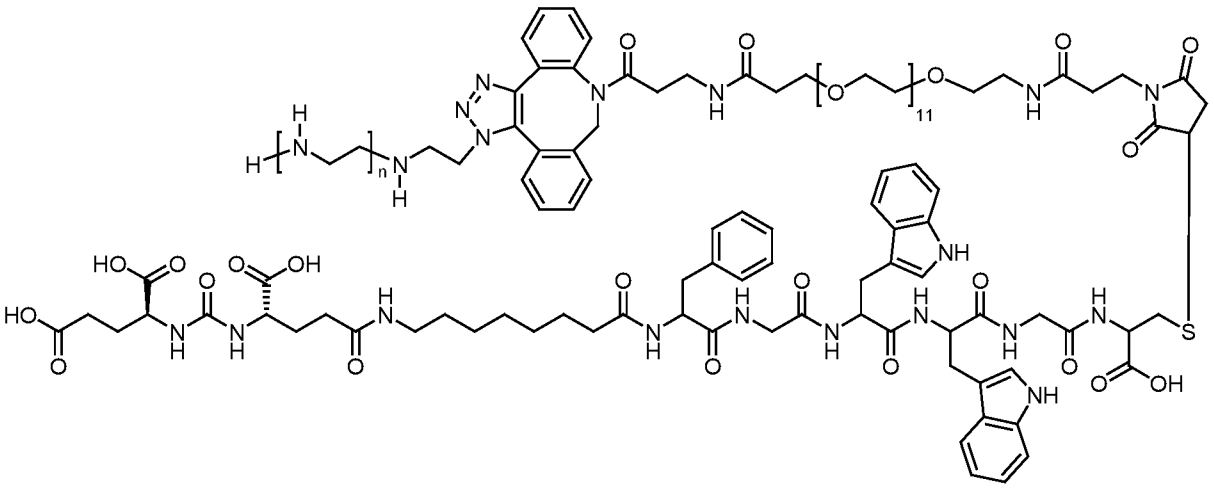
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



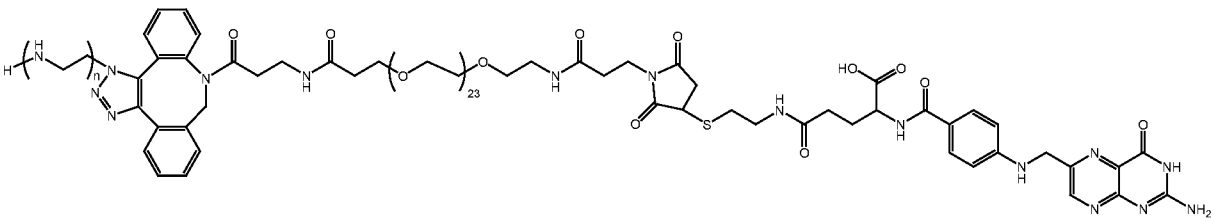
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



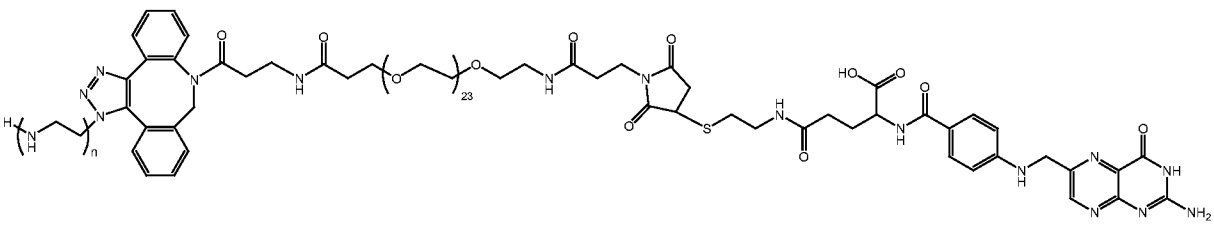
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



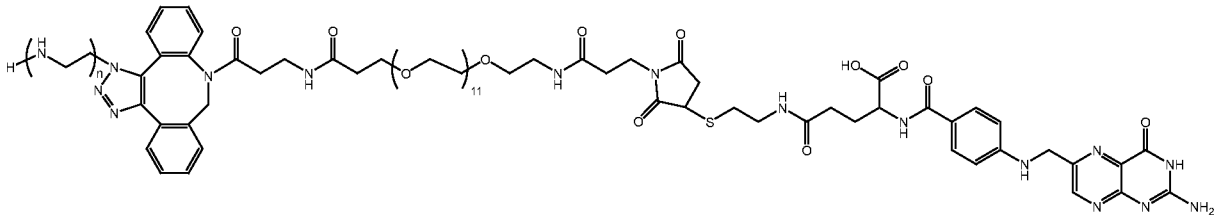
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

In some embodiments, the composition comprises a conjugate of the formula:



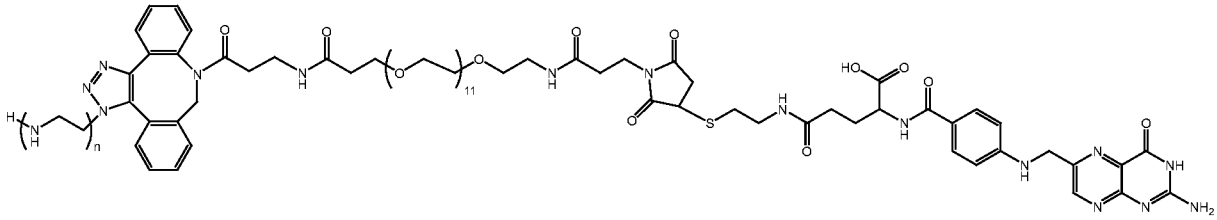
preferably wherein n is between about 400 and 580 with a dispersity about 1.2 or less.

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 1a, Compound 1b, Compound 4a, Compound 4b, Compound 7a, Compound 7b, Compound 10a, Compound 10b, Compound 14, Compound 17a, Compound 17b, Compound 18, Compound 19, Compound 22a, Compound 22b, Compound 28a, Compound 28b, Compound 31a, Compound 31b, Compound 38a, Compound 38b, Compound 43, Compound 47a, Compound 47b, Compound 51a, Compound 51b, Compound 56a, Compound 56b, Compound 62a, Compound 62b, Compound 70a, Compound 70b, Compound 72a, Compound 72b, Compound 75a, Compound 75b, Compound 78a and/or Compound 78b.

In a preferred embodiment, the composition comprises a conjugate selected from Compound 1a, Compound 1b, Compound 4a, Compound 4b, Compound 7a, Compound 7b, Compound 10a, Compound 10b, Compound 14, Compound 17a, Compound 17b, Compound 18, Compound 19, Compound 22a, Compound 22b, Compound 28a, Compound 28b, Compound 31a, Compound 31b, Compound 38a, Compound 38b, Compound 43, Compound 47a, Compound 47b, Compound 51a, Compound 51b, Compound 56a, Compound 56b, Compound 62a, Compound 62b, Compound 70a, Compound 70b, Compound 72a, Compound 72b, Compound 75a, Compound 75b, Compound 78a and/or Compound 78b.

In a preferred embodiment, the composition comprises a conjugate comprising Compound 1a, and/or Compound 1b. In some embodiments, the composition comprises a conjugate comprising Compound 4a and/or Compound 4b. In some embodiments, the composition comprises a conjugate comprising Compound 7a and/or Compound 7b. In some embodiments, the composition comprises a conjugate comprising Compound 10a and/or

Compound 10b. In some embodiments, the composition comprises a conjugate comprising Compound 14. In some embodiments, the composition comprises a conjugate comprising Compound 17a and/or Compound 17b. In some embodiments, the composition comprises a conjugate comprising Compound 18. In some embodiments, the composition comprises a conjugate comprising Compound 19. In some embodiments, the composition comprises a conjugate comprising Compound 22a and/or Compound 22b. In some embodiments, the composition comprises a conjugate comprising Compound 28a and/or Compound 28b. In some embodiments, the composition comprises a conjugate comprising Compound 31a and/or Compound 31b. In some embodiments, the composition comprises a conjugate comprising Compound 38a and/or Compound 38b. In some embodiments, the composition comprises a conjugate comprising Compound 43. In some embodiments, the composition comprises a conjugate comprising Compound 47a and/or Compound 47b. In some embodiments, the composition comprises a conjugate comprising Compound 51a and/or Compound 51b. In some embodiments, the composition comprises a conjugate comprising Compound 56a and/or Compound 56b. In some embodiments, the composition comprises a conjugate comprising Compound 62a and/or Compound 62b. In some embodiments, the composition comprises a conjugate comprising Compound 70a and/or Compound 70b. In some embodiments, the composition comprises a conjugate comprising Compound 72a and/or Compound 72b. In some embodiments, the composition comprises a conjugate comprising Compound 75a and/or Compound 75b. In some embodiments, the composition comprises a conjugate comprising Compound 78a and/or Compound 78b.

In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 1a, and/or Compound 1b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 4a and/or Compound 4b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 7a and/or Compound 7b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 10a and/or Compound 10b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 14. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 17a and/or Compound 17b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 18. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 19. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 22a

and/or Compound 22b. 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 31a and/or Compound 31b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 38a and/or Compound 38b. 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 47a and/or Compound 47b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 51a and/or Compound 51b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 56a and/or Compound 56b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 62a and/or Compound 62b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 70a and/or Compound 70b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 72a and/or Compound 72b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 75a and/or Compound 75b. In a preferred embodiment, the composition comprises a conjugate, wherein said conjugate is Compound 78a and/or Compound 78b.

Polyplexes

The inventive compositions further comprise a polyanion, preferably wherein said polyanion is a nucleic acid, and wherein said polyanion and said conjugate preferably form a polyplex. 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 , preferably tumor cell or tumortissue causing the production of chemokines, as shown herein. The production of chemokines will attract immune cells to the tumor site.

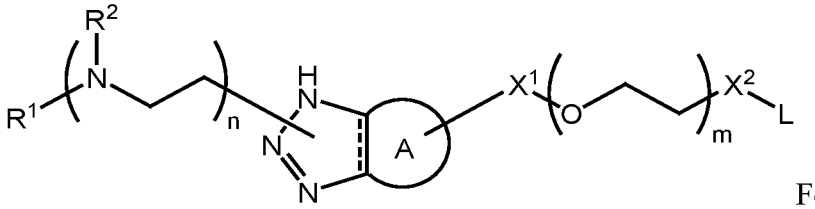
The inventive polyplex provides efficient delivery of the the polyanion and, preferably the nucleic acid, into cells harboring the target cell surface receptor. As described herein, the targeting fragment comprised by the inventive polyplex is capable of binding to the target cell surface receptor.

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.

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).

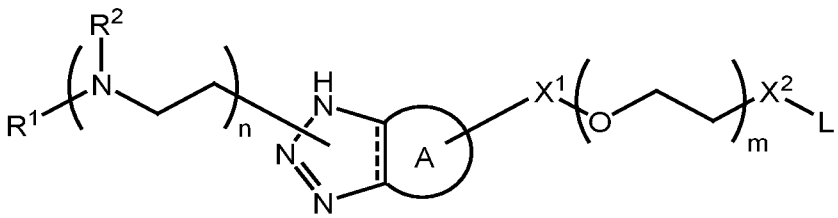
In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, or a pharmaceutically acceptable salt, solvate, hydrate, tautomer or enantiomer thereof, and a nucleic acid, wherein said nucleic acid is preferably non-covalently bound to said conjugate



Formula I,

wherein A, R^1, R^2, X^1, X^2 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, 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;

m is a discrete number of repeating units m of 2 to 100, preferably of a discrete number of repeating units m of 4 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;

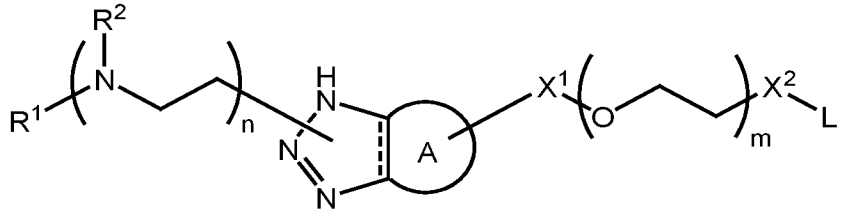
Ring A is a 5 to 10-membered cycloalkyl, cycloalkenyl, heterocycloalkyl, or heterocycloalkenyl, optionally substituted at any position with one or more R^{A1}; R^{A1} is independently selected from C_1-C_6 alkyl, C_1-C_6 alkoxy, oxo, or halogen; or two R^{A1}, together with the atoms to which they are 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, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. 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 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:

$\equiv\equiv$ 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, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. 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).

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.

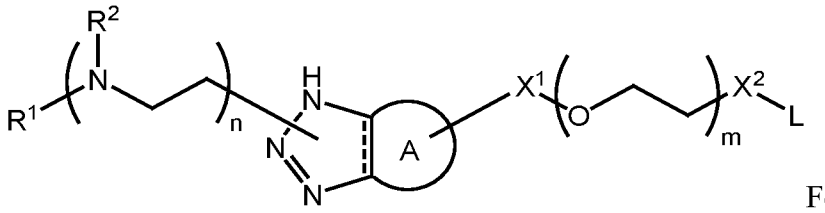
"Ribonucleotide" relates to a nucleotide with a hydroxyl group at the 2'-position of a β-D-ribofuranosyl group. The term "RNA" as used herein comprises double stranded RNA (dsRNA) and single stranded RNA (ssRNA). The term "RNA" further includes isolated RNA such as partially or completely purified RNA, 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



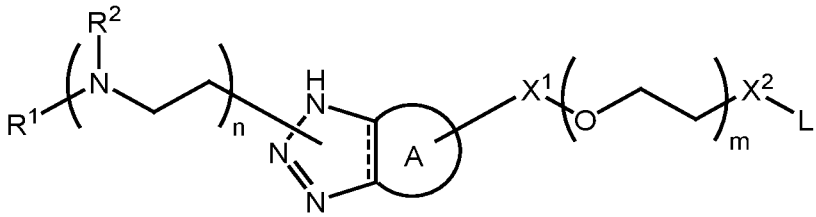
Formula I,

wherein A, R^1, R^2, X^1, X^2 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, 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 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 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 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 an RNA 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 mRNA have a size of bout 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 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.

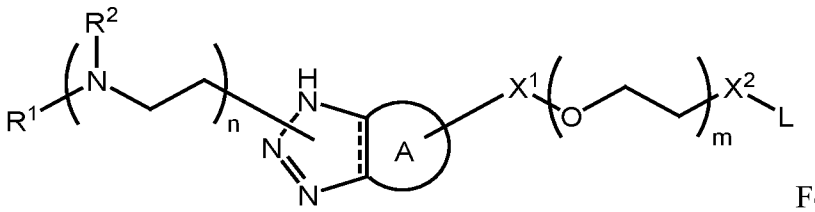
With respect to RNA, and in particular with respect to mRNA, the term "expression" or "translation" relates to the process, typically in the ribosomes of a cell, by which a strand of mRNA directs the assembly of a sequence of amino acids to make a peptide or protein. The term "expression" is used in its most general meaning and comprises production of RNA and/or

protein.

A "pharmaceutically active peptide or protein" or "therapeutic peptide or protein" is a peptide ora 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.

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.

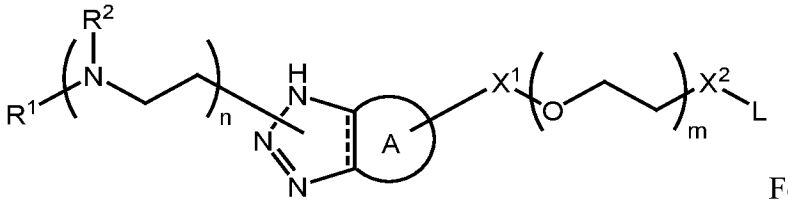


Formula I

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. 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, 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, 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 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 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.

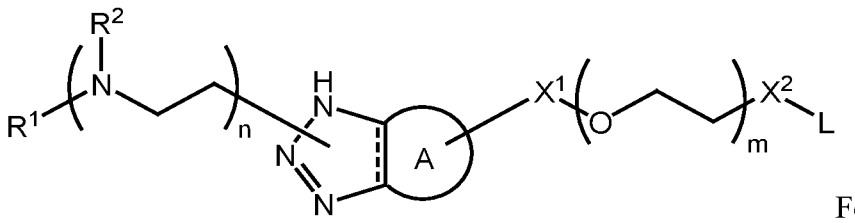
In some embodiments the non-self-antigen is a virus antigen. In some embodiments the non-self-antigen is a polypeptide or a protein from a fungus. In some embodiments the non-self-antigen is a polypeptide or protein from a unicellular eukaryotic parasite.

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 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.

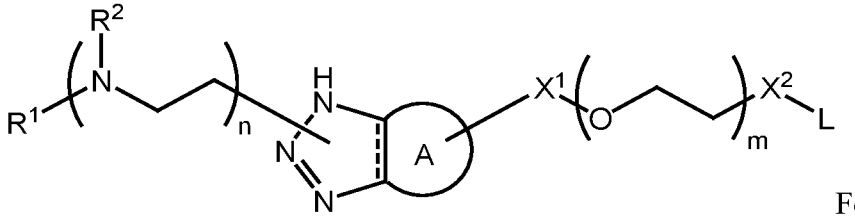
In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, and a nucleic acid, wherein said nucleic acid is preferably non-covalently bound to said conjugate, and wherein said nucleic acid is a pharmaceutically active nucleic acid.



Formula I

wherein A, R^1, R^2, X^1, X^2 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 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 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 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.

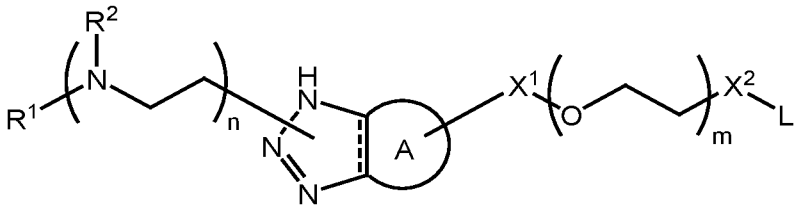
Negatively Charged Polyanions Used to Form Polyplexes

In some embodiments, the triconjugates of the present disclosure can form polyplexes with polyanions and anionic polymers. 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 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, 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).

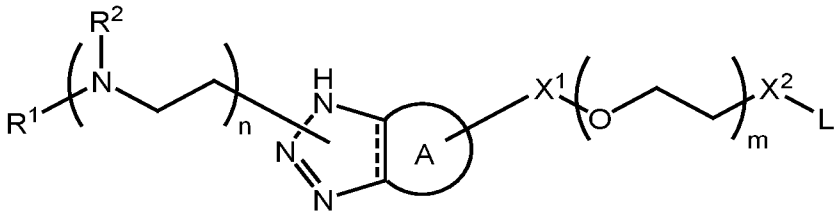
In another aspect, the present invention provides a polyplex comprising a conjugate of Formula I*, preferably of Formula I, and poly(IC), wherein said poly(IC) is preferably non-covalently bound to said conjugate



Formula I

wherein A, R^1, R^2, X^1, X^2 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, 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 2 to 100, preferably of a discrete number of repeating units m of 4 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 $-(NR^2\text{-}CH_2\text{-}CH_2)_n-$ 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 $-OSO_3H$;

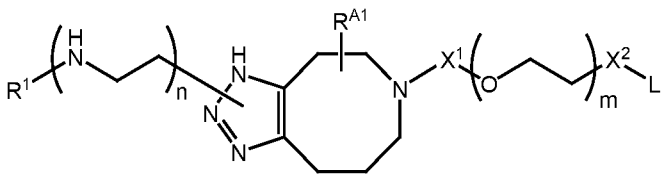
X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

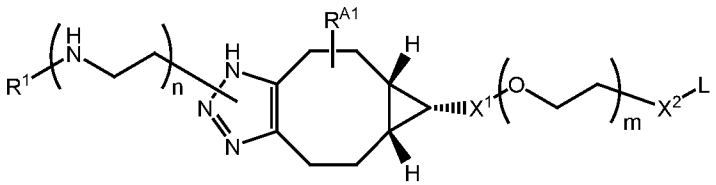
L is a targeting fragment, wherein preferably said targeting fragment is capable of binding to a cell, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is $-CH_3$.

In a preferred embodiment, said Ring A is cyclooctene, succinimide, or 7- to 8-membered heterocycloalkenyl, wherein the heterocycloalkyl or heterocycloalkenyl comprises one or two heteroatoms selected from N, O and S, and wherein each cyclooctene, heterocycloalkyl 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 $-OSO_3H$.

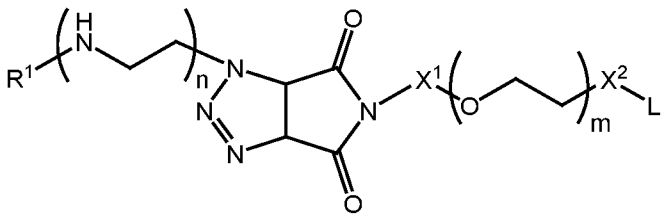
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



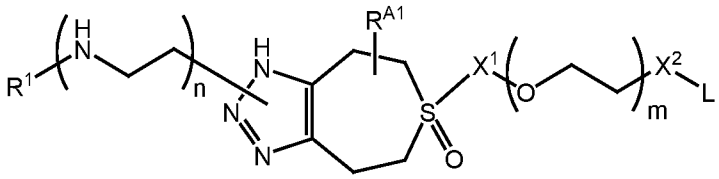
Formula IA,



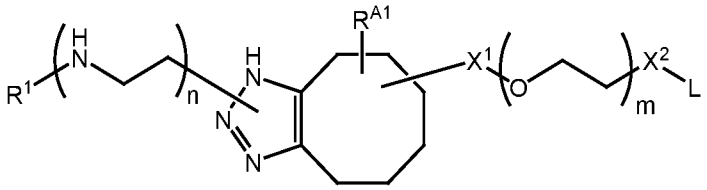
Formula IB,



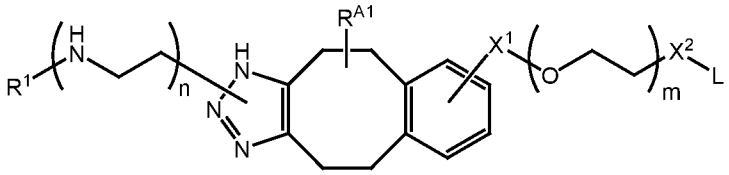
Formula IC,



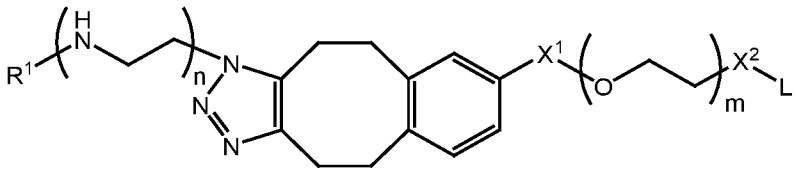
Formula ID,



Formula IE,



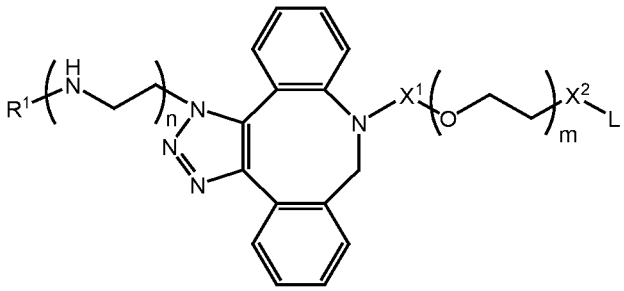
Formula IH, and



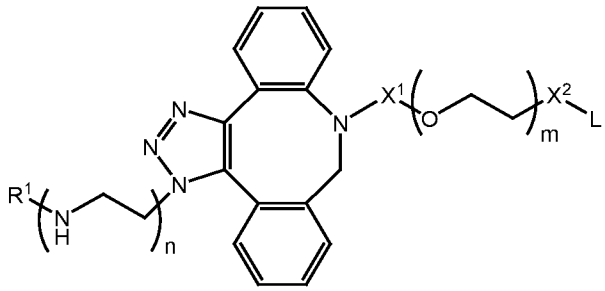
Formula IH-1,

wherein R^1, R^{A1}, X^1, X^2 and L 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 and L, or collectively to some or all of R^1, R^{A1}, X^1, X^2 and L.

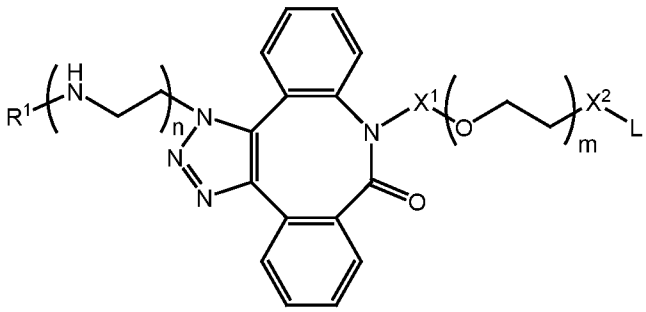
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



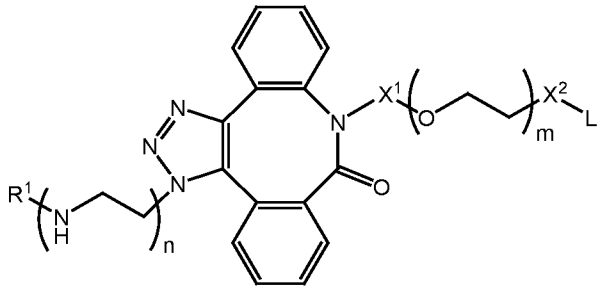
Formula IA-3,



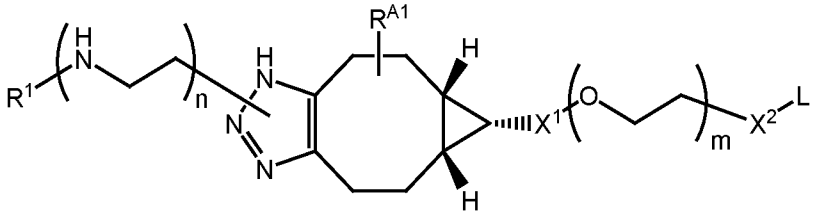
Formula IA-4,



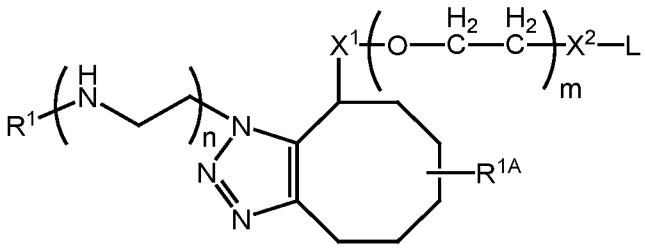
Formula IA-9,



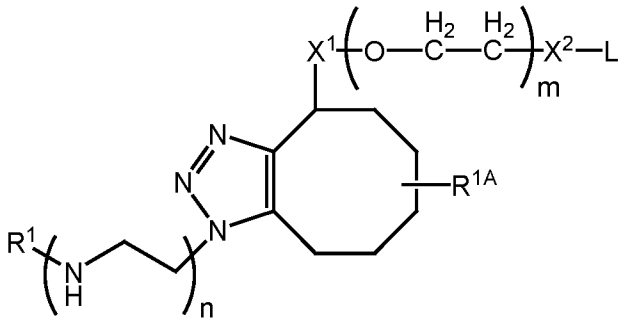
Formula IA-10,



Formula IB,



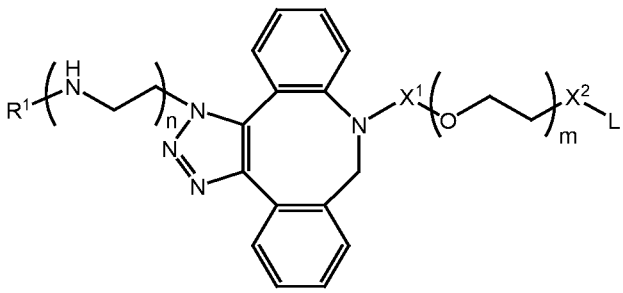
Formula IE-13, and



Formula IE-14,

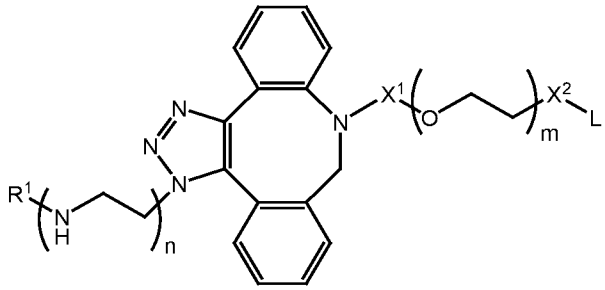
wherein R^1, R^{A1}, X^1, X^2 and L are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2 and L, or collectively to some or all of R^1, R^{A1}, X^1, X^2 and L.

In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IA-3,

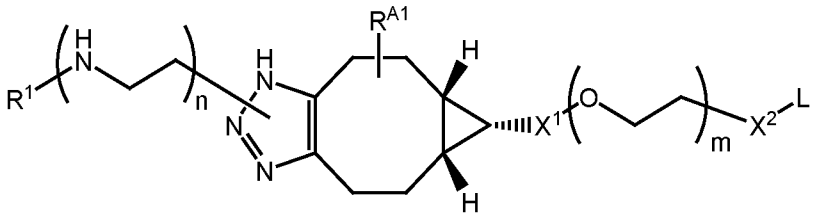
and



Formula IA-4,

wherein R^1, X^1, X^2 and L 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 and L, or collectively to some or all of R^1, X^1, X^2 and L.

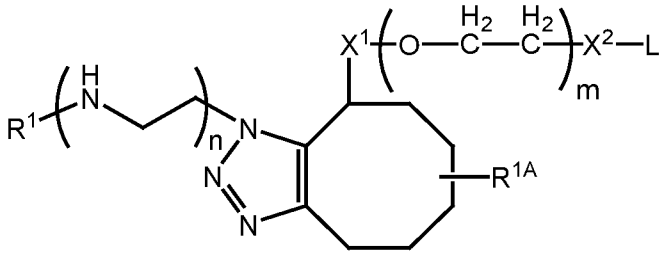
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



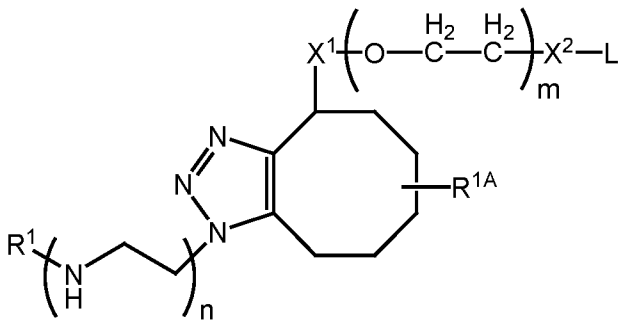
Formula IB,

wherein R^1, R^{A1}, X^1, X^2 and L 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 and L, or collectively to some or all of R^1, R^{A1}, X^1, X^2 and L.

In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IE-13, and



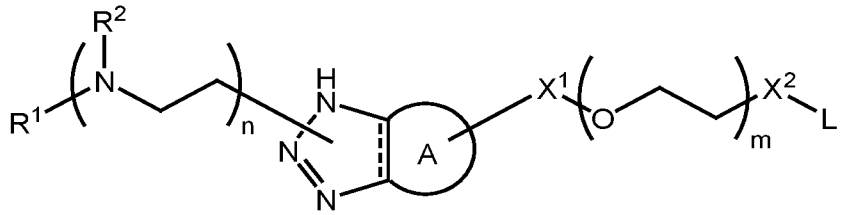
Formula IE-14,

wherein R^1, R^{A1}, X^1, X^2 and L 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 and L, or collectively to some or all of R^1, R^{A1}, X^1, X^2 and L.

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;

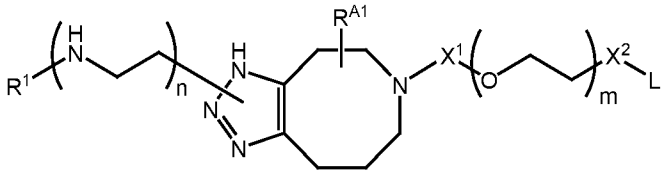
X^1 is a divalent covalent linking moiety;

X^2 is a divalent covalent linking moiety; and

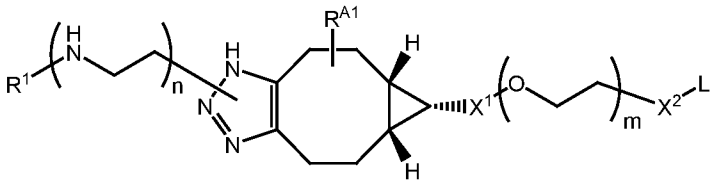
L is a targeting fragment, wherein preferably said targeting fragment is capable of binding to a cell, and wherein further preferably said targeting fragment is capable of binding to a cell surface receptor. In a preferred embodiment, said R^1 is -H. In a preferred embodiment, said R^1 is -CH_3.

In a preferred embodiment, said Ring A is cyclooctene, succinimide, or 7- to 8-membered heterocycloalkenyl, wherein the heterocycloalkyl or heterocycloalkenyl comprises one or two heteroatoms selected from N, O and S, and wherein each cyclooctene, heterocycloalkyl 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 $-OSO_3H$.

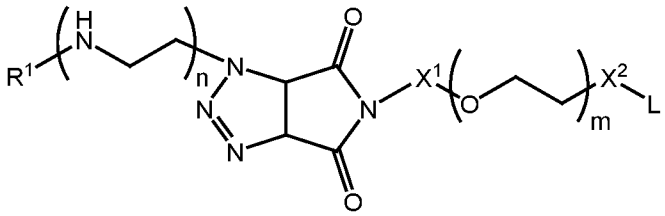
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



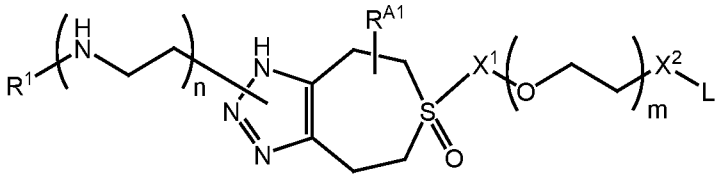
Formula IA,



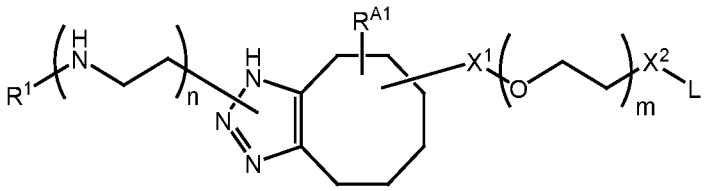
Formula IB,



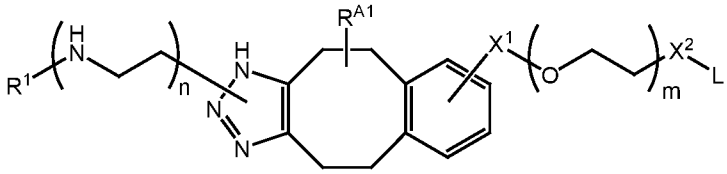
Formula IC,



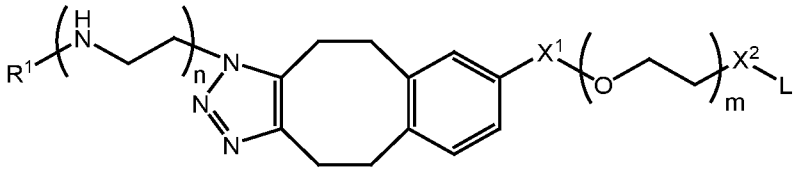
Formula ID,



Formula IE,



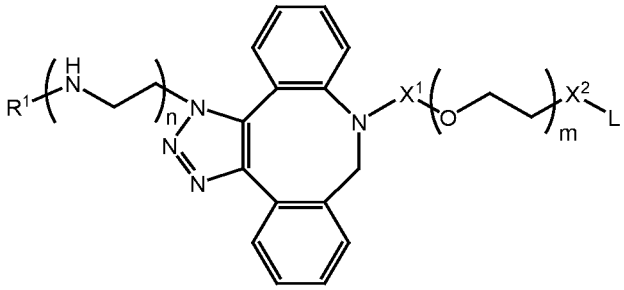
Formula IH, and



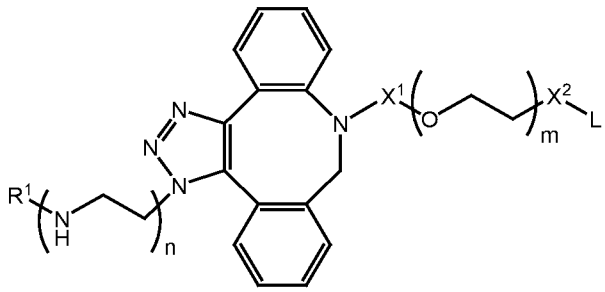
Formula IH-1,

wherein R^1, R^{A1}, X^1, X^2 and L 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 and L, or collectively to some or all of R^1, R^{A1}, X^1, X^2 and L.

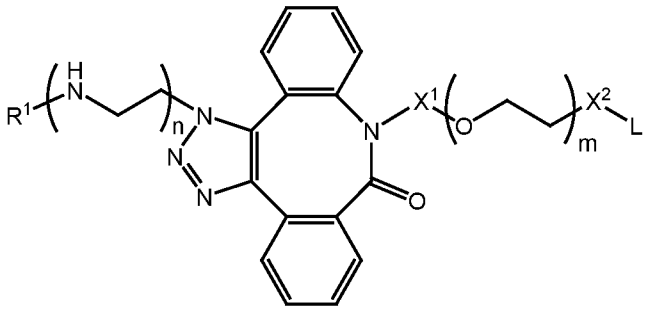
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



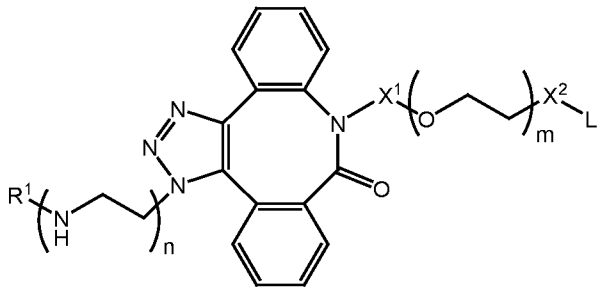
Formula IA-3,



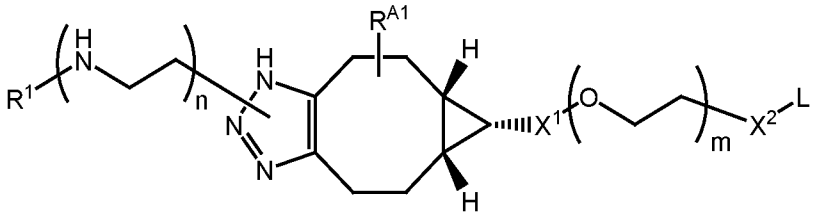
Formula IA-4,



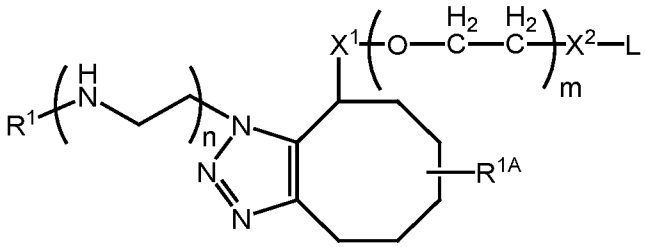
Formula IA-9,



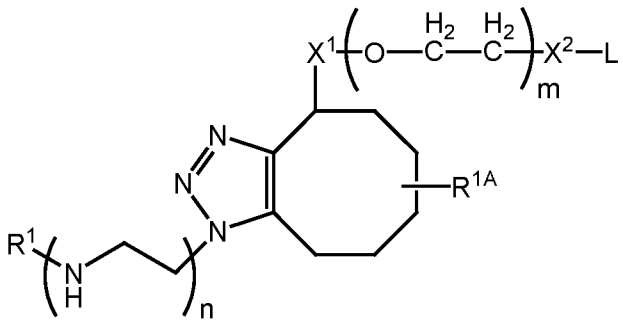
Formula IA-10,



Formula IB,



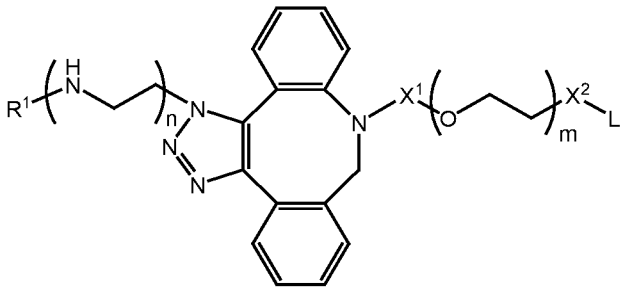
Formula IE-13, and



Formula IE-14,

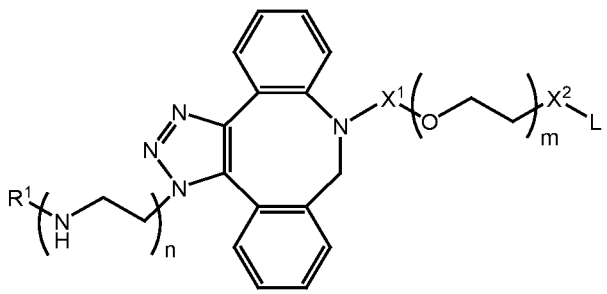
wherein R^1, R^{A1}, X^1, X^2 and L are as defined herein, preferably as defined in any embodiment described herein, be it individually related to each parameter R^1, R^{A1}, X^1, X^2 and L, or collectively to some or all of R^1, R^{A1}, X^1, X^2 and L.

In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



Formula IA-3,

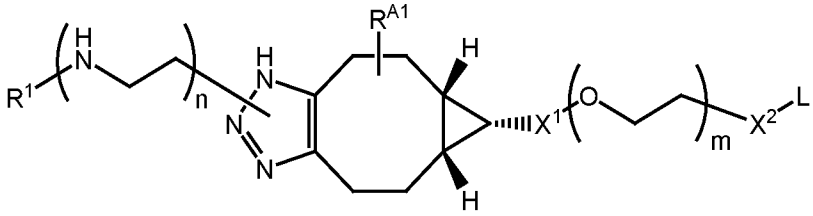
and



Formula IA-4,

wherein R^1, X^1, X^2 and L 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 and L, or collectively to some or all of R^1, X^1, X^2 and L.

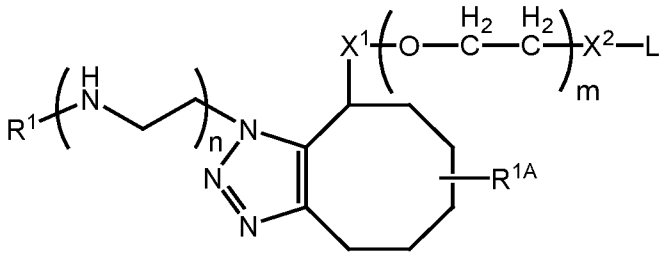
In a preferred embodiment said conjugate of Formula I is a conjugate selected from:



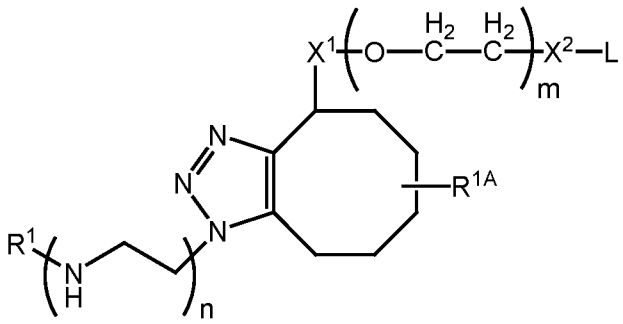
Formula IB,

wherein R^1, R^{A1}, X^1, X^2 and L 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 and L, or collectively to some or all of R^1, R^{A1}, X^1, X^2 and L.

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 and L 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 and L, or collectively to some or all of R^1, R^{A1}, X^1, X^2 and L.

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.

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 hEGF) polyplexed with a polyanion such as a cytotoxic agent (e.g., a nucleic acid such double stranded RNA (dsRNA such as poly(IC)). As shown in the Examples below, 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 an acetate solution at pH 4-4.5 containing 5% glucose e.g., at room temperature).

The particle size distribution such as the z-average diameter 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 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 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 or 6. 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 100 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 100 nm. In another preferred embodiment, said polyplexes have a z-average diameter of between around 200 nm and around 100 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 18 mV, e.g. between 18 mV and 50 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.

FIGs 1A, 1B and 1C show the z-average diameter of polyplexes disclosed herein as a function of N/P ratio. FIG 1A shows that LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) polyplexes with an N/P ratio of 2.4 had an z-average diameter over 200 nm (i.e., 306 nm). In contrast, FIG 1B and 1C show that LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) polyplexes with a N/P ratio of 4 or 5.6 had an z-average diameter less than 200 nm (i.e., 116 nm and 107 nm, respectively). Without wishing to be bound by theory, the Examples and figures herein show that the size of the polyplexes disclosed herein can be controlled by adjusting the N/P ratio.

FIGs 3 demonstrate that triconjugates that do not comprise a targeting fragment can form polyplexes of similar z-average diameter and dispersity as conjugates with a targeting fragment. FIG 3 shows DLS characterization of LPEI-*l*-PEG_{23}-OMe:poly(IC) at an N/P ratio of 4. The polyplex shown in FIG. 3 comprises a PEG fragment terminated with -OMe and does not have a targeting fragment. However, the polyplexes shown in FIG. 3 have a similar z-average size distribution and ζ-potential (107 nm and 34.1 mV) as those having a targeting fragment such as hEGF.

FIG. 4 shows DLS characterization of LPEI-*l*-PEG_{12}-hEGF:poly(IC) at an N/P ratio of 4. The polyplexes have a z-average diameter of 156 nm and a ζ-potential of 38.3 mV.

FIG. 5 shows DLS characterization of a polyplex formed with a DUPA-modified LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) at an N/P ratio of 4. As shown in FIG. 5, the z-average diameter of the polyplexes is about 120 nm and the ζ-potential is 31.1 mV.

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.

Cytotoxic Activity of the Polyplexes

The present invention relates to polyplexes of conjugates comprising LPEI, PEG, and targeting fragments such as hEGF, DUPA, HER2 or folate, 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 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 EGFR or 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). Moreover, the cytotoxic and/or immunostimulatory activity of the polyplexes can be tailored by the selection of an appropriate polyanion. For example, poly(Glu) does not exhibit an immunostimulatory or cytotoxic effect, in contrast to poly(IC), and was thus used as a control for comparison in the cytotoxicity examples described herein.

Without wishing to be bound by theory, the linear conjugates of the present invention can include targeting fragments that help increase relative uptake of the triconjugate:poly(IC) polyplexes. For example, conjugates (and the resulting polyplexes) that contain human epidermal growth factor (hEGF) can be taken up at higher concentrations in cells that highly express human epidermal growth factor receptor (EGFR) as compared to cells that have lower EGFR expression levels. Similarly, 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), and conjugates (and the resulting polyplexes) that contain the targeting fragment folate, can be taken up at greater rates in cells that have high expression level of folate receptor. 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 evidenced by the high cytotoxicity of the inventive triconjugate:nucleic acid polyplexes. In preferred embodiments, the high cytotoxicity of the polyplexes is believed to be caused by poly(IC).

Moreover, the Examples herein demonstrate that the inventive polyplexes were significantly more cytotoxic in A431 cells that expressed hEGFR at high (i.e., 10^6 molecules/cell) levels than in cells that expressed hEGFR at low (i.e., 10^3 molecules/cell) 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. In preferred embodiments, cytotoxicity of the inventive triconjugate:nucleic acid polyplexes is due to primarily the delivery of the selected nucleic acid (e.g., poly(IC)). In preferred embodiments, the cytotoxicity of the inventive polyplexes can be increased by adding a targeting fragment to the inventive triconjugates.

As described in the Examples, the polyplexes comprising LPEI-*l*-PEG:poly(IC) in accordance with the present invention are not only at least as potency and exhibit at least a similar cytotoxic activity against cells that have high surface expression of EGFR compared to the prior art random, branched polyplexes comprising LPEI, PEG, targeting fragment and poly(IC), but the inventive polyplexes show even an increase in their biological activity such as potency and selectivity resulting from the targeted nucleic acid delivery. Moreover, the results in FIGs 6A-10B demonstrate that LPEI-*l*-PEG_n-hEGF:poly(IC) induces potent and selective decrease in cell survival in EGFR overexpressing cells. Little to no significant cell death was observed in A431 cells when poly(IC) was replaced by poly(Glu) or when non-targeted polyplex were used.

Example 24 demonstrates that selective delivery of LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(pIC) decreases the survival of PSMA overexpressing cells. Cancer cell lines with differential expression of PSMA (PC-3: low PSMA expression; and LNCaP: high PSMA expression) were treated with LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(Glu) polyplexes for 72 h. Thus, FIG 11A 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 for either polyplex 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 11B 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(IC) exhibited unspecific cytotoxic activity at high concentrations. LPEI-*l*-[N_3:DBCO]-PEG_{24}-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*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) inhibited PC-3 cell survival with an IC_{50} value of

0.24 μg/mL.

FIGs 11A and 11B show that the inventive LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) polyplex treatment selectively induces cancer cell death in PSMA-overexpressing cells with high efficacy and selectivity as compared to control polyanion, poly(Glu) treatment.

FIGs 6A-11B demonstrate that the inventive polyplexes disclosed herein can be selective for treating diseases such as cancers that overexpress a specific cell surface receptor or receptors. For example, as shown in FIGs 6A-10B, polyplexes containing a hEGF targeting fragment selectively target cells that overexpress EGFR. Similarly, as shown in FIGs 11A-11B, polyplexes containing a DUPA targeting fragment selectively target cells that overexpress PSMA. One of skill in the art will understand that the polyplexes disclosed herein can be modified to contain any suitable targeting fragments, including but not limited to those described herein, to selectively target cell types that overexpress other cell surface receptors and/or antigens.

Immunostimulatory Activity of the Polyplexes

As shown below in Example 11, the immunostimulatory activity of LPEI-*l*-PEG_{24}-hEGF:poly(IC) was measured using an IP-10 ELISA assay in cell lines with high expression of EGFR (A431) and low expression of EGFR (MCF7). As seen in FIG. 17, IP-10 secretion strongly and selectively increased in a dose dependent manner in A431 cells. Only a very slight increase was observed in MCF7 cells at the highest concentrations. These results demonstrate that the polyplexes described herein can be used to induce an immune response (e.g., a poly(IC)-induced cytokine secretion) selectively in cell types that overexpress a particular cell surface receptor (e.g., EGFR).

Target Engagement of Targeted Polyplexes

FIG. 18 is a Western Blot image showing EGFR target engagement of LPEI-*l*-PEG_{24}-EGF:poly(IC) polyplexes.

Treatment of NIH3T3 cells with both carrier LPEI-*l*-PEG_{24}-EGF (0.04 μg/ml) and polyplex LPEI-*l*-PEG_{24}-EGF:poly(IC), (0.0615 μg/ml poly(IC) in polyplexes), induced EGFR protein phosphorylation (P-EGFR) after 30 minutes as a result of EGF ligand binding to EGFR. Protein levels are shown using Western Blot imaging with serum starved condition as negative control and hEGF treatment as positive control. Tubulin demonstrates equal loading of total protein. Without wishing to be bound by theory, FIG. 18 demonstrates that both the triconjugates and the polyplexes described herein can effectively bind to and target specific cell

surface receptors such as EGFR.

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. 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. In another aspect, the present invention provides a method of treating a disease or disorder such as a 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 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 and/or antigen may be, but is not limited to, EGFR; HER2; an integrin (e.g., an RGD integrin); a sigma-2 receptor; Trop-2; folate receptor; prostate-specific membrane antigen (PSMA); p32 protein; a somatostatin receptor such as somatostatin receptor 2 (SSTR2); an insulin-like growth factor 1 receptor (IGF1R); a vascular endothelial growth factor receptor (VEGFR); a platelet-derived growth factor receptor (PDGFR); and/or a fibroblast growth factor receptor (FGFR).

In some embodiments, the cancer can be characterized by cells that have increased expression (e.g., overexpression) of EGFR. In some preferred embodiments, cancers characterized by cells that have increased expression of EGFR can be treated with polyplexes comprising an EGFR-targeting fragment such as hEGF. In certain embodiments, the cancer characterized by EGFR-overexpressing cells is an adenocarcinoma, squamous cell carcinoma, lung cancer (e.g., non-small-cell-lung-carcinoma), breast cancer, glioblastoma, head and neck

cancer (e.g., head and neck squamous cell carcinoma), renal cancer, colorectal cancer, ovarian cancer, cervical cancer, bladder cancer or prostate cancer, and/or metastases thereof.

In certain embodiments, the cancer can be characterized by cells that have increased expression (e.g., overexpression) of HER2. In some preferred embodiments, cancers characterized by cells that have increased expression of HER2 can be treated with polyplexes comprising a HER2-targeting fragment such as anti-HER2 peptide (e.g., an anti-HER2 antibody or affibody). In some embodiments, the cancer characterized by HER2-overexpressing cells is breast cancer, ovarian cancer, stomach (gastric) cancer, and/or uterine cancer (e.g., aggressive forms of uterine cancer, such as uterine serous endometrial carcinoma) and/or metastases thereof. In certain embodiments, the HER2 overexpressing cells are treatment-resistant cells (e.g., Herceptin/trastusumab resistant cells). Thus, the polyplex of the present invention may be for use in the treatment of Herceptin/trastusumab resistant cancer, i.e. cancer comprising cells that do not respond, or respond to a lesser extent to exposure to Herceptin/trastusumab.

In some embodiments, the cancer can be characterized by cells that have increased expression (e.g., overexpression) 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) 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.

In some embodiments, the cancer can be characterized by cells that have increased expression (e.g., overexpression) of folate receptor. In some preferred embodiments, cancers characterized by cells that have increased expression of folate receptor can be treated with polyplexes comprising folate and/or folic acid as a targeting fragment. In certain embodiments, the cancer characterized by folate receptor-overexpressing cells is gynecological, breast, cervical, uterine, colorectal, renal, nasopharyngeal, ovarian, endometrial cancers and/or

metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression (e.g., overexpression) of somatostatin receptors such as somatostatin receptor 2 (SSTR2). In some embodiments, cancers characterized by increased expression of SSTR2 can be treated with polyplexes comprising a somatostatin receptor-targeting fragment such as somatostatin and/or octreotide. In certain embodiments, cancers characterized by increased expression of somatostatin receptors (e.g., SSTR2) include colorectal cancer and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression of integrins (e.g., RGD integrins such as $\alpha_v\beta_6$ integrin or $\alpha_v\beta_8$ integrin). In some embodiments, cancers characterized by increased expression of integrins such as RGD integrins can be treated with polyplexes comprising an integrin-targeting fragment such as arginine-glycine-aspartic acid (RGD)-containing ligands (e.g., cyclic RGD ligands). In some preferred embodiments, the integrin-targeting fragment can be a peptide such as SFITGv6, SFFN1, SFTNC, SFVTN, SFLAP1, SFLAP3, A20FMDV2 (see, e.g., Roesch et al., *J. Nucl. Med.* **2018**, *59* (11) 1679-1685). In some embodiments, the integrin-targeting fragment can be an anti-integrin antibodies such as anti $\alpha_v\beta_6$ integrin antibodies, anti-integrin diabodies, or knottins. In some embodiments, the integrin-targeting fragment can be latent transforming growth factor-ß (TGFß). In some embodiments, cancer cells characterized by increased expression of integrins such as RGD integrins can include solid tumor, breast cancer, ovarian cancer, cervical cancer, pancreatic cancer, non-small cell lung cancer (NSCLC), colon cancer, oral squamous cell cancer, astrocytoma, head and neck squamous cell carcinoma and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that exist in a low pH microenvironment. In some embodiments, cancers characterized by a low pH microenvironment can be treated with polyplexes comprising low pH insertion peptides (pHLIPs) as a targeting fragment. In some preferred embodiments, cancers characterized by cells exist in a low pH microenvironment include breast cancer and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression of asialoglycoprotein receptors. In some embodiments, cancers characterized by increased expression of asialoglycoprotein receptors can be treated with polyplexes comprising an asialoglycoprotein receptor-targeting fragment such as asialoorosomucoid. In certain embodiments, the cancer characterized by increased expression of asialoglycoprotein receptors is liver cancer, gallbladder cancer, stomach cancer and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression of insulin receptors. In some embodiments, cancers characterized by increased expression of insulin receptors can be treated with polyplexes comprising an insulin-receptor targeting fragment such as insulin. In certain embodiments, the cancer characterized by insulin-receptor overexpressing cells is breast cancer, prostate cancer, endometrial cancer, ovarian cancer, liver cancer, bladder cancer, lung cancer, colon cancer, thyroid cancer and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression of mannose-6-phosphate receptors (e.g., monocytes). In some embodiments, cancers characterized by increased expression of mannose-6-phosphate receptors can be treated with polyplexes comprising a mannose-6-phosphate receptor targeting fragment such as mannose-6-phosphate. In some embodiments, the cancer characterized by overexpression of mannose-6-phosphate receptor is leukemia.

In some embodiments, the cancer can be characterized by cells that have increased expression of mannose receptors. In some embodiments, cancers characterized by increased expression of mannose receptors can be treated with polyplexes comprising a mannose-receptor targeting fragment such as mannose. In some embodiments, cancers characterized by increased expression of mannose receptors include gastric cancer and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression of glycosides such as Sialyl Lewis^x antigens. In some embodiments, cancers characterized by increased expression of Sialyl Lewis^x antigens can be treated with polyplexes comprising Sialyl Lewis^x antigen targeting fragments such as E-selectin.

In some embodiments, the cancer can be characterized by cells that have increased expression of N-acetyllactosamine. In some embodiments, cancers characterized by increased expression of N-acetyllactosamine can be treated with polyplexes comprising an N-acetyllactosamine targeting fragment.

In some embodiments, the cancer can be characterized by cells that have increased expression of galactose. In some embodiments, cancers characterized by increased expression of galactose can be treated with polyplexes comprising a galactose targeting fragment. In some embodiments, cancers characterized by increased expression of galactose include colon carcinoma and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression of sigma-2 receptors. In some embodiments, cancers characterized by increased

expression of sigma-2 receptors can be treated with polyplexes comprising sigma-2 receptor agonists, such as N,N-dimethyltryptamine (DMT), sphingolipid-derived amines, and/or steroids (e.g., progesterone). In some embodiments, cancers characterized by increased expression of sigma-2 receptors include pancreatic cancer, lung cancer, breast cancer, melanoma, prostate cancer, ovarian cancer and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression of the mitochondrial protein p32. In some embodiments, cancers characterized by increased expression of p32 can be treated with polyplexes comprising p32-targeting ligands such as anti-p32 antibody or p32-binding LyP-1 tumor-homing peptide. In some embodiments, cancers characterized by increased expression of p32 include glioma, breast cancer, melanoma, endometrioid carcinoma, adenocarcinoma, colon cancer and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression of Trop-2. In some embodiments, cancers characterized by increased expression of Trop-2 can be treated with polyplexes comprising a Trop-2 targeting fragment such as an anti-Trop-2 antibody and/or antibody fragment. In some embodiments, cancers characterized by increased expression of Trop-2 include breast cancer, squamous cell carcinoma, esophageal squamous cell carcinoma (SCC), pancreatic cancer, hilar cholangiocarcinoma, colorectal cancer, bladder cancer, cervical cancer, ovarian cancer, thyroid cancer, non-small-cell lung cancer (NSCLC), hepatocellular cancer, small cell lung cancer, prostate cancer, head and neck cancer, renal cell cancer, endometrial cancer, glioblastoma, gastric cancer and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression (e.g., overexpression) of insulin-like growth factor 1 receptor. In some preferred embodiments, cancers characterized by cells that have increased expression of insulin-like growth factor 1 receptor can be treated with polyplexes comprising an insulin-like growth factor 1 receptor-targeting fragment, such as insulin-like growth factor 1. In some embodiments, the cancer characterized by insulin-like growth factor 1 receptor overexpressing cells is breast cancer, prostate cancer, lung cancer and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression (e.g., overexpression) of VEGF receptor. In some preferred embodiments, cancers characterized by cells that have increased expression of VEGF receptor can be treated with polyplexes comprising a VEGF receptor-targeting fragment such as VEGF.

In some embodiments, the cancer can be characterized by cells that have increased

expression (e.g., overexpression) of platelet-derived growth factor receptor. In some preferred embodiments, cancers characterized by cells that have increased expression of platelet-derived growth factor receptor can be treated with polyplexes comprising an platelet-derived growth factor receptor-targeting fragment such as platelet-derived growth factor. In some preferred embodiments, cancers characterized by cells that have increased expression of platelet-derived growth factor receptor include breast cancer and/or metastases thereof.

In some embodiments, the cancer can be characterized by cells that have increased expression (e.g., overexpression) of fibroblast growth factor receptor. In some preferred embodiments, cancers characterized by cells that have increased expression of fibroblast growth factor receptor can be treated with polyplexes comprising a fibroblast growth factor receptor-targeting fragment such as fibroblast growth factor.

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
ASGPr	Asialoglycoprotein Receptor
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
LPEI-*l*-PEG-hEGF	Linear polyethyleneimine-PEG-human epidermal growth factor conjugate
MADOPA	N^{10}-Methyl-4-amino-4-deoxypteroic acid
MAL	Maleimide
MCC	4-(N-maleimidomethyl)-cyclohexane-1-carboxy linker
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)
SSTR2	Somatostatin receptor 2
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., hEGF) 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_{24}-hEGF" is an unbranched (i.e., linear) conjugate comprising LPEI connected to a 24-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 hEGF.

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) was 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_4-TFP (Product No. PEG6740, $C_{37}H_{38}F_4N_2O_8$; Mw 714.7), DBCO-PEG_{12}-TFP (Product No. JSI-A1201-068, $C_{53}H_{70}F_4N_2O_8$; Mw 1067.12),

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), 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, hEGF peptides, and MCC-hEGF ($C_{282}H_{409}N_{79}O_{86}S_7$; Mw 6435) were synthesized by CBL Patras S.A. (Greece). Cys-GE-11 peptide (sequence: Cys-Tyr-His-Trp-Tyr-Gly-Tyr-Thr-Pro-Gln-Asn-Val-Ile; CYHWYGYTPQNVI, SEQ ID NO: 6) was custom synthesized by GenScript Biotech(Netherlands)B.V. HER2 affibody was purchased from Abcam (Anti-ErbB2 / HER2 Affibody® Molecule, Product No. ab31889). 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. Tris-$GalNAc_3$-Ala-PEG_3-NH_2 ($C_{73}H_{32}N_{12}O_{32}$; Mw 1689.9) was purchased from Sussex Research Laboratories Inc. (Canada) (Product No. MV100017) 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.

UV spectrophotometry of samples comprising hEGF. Measurements of hEGF content in reagent solutions and in conjugated samples were performed on a microplate reader (Spectramax Paradigm, Molecular Devices) using Brand® pureGrade UV-transparent microplates at 280 nm. UV absorption of a 100 mL solution of sample in its buffer was measured and the absorbance of the sample was corrected by subtracting the absorbance of buffer solution alone (blank). ε (280 nm) of hEGF was calculated with the following formula:

$\varepsilon_{(280\ nm)}$ = (#Trp)*(5500) + (#Tyr)*(1490) + (#cystine)*(125) = 2*(5500) + 5*(1490) + 2*(125) = 18'700 $cm^{-1}\cdot M^{-1}$.

The concentration of total hEGF was calculated using the formula:

c(hEGF) [mol/L] = A_{280} [AU]/ (ε_{280} [L*mol^{-1}*cm^{-1}]*0.28 cm).

UV spectrophotometry of samples comprising HER2. For measurements of HER2 (e.g., DBCO-PEG_{24}-HER2 or LPEI-PEG_{24}-HER2 content in samples), UV spectrophotometry was

performed on a Thermofischer Nanodrop One C device at 280 nm. 2 mL of the sample were analysed and the absorbance of the sample was corrected for by subtracting the absorbance of 2 mL of the appropriate buffer solution alone (blank). ε (280 nm) of HER2 was 16600 $cm^{-1} \cdot M^{-1}$. The concentration of total HER2 was calculated using this formula:

c(HER2) [mol/L] = A_{280} [AU]/ (ε_{280} [L*mol^{-1}*cm^{-1}]*1 cm).

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(DBCO) [mol/L] = A_{309} [AU]/ (ε_{309} [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-*l*-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(LPEI total) [mg/L] = ($A_{corr,\ average}$ [AU] / k [L*mg^{-1}]) * (200/8) * 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 37) • 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 37) 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 31a and 31b) • 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 31a and 31b) • 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_{24}-hEGF) were complexed with nucleic acids (e.g., poly(IC)) to form polyplexes (e.g., LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC)). The polyplexes were characterized by measuring the molar ratio of nitrogen in LPEI to phosphorous in poly(IC) (referred to herein as the N/P ratio). Polyplex size 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 were transferred into a quartz cuvette using particle RI of 1.59 and absorption of 0.01 in HBG at 25° C with viscosity of (0.98 mPa) and RI of 1.330. Measurements were made using a 173° Backscatter angle of detection previously equilibrated to 25° C for 60 seconds in triplicate, each with 5 runs and automatic run duration, without delay between measurements. Each measurement was performed seeking optimum position with an automatic attenuation selection. Data was

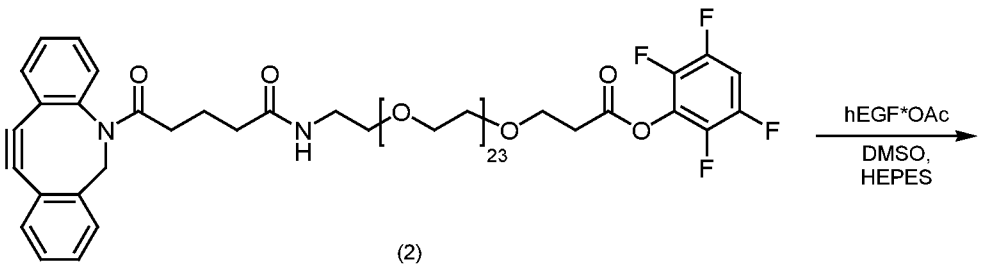
analyzed using a General-Purpose model with normal resolution. The calculations for particle size and PDI are determined according to the ISO standard document ISO 22412:2017. The ζ-potential of polyplexes was measured by phase-analysis light scattering (PALS) (for example in HBG buffer at 25 °C), and/or electrophoretic light scattering (ELS) as described by instrument supplier (https://www.malvernpanalytical.com/en/products/technology/light-scattering/electrophoretic-light-scattering).

EXAMPLE 1

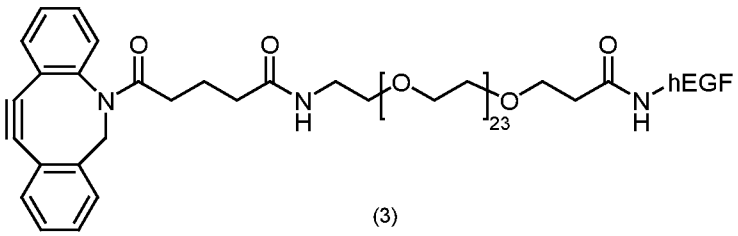
SYNTHESIS OF LPEI-*L*-[N_3:DBCO]-PEG_{24}-hEGF (COMPOUNDS 1a AND 1b)

LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF was synthesized as a mixture of regioisomers 1a and 1b in two steps according to the schemes below. In the first step, human epidermal growth factor (hEGF) was coupled to dibenzoazacyclooctyne-24(ethylene glycol)-propionyl 2,3,5,6-tetrafluorophenol ester (DBCO-PEG_{24}-TFP; Compound 2) in 20 mM HEPES buffer to produce DBCO-PEG_{24}-hEGF (Compound 3). In the second step, DBCO-PEG_{24}-hEGF was conjugated to LPEI-N_3 to produce LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF (Compounds (1a and 1b).

Step 1: Synthesis of DBCO-PEG_{24}-hEGF (Compound 3)



(2)



(3)

Human epidermal growth factor (hEGF acetate salt, 152.6 mg, 24.5 mmol; MW=6216.01g/mol; CBL Patras, Greece) was weighed in a 250 mL round-bottom flask. 75 mL of 20 mM HEPES (pH 7.4) were added to the hEGF powder to obtain a 2 mg/mL solution of hEGF protein. The solution was agitated by magnetic stirring for 10 minutes until complete dissolution of the protein. The pH was adjusted to pH 7.5 with 150 mL of 1M NaOH and 60 mL of 5M NaOH. The purity of the solution was determined by UV spectrophotometry at 280

nm and the effective concentration of protein was found to be 0.23 mM (17.2 mmol).

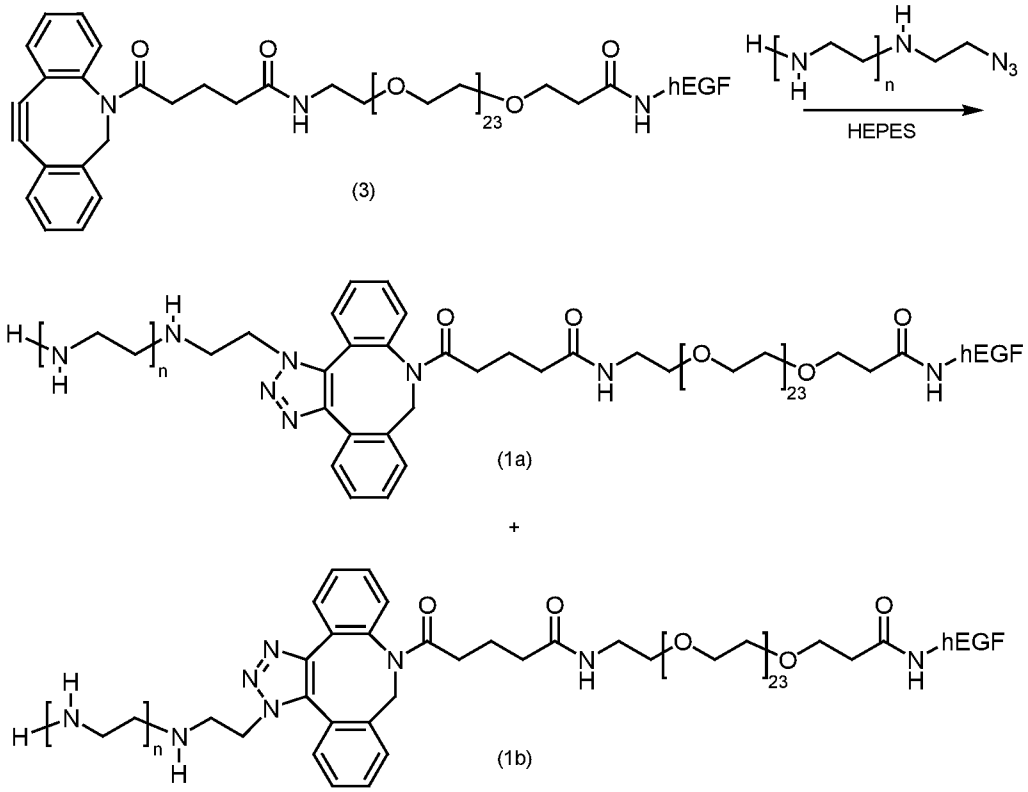
Dibenzoazacyclooctyne-24(ethylene glycol)-propionyl 2,3,5,6-tetrafluorophenol ester (DBCO-PEG_{24}-TFP; Compound 2; 100.2 mg, 62.8 mmol, MW=1,595.75 g/mol; Iris Biotech, Germany) was weighed in a 15 mL Falcon tube and dissolved in 6.0 mL DMSO to form a 10 mM stock solution. The purity of the DBCO-PEG_{24}-TFP solution was measured by UV spectrophotometry at 309 nm after a 40-fold dilution with DMSO. The effective concentration of DBCO-PEG_{24}-TFP was found to be 9.32 mM (89%, 55.9 mmol).

DBCO-PEG_{24}-TFP (Compound 2; 3.68 mL, 34.3 mmol, 2.0 eq of the stock solution) was slowly added to the hEGF solution under magnetic stirring at room temperature. After 2.5 hours an additional 0.92 mL of the DBCO-PEG-TFP (Compound 2) stock solution (8.6 mmol, 0.5 eq) were added to the reaction mixture. The solution was left to react for a further 30 minutes. The reaction mixture was transferred into two 50 mL Falcon tubes and kept at 4 °C for 2 hours prior to purification.

The reaction mixture (79 mL) was purified in 4 runs using the Waters preparative chromatography system. Before each run, the solutions were supplemented with acetonitrile to reach 10% ACN in order to have the same composition as the eluant at the start of the preparative chromatography. Pooled fractions were collected for lyophilization. A total of about 273 mL of isolated DBCO-PEG_{24}-hEGF (Compound 3) were recovered in 50 mL Falcon tubes (3.4-fold dilution). The four pools were mixed and the combined samples were analyzed by C8-RP-HPLC and stored under argon at -80 °C prior to lyophilization.

The isolated DBCO-PEG_{24}-hEGF (Compound 3) was cooled in liquid nitrogen for about 3 min before lyophilization. A fluffy lyophilizate (70 mg, 46% yield in hEGF, 89% yield in DBCO, $[(M+6H)^{6+}]/6$=1274.42, monoisotopic mass [Da] measured 7640.47, monoisotopic mass [Da] calculated 7640.47) was recovered and stored under argon at -80 °C.

Step 2: Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF (Compounds 1a and 1b)



DBCO-PEG$_{24}$-hEGF lyophilisate (Compound 3; ~43 mg) was weighed into a 15 mL Falcon tube and dissolved in 5.4 mL of 20 mM HEPES (pH 6.5; 8 mg/mL solution). The pH after dissolution was 3.9 and was adjusted to pH 4.5 with 3 µL of 5M NaOH. As the solution became cloudy, 15 µL of HCl 1M were used to re-dissolve the precipitate and the solution became clear again. The final pH of the solution was 3.7. The solution was filtered using 0.45 µm nylon filters (13 mm nylon membrane from Exapure, Germany) to give ~4.7 mL of DBCO-PEG$_{24}$-hEGF (Compound 3) solution. The effective concentration of DBCO-PEG$_{24}$-hEGF (Compound 3) was measured by UV spectrophotometry at 309 nm after a 20-fold dilution with H_2O. The assay gave a compound content of ~86% with a concentration of 0.89 mM (4.2 µmol).

LPEI-N_3 (199.5 mg) was weighed in a 50 mL Falcon tube and dissolved in 10 mL MilliQ water pH 2.2 (20 mg/mL solution). 350 µL of 1M HCl were added to help solubilize the LPEI-N_3. The solution was sonicated for about three minutes and heated to 70 °C until the LPEI-N_3 was completely dissolved. The measured pH was 7.8 and 800 µL of 1M HCl + 300 µL of 1M NaOH were used to adjust the pH to 4.6. The concentration of LPEI-N_3 was measured by copper assay and a purity of ~69% was found. The effective concentration of the solution was 0.55 mM.

In a 50 mL Falcon tube, DBCO-PEG$_{24}$-hEGF (Compound 3) solution (4.7 mL, 4.2 µmol), LPEI-N_3 solution (7.6 mL,4.2 µmol) and a NaCl solution (400 µL, 4.8 M) were mixed and left to react on a Stuart rotator at 20 rpm at room temperature. Samples were regularly taken

for analytical HPLC monitoring of the reaction at 240 nm and 309 nm. After 95 hours no significant further conversion was evident and the reaction was stopped. Based on the decrease of the peak area, 55-60% of DBCO-PEG_{24}-hEGF (Compound 3) was consumed. About 12.5 mL of solution were recovered and the pH was measured to be 4.9. The solution was stored at -80 °C under argon prior to purification.

The reaction mixture (about 12.5 mL) was brought to room temperature and treated with 1.4 mL of acetonitrile and 15 µL TFA. The solution was filtered with 0.45 µM filters before purification using PuriFlash RP preparative chromatography. The fractions containing pure products were lyophilized, weighed, and analyzed by RP-HPLC, copper assay, and UV spectrophotometry at 280 nm. The retention time of the LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF (Compounds 1a and 1b) in the analytical RP-HPLC analysis was 5.6-5.8 min. 29 mg of a mixture of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF (Compounds 1a and 1b) trifluoroacetate, each with a LPEI:hEGF ratio of 1:1 and no further impurities was isolated (12% overall yield in LPEI).

Step 3: Exchanging TFA salt for HEPES Buffer

To exchange TFA with HEPES, 11.5 mg of lyophilized LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF (Compounds 1a and 1b) trifluoroacetate (w_{LPEI} = 26%, ~3 mg in total LPEI) were dissolved in 1.0 mL, 20 mM HEPES (pH 7.2) in a 2 mL Eppendorf tube. The initial pH was 3.5 and was adjusted to pH 7.2 with 8 µL of 5 M NaOH and 9 µL of 1 M HCl. An additional 483 µL of 20 mM HEPES (pH 7.2) was added to give a final volume of about 1.5 mL. The total concentration of LPEI was about 2 mg/mL. Three centrifugal filters were filled with 450 µL (1350 µL in total) of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF trifluoroacetate. The tubes were each centrifuged once at 14,000 g for 30 minutes. The supernatant was decanted, and the pellet re-suspended in 20 mM HEPES buffer (pH 7.2) at 25 °C. The tubes were centrifuged again at 14,000 g for 30 minutes and the supernatant was decanted. The pellet was re-suspended in 20 mM HEPES buffer (pH 7.2) and re-centrifuged two additional times. About 1.3 mL of the solution of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF (Compounds 1a and 1b) as a HEPES salt were recovered at a concentration of 2.1 mg/mL of total LPEI.

Step 4: Exchanging TFA salt for Acetate Buffer

To exchange TFA with acetate, 12.5 mg of lyophilized LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF (Compounds 1a and 1b) trifluoroacetate (w_{LPEI} = 26%, ~3 mg in total LPEI) were dissolved in 1.3 mL, 50 mM acetate buffer (pH 4.5) in a 2.0 mL Eppendorf tube. The initial pH was 4.0 and was adjusted to pH 4.5 with 3.5 µL of 5 M NaOH. The total concentration of LPEI

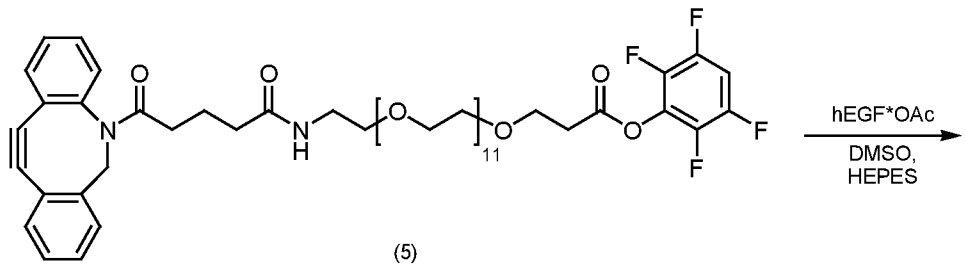
was about 2 mg/mL. Four centrifugal filters were filled with 325 µL (1300 µL in total) of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF trifluoroacetate. The tubes were each centrifuged once at 14,000 g for 30 minutes. The supernatant was decanted, and the pellet re-suspended in 50 mM acetate buffer (pH 4.5) at 4°C. The tubes were centrifuged again at 14,000 g for 30 minutes and the supernatant was decanted. The pellet was re-suspended in 50 mM Acetate buffer (pH 4.5) and re-centrifuged two additional times. About 1.4 mL of the solution of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF (Compounds 1a and 1b) as an acetate salt were recovered at a concentration of 2.3 mg/mL of total LPEI.

EXAMPLE 2

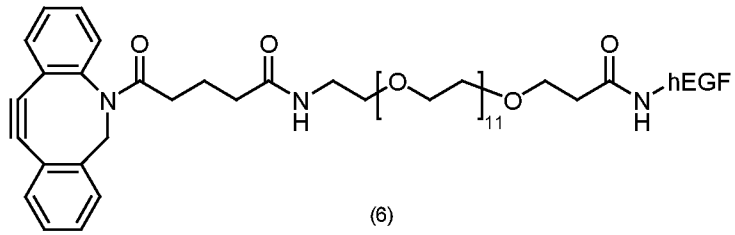
SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF (COMPOUNDS 4a AND 4b)

LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF was synthesized as a mixture of regioisomers 4a and 4b in two steps according to the schemes below. In the first step, human epidermal growth factor (hEGF) was coupled to dibenzoazacyclooctyne-12(ethylene glycol)-propionyl 2,3,5,6-tetrafluorophenol ester (DBCO-PEG_{12}-TFP; Compound 5) in 20 mM HEPES buffer to produce DBCO-PEG_{12}-hEGF (Compound 6). In the second step, DBCO-PEG_{12}-hEGF (Compound 6) was conjugated to LPEI-N_3 to produce LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF (Compounds 4a and 4b).

Step 1: Synthesis of DBCO-PEG_{12}-hEGF (Compound 6)



(5)



(6)

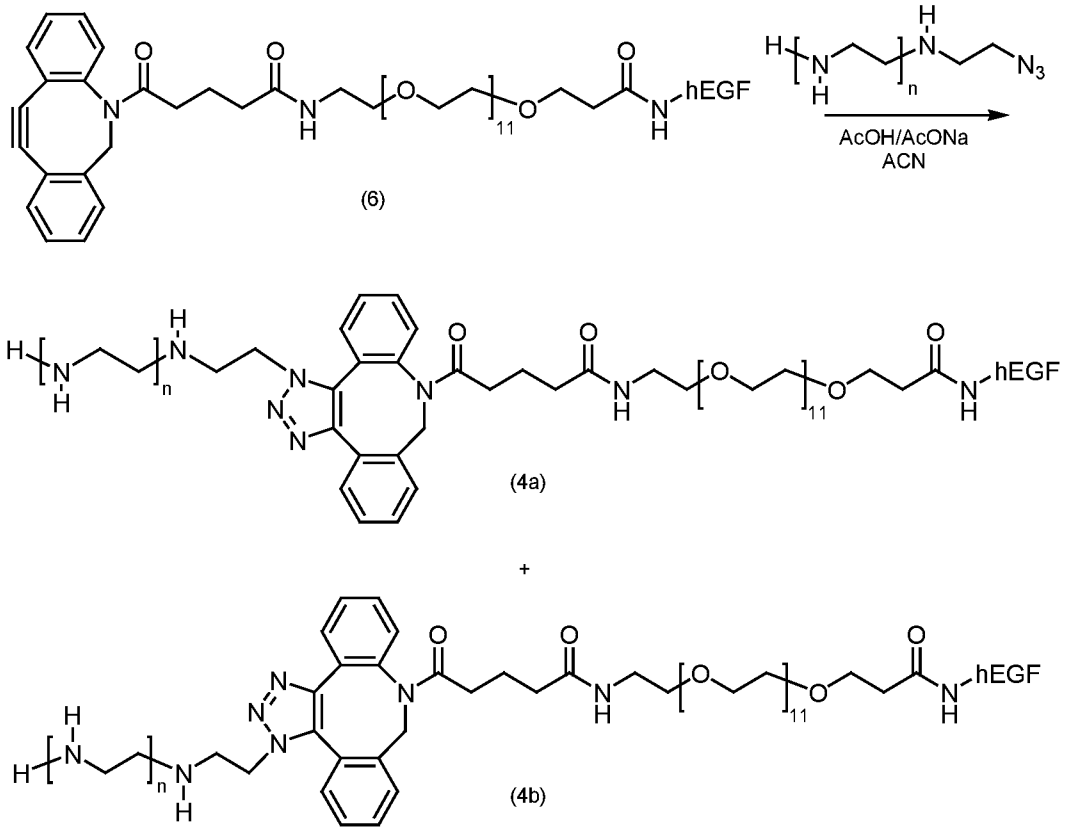
56 mg of dibenzoazacyclooctyne-12(ethylene glycol)-propionyl 2,3,5,6-tetrafluorophenol ester (DBCO-PEG_{12}-TFP; Compound 5; assay 96.3%; 51 µmol pure product) were weighed in a 5 mL Eppendorf tube and dissolved in 2.6 mL DMSO (~20 mM stock

solution, pure product). The solution was manually mixed to dissolve DBCO-PEG_{12}-TFP.

148 mg (crude mass) of hEGF (Lot 5263, 87.1% peptide content; 21 µmol) were weighed in a 100 mL round-bottom flask and dissolved in 75 mL 20 mM HEPES, pH 7.4. The solution was agitated by magnetic stirring for about 10 minutes to dissolve the protein and the pH of the solution was adjusted to 7.5 with 100 µL 5 M NaOH and 16 µL 6 M HCl.

2.08 mL of DBCO-PEG_{12}-TFP (Compound 5) stock solution (2 eq, 42 µmol) were slowly added to the hEGF solution with stirring. After about 15 minutes, 8.6 mL of ACN were added to the reaction mixture (10% of final volume). After about 50 minutes, an additional 0.52 mL of DBCO-PEG_{12}-hEGF stock solution (0.5 eq Compound 5; 10 µmol) were added to the reaction mixture and further stirred for 3 hours. The slightly cloudy reaction mixture was centrifuged at 15,000 g for five minutes prior to purification. 86 mL of reaction mixture (10% acetonitrile) were purified in one run using the PuriFlash RP-preparative Column system. Pooled fractions were collected and lyophilized (47 mg, 31% yield of Compound 6; $[(M+5H)^{5+}]/5$=1186.37, (monoisotopic mass [Da] measured 7112.16, monoisotopic mass [Da] calculated 7112.18)).

Step 2: LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF (Compounds 4a and 4b)



DBCO-PEG_{12}-hEGF lyophilisate (Compound 6; 46 mg, 6.5 µmol) was dissolved in a mixture of 20 mL of 50 mM acetate, pH 4.0, and 2.2 mL acetonitrile (10% acetonitrile final

volume). The pH of the solution was pH 4.2 and adjusted to 4.0 with 6 µL 6 M HCl. The final concentration of DBCO-PEG_{12}-hEGF (Compound 6) in solution was 2.3 mg/mL.

LPEI-N_3 (204 mg) were weighed in a 15 mL Falcon tube and dissolved in 10 mL 50 mM acetate, pH 4.0. The solution was heated to about 70 °C for about 2 minutes and 360 µL of 6 M HCl were added to help solubilize the LPEI-N_3 and to adjust the pH to 4.0 (19.7 mg/mL). The concentration of LPEI-N_3 (MW= 22 kDa) was measured by copper assay and a purity of about 85% was determined. The effective concentration of the solution was 16.8 mg/mL (7.9 µmol of LPEI-N_3 in solution).

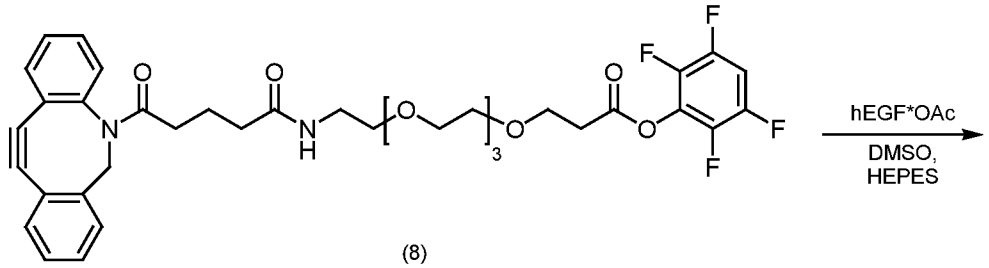
The LPEI-N_3 solution (7.9 µmol, 1.2 eq) was transferred to a 100 mL round-bottom flask equipped with a magnetic stirrer, and a DBCO-PEG_{12}-hEGF (Compound 6) solution (6.5 µmol, 1.0 eq) was added. The reaction mixture was stirred at room temperature and protected from light for about 45 hours. Samples were regularly taken for monitoring and were diluted 10-fold with acetonitrile/H_2O (1:9) before injection. The reaction mixture (about 35 mL) was adjusted to contain about 6% (vol.) acetonitrile, and purified using the PuriFlash Pump injection system coupled to a preparative HPLC column. The pooled fractions containing pure products were lyophilized, weighed, and analyzed by RP-HPLC, copper assay, and UV spectrophotometry at 280 nm. 47 mg of a mixture of LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF (Compounds 4a and 4b), each with a LPEI:hEGF ratio of 1:1 and no further impurities was isolated (7% overall yield in LPEI). Retention times of the LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF in the analytical RP-HPLC analysis was 5.5-6.2 min.

EXAMPLE 3

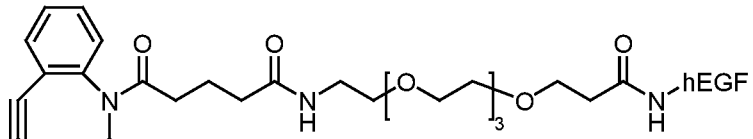
SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF (COMPOUNDS 7a AND 7b)

LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF was synthesized as a mixture of regioisomers 7a and 7b in two steps according to the schemes below. In the first step, human epidermal growth factor (hEGF) was coupled to dibenzoazacyclooctyne-4(ethylene glycol)-propionyl 2,3,5,6-tetrafluorophenol ester (DBCO-PEG_4-TFP; Compound 8) in 20 mM HEPES buffer to produce DBCO-PEG_4-hEGF (Compound 9). In the second step, DBCO-PEG_4-hEGF (Compound 9) was conjugated to LPEI-N_3 to produce LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF (Compounds 7a and 7b).

Step 1: Synthesis of DBCO-PEG_4-hEGF (Compound 9)



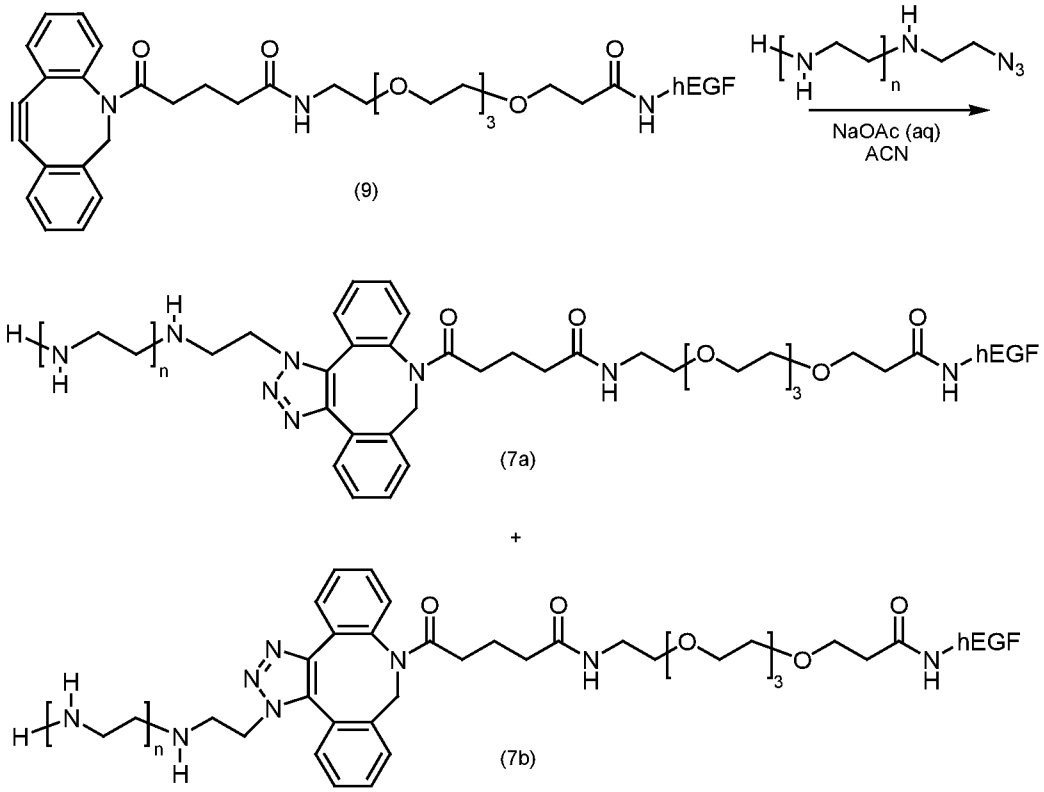
(8)



(9)

A solution of hEGF (150 mg, peptide content 87.1%, 24 µmol pure peptide, 1.0 eq, 0.29 mM) in 20 mM HEPES pH 7.5 / ACN (9:1) (83.6 mL) was mixed with a solution of DBCO-PEG_4-TFP (Compound 8; 43 µmol,1.8 eq, 20 mM) in DMSO (2.2 mL). The reaction mixture was incubated in a 100 mL round-bottom flask under magnetic stirring at room temperature and was monitored by RP-C_8-HPLC. After 25 minutes, the reaction mixture was supplemented with acetonitrile (10 mL) and after one and a half hours, the mixture was supplemented with additional DBCO-PEG_4-TFP (12 µmol, 0.5 eq, 20 mM). After a total of three hours, reaction mixture was stored at 4°C overnight. The reaction mixture was adjusted to 10% ACN and DBCO-PEG_4-hEGF was isolated following RP-C_{18} preparative HPLC and lyophilization of pooled fractions. A solid (59 mg) was recovered and analyzed by HPLC – ESI^+ qTOF mass spectrometry. The solid contained DBCO-PEG_4-hEGF (calculated monoisotopic mass: 6759.95 Da; measured: 6760.02 Da).

Step 2: Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF (Compounds 7a and 7b)



LPEI-N_3 solution (10 mL, 6.4 µmol, 1.0 eq, 0.64 mM) in 50 mM acetate buffer pH 4.0 was slowly added a solution of DBCO-PEG_4-hEGF (6.6 µmol, 1.0 eq, 0.30 mM) in 50 mM acetate pH 4.0 / ACN (9:1) (22.3 mL). The reaction mixture was incubated in a 100 mL round-bottom flask under magnetic stirring at room temperature and protected from light and was monitored by RP-C_8-HPLC. After 45 hours of reaction, LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF was isolated as a mixture of regioisomers 7a and 7b using RP-C_{18} preparative HPLC. Pooled fractions were lyophilized (47 mg, fluffy white solid) and characterized by RP-C_8-HPLC, copper assay and spectrophotometry at 280 nm for determination of the hEGF content. Lyophilisate had a weight percentage in LPEI of 26%w/w and a LPEI to hEGF ratio of 1/1.0.

Step 3: Preparation of LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF-HEPES salt

LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF (Compounds 7a and 7b) TFA salt (22.9 mg, w_{LPEI} = 26%, 6.0 mg in total LPEI) were dissolved in 2.5 mL 20 mM HEPES pH 7.2. Six centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 420 µL of LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF 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 449 µL of LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF-HEPES salt solution were recovered and supplemented with 1.2 mL 20 mM HEPES, pH 7.2. The concentration of the solution was determined by copper assay (3.6 mg/mL in total LPEI, ratio LPEI/hEGF = 1/1.0).

Step 4: Preparation of LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF-acetate salt

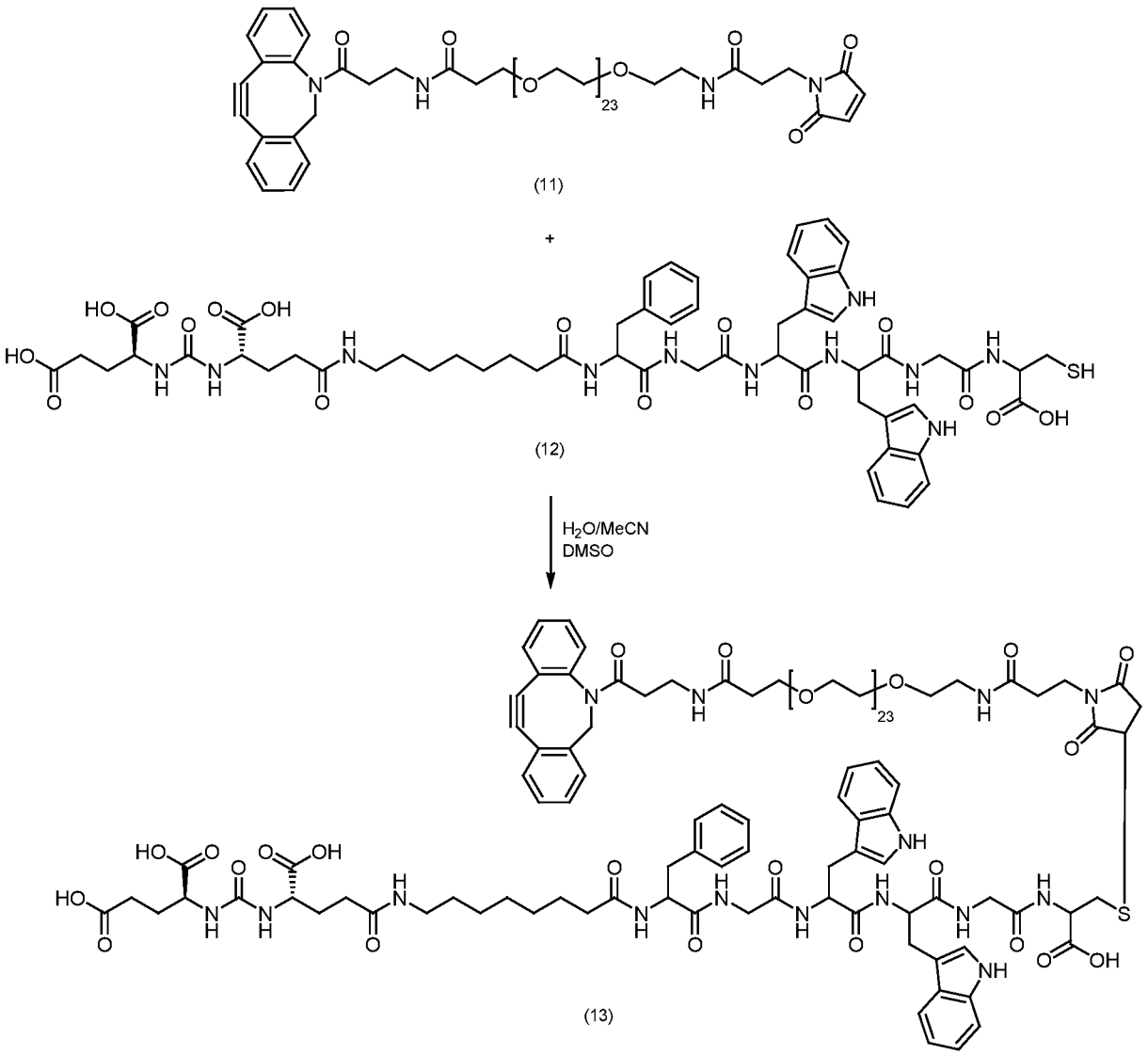
LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF (Compounds 7a and 7b) TFA salt (13.2 mg, w_{LPEI} = 26%, 3.6 mg in total LPEI) were dissolved in 1.7 mL 50 mM acetate pH 4.3. Four centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 425 µL of LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF solution each, centrifuged 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 211 µL of LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF-acetate salt solution were recovered and supplemented with 1.2 mL 50 mM acetate pH 4.3. The concentration of the solution was determined by copper assay (2.2 mg/mL in total LPEI, ratio LPEI/hEGF = 1/1.0).

EXAMPLE 4

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA (COMPOUNDS 10a AND 10b)

LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA was synthesized as a mixture of regioisomers 10a and 10b in two steps according to the schemes below. In the first step, DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12; 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 11) by Michael addition to prepare DBCO-PEG_{24}-DUPA (Compound 13). In the second step, DBCO-PEG_{24}-DUPA (Compound 13) was conjugated to LPEI-N_3 to produce LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA (Compounds 10a and 10b).

Step 1: Synthesis of DBCO-PEG$_{24}$-DUPA (Compound 13)

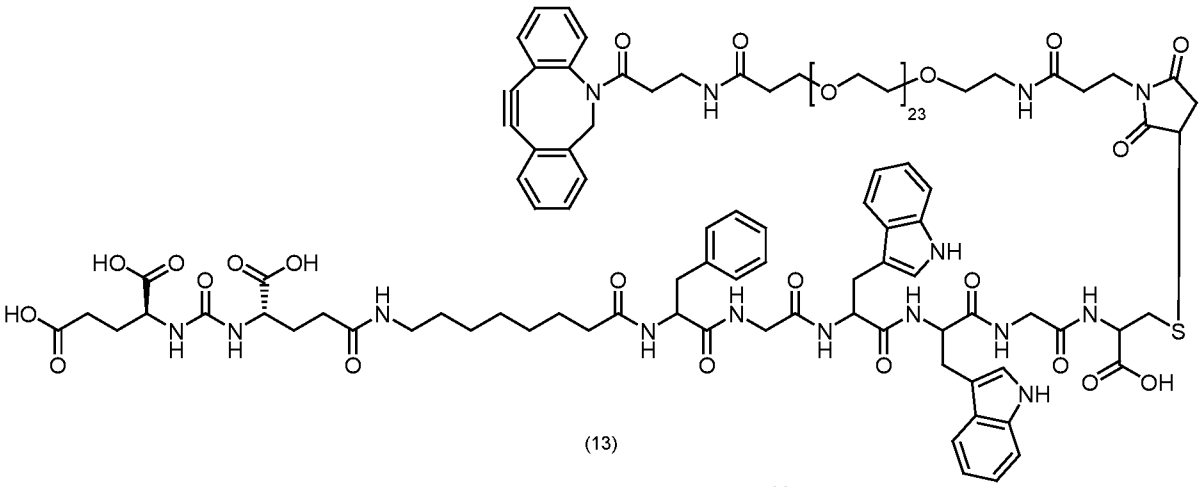


18.06 mg (crude mass) of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12; 15 µmol pure theoretical peptide content) were weighed in a 50 mL Falcon tube and dissolved in 9 mL H_2O/25% ACN (2.0 mg/mL stock solution). The solution was sonicated for about 15 seconds to help dissolve the DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12). The pH of the solution was adjusted to 3.5 with 8.5 µL 6 M HCl.

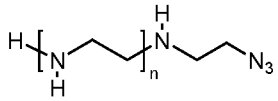
21.38 mg (crude mass) of DBCO-PEG$_{24}$-MAL (Compound 11; 13 µmol pure product) were weighed in a 1.5 mL Eppendorf tube and dissolved in 650 µL DMSO (20 mM pure product). In the 50 mL Falcon tube containing the Compound 12 solution (15 µmol, 1.5 eq), 500 µL of the DBCO-PEG$_{24}$-MAL (Compound 11) stock 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 11). The identity of the DBCO-PEG_{24}-DUPA (Compound 13) 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.

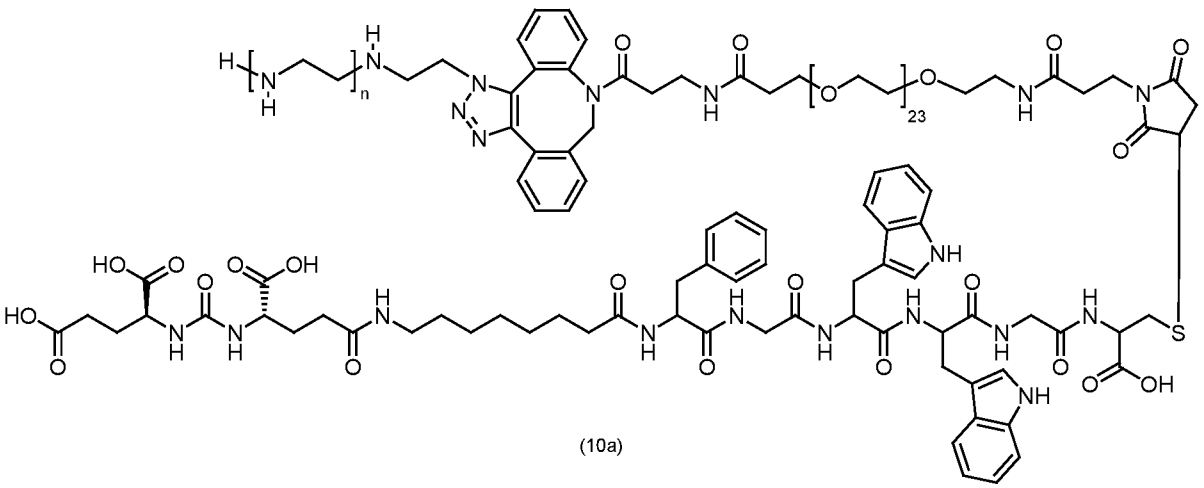
Step 2: Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA (Compounds 10a and 10b)



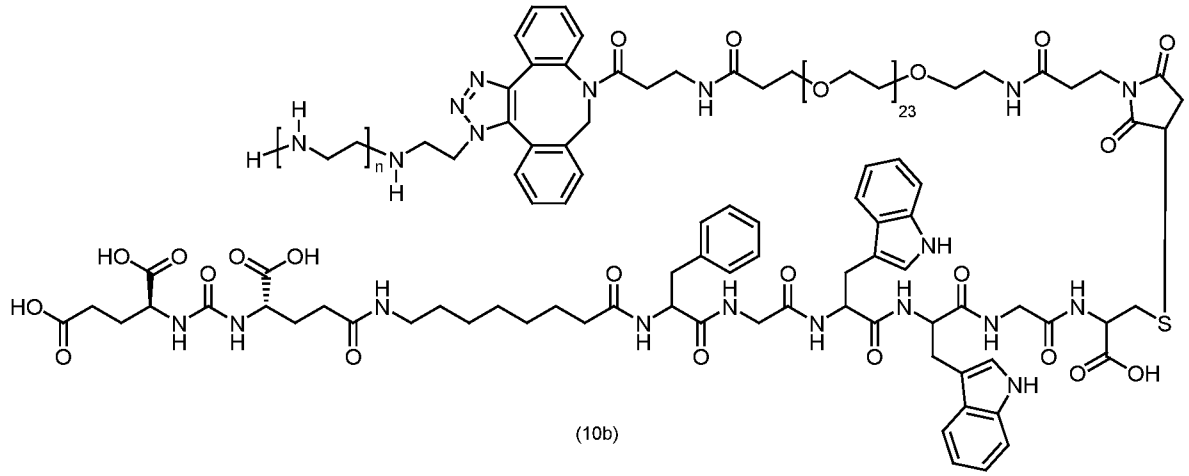
(13)



NaOAc (aq), ACN



(10a)



(10b)

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 13) 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.

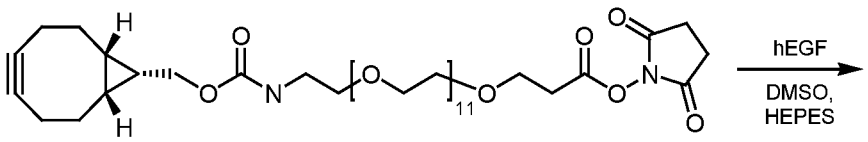
The pooled fractions containing pure Compounds 10a and 10b were lyophilized, weighed, and analyzed by RP-HPLC, copper assay, and UV spectrophotometry at 280 nm. 28 mg of LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA (Compounds 10a and 10b), each with a LPEI:DUPA ratio of 1:1 and no further impurities was isolated (7% overall yield in LPEI). The retention time of the LPEI-*l*-[N_3:DBCO]-PEG_{24}- DUPA (Compounds 10a and 10b) in the analytical RP-HPLC analysis was 5.4-6.4 min with a maximum at 5.5 min.

EXAMPLE 5

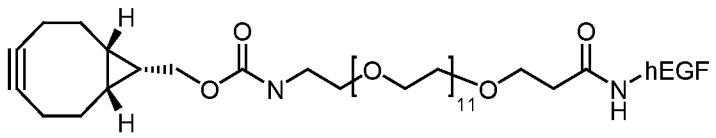
SYNTHESIS OF LPEI-*l*-[N_3:BCN]-PEG_{12}-hEGF (COMOPUND 14)

LPEI-*l*-[N_3:BCN]-PEG_{12}-hEGF (Compound 14) is synthesized in two steps according to the schemes below. In the first step, human epidermal growth factor (hEGF) is coupled to *endo*-BCN-PEG_{12}-NHS ester (Compound 15) in 20 mM HEPES buffer to produce *endo*-BCN-PEG_{12}-hEGF (Compound 16). In the second step, *endo*-BCN-PEG_{12}-hEGF (Compound 16) is conjugated to LPEI-N_3 to produce LPEI-*l*-[N_3:BCN]-PEG_{12}-hEGF (Compound 14).

Step 1: Synthesis of *endo*-BCN-PEG_{12}-hEGF



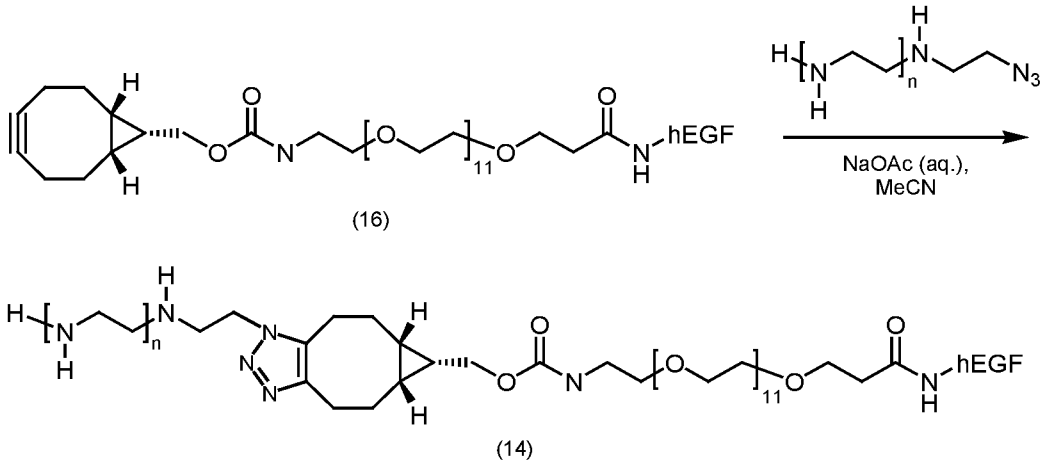
(15)



(16)

Endo-BCN-PEG_{12}-NHS (Compound 15; 21.8 mg, 23.9 µmol, assay 97.7%) were weighed in a 5 mL Eppendorf tube and dissolved in 2.4 mL DMSO (10 mM stock solution, pure product). The solution was manually agitated to aid dissolution. hEGF (157 mg, 22.0 µmol, 87.1% peptide content) was weighed in a 100 mL round-bottom flask and dissolved using 75 mL 20 mM HEPES, pH 7.4. The solution was agitated by magnetic stirring for about 10 minutes and adjusted to pH 7.4 with 60 µL 5 M NaOH. *Endo*-BCN-PEG_{12}-NHS (Compound 15) stock solution (2.2 mL, 22.0 µmol, 1.0 eq) was slowly added to the magnetically stirred hEGF solution (22.0 µmol, 1.0 eq). After ~4 hours the reaction mixture was diluted to 10% ACN prior to PuriFlash purification. Pooled fractions from the preparative chromatography were analyzed by C8-RP-HPLC and lyophilized to give 43 mg *endo*-BCN-PEG_{12}-hEGF (Compound 16). The resulting Compound 16 lyophilizate was dissolved in 5.0 mL of 85% v/v 50 mM acetate (pH 4.0) containing 15% v/v ACN and further purified using 3 NAP-25 columns to remove hydrolyzed *endo*-BCN-PEG_{12}-OH impurity (identified by RP-C8-HPLC-MS (Single quadrupole, positive ionization)).

Step 2: Synthesis of LPEI-*l*-[N_3:BCN]-PEG_{12}-hEGF



(16)

(14)

2.9 mL of LPEI-N_3 from a 0.77 mM stock solution (2.9 mL, 2.2 µmol, 1.5 eq) was slowly added to a solution of *endo*-BCN-PEG_{12}-hEGF (Compound 16; 7 mL,1.5 µmol in peptide content, 1.0 eq) previously dissolved in 85% v/v 50 mM acetate, pH 4.0, 15% v/v ACN. The mixture was shaken for a total of 95 hours (40°C) on a thermoshaker and protected from light. After ~70 hours, an additional 0.85 mL (0.65 µmol, 0.4 eq) of the LPEI-N_3 stock solution were added to the reaction mixture and the pH was adjusted to pH 4.0 using 5 M NaOH. Preparative chromatography was performed using an Agilent 1260 Infinity II preparative system to isolate the trifluoroacetate salt of Compound 14, which was subsequently lyophilized.

Step 3: Preparation of LPEI-*l*-[N_3:BCN]-PEG_{12}-hEGF (Compound 14) acetate salt:

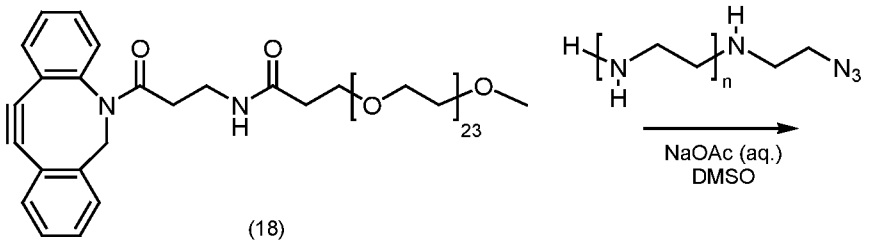
The lyophilized LPEI-*l*-[N_3:BCN]-PEG_{12}-hEGF-TFA salt produced above (~50 mg) was mixed and solubilized with 4.5 mL 50 mM acetate (pH 4.5). The pH was adjusted to pH 4.3 using 5 M NaOH. Ten centrifugal filters (Amicon Ultra – 0.5 mL, Merck Millipore Ltd.) were filled with 450 µL of LPEI-*l*-[N_3:BCN]-PEG_{12}-hEGF TFA salt solution each. They were centrifugated one time at 14'000 g for 30 minutes to remove buffer and then three times against 450 µL 50 mM acetate, pH 4.3 at 4°C. About 574 µL of the concentrated solution of LPEI-*l*-[N_3:BCN]-PEG_{12}-hEGF (Compound 14) acetate salt were recovered after buffer exchange and were supplemented with 3.0 mL 50 mM acetate, pH 4.3. A copper assay was performed on the final LPEI-*l*-[N_3:BCN]-PEG_{12}-hEGF (Compound 14) acetate salt solution (~3.5 mL) and a concentration of 2.1 mg/mL total LPEI was determined (ratio LPEI/hEGF = 1/0.9).

EXAMPLE 6

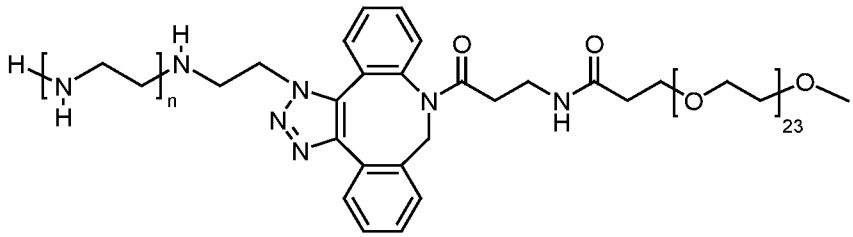
SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3 (COMPOUNDS 17a AND 17b)

LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3 was synthesized in one step as a mixture of regioisomers 17a and 17b according to the scheme below. DBCO-PEG_{23}-OCH_3 (Compound

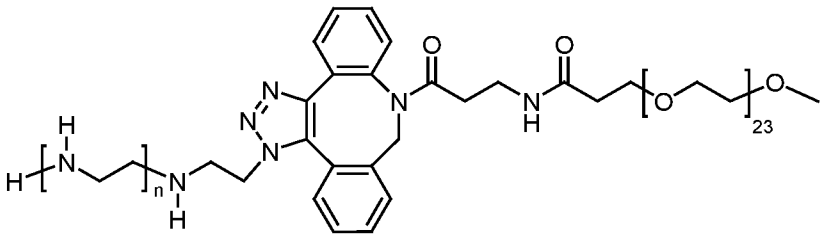
18) was coupled to LPEI-N_3 and purified over a 10 KDa filter using small scale, size exclusion centrifugation.



(18)



(17a)



(17b)

Step 1: Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3 (Compounds 17a and 17b)

DBCO-PEG_{23}-OCH_3 (Compound 18, 3.25 mg, 2.4 µmol, assay 98.9%) was weighed in a 1.5 mL Eppendorf tube and dissolved in 116 µL of DMSO (21 mM pure product). LPEI-N_3 (14.4 mg, MW = 22 kDa) was weighed in a 1.5 mL Eppendorf tube and dissolved in 400 µL of 50 mM acetate buffer (pH 4.0). 6 M HCl (19 µL) was added to aid dissolution and to adjust to pH 3.5. Total LPEI concentration was measured by copper assay (25.1 mg/mL, 1.14 mM).

The LPEI-N_3 solution (400 µL, 0.46 µmol, 1.0 eq) was transferred to a 1.5 mL Eppendorf tube and the DBCO-PEG_{23}-OCH_3 (Compound 18) solution (29 µL, 0.60 µmol, 1.3 eq) was added to the reaction mixture and the resultant solution was kept at 40°C for about 3 days.

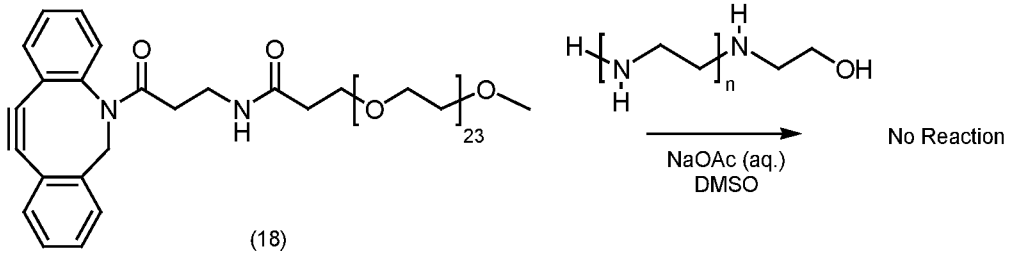
The reaction mixture was purified over an Amicon centrifugal filter (10 kDa MWCO) against 50 mM acetate buffer (pH 4.0). Purified LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3 solution was further diluted with 2.8 mL of 50 mM acetate buffer (pH 4.0). The total LPEI content of the LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3 (Compound 17a and 17b) solution (~3 mL) was measured by copper assay and found to be 1.3 mg/mL total LPEI. Based on the copper assay, the overall yield of reaction and purification was 39%.

COMPARATIVE EXAMPLE 1

NO CYCLOADDITION REACTION BETWEEN LPEI-OH AND DBCO-PEG$_{23}$-OCH$_3$ (COMOPUND 18)

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 18) at pH 4 under the conditions set forth above in Example 6.

Step 1: Treatment of DBCO-PEG$_{23}$-OCH3 with LPEI-OH



(18)

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 18) 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)).

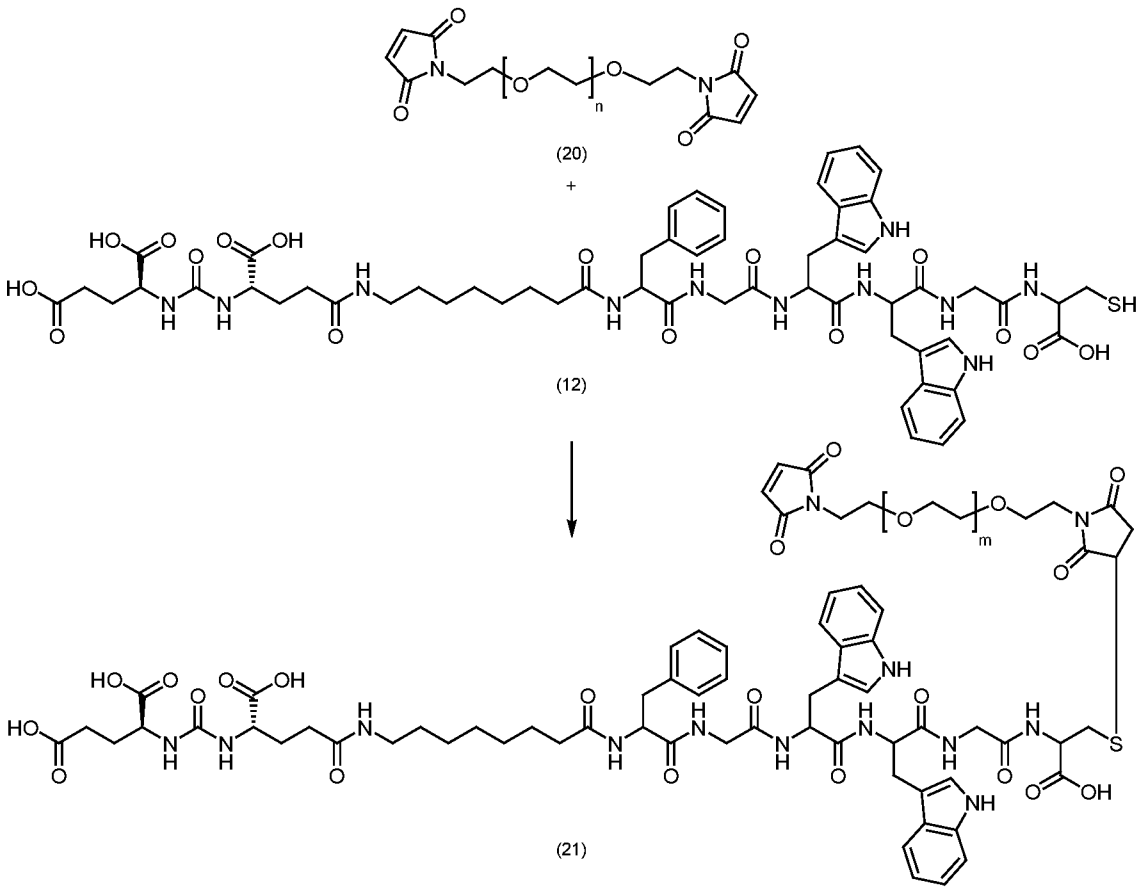
EXAMPLE 7

SYNTHESIS OF LPEI-*l*-[N$_3$:MAL]-PEG$_{2K}$-DUPA (COMPOUND 19)

LPEI-*l*-[N$_3$:MAL]-PEG$_{2K}$-DUPA (Compound 19), wherein the PEG fragment is a

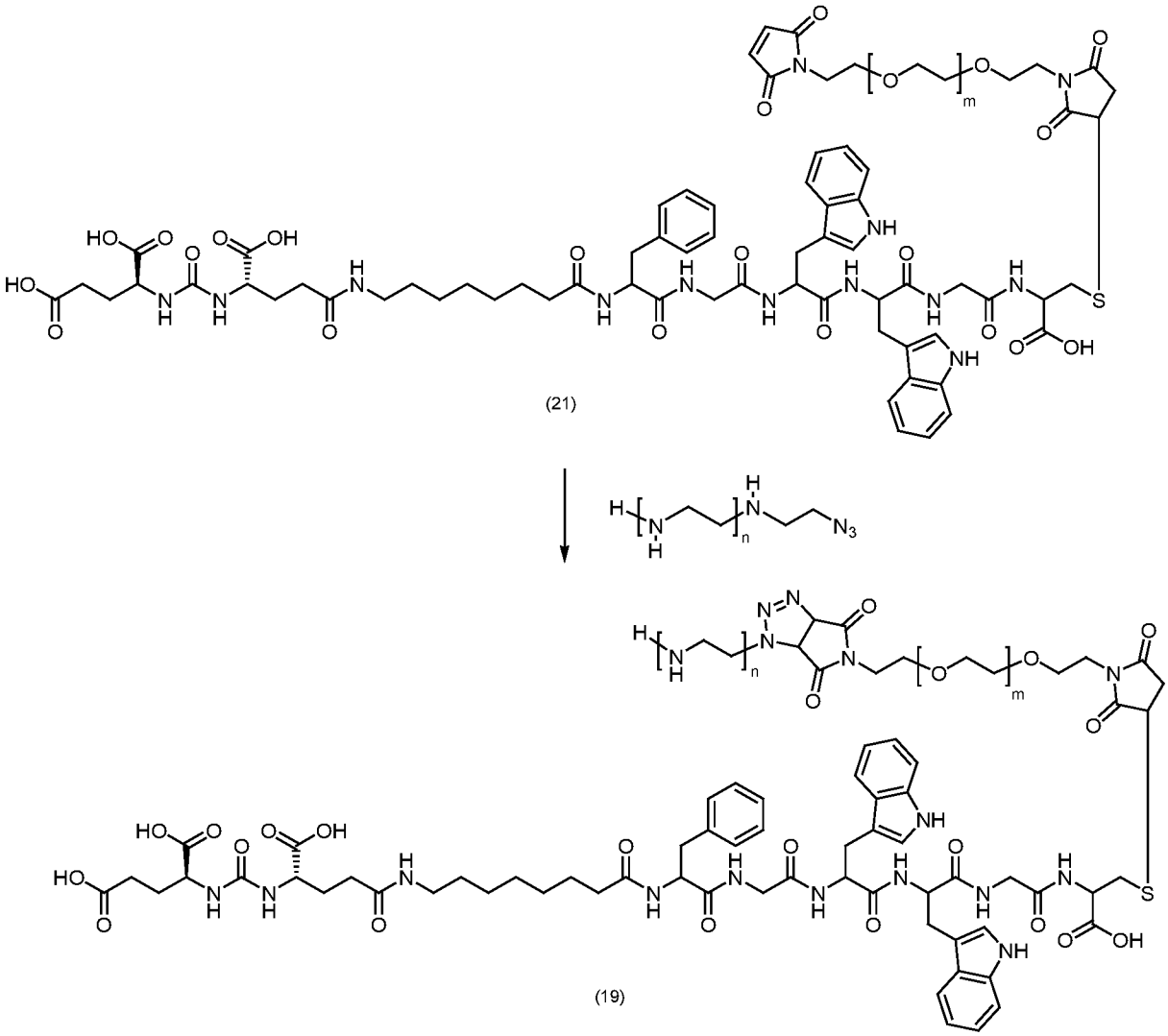
polydisperse fragment with a molecular weight of about 2,000, is synthesized in two steps according to the scheme below. In the first step, DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12; SEQ ID NO:4) (see Example 4), is coupled with half equivalent of MAL-PEG_{2K}-MAL (Compound 20) to prepare MAL-PEG_{2K}-DUPA (Compound 21). In the second step, MAL-PEG_{2K}-DUPA (Compound 21) is subjected to a 1,3-dipolar cycloaddition reaction with LPEI-N_3 according to the procedure taught by Zhu *et al.*, *Macromol. Res.* **24**, 793–799 (2016) to produce LPEI-*l*-[N_3:MAL]-PEG_{2K}-DUPA (Compound 19).

Step 1: Synthesis of MAL-PEG_{2K}-DUPA (Compound 21)



DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12; SEQ ID NO:4) is coupled with 0.5 equivalents of MAL-PEG_{2K}-MAL (Compound 20) to prepare MAL-PEG_{2K}-DUPA (Compound 21) according to the procedure of Example 4.

Step 2: Synthesis of LPEI-*l*-[N_3:MAL]-PEG_{2K}-DUPA (Compound 19)



(21)

(19)

MAL-PEG$_{2K}$-DUPA (Compound 21) is subjected to a 1,3-dipolar cycloaddition reaction with LPEI-N$_3$ according to the procedure taught by Zhu *et al.*, *Macromol. Res.* **24**, 793–799 (2016) to produce LPEI-*l*-[N$_3$:MAL]-PEG$_{2K}$-DUPA (Compound 19).

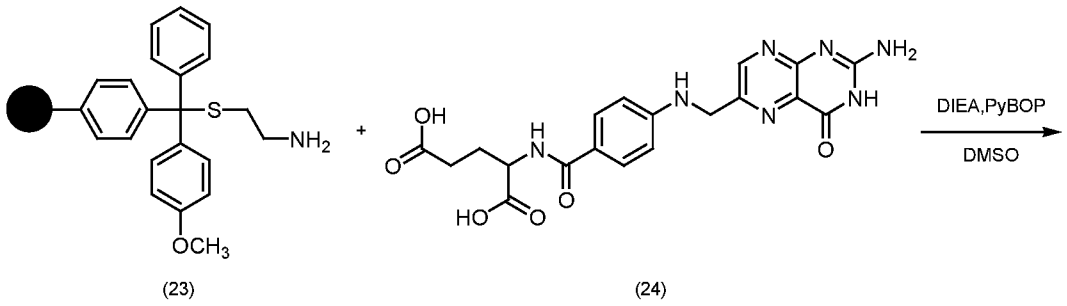
EXAMPLE 8

SYNTHESIS OF LPEI-*l*-[N$_3$:DBCO]-PEG$_{24}$-Folate (COMPOUNDS 22a AND 22b)

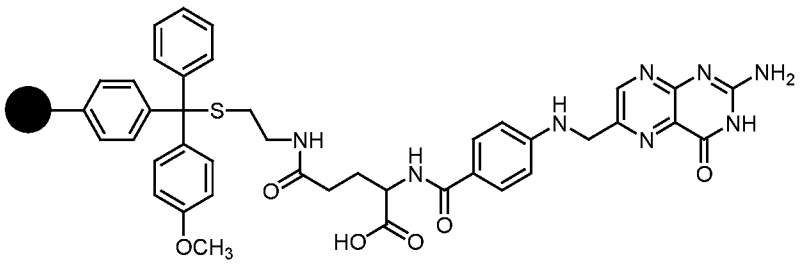
LPEI-*l*-[N$_3$:DBCO]-PEG$_{24}$-Folate was synthesized as a mixture of regioisomers 22a and 22b in a multi-step procedure according to the schemes below. In the first step, folic acid (Compound 24) was functionalized at the gamma-Glu residue with a cysteamine spacer using a solid phase synthesis approach, analogous to that described by Atkinson *et al.*, (*J. Biol. Chem.* **276**(30) 27930-35 (2001)). The resultant folate-thiol (Compound 26) was coupled to dibenzoazacyclooctyne-24(ethylene glycol)-maleimide (DBCO-PEG$_{24}$-MAL; Compound 11) by Michael addition. In a next step, DBCO-PEG$_{24}$-Folate (Compound 27) was added to LPEI-

N_3 in a [2+3] cycloaddition reaction to produce LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate (Compounds 22a and 22b).

Step 1: Folic Acid Loading to Solid Phase Resin



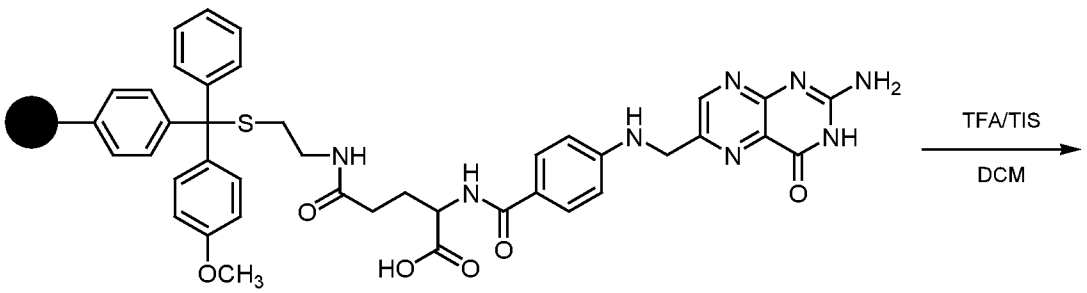
(23) (24)



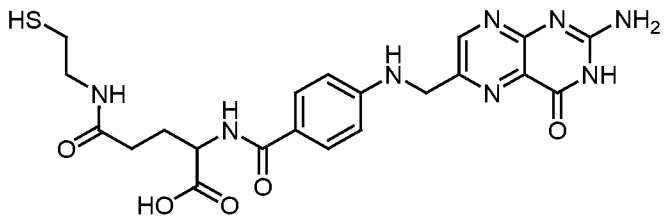
(25)

20 mL of DMSO was heated at 50°C in a 50 mL Erlenmeyer and folic acid (Compound 24; 881.4 mg, 2.0 mmol, 5.0 eq) was slowly added under magnetic stirring. Dry cysteamine 4-methoxytrityl resin (Compound 23; 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



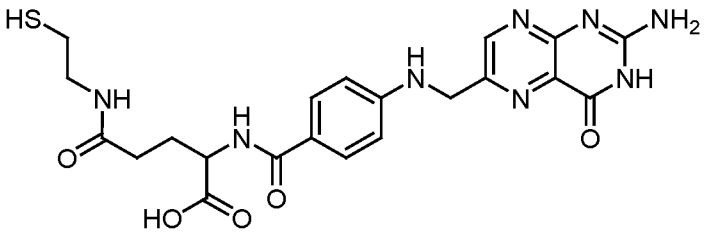
(25)



(26)

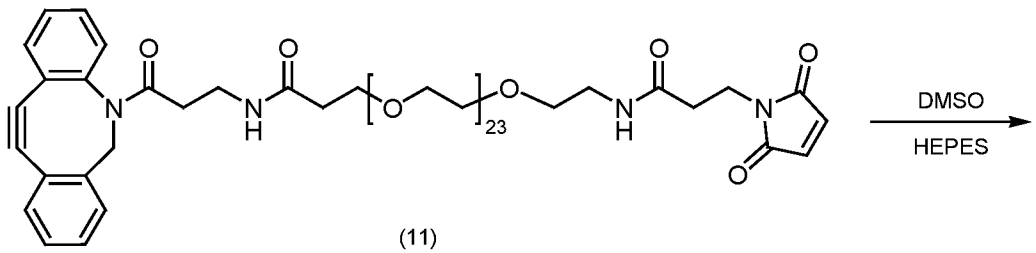
10 mL of DCM/TFA/TIS (92/3/5 v/v/v) was added to the folate-modified resin (Compound 25) of Step 1 in the glass column and the mixture was kept for 30 min with occasional swirling of the flask. The resin was filtered and washed (10 mL DCM/TFA (95/5 v/v) and the filtrate and washings were recovered and 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 26) crude product was dried overnight under reduced pressure and confirmed by mass spectrometry. The thiol content of the crude Compound 26 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 27)

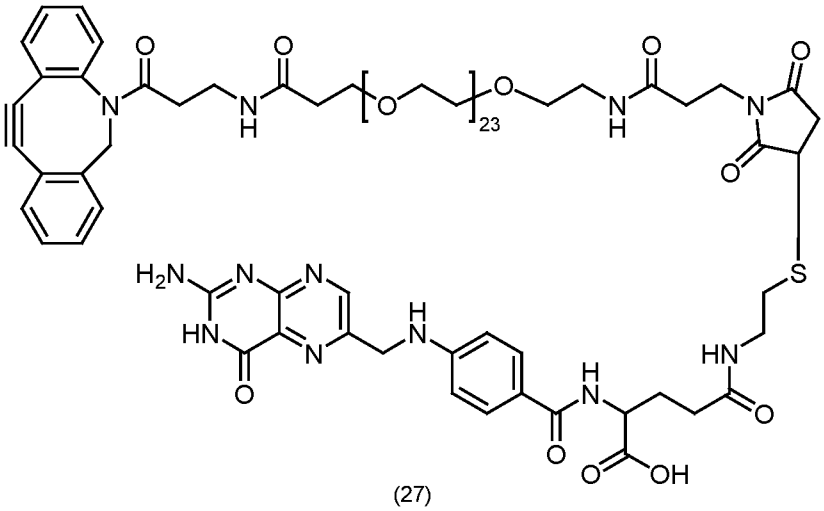


(26)

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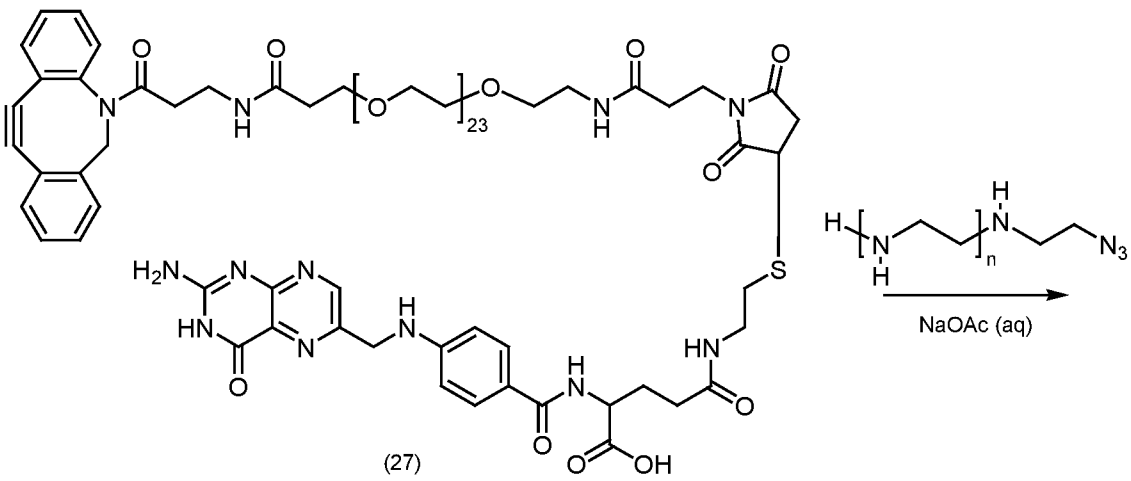
(11)



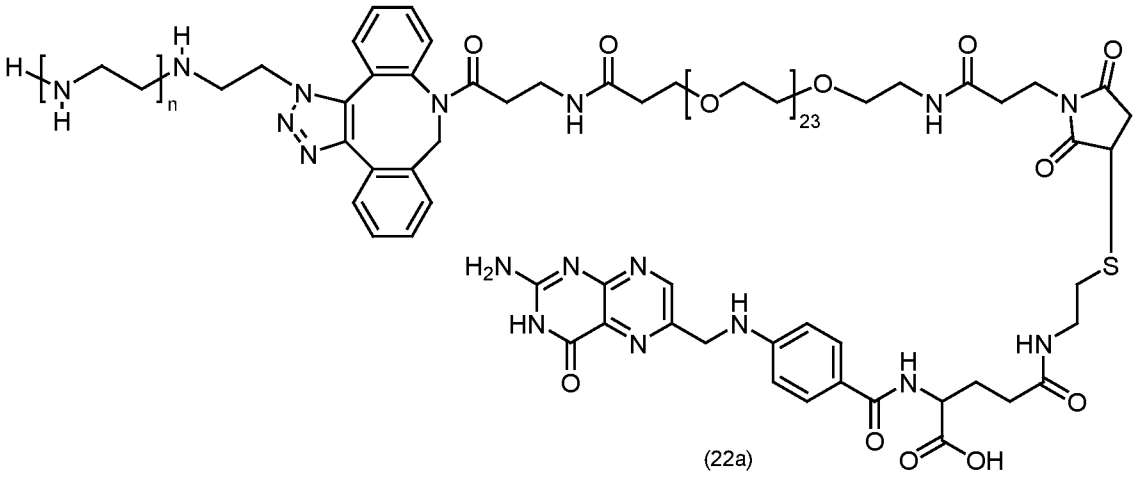
(27)

The folate-thiol (Compound 26) 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 26 and diluted with 72 mL of 20 mM HEPES (pH 7.4). DBCO-PEG_{24}-MAL (Compound 11; *see* Example 4) (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 26) solution (29.4 µmol, 1.7 eq), the DBCO-PEG_{24}-MAL (Compound 11) 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 27) 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.

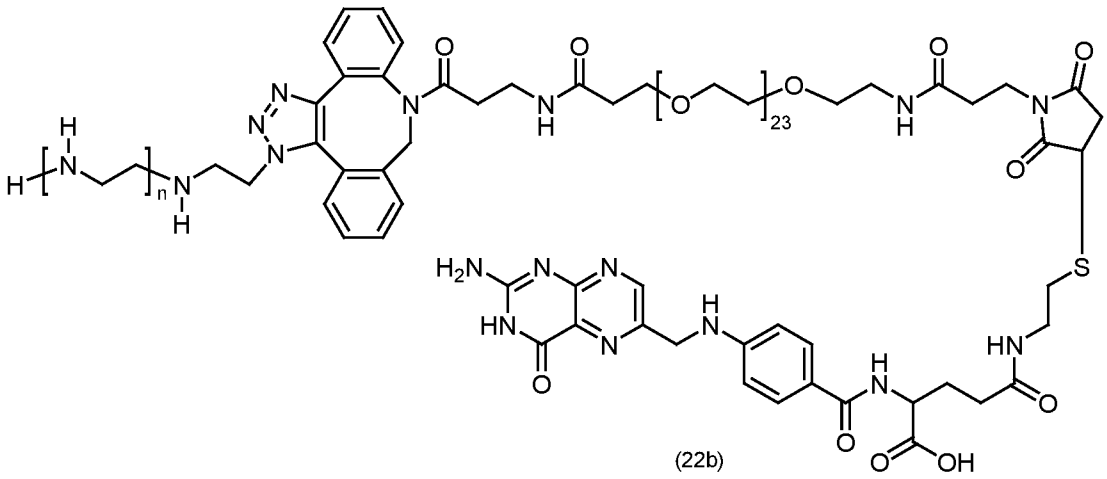
Step 4: Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate (Compounds 22a and 22b)



(27)



(22a)



(22b)

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 27; 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.

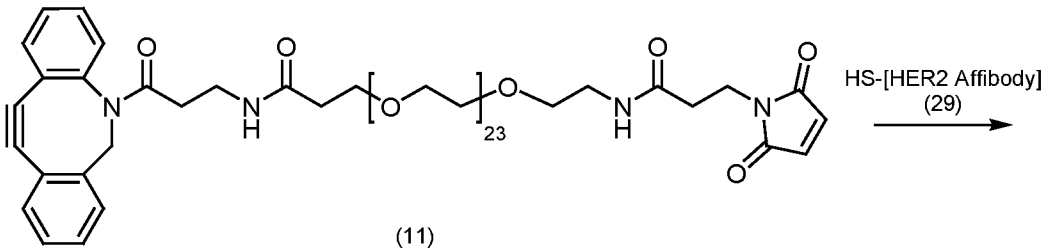
Crude LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate was purified by preparative chromatography using a Puriflash system and isolated as a mixture of regioisomers 22a and 22b. Pooled fractions were measured for total LPEI content using the copper assay and for folate content by spectrophotometry (360 nm, ε = 6'765 $M^{-1}cm^{-1}$). Yield: 19 mg in LPEI content (copper assay); LPEI/folate ratio 1:1.

EXAMPLE 9

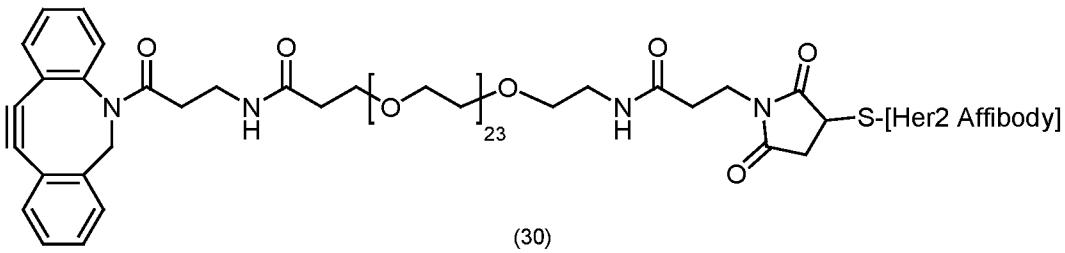
SYNTHESIS LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-AFFIBODY (COMPOUNDS 28a AND 28b)

LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-affibody was synthesized as a mixture of regioisomers 28a and 28b using a procedure analogous to the above method description for LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA of Example 4, using a commercial cysteine-terminally modified affibody (Compound 29) in a Michael addition reaction to DBCO-PEG_{24}-MAL (Compound 11). The resulting DBCO-PEG_{24}-HER2-affibody (Compound 30) was coupled to LPEI-N3 in a [2+3] cycloaddition reaction to produce LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-affibody (Compounds 28a and 28b).

Step 1: Synthesis of DBCO-PEG_{24}-HER2



(11)



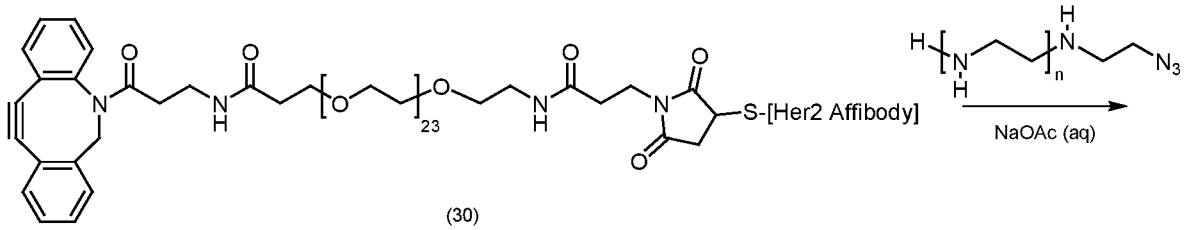
(30)

HER2 affibody (Compound 29; 4 mg, 0.29 µmol, Mw = 14kDa) were weighed in a 5 mL Eppendorf tube. To reduce potential disulfide bonds within the HER2 affibody, a 0.5 M stock solution of DTT was prepared and was added to the HER2 affibody to a 20 mM final concentration of HER2 affibody. The reaction mixture was incubated for about 5 hours at room temperature. After reduction, DTT was removed with Sephadex G-25 columns with 20 mM HEPES (pH 7.4) as elution buffer. About 3.6 mg of purified HER2 affibody were recovered after NAP purification. Yield after NAP purification was estimated to be 90%.

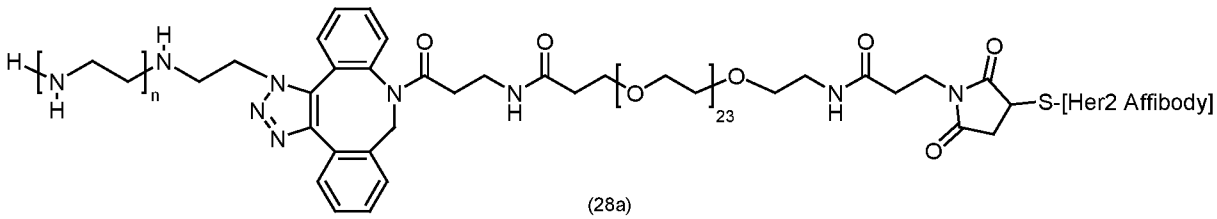
A DBCO-PEG_{24}-MAL (Compound 11) stock solution was prepared by weighing 4.4 mg (crude mass) of Compound 11 in a 1.5 mL Eppendorf tube and adding 132 µL of DMSO to prepare a 20 mM stock solution. DBCO-PEG_{24}-MAL (Compound 11; 15 µL, 0.31 µmol, 1.2 eq) stock solution was slowly added to the purified HER2-affibody solution (0.26 µmol, 1.0 eq). The reaction mixture was incubated at room temperature on a Stuart rotator for about two hours and the reaction was monitored by RP-C8-HPLC at 280 nm and 309 nm.

The reaction mixture was purified with Amicon filters (10 kDa MWCO) to remove excess of DBCO-PEG$_{24}$-MAL (Compound 11) from the DBCO-PEG$_{24}$-HER2-affibody conjugate (Compound 30). Fourteen centrifugal filters (Amicon Ultra – 0.5 mL, Merck Millipore Ltd.) were each filled with 429 µL of the reaction mixture. They were centrifugated one time at 14'000 g for 30 minutes to exchange buffer and remove residual DBCO-PEG$_{24}$-MAL (Compound 11) and then three times against 50 mM acetate buffer, pH 4.0 at 20°C. A concentrated solution of DBCO-PEG$_{24}$-HER2-affibody (Compound 30; 243 µL) was recovered after buffer exchange and supplemented with 1.0 mL 50 mM Acetate, pH 4.0. A total of ~1.24 mL of purified DBCO-PEG$_{24}$-HER2-affibody (Compound 30) solution was obtained after the NAP purification step. The purified solution was analyzed by RP-C$_8$-HPLC and spectrophotometry at 309 nm with Nanodrop One C and a concentration of 118 µM of DBCO was measured (~0.15 µmol).

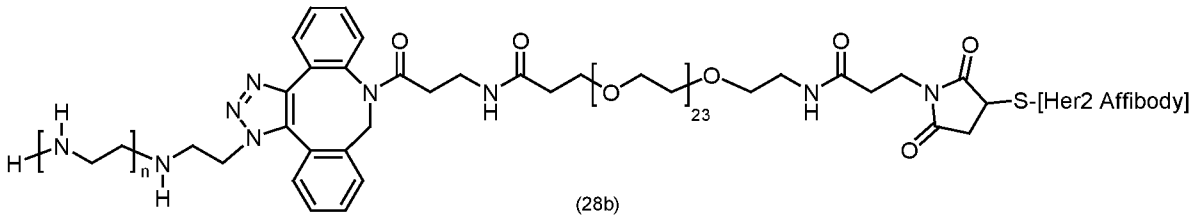
Step 2: Synthesis of LPEI-*l*-[N$_3$:DBCO]-PEG$_{24}$-HER2



(30)



(28a)



(28b)

LPEI-N$_3$ (7.4 mg 0.34 µmol, based on LPEI 72% (Cu assay) were weighed in a 15 mL Falcon tube and dissolved in 0.4 mL of 50 mM acetate buffer (pH 4.0). The solution was acidified, heated to 70°C, sonicated to fully dissolve LPEI particles, adjusted to pH 4.0 with a total of 15 µL of 6 M HCl, and degassed with argon. LPEI-N$_3$ from the stock solution (333 µL, 0.28 µmol, 2.0 eq) was slowly added to the DBCO-PEG$_{24}$-HER2 (Compound 29) solution (0.14 µmol, 1 eq). The reaction mixture was incubated for about 72 hours on a Stuart rotator. Additional LPEI-N$_3$ from a stock solution (215 µL, 0.14 µmol, 1.0 eq) was added to the reaction

mixture and the solution was incubated for about 24 hours at 35°C on a thermoshaker and monitored by RP-C_8-HPLC at 240 nm, 280 nm, and 309 nm with an ELSD detector. Prior to preparative chromatography, the percentage of acetonitrile of the reaction mixture was adjusted to 10% (final volume) with 189 µL of ACN and to 1% TFA (final volume) with 19 µL of TFA. The solution (~1.7 mL) was supplemented with 1.0 mL of 90% v/v H_2O (0.1% TFA)/ 10% v/v ACN (0.1% TFA) and the total volume of sample was injected into the Agilent Prep-HPLC system. Pooled fractions were lyophilized to yield 4 mg LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-affibody as a mixture of regioisomers 28a and 28b (overall yield in LPEI = 6.9%; overall yield in anti-HER2 affibody = 14%; 16% w/w in LPEI; ratio LPEI:DUPA = 1/1.4).

Step 3: Preparation of HEPES salt form

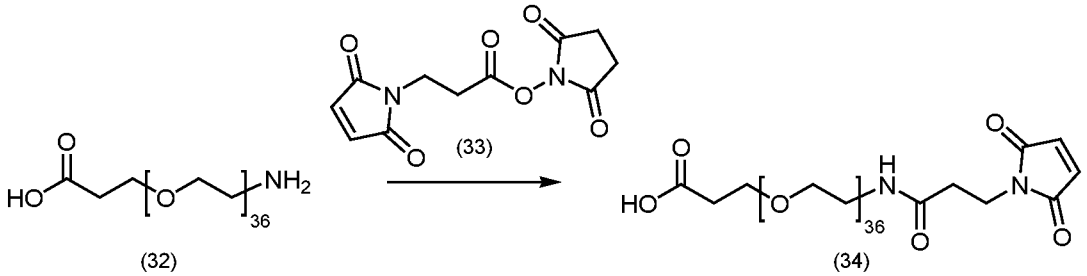
The lyophilized LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-affibody (Compounds 28a and 28b)was dissolved in 0.8 mL 20 mM HEPES pH 7.2 in a 1.5 mL Eppendorf tube. The pH was adjusted to pH 7.2 with 5 M NaOH / 1 M HCl. Two centrifugal filters (Amicon Ultra – 0.5 mL, Merck Millipore Ltd.) were each filled with 400 µL of LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-affibody (Compounds 28a and 28b) solution. They were centrifugated one time at 14'000 g for 30 minutes to remove buffer and then three times against 20 mM HEPES, pH 7.2 at 4°C. A concentrated solution of LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-affibody HEPES salt (~146 µL) was recovered after buffer exchange and supplemented with 170 µL 20 mM HEPES, pH 7.2. A copper assay was performed on the final HEPES salt solution (~0.3 mL) and a concentration of 1.7 mg/mL total LPEI was measured.

EXAMPLE 10

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (COMPOUNDS 31a AND 31b)

LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA was synthesized as a mixture of regioisomers 31a and 31b according to the schemes below. In a first step, HOOC-PEG_{36}-NH_2 (Compound 32) was coupled to N-succinimidyl 3-maleimidopropionate (Compound 33) by amine formation to produce HOOC-PEG_{36}-MAL (Compound 34). In a next step, HOOC-PEG_{36}-MAL (Compound 34) was coupled to DBCO-NH_2 (Compound 35) by amine formation to produce DBCO-PEG_{36}-MAL (Compound 36). In a next step, DBCO-PEG_{36}-MAL (Compound 36) was coupled to DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12; SEQ ID NO:4) by a Michael addition to produce DBCO-PEG_{36}-DUPA (Compound 37). In a next step, DBCO-PEG_{36}-DUPA (Compound 37) 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 31a and 31b.

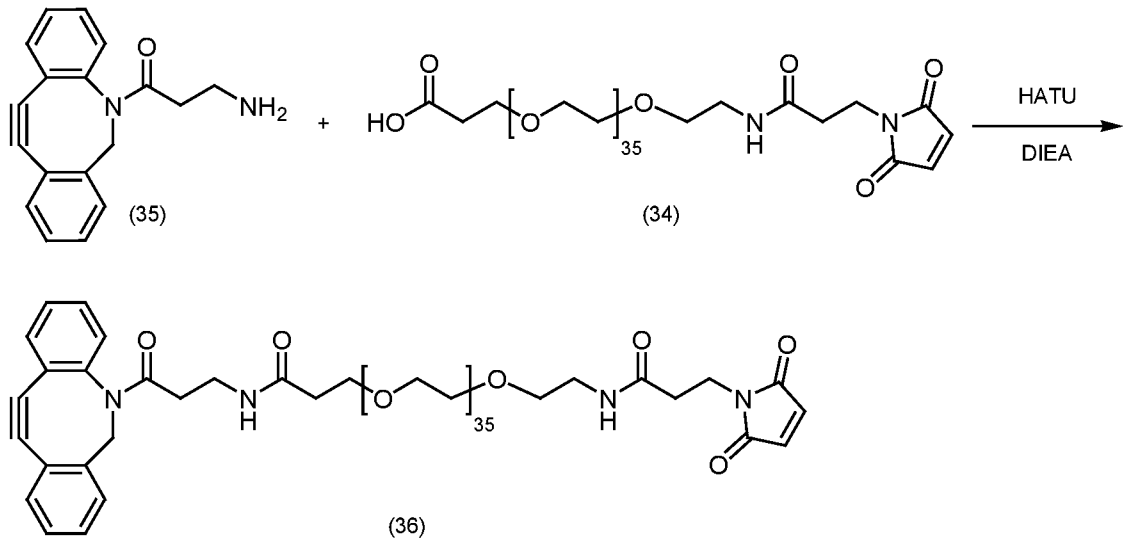
Step 1: Synthesis of HOOC-PEG$_{36}$-MAL (Compound 34)



Stock solutions were prepared as follows: HOOC-PEG$_{36}$-NH$_2$ (Compound 32) 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 33) 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 34) were recovered and analyzed by RP-C8-HPLC; qTOF mass spectrometry (calculated monoisotopic mass: 1'825.02 Da; measured: 1'825.02 Da).

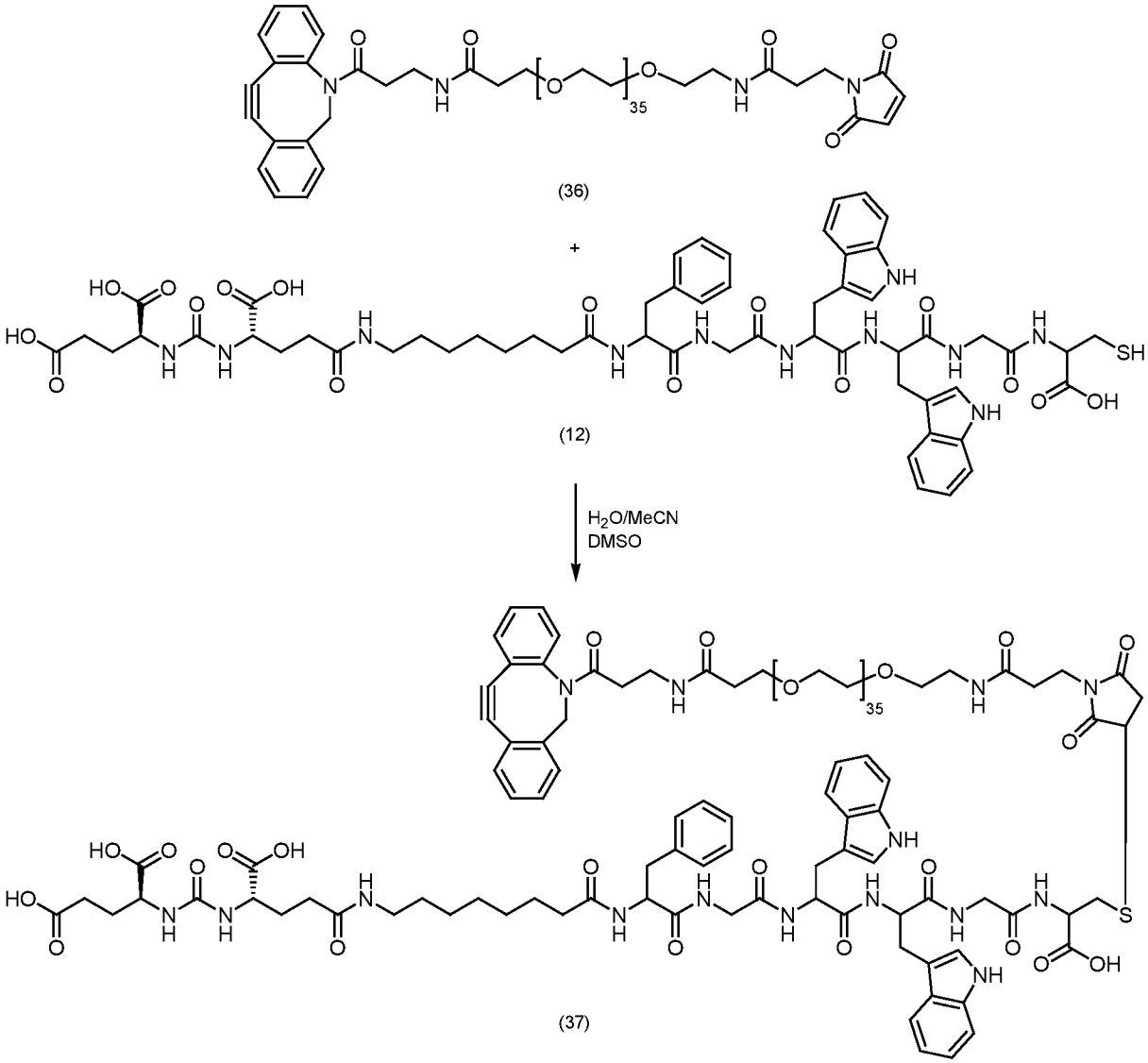
Step 2: Synthesis of DBCO-PEG$_{36}$-MAL (Compound 36)



A stock solution of HOOC-PEG_{36}-MAL was prepared by dissolving 458 mg (crude mass) of HOOC-PEG_{36}-MAL (Compound 34) 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 35) 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 34), 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 35; 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 36), 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).

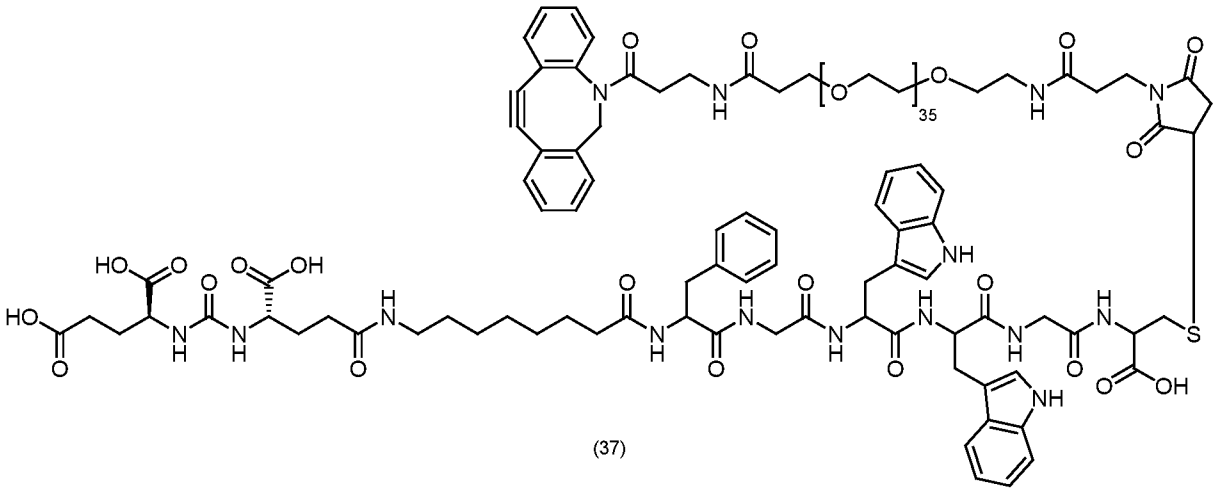
Step 3: Synthesis of DBCO-PEG_{36}-DUPA



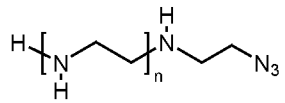
A stock solution of DBCO-PEG$_{36}$-MAL (Compound 36) was prepared by dissolving 548 mg in a 50 mL Falcon tube and dissolving in 10 mL DMSO (26.3 mM stock solution). A stock solution of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12; SEQ ID NO:4) was prepared by weighing 318 mg in a 250 mL round-bottom 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 12. 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 12 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 12 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 37). qTOF mass spectrometry (calculated monoisotopic mass: 3'280.60 Da; measured: 3'280.64 Da)

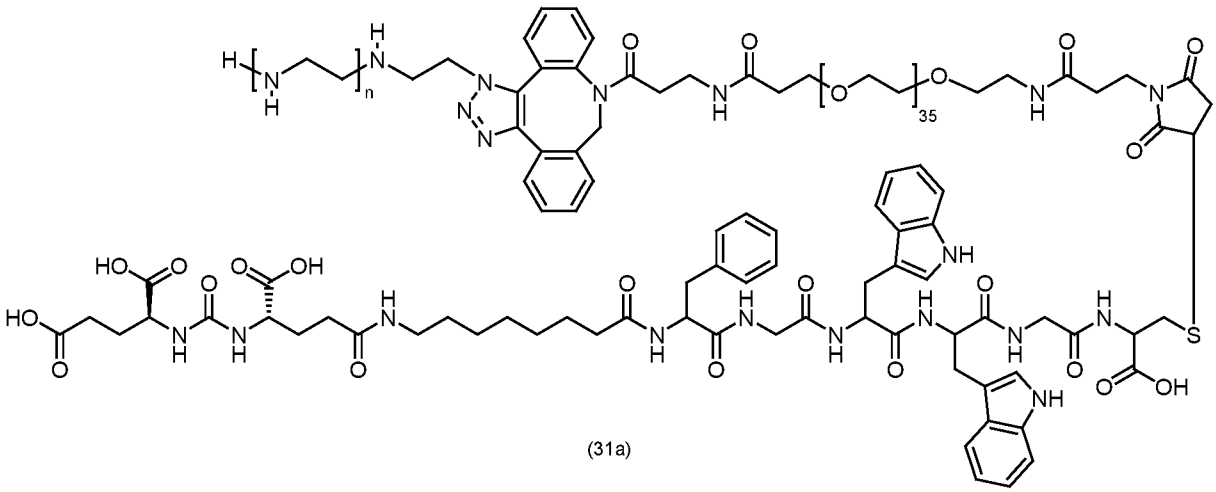
Step 4: Synthesis of LPEI-*l*-[N$_3$:DBCO]-PEG$_{36}$-DUPA



(37)

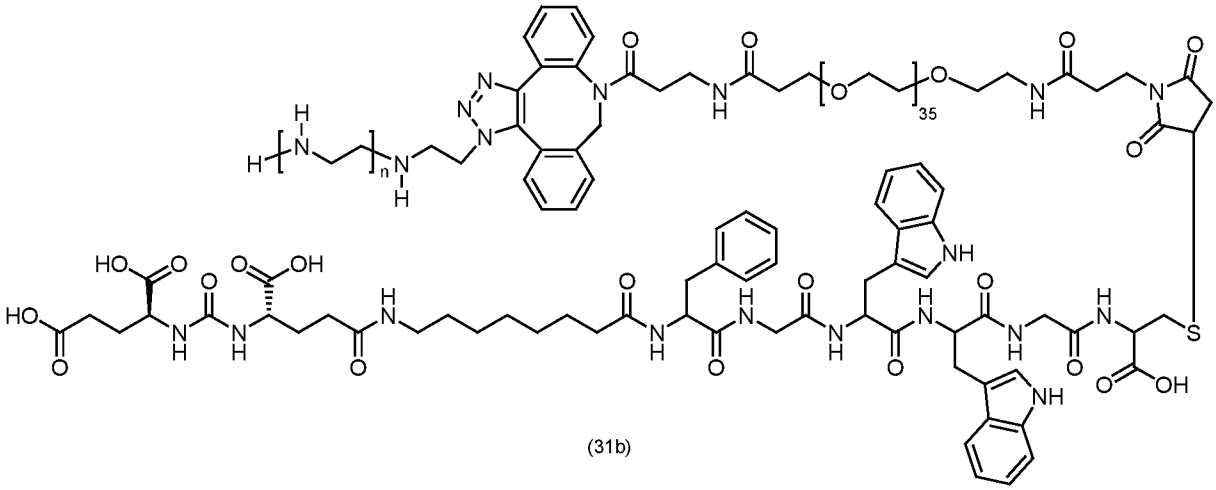


NaOAc (aq.)



(31a)

+



(31b)

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 37) 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 31a and 31b. 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 31a and 31b) 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 31a and 31b) 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 31 and 31b) acetate salt

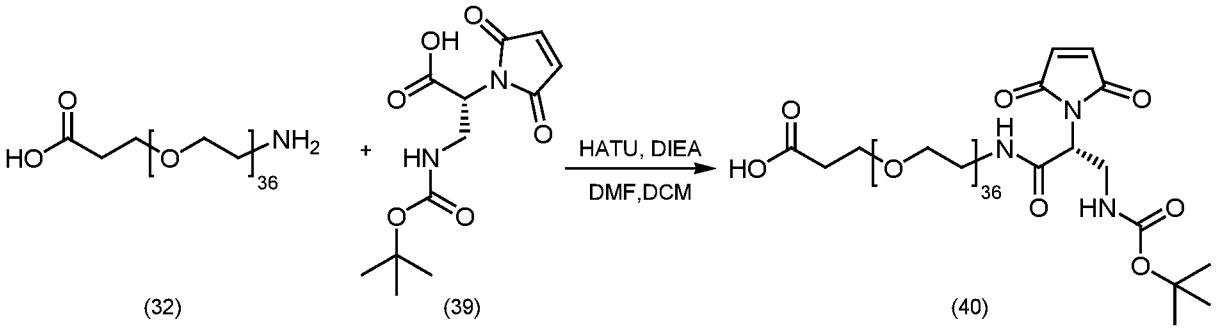
Lyophilized LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA-TFA salt (4.9 mg, w_{LPEI} = 34%, ~1.7 mg in total LPEI) was dissolved in 0.8 mL 50 mM acetate pH 4.3 in a 1.5 mL Eppendorf tube. The pH was adjusted to pH 4.5 with 3.0 µL 5 M NaOH. Two centrifugal filters (Amicon Ultra – 0.5 mL, 3kDa MWCO) were filled with 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 31a and 31b) - acetate salt solution (~0.6 mL) and a concentration of 2.0 mg/mL total LPEI was determined.

EXAMPLE 11

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA (COMPOUNDS 38a AND 38b)

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA was synthesized as a mixture of regioisomers 38a and 38b according to the schemes below. In the first step, HOOC-PEG_{36}-NH_2 (Compound 32) was condensed with Mal-L-Dap(Boc)-OH (Compound 39) to give HOOC-PEG_{36}-(Boc)-MAL (Compound 40). Compound 40 was subsequently condensed with DBCO-NH_2 (Compound 35) and deprotected to give DBCO-PEG_{36}-(NH_2)-MAL (Compound 41). Compound 41 was reacted with DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12) via Michael Addition and cyclized with LPEI-N_3 to produce compounds 38a and 38b.

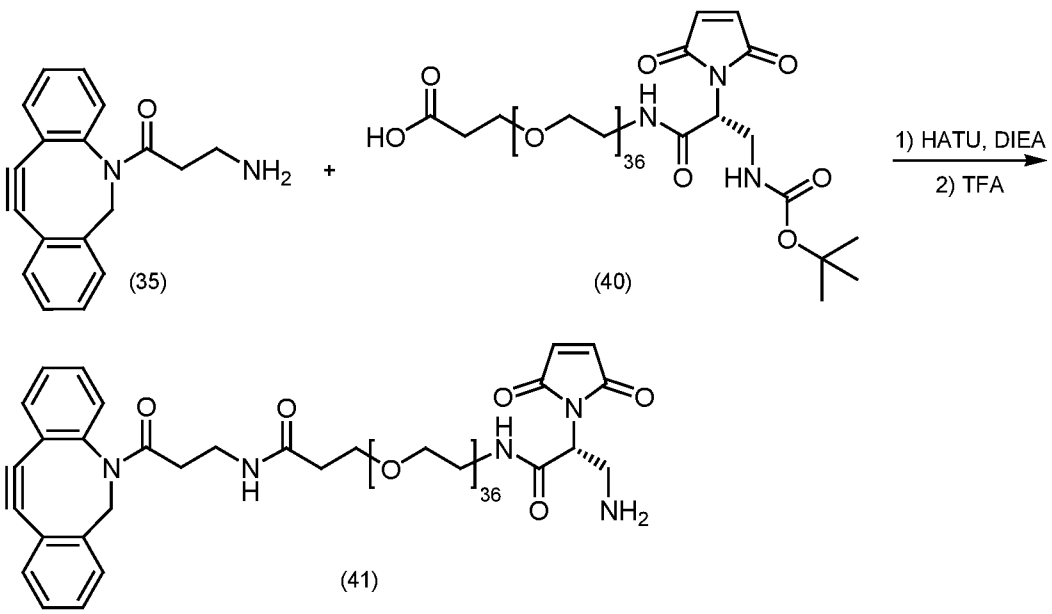
Step 1. Synthesis of HOOC-PEG_{36}-(Boc)-MAL (Compound 40)



(32) (39) (40)

A solution Mal-L-Dap(Boc)-OH (N-α-Maleimido-N-β-t-butyloxycarbonyl-L-2,3-diaminopropionic acid DCHA salt; Compound 39; 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 32, 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 40) was recovered and analyzed by HPLC – ESI^+ qTOF mass spectrometry (calculated monoisotopic mass: 1940.08 Da; measured: 1940.10 Da). The crude Compound 40 was used without further purification in the next step.

Step 2. Synthesis of DBCO-PEG_{36}-(NH_2)-MAL (Compound 41)



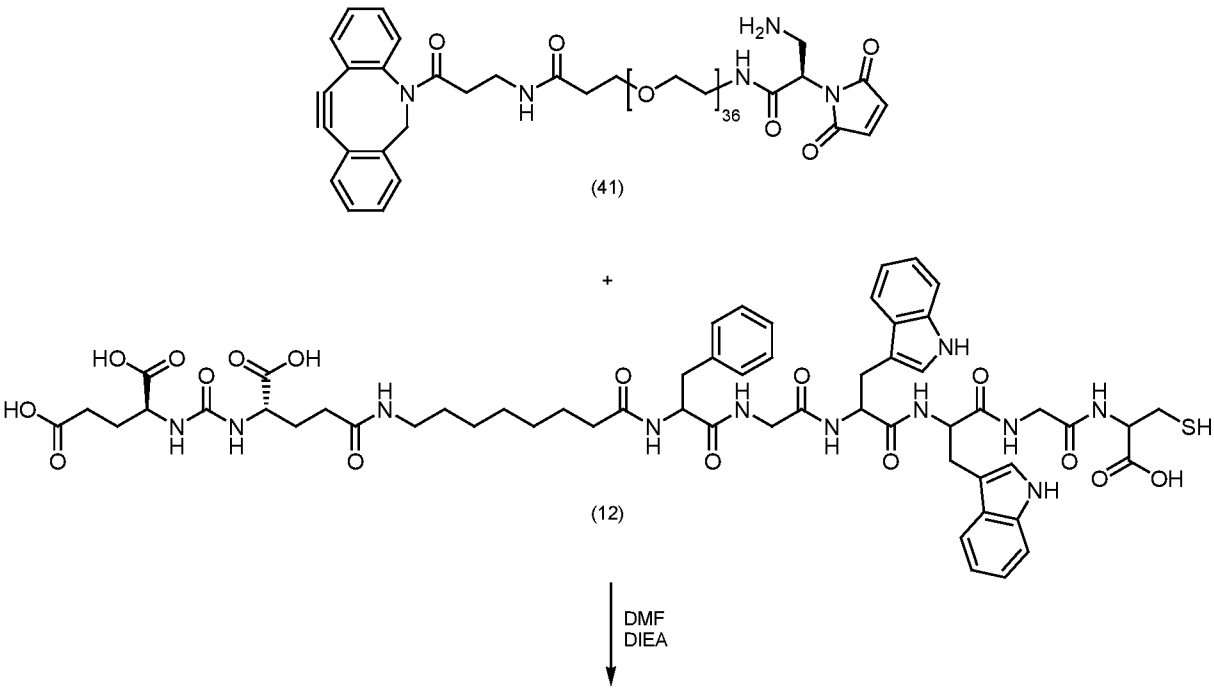
(35) (40) (41)

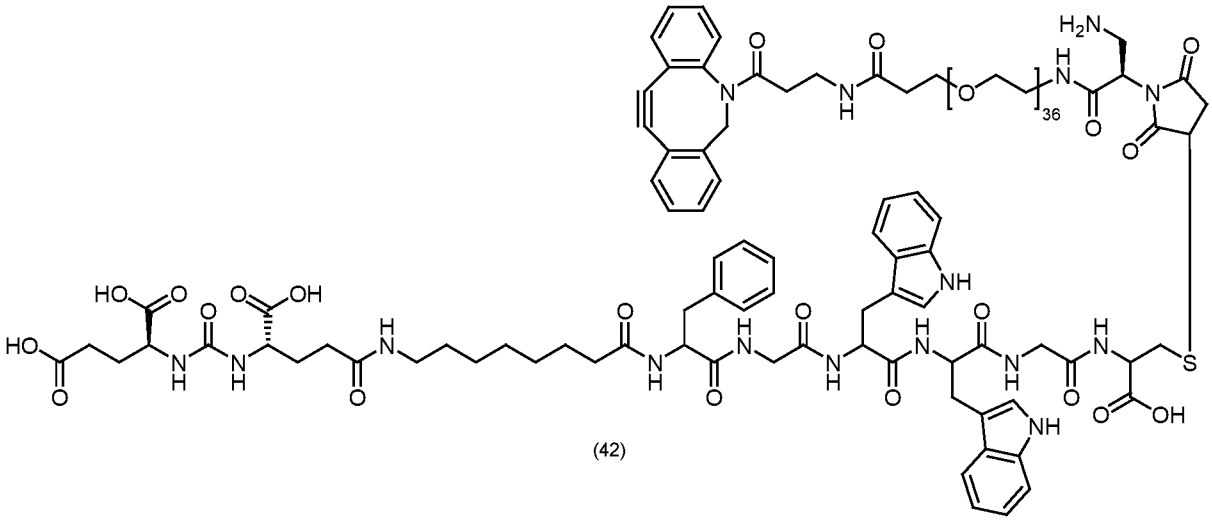
HATU (35 µmol, 0.9 eq, 208 mM) in DMF (169 µL) was added to a solution of HOOC-

PEG_{36}-(Boc)-MAL (Compound 40; 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 35; 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 41) 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 42)

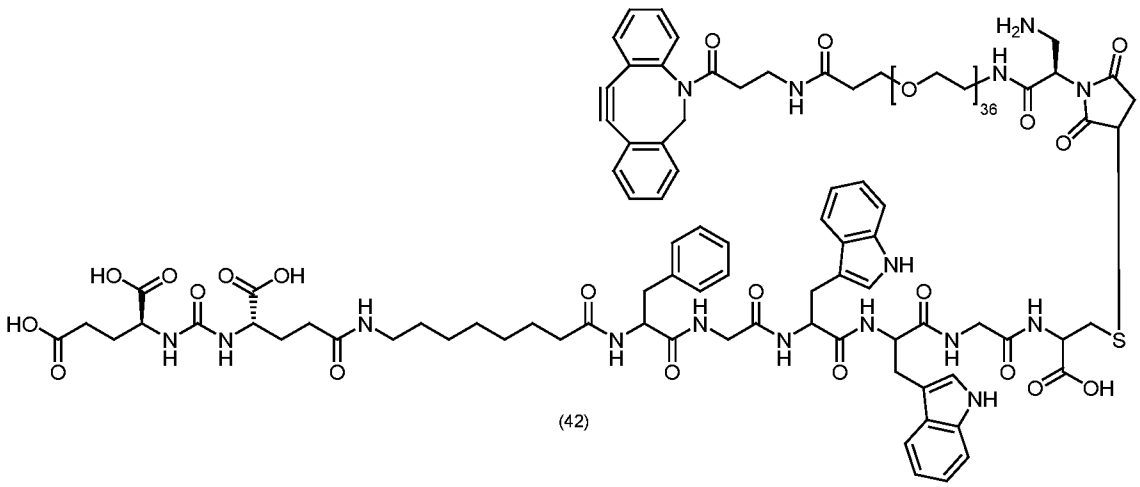




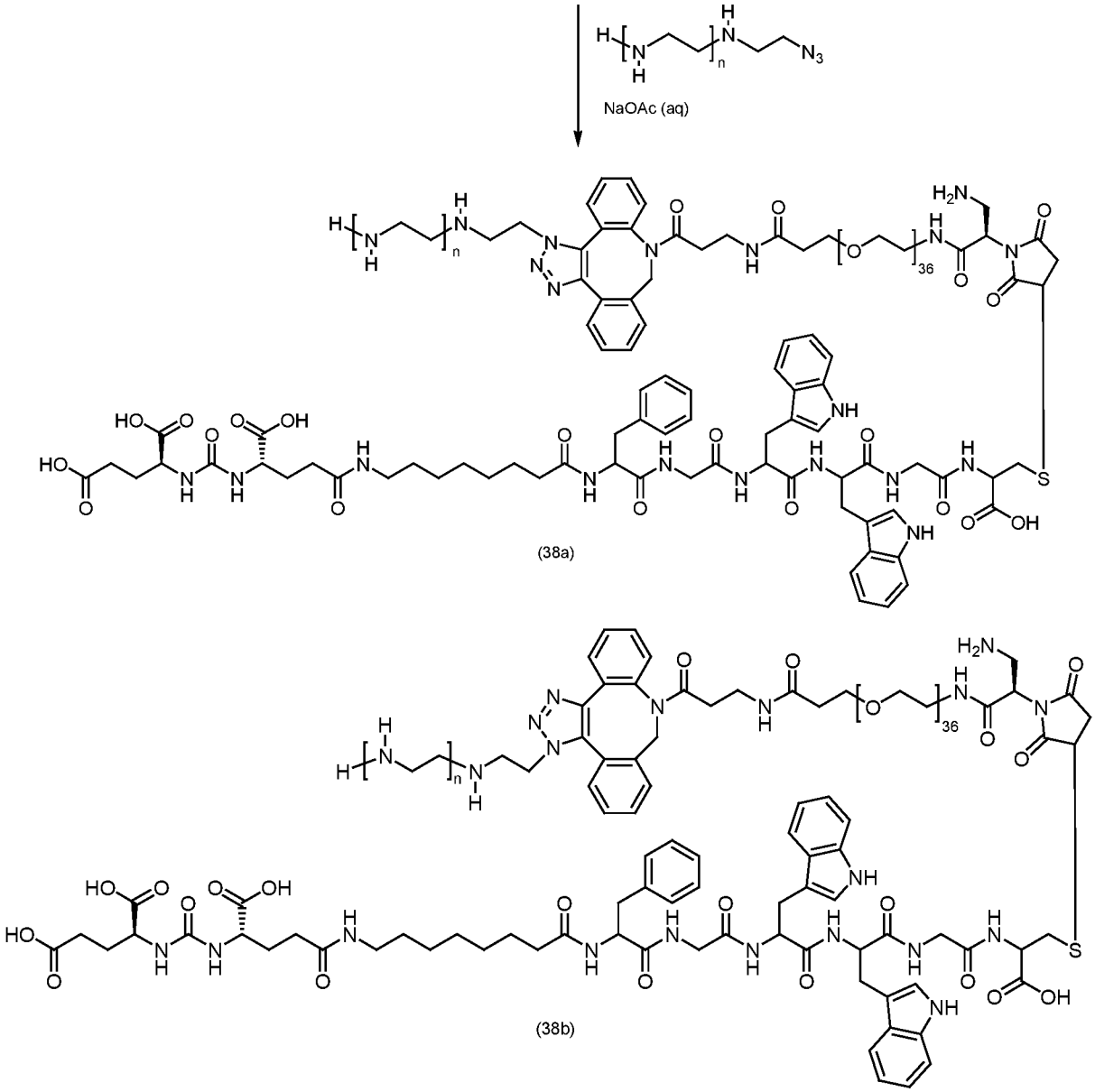
(42)

A solution of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12; 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 41; 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 42) 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 38a and 38b)



(42)



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 42; 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 38a and 38b 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 38a and 38b) HEPES salt

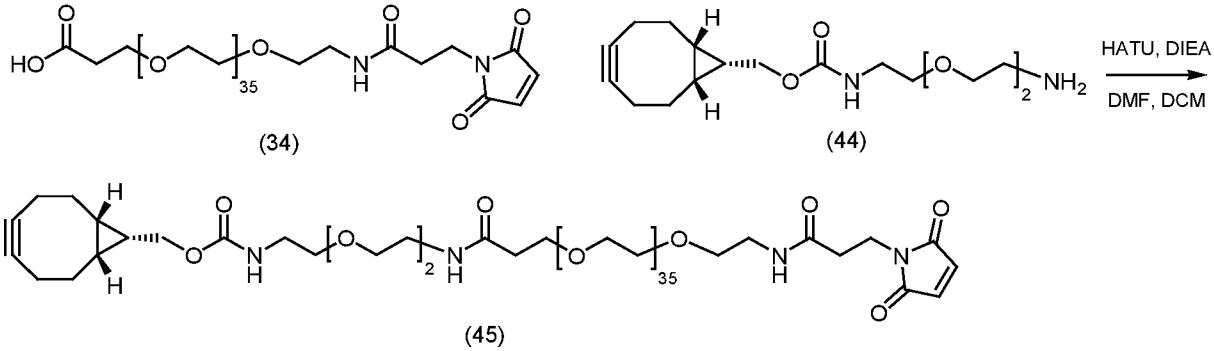
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA (Compounds 38a and 38b) TFA salt (21.9 mg, w_{LPEI} = 32%, 7.0 mg in total LPEI) were dissolved in 1.2 mL 20 mM HEPES pH 7.5. Three centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)-MAL-S]-DUPA 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 12

SYNTHESIS OF LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA (COMPOUND 43)

LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA (Compound 43) was synthesized according to the schemes below. Endo-BCN-PEG_{36}-MAL (Compound 45) was prepared by condensing HOOC-PEG_{36}-MAL (Compound 34) with endo-BCN-PEG_2-NH_2 (Compound 44). In a next step, Compound 45 was condensed with Compound 12, and the resulting endo-BCN-PEG_{36}-DUPA (Compound 46) was reacted with LPEI-N_3 to give Compound 43.

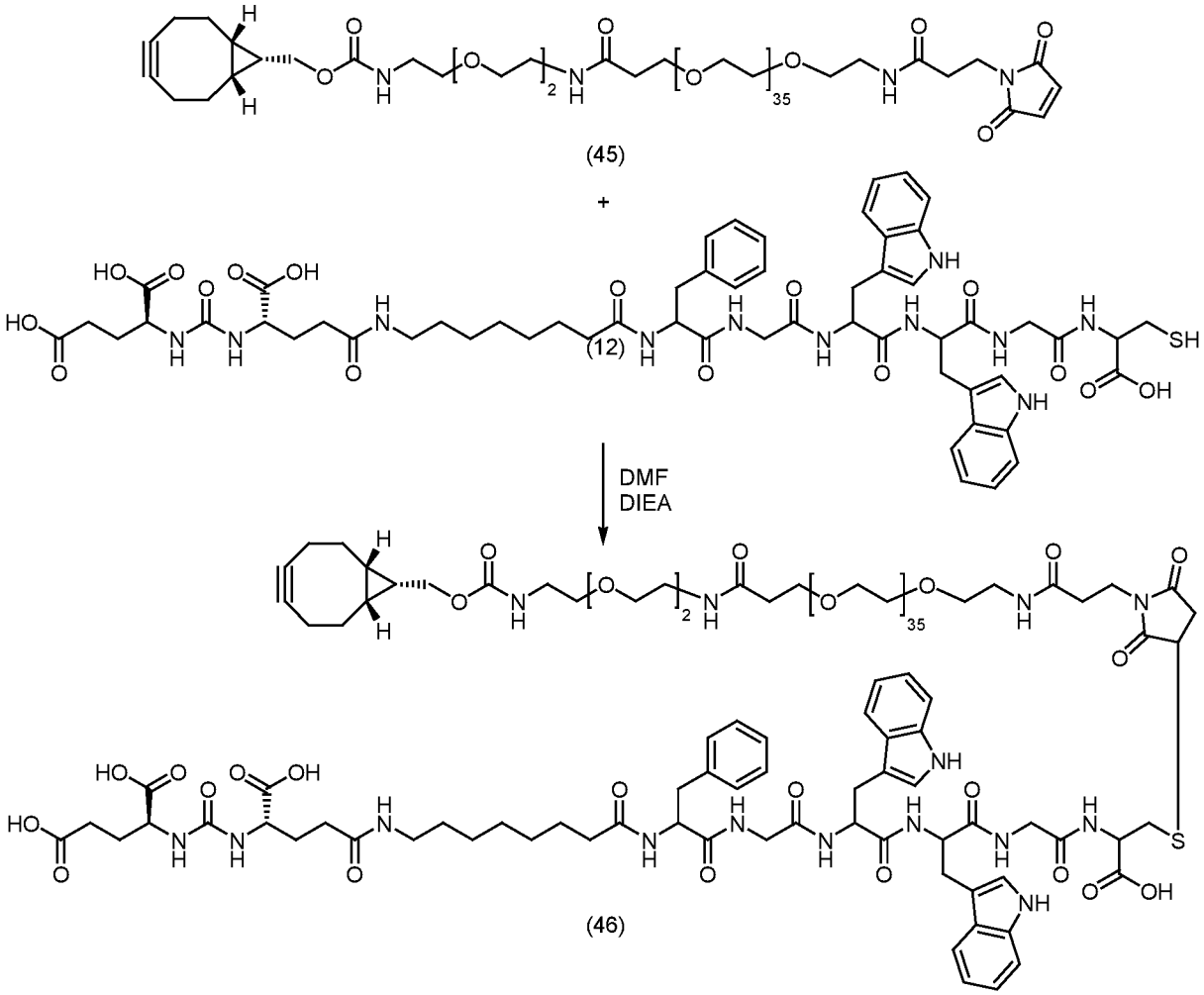
Step 1. Synthesis of *endo*-BCN-PEG_{36}-MAL (Compound 45)



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 34; see Example 10; 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 44; 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 45; 61 mg) was isolated and analysed by RP-C_8-HPLC coupled with ESI^+-qTOF

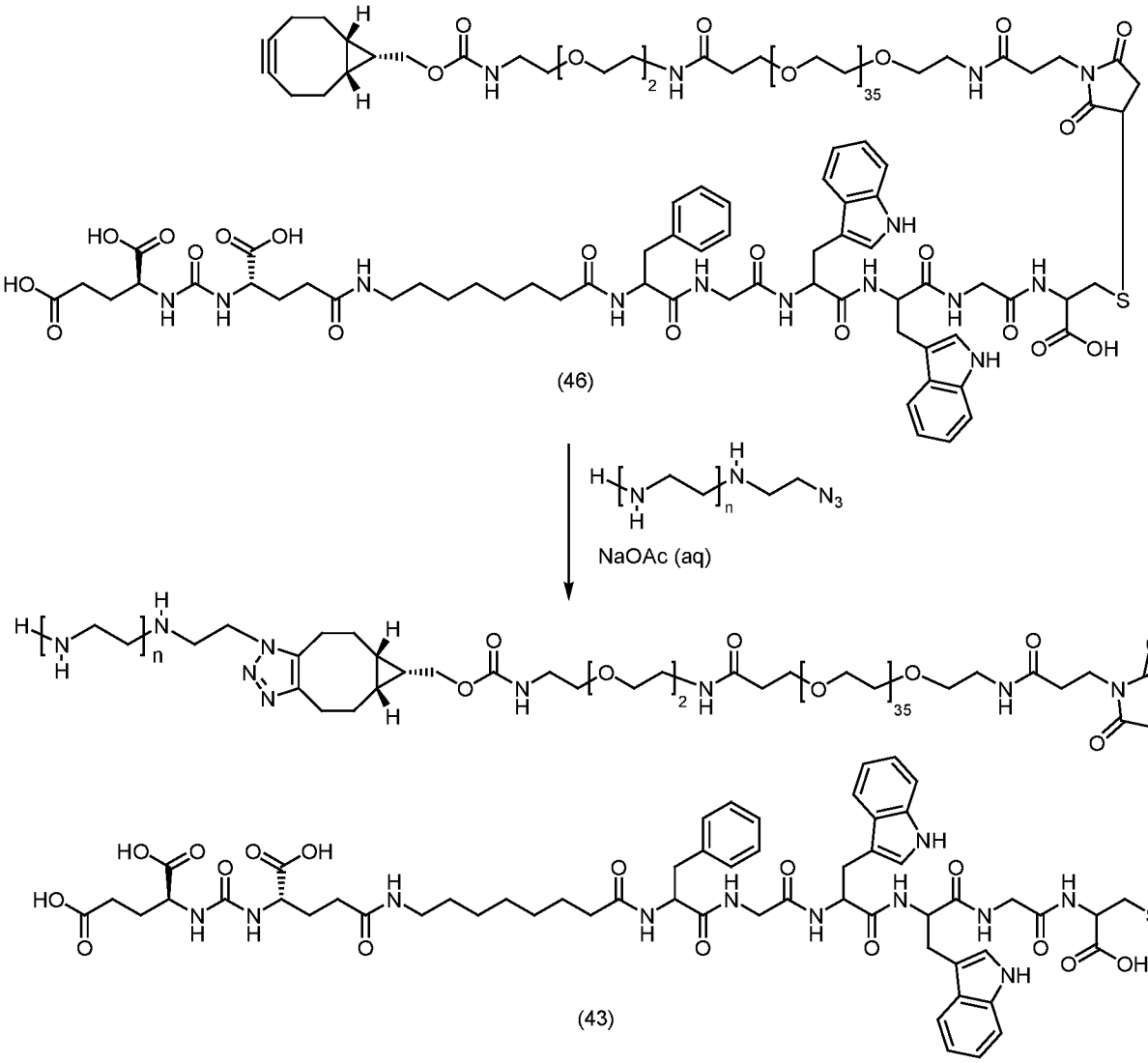
mass spectrometry (Calculated monoisotopic mass: 2'131.21 Da; measured: 2'131.22 Da) and used in the next step without further purification.

Step 2. Synthesis of *endo*-BCN-PEG$_{36}$-DUPA (Compound 46)



A solution of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12; 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 45; 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$_{36}$-DUPA (Compound 46). A sample was analyzed by HPLC ESI$^+$ qTOF mass spectrometry (*endo*-BCN-PEG$_{36}$-DUPA: calculated monoisotopic mass: 3328.69 Da; measured: 3328.72 Da).

Step 3. Synthesis of LPEI-*l*-[N$_3$;BCN]-PEG$_{36}$-DUPA (Compound 43)



endo-BCN-PEG$_{36}$-DUPA (Compound 46; 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$_{36}$-DUPA (Compound 43) 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$_{36}$-DUPA (Compound 43) HEPES salt

24.8 mg of LPEI-*l*-[N_3:BCN]-PEG$_{36}$-DUPA (Compound 43) 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}-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}-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}-DUPA (Compound 43) Acetate salt

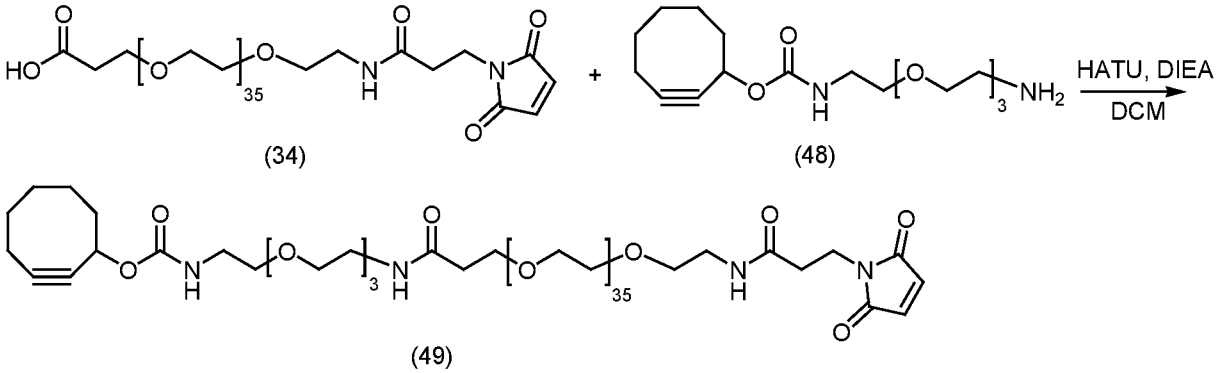
5.5 mg of LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA (Compound 43) TFA salt (w_{LPEI} = 31%, ~1.7 mg in total LPEI) were dissolved in 0.8 mL 50 mM acetate, pH 4.0. Two centrifugal filters (Amicon Ultra – 0.5 mL, 3kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA solution each. They were centrifuged 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}-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 13

SYNTHESIS OF LPEI-*l*-[N_3:SCO]-PEG_{36}-DUPA (COMPOUNDS 47a AND 47b)

LPEI-*l*-[N_3:SCO]-PEG_{36}-DUPA was synthesized as a mixture of regioisomers 47a and 47b according to the schemes below. SCO-PEG_{36}-MAL (Compound 49) was prepared by condensing HOOC-PEG_{36}-MAL (Compound 34) with SCO-PEG_3-NH_2 (Compound 48). Compound 49 was reacted with Compound 12 via Michael Addition, and the resulting SCO-PEG_{36}-DUPA (Compound 50) was reacted with LPEI-N_3 to synthesize Compounds 47a and 47b.

Step 1. Synthesis of SCO-PEG_{36}-MAL (Compound 49)

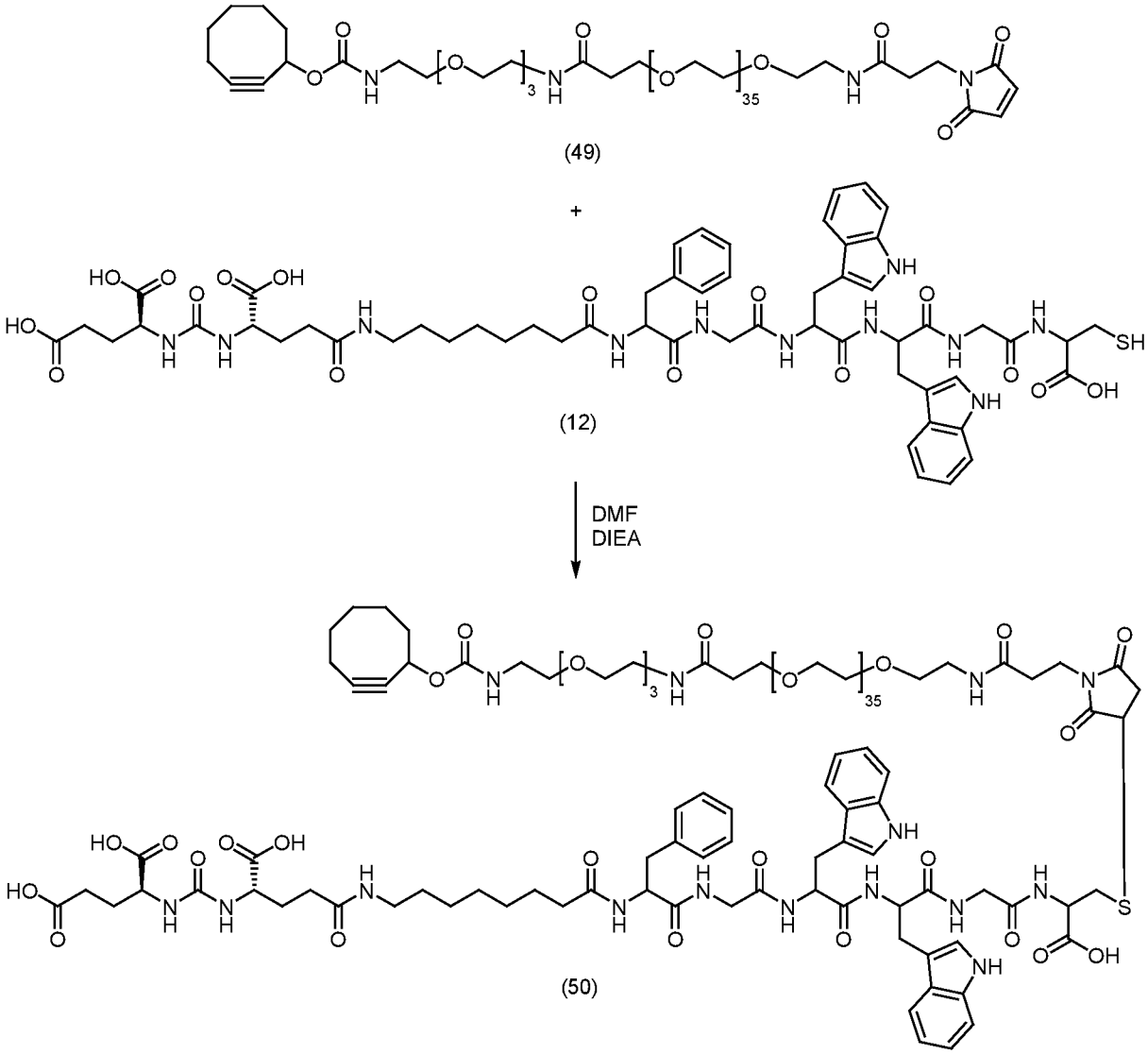


(34) (48)

(49)

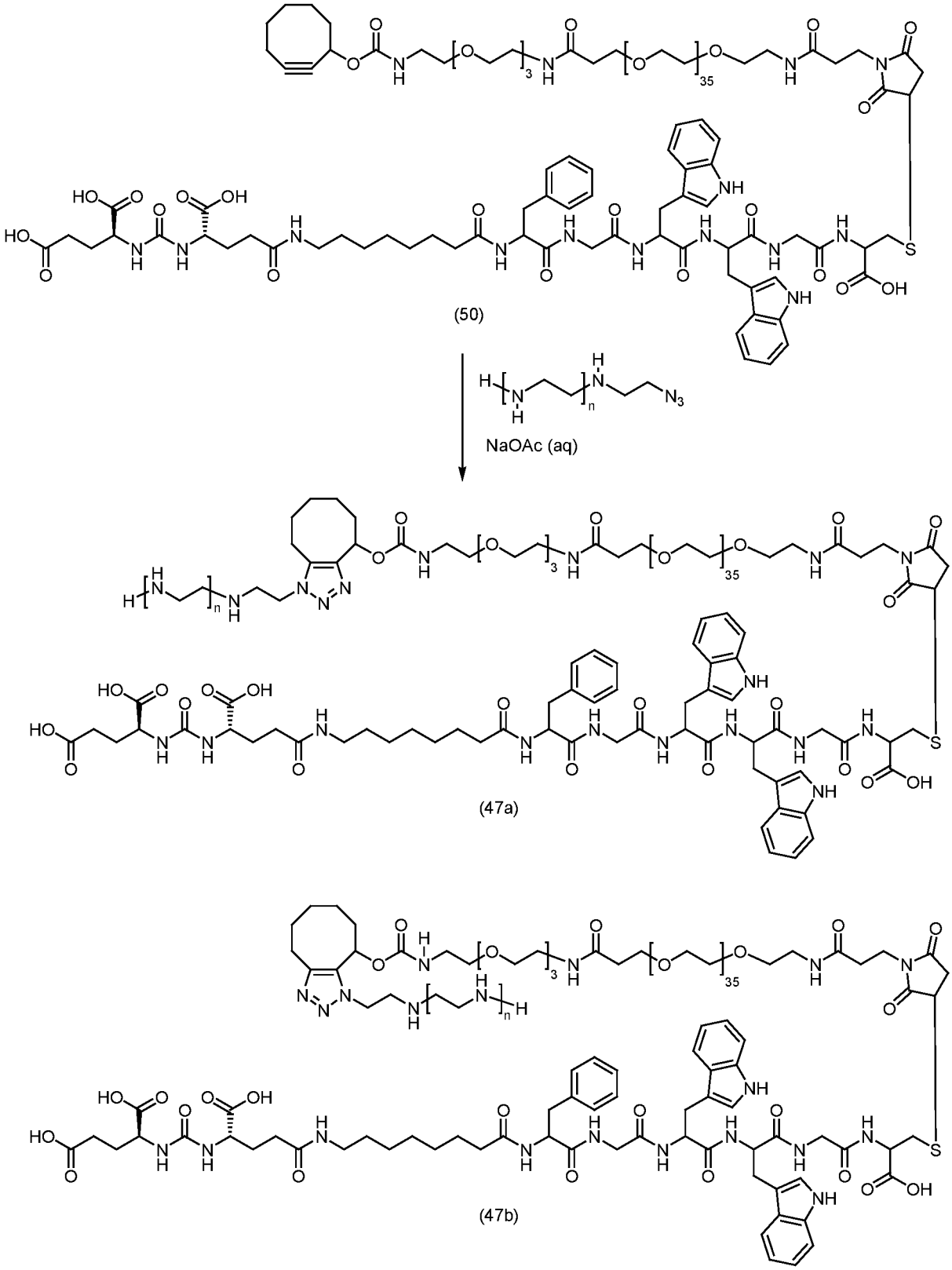
A solution of HATU (25 µmol, 0.9 eq, 147 mM) in DMF (69 µL) was added to HOOC-PEG_{36}-MAL (Compound 34; 28 µmol, 1.0 eq, 70 mM) in DCM followed by DIEA (9.6 µL, 56 µmol, 2.0 eq). To the reaction mixture was added a solution of SCO-PEG_3-NH_2 (Compound 48; 22 µmol, 0.8 eq, 137 mM) in DCM (166 µL). The 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 49) 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$_{36}$-DUPA (Compound 50)



A solution of DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12; 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 49; 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 12 (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$_{36}$-DUPA (Compound 50) were lyophilized and analyzed by HPLC-ESI$^+$ qTOF mass spectrometry (SCO-PEG$_{36}$-DUPA calculated monoisotopic mass: 3346.70 Da; measured: 3346.71 Da).

Step 3: Synthesis of LPEI-*l*-[N_3:SCO]-PEG_{36}-DUPA (Compounds 47a and 47b)



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}-DUPA (Compound 50) 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}-DUPA as a mixture of regioisomers 47a and 47b. 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}-DUPA (Compounds 47a and 47b) HEPES salt

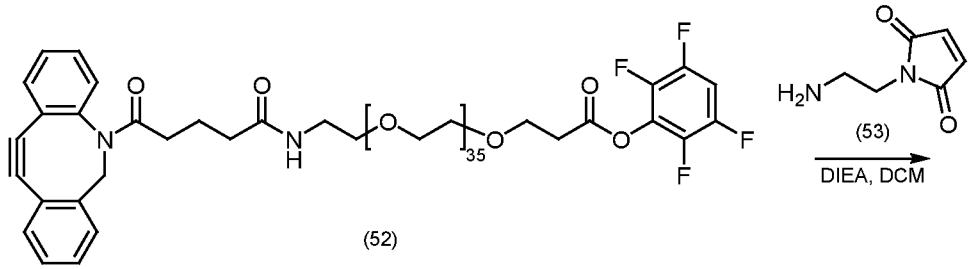
23.2 mg of LPEI-*l*-[N_3:SCO]-PEG_{36}-DUPA (Compounds 47a and 47b) TFA salt (w_{LPEI} = 26%, 6.0 mg in total LPEI) were dissloved in 1.2 mL 20 mM HEPES pH 7.4. Three centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:SCO]-PEG_{36}-DUPA solution each, centrifuged 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}-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 14

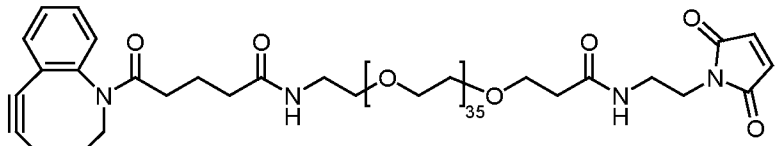
SYNTHESIS OF LPEI-*l*-[N_3:DBCO]CONH-PEG_{36}-DUPA COMPOUNDS 51a AND 51b)

LPEI-*l*-[N_3:DBCO]CONH-PEG_{36}-DUPA was synthesized as a mixture of regioisomers 51a and 51b according to the schemes below. DBCO-PEG_{36}-[CONH]-MAL (Compound 54) was prepared by condensing DBCO-PEG36-TFP (Compound 52) with NH2-MAL (Compound 53). The resulting DBCO-PEG_{36}-[CONH]-MAL (Compound 54) was condensed with Compound 12 and reacted with LPEI-N_3 to give Compounds 51a and 51b.

Step 1. Synthesis of DBCO-PEG_{36}-[CONH]-MAL (Compound 54)



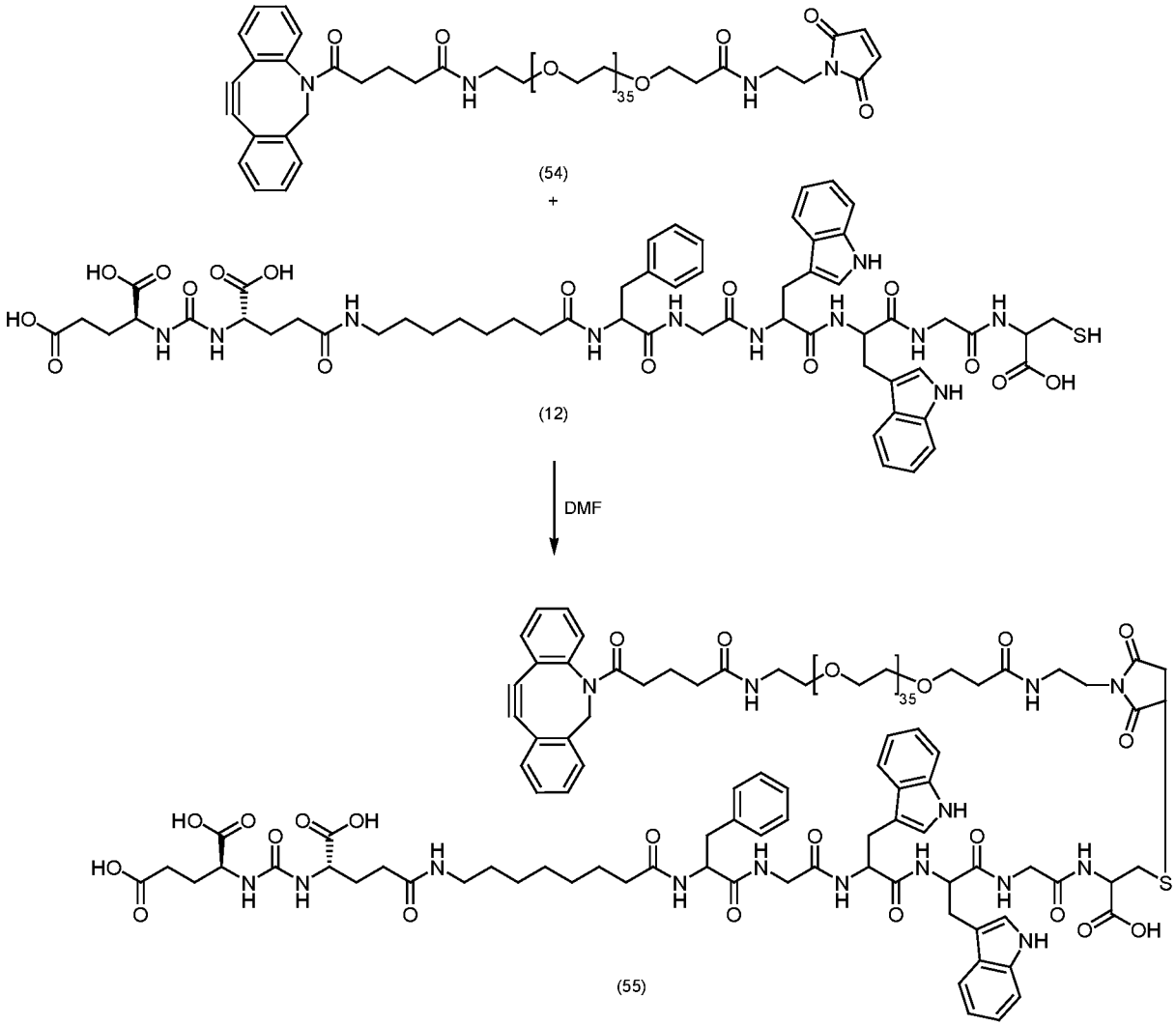
(52)



(54)

A solution of DBCO-PEG_{36}-TFP (Compound 52; 24 µmol, 1.0 eq, 60 mM) in DCM (0.40 mL) was mixed with a solution of NH_2-MAL (Compound 53; 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-PEG_{36}-[CONH]-MAL (Compound 54; calculated monoisotopic mass: 2097.15 Da; measured: 2097.16 Da).

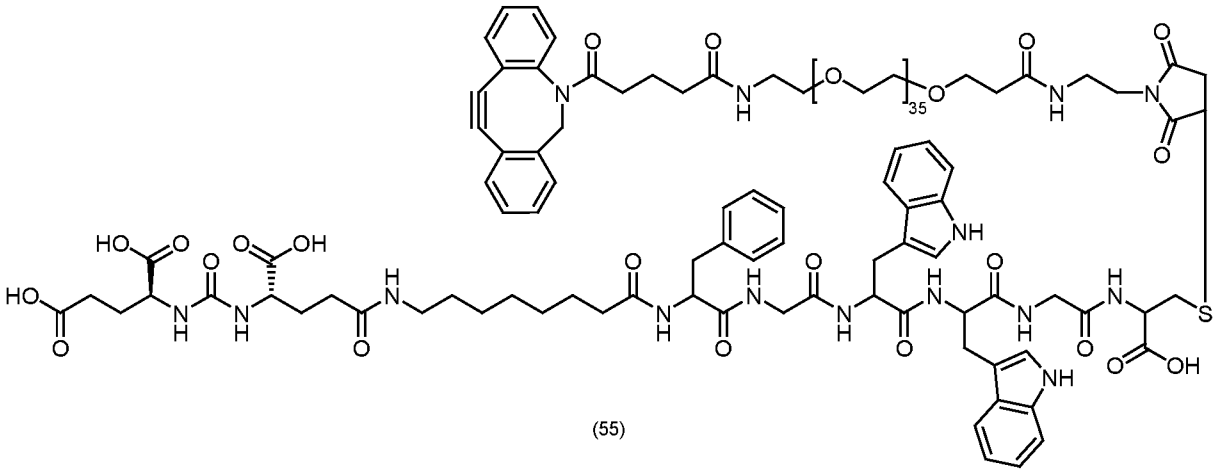
Step 2. Synthesis of DBCO-PEG$_{36}$-[CONH]-DUPA (Compound 55)



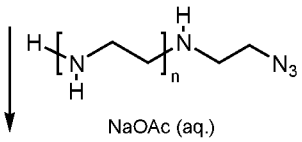
A solution of DBCO-PEG$_{36}$-[CONH]-MAL (Compound 54; 24 µmol, 1.0 eq, 120 mM) in DMF (0.20 mL) was mixed with a DUPA-Aoc-Phe-Gly-Trp-Trp-Gly-Cys (Compound 12; SEQ ID NO:4) (17 µmol, 0.7 eq, 123 mM) in DMF (137 µL). The reaction mixture was incubated on a Stuart rotator at room temperature and protected 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 55) 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-PEG$_{36}$-[CONH]-

DUPA (Compound 55; 36 mg) calculated monoisotopic mass: 3294.64 Da; measured: 3294.65 Da).

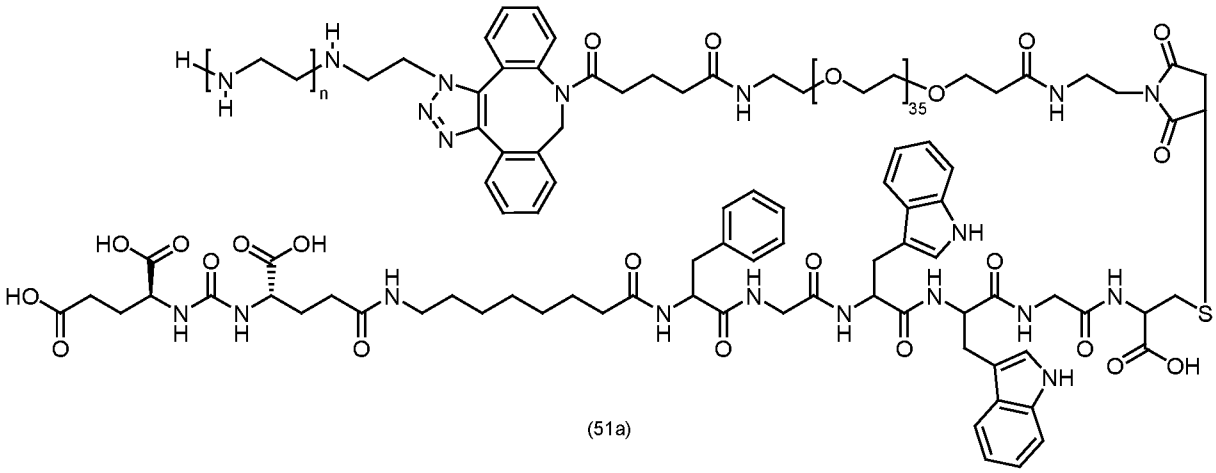
Step 3. Synthesis of LPEI-*l*-[N_3:DBCO]CONH-PEG_{36}-DUPA (Compounds 51a and 51b)



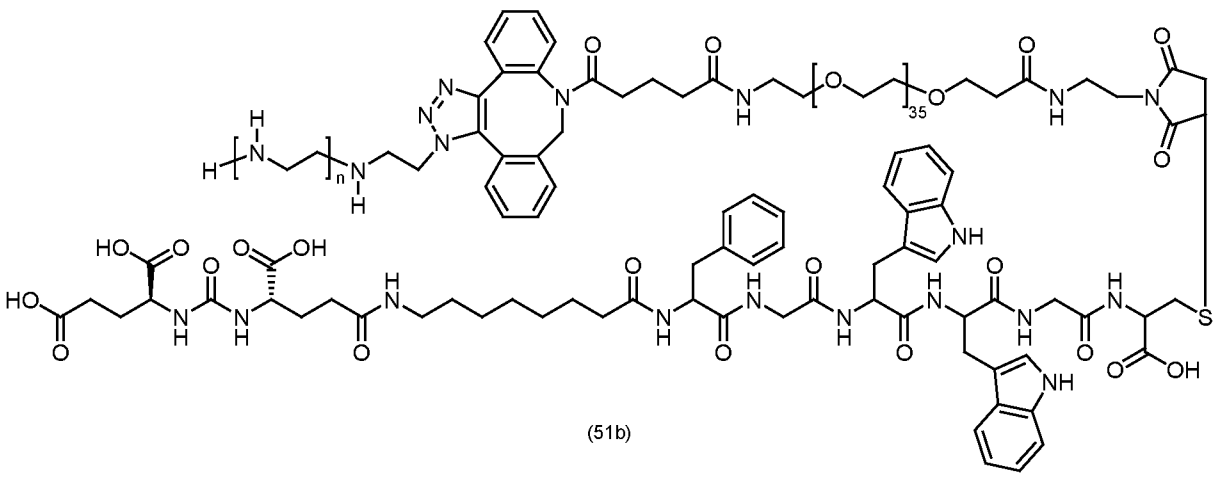
(55)



NaOAc (aq.)



(51a)



(51b)

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-PEG_{36}-[CONH]-DUPA (Compound 55; 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]-PEG_{36}-[CONH]-DUPA (87 mg) as a mixture of regioisomers 51a and 51b 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]-PEG_{36}-[CONH]-DUPA (Compounds 51a and 51b) HEPES salt

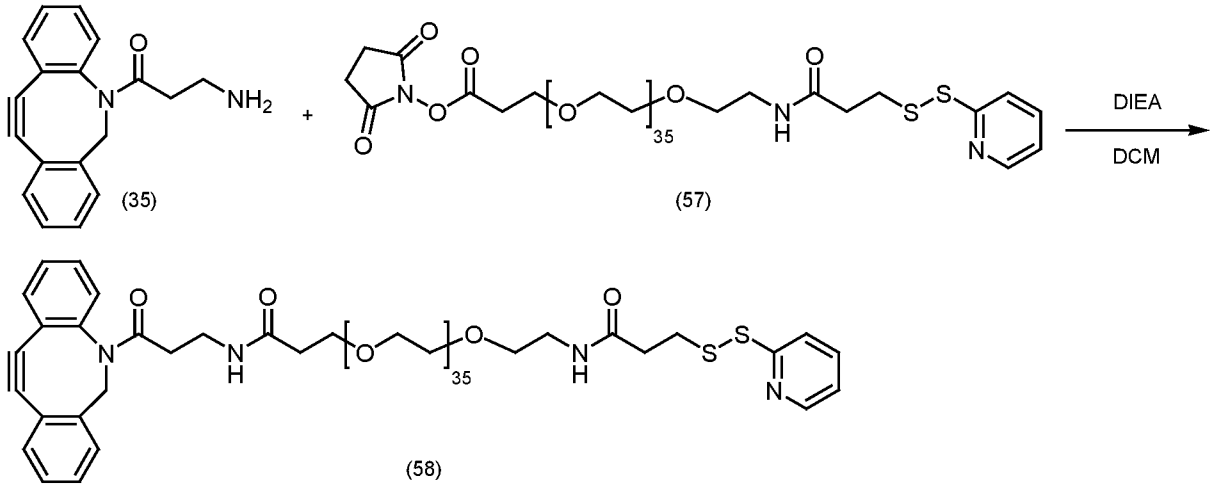
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[CONH]-DUPA (Compounds 51a and 51 b) TFA salt (20.8 mg, w_{LPEI} = 30%, 6.2 mg in total LPEI) was dissolved in 1.2 mL 20 mM HEPES pH 7.2. Three centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[CONH]-DUPA solution 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]-PEG_{36}-[CONH]-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 LPEId.

EXAMPLE 15

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA (COMPOUNDS 56a AND 56b)

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA was prepared as a mixture of regioisomers 56a and 56b according to the schemes below. DBCO-PEG_{36}-SH (Compound 59) was prepared by condensing DBCO-NH_2 (Compound 35) with NHS-PEG_{36}-OPSS (Compound 57) and subsequent reduction. Compound 59 was then condensed with DUPA-MAL (Compound 60) and reacted with LPEI-N_3 to give Compounds 56a and 56b.

Step 1. Synthesis of DBCO-PEG$_{36}$-SPDP (Compound 57)

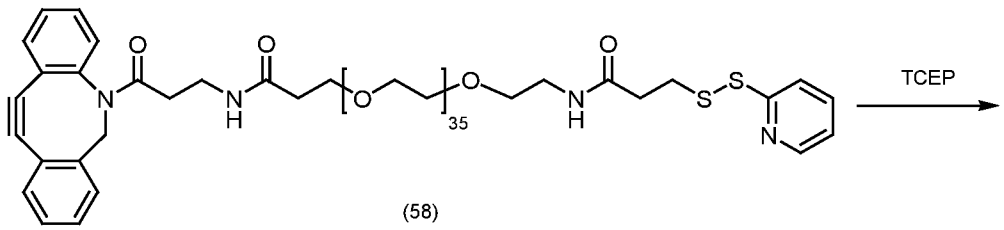


(35) (57)

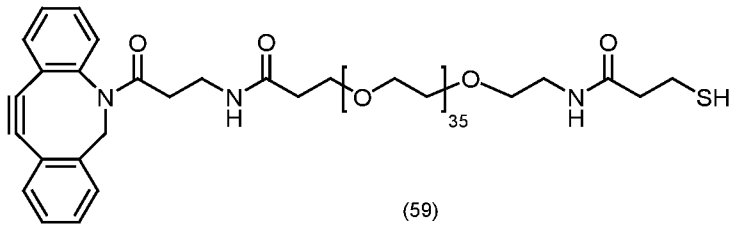
(58)

A solution of NHS-PEG$_{36}$-OPSS (Compound 57; 49 µmol, 1.0 eq, 123 mM) in DCM (0.40 mL) was mixed with a solution containing DBCO-NH$_2$ (Compound 35; 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 58) 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 59)



(58)

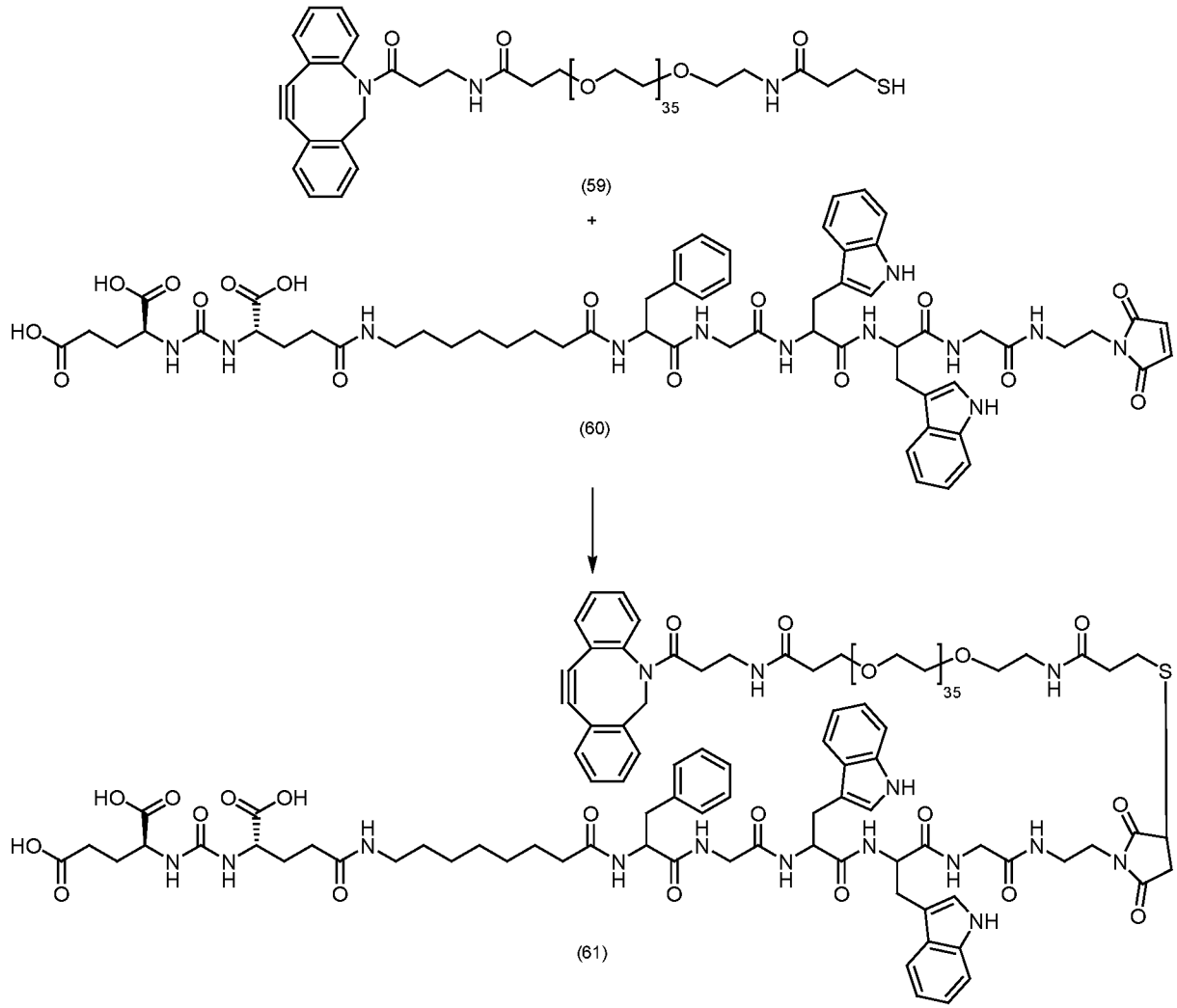


(59)

A solution of DBCO-PEG$_{36}$-OPSS (Compound 58; 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 59) was used without further purification in the next step.

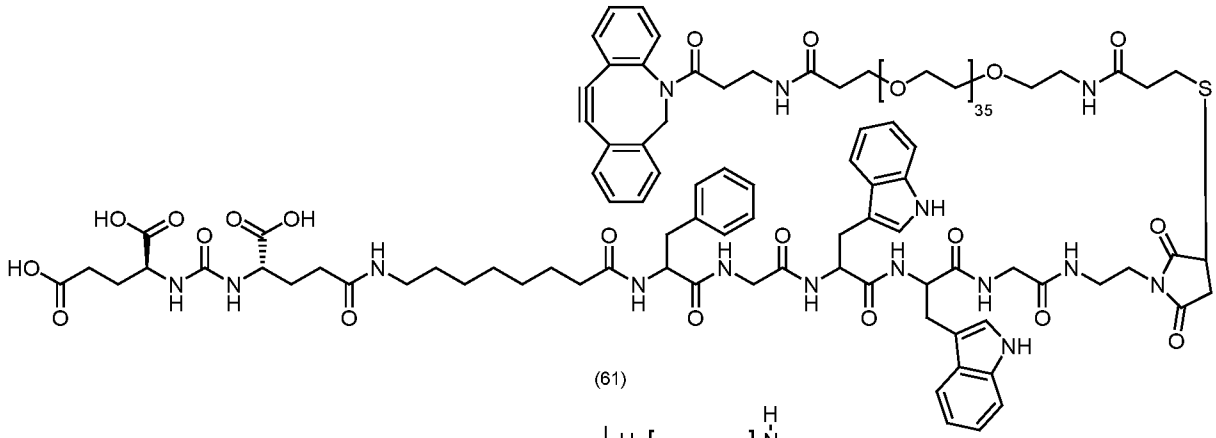
Step 3. Synthesis of DBCO-PEG_{36}-[S-MAL]-DUPA (Compound 61)



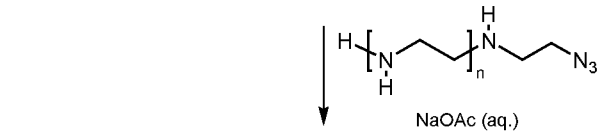
A solution of DUPA-MAL (Compound 60; 4.0 µmol, 1.0 eq, 2.5 mM) in 20 mM HEPES pH 7.4 (1.6 mL) was added to the solution of DBCO-PEG_{36}-SH (Compound 59; 364 µL, 4.0 µmol, 1.0 eq) prepared in Step 2 and the reaction mixture was incubated on a Stuart rotator at room temperature and monitored by RP-C_8-HPLC. 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 61) 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).

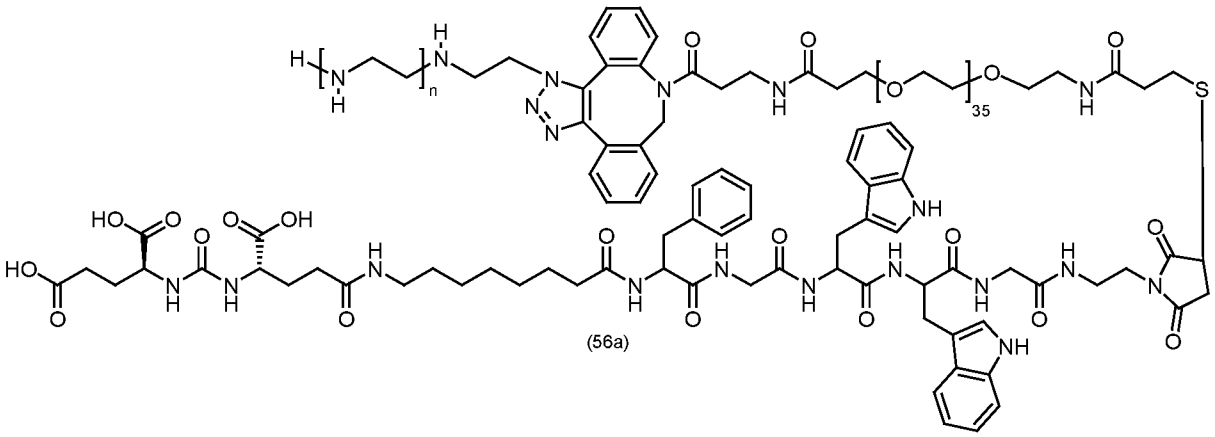
Step 4. Synthesis of LPEI-*l*-[N$_3$:DBCO]-PEG$_{36}$-[S-MAL]-DUPA (Compounds 56a and 56b)



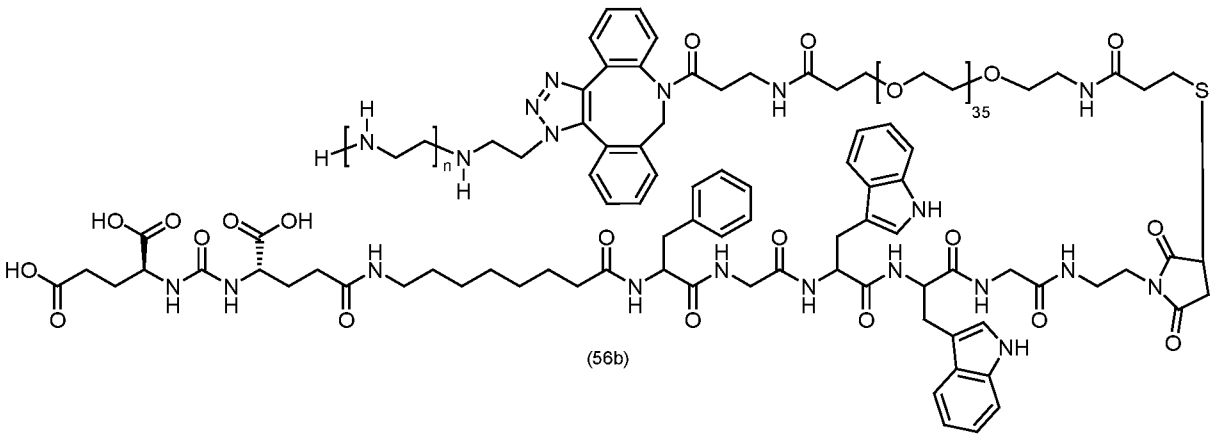
(61)



NaOAc (aq.)



(56a)



(56b)

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 61; 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 56a and 56b 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 56a and 56b) HEPES salt

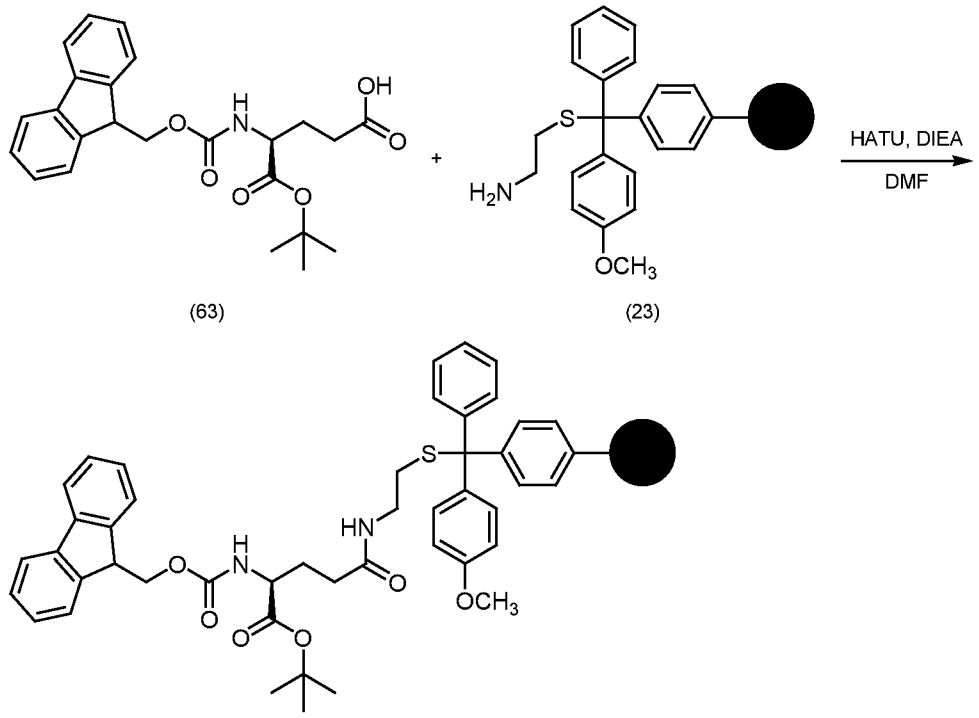
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA (Compound 56a and 56b) TFA salt (24.9 mg, w_{LPEI} = 28%, 7.0 mg in total LPEI) was dissolved in 0.8 mL 20 mM HEPES pH 7.2. Two centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA solution 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 56a and 56b) 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 16

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX (COMPOUNDS 62a AND 62b)

Compounds 62a and 62b were synthesized as a mixture of regioisomers 62a and 62b according to the schemes below. Thiol-modified methotrexate MTX-SH (Compound 68) was prepared using solid phase synthesis. Compound 68 was condensed via Michael addition with DBCO-PEG_{36}-MAL (Compound 36), and the resulting DBCO-PEG_{36}-MTX was reacted with LPEI-N_3 to give Compounds 62a and 62b.

Step 1. Synthesis of Fmoc-Glu-(OtBu)-cysteamine-4-methoxy trityl resin (Compound 64)

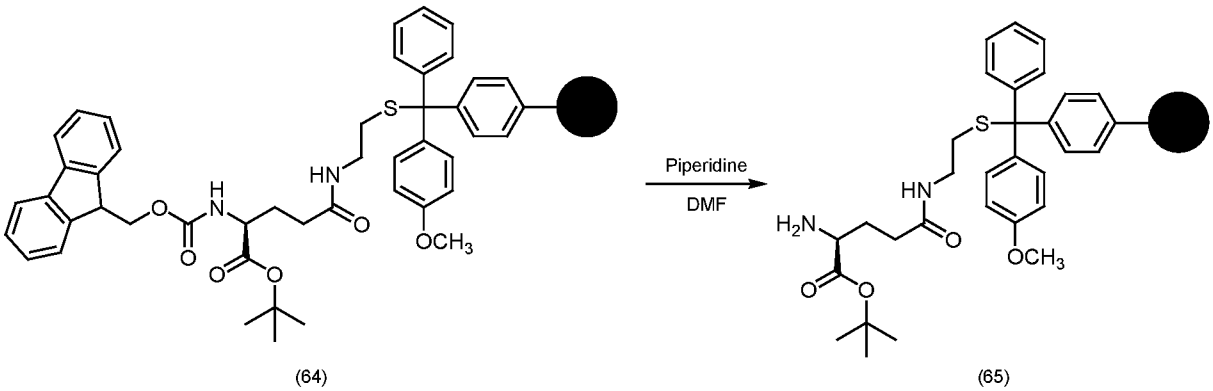


(63) (23)

(64)

A solution of Fmoc-Glu-(OtBu) (Compound 63; 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 23; 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 64) 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-methoxy trityl resin (Compound 65)

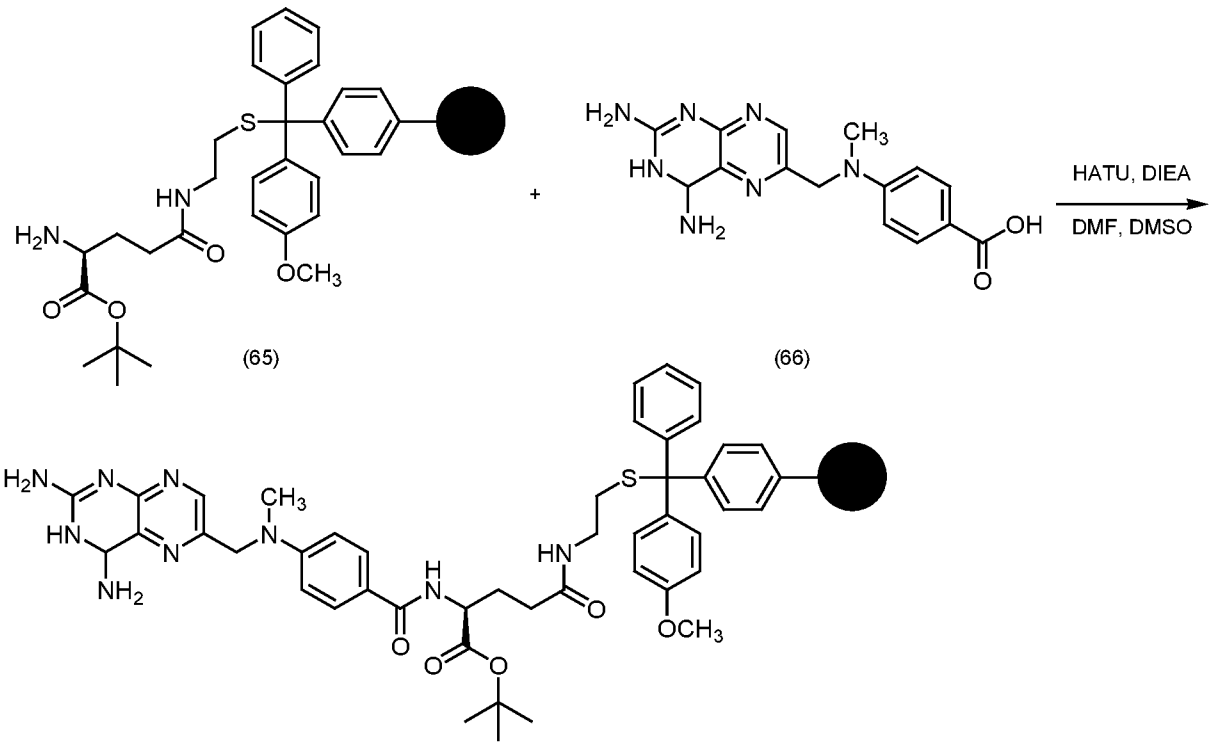


(64) (65)

A solution of 25% piperidine in DMF (5 mL) was added to the Fmoc-Glu-(OtBu)-cysteamine-4-methoxy trityl resin (Compound 64) 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 65).

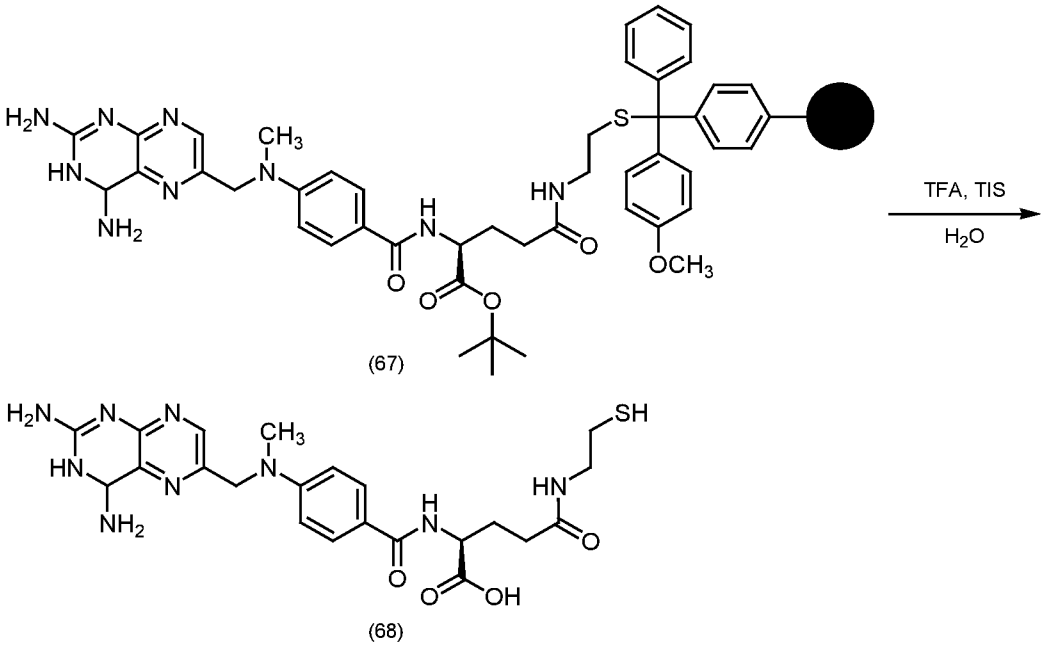
Step 3. Synthesis of MTX-4-methoxy trityl resin (Compound 67)



(65) (66) (67)

A solution of N^{10}-Methyl-4-amino-4-deoxypteroic acid (MADOPA; Compound 66; 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 65) 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 67).

Step 4. Synthesis of MTX-SH (Compound 68)

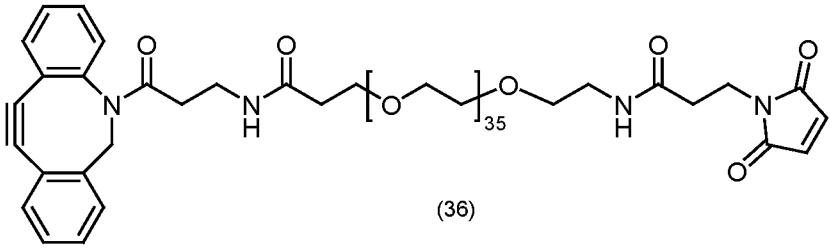


(67)

(68)

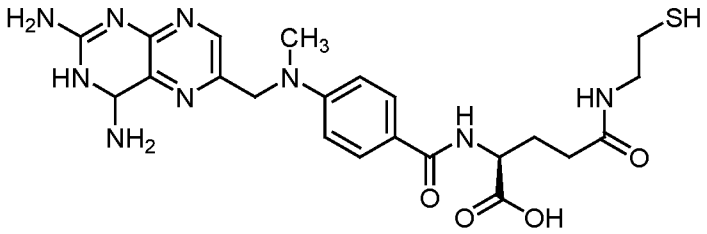
A solution of TFA/TIS/H_2O (95:2.5:2.5) (4 mL) was added to the MTX-4-methoxy trityl resin (Compound 67) prepared in Step 3. The reaction mixture was incubated for one hour on a shaker at room temperature. The resin was filtered, and the filtrate was recovered and concentrated under nitrogen flow for 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 68) 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 69)



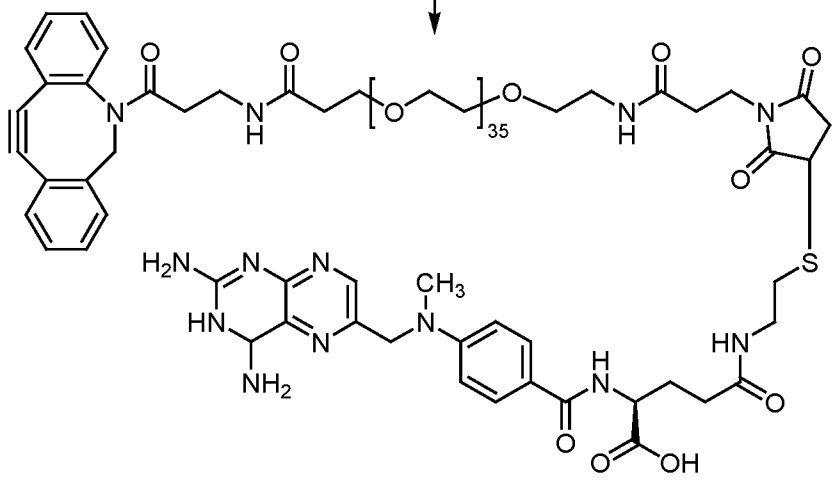
(36)

+



(68)

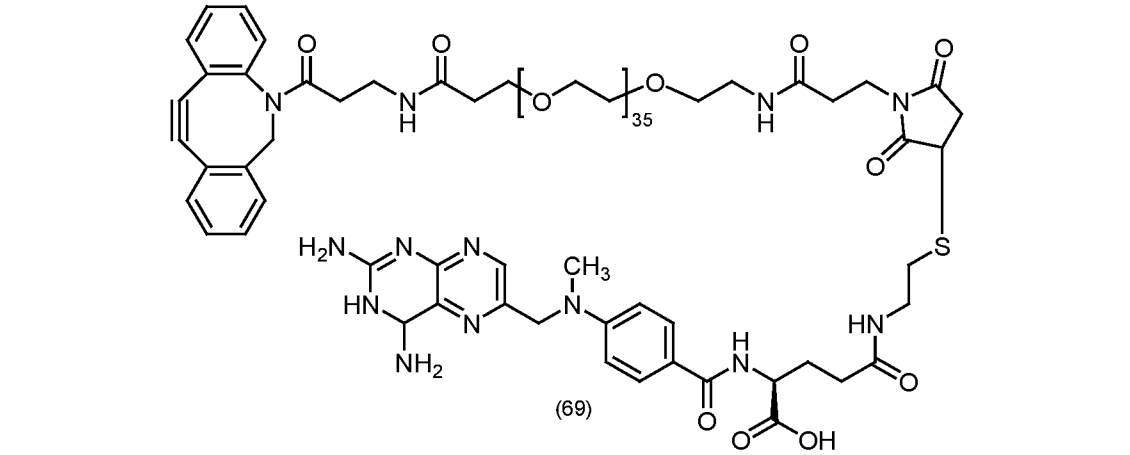
DMSO, HEPES



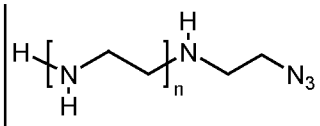
(69)

A solution of MTX-SH (Compound 68; 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 36; 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 69; 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).

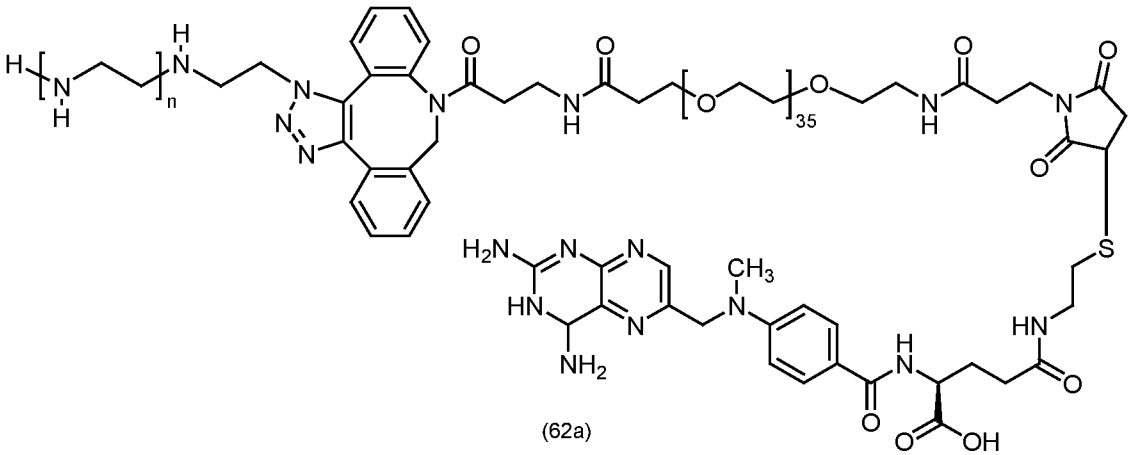
Step 6. Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX (Compounds 62a and 62b)



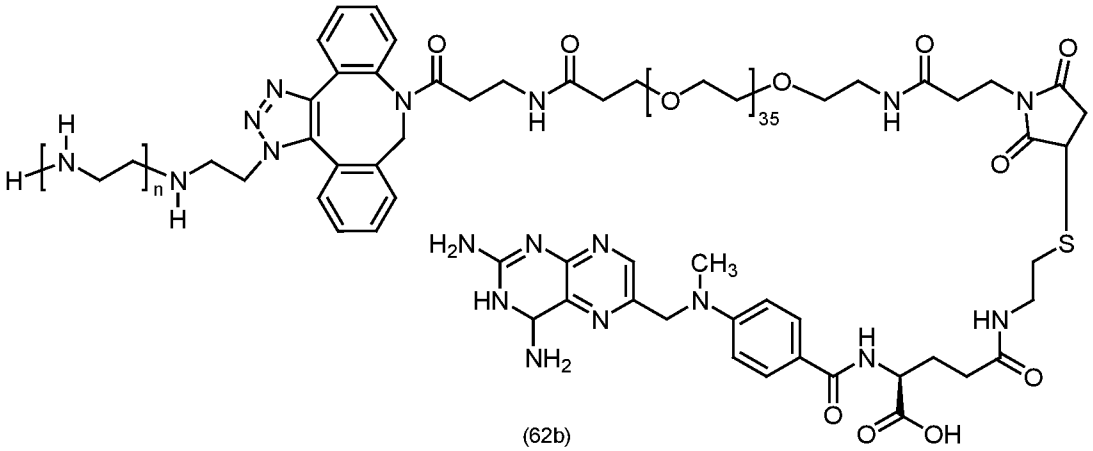
(69)



NaOAc (aq)



(62a)



(62b)

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 69; 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 62a and 62b 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 62a and 62b) HEPES salt

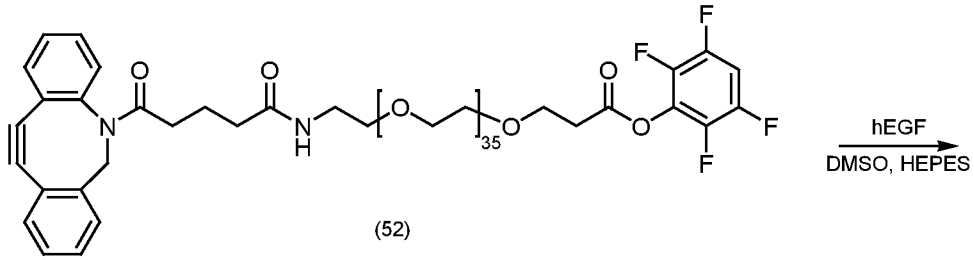
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX (Compounds 62a and 62b) TFA salt (23.8 mg, w_{LPEI} = 34%, 8.1 mg in total LPEI) were dissolved in 0.8 mL 20 mM HEPES pH 7.2. Two centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX solution 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 17

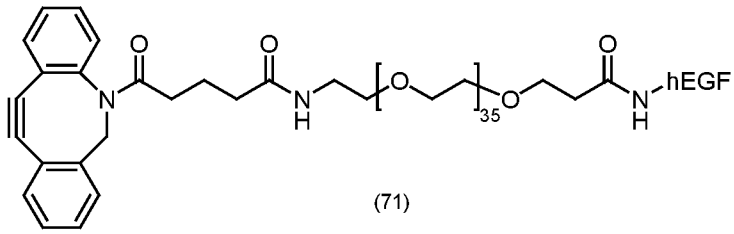
SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF (COMPOUNDS 70a AND 70b)

LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF was prepared as a mixture of regioisomers 70a and 70b according to the schemes below. DBCO-PEG_{36}-TFP (Compound 52) was condensed with hEGF, and the resulting DBCO-PEG_{36}-hEGF (Compound 71) was reacted with LPEI-N_3 to give Compounds 70a and 70b.

Step 1. Synthesis of DBCO-PEG_{36}-hEGF (Compound 71)



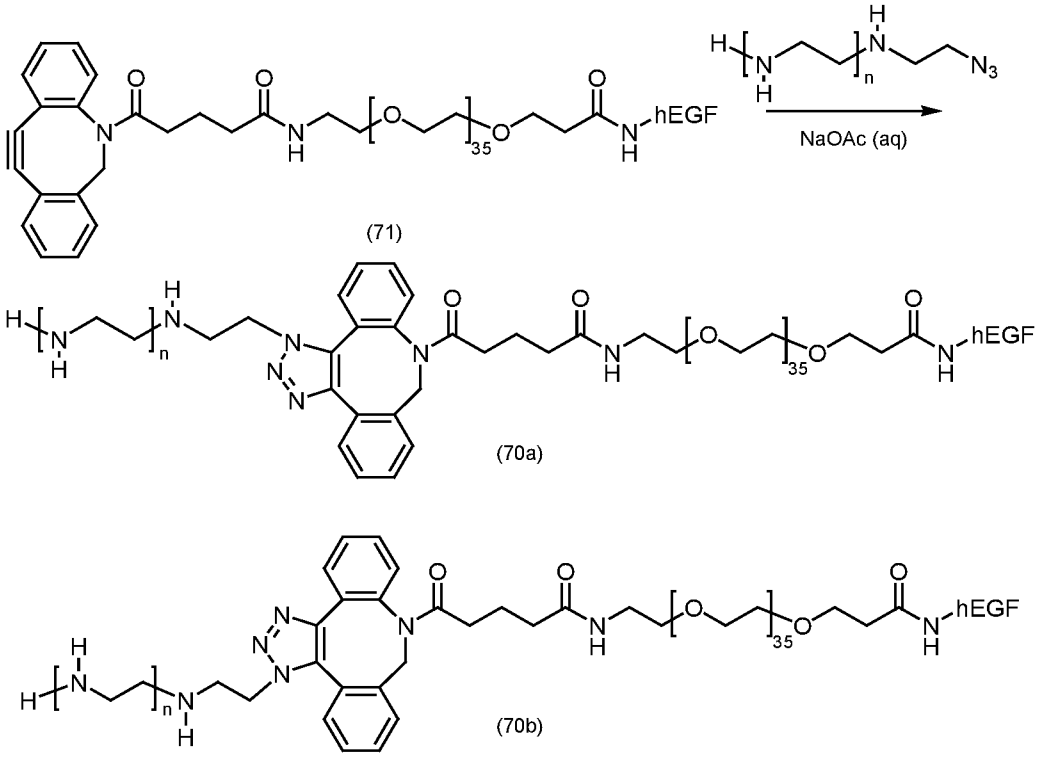
(52)



(71)

A solution of DBCO-PEG$_{36}$-TFP (Compound 52; 128 µmol, 1.4 eq, 64 mM) in DMSO (2.0 mL) was slowly added to a solution of hEGF (92 µmol, 1.0 eq, 2.6 mM) in 20 mM HEPES pH 7.5 (35 mL). The reaction mixture was stirred in a round-bottom flask and the reaction was monitored by RP-C$_8$-HPLC. After one hour, an additional amount of DBCO-PEG$_{36}$-TFP (140 µL, 9 µmol, 0.1 eq, 64 mM) was added. After a total of 1.5 hrs, acetonitrile (4 mL) was added to the reaction mixture and the pH adjusted to 3.5. DBCO-PEG$_{36}$-hEGF (Compound 71) was isolated following RP-C$_{18}$ preparative HPLC. Pooled fractions were lyophilized to give 310 mg of a fluffy white solid which was analyzed by RP-HPLC-ELSD and RP-HPLC – ESI$^+$ qTOF mass spectrometry (DBCO-PEG$_{36}$-hEGF calculated monoisotopic mass: 8168.79 Da; measured: 8168.80 Da).

Step 2. Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF (Compounds 70a and 70b)



LPEI-N_3 solution (24 mL, 23 µmol, 1.0 eq, 0.94 mM) in 50 mM acetate buffer pH 4.0 was slowly added to a solution of DBCO-PEG_{36}-hEGF (Compound 71; 16 mL, 22 µmol, 1.0 eq) in 50 mM acetate buffer pH 4.0. The reaction mixture was stirred in a round-bottom flask and monitored by RP-C_8-HPLC. After a total of 72 hours, acetonitrile (4 mL) and TFA (400 µL) were added to the reaction mixture. LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF was isolated as a mixture of regioisomers 70a and 70 b using RP-C_{18} preparative HPLC. Pooled fractions were lyophilized (505 mg) and characterized by RP-C_8-HPLC, copper assay and spectrophotometry at 280 nm for determination of the hEGF content.

The lyophilizate was dissolved in 50 mM acetate, pH 4.5 and processed by TFF (10 kDa MWCO membrane) to remove TFA residues. A solution of LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF (Compounds 70a and 70b) acetate (42 mL) was recovered and characterized by RP-C_8-HPLC, copper assay and spectrophotometry at 280 nm for determination of the hEGF content. The solution had a concentration of 2.6 mg/mL in total LPEI and a LPEI to hEGF ratio of 1/1.0.

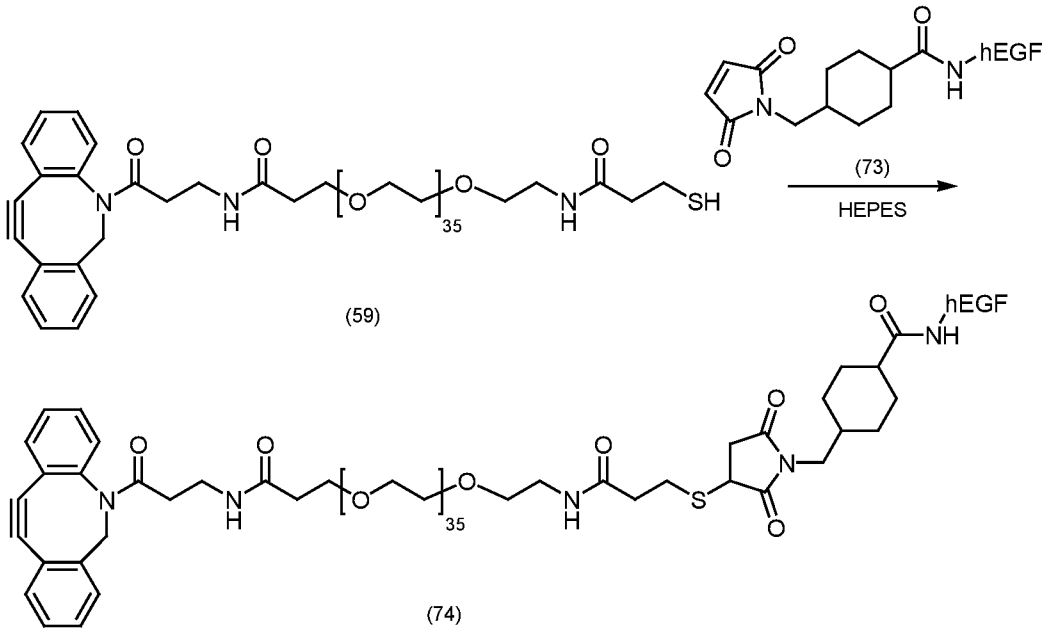
EXAMPLE 18

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-hEGF (COMPOUNDS 72a AND 72b)

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-hEGF was prepared as a mixture of regioisomers

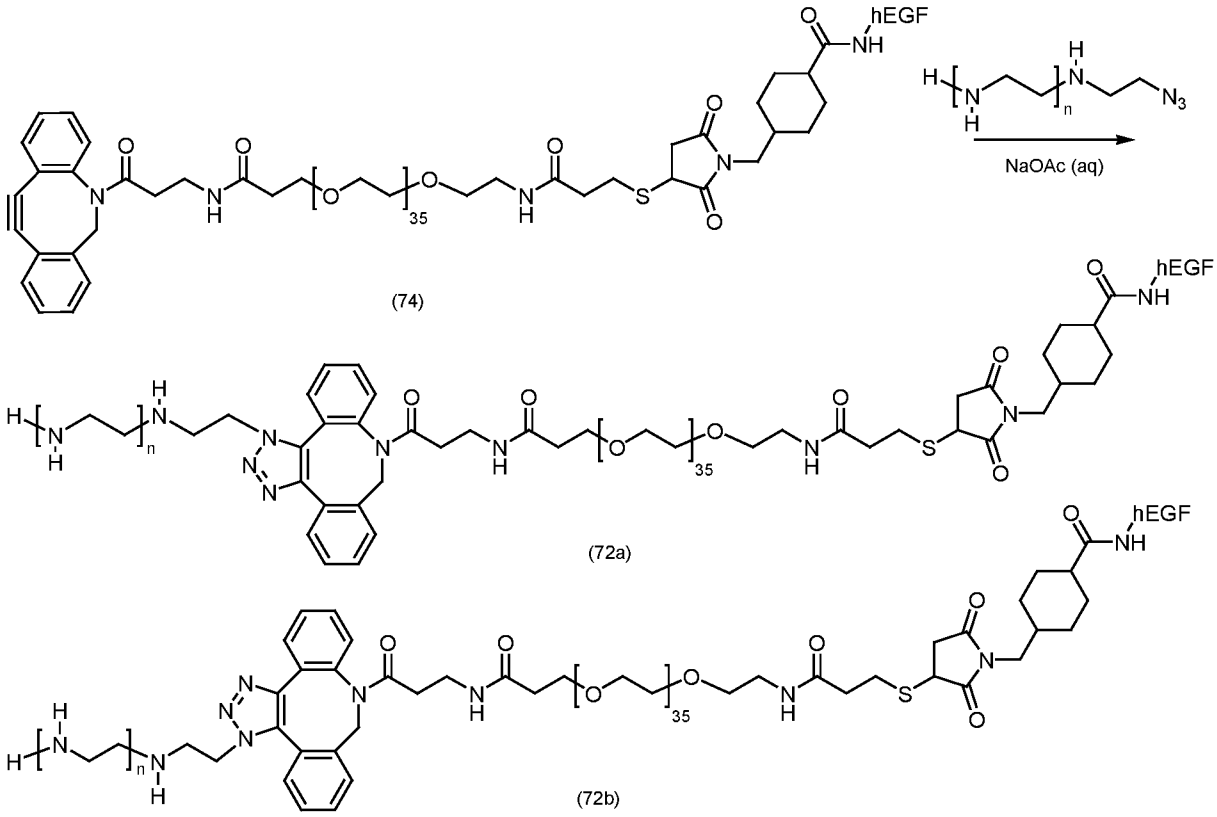
72a and 72b according to the schemes below. DBCO-PEG_{36}-[S-MAL]-hEGF (Compound 74) was prepared by condensing hEGF with MCC-hEGF (Compound 73). The resulting DBCO-PEG_{36}-[S-MAL]-hEGF (Compound 74) was reacted with LPEI-N_3 to give Compounds 72a and 72b.

Step 1. Synthesis of DBCO-PEG_{36}-[S-MAL]-hEGF (Compound 74)



A solution of DBCO-PEG_{36}-SH (Compound 59) (230 µL, 3.0 µmol, 1.0 eq) was prepared as described in Example 15. A solution of MCC-hEGF (Compound 73; 2.9 µmol, 1.0 eq, 0.58 mM based on 77% measured peptide content; CBL Patras S.A. (Greece)) in 20 mM HEPES pH 7.2 (5.0 mL) was added and the reaction mixture was incubated on a Stuart rotator at room temperature and was monitored by RP-C_8-HPLC. After 15 min, an additional amount of DBCO-PEG_{36}-SH solution (20 µL, 0.3 µmol, 0.1 eq) was added. After a total of 30 minutes, acetonitrile (0.56 mL) was added and the reaction mixture was purified by RP-C_{18} preparative HPLC. DBCO-PEG_{36}-[S-MAL]-hEGF (Compound 74) was isolated and pooled fractions containing Compound 74 were lyophilized. The lyophilisate (15 mg) was analyzed by RP-HPLC-ELSD and RP-HPLC – ESI^+ qTOF mass spectrometry (DBCO-PEG_{36}-[S-MAL]-hEGF calculated monoisotopic mass: 8450.90 Da; measured: 8450.97 Da).

Step 2. Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-hEGF (Compounds 72a and 72b)



LPEI-N_3 solution (2.5 mL, 2.0 µmol, 1.2 eq, 0.84 mM) in 50 mM acetate buffer pH 4.0 was slowly added to 4.0 mL of a solution of DBCO-PEG_{36}-[S-MAL]-hEGF (Compound 74; 1.7 µmol, 1.0 eq, 0.43 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 (0.72 mL) and TFA (73 µL). LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-hEGF was isolated as a mixture of regioisomers 72a and 72b using RP-C_{18} preparative HPLC and characterized by RP-C_8-HPLC, copper assay and spectrophotometry at 280 nm for determination of the hEGF content. The lyophilisate had a weight percentage in LPEI of 25% w/w and a LPEI to hEGF ratio of 1/1.09.

Step 3. Preparation of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-hEGF (Compounds 72a and 72b) HEPES salt

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-hEGF (Compounds 72a and 72b) TFA salt (26.4 mg, w_{LPEI} = 25%, 6.6 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]-hEGF 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 212 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-hEGF (Compounds 72a and 72b) 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 2.3 mg/mL in total LPEI.

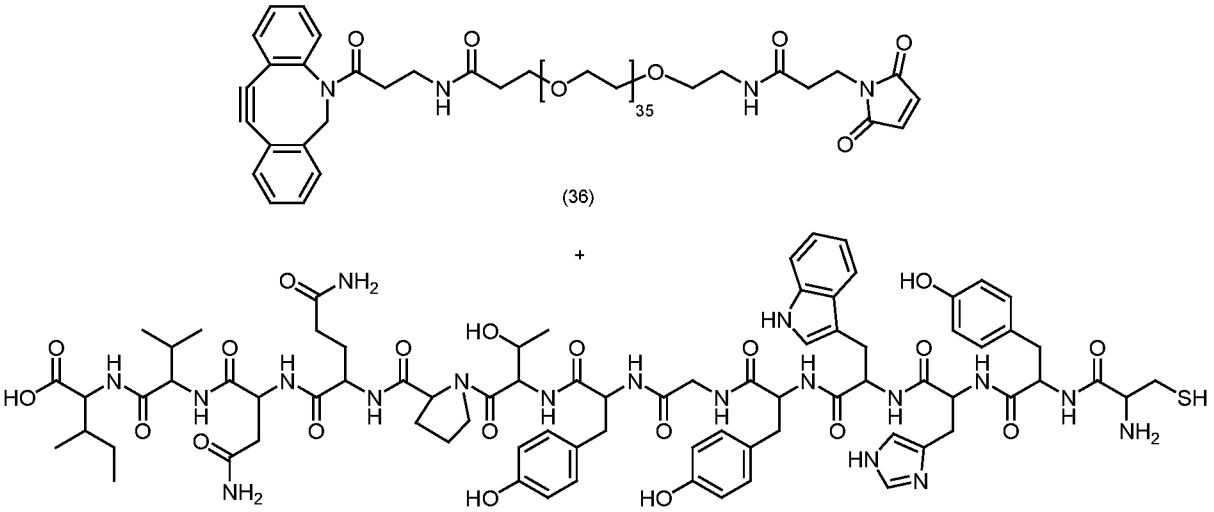
EXAMPLE 19

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-CysGE11 (COMPOUNDS 75a AND 75b)

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-GE11 was synthesized as a mixture of regioisomers 75a and 75b in two steps according to the schemes below. In the first step, human peptide Cys-GE11 (Compound 76) was coupled to (DBCO-PEG_{36}-MAL; Compound 36) in 20 mM HEPES buffer to produce DBCO-PEG_{36}-[MAL-S]-CysGE11 (Compound 77). In the second step, DBCO-PEG_{36}-[MAL-S]-CysGE11 was conjugated to LPEI-N_3 to produce LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-CysGE11 (Compounds (75a and 75b).

Step 1.Synthesis of DBCO-PEG_{36}-[MAL-S]-CysGE11

A solution of CysGE11 peptide (Compound 76; 6.5 µmol, 1.0 eq, 0.93 mM) in 20 mM HEPES pH 7.4 (7.0 mL) was mixed with a solution of TCEP (6.5 µmol, 1.0 eq, 85 mM) in 20 mM HEPES pH 7.4 (76 µL). A solution of DBCO-PEG_{36}-MAL (Compound 36; 7.8 µmol, 1.2 eq, 24 mM) in DMSO (0.32 mL) was then added and the reaction mixture was incubated on a Stuart rotator at room temperature. After a total of 30 minutes, acetonitrile (0.8 mL) was added to the reaction mixture which was purified by RP-C_{18} preparative HPLC. Lyophilization of pooled fractions yielded DBCO-PEG_{36}-[MAL-S]-CysGE11 (Compound 77) as a solid (13 mg; calculated monoisotopic mass: 3725.85 Da; measured: 3725.90 Da).

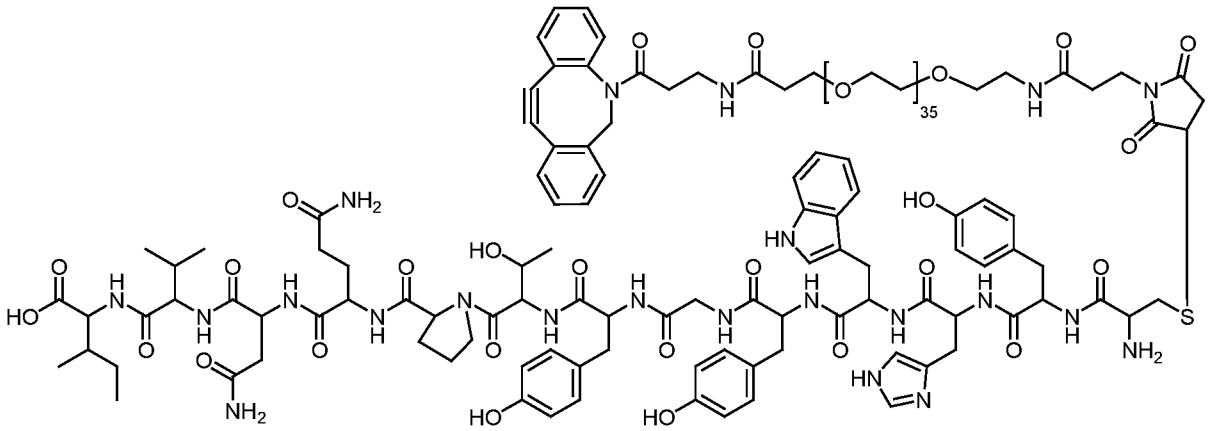


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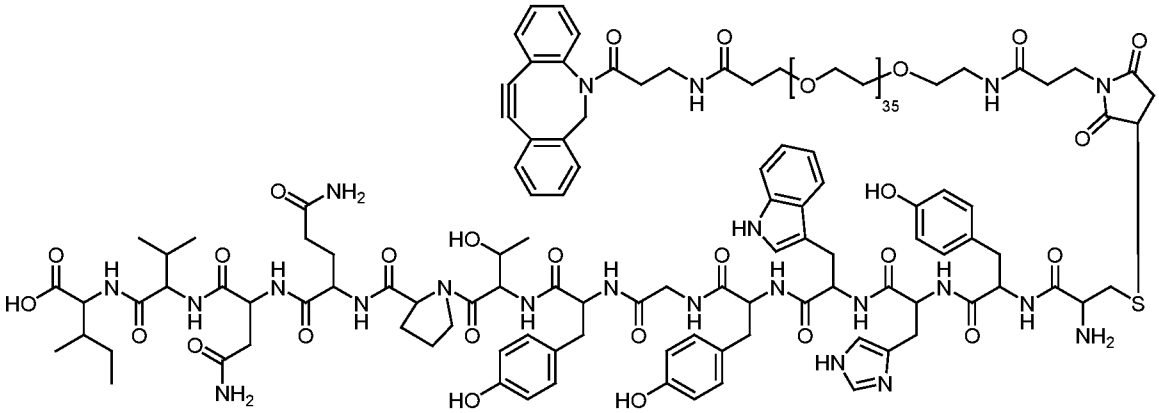
(76)

H_2O/MeCN
DMSO

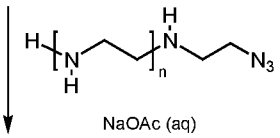


(77)

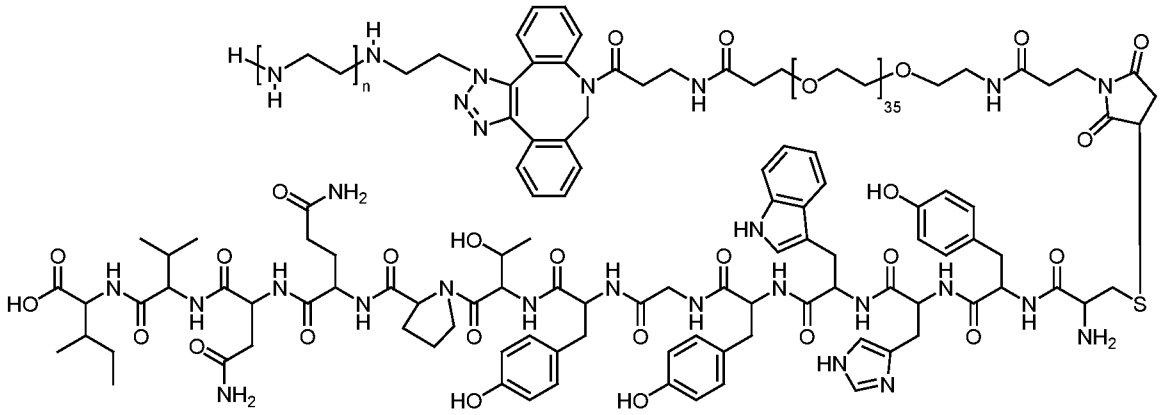
Step 2. Synthesis of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-CysGE11



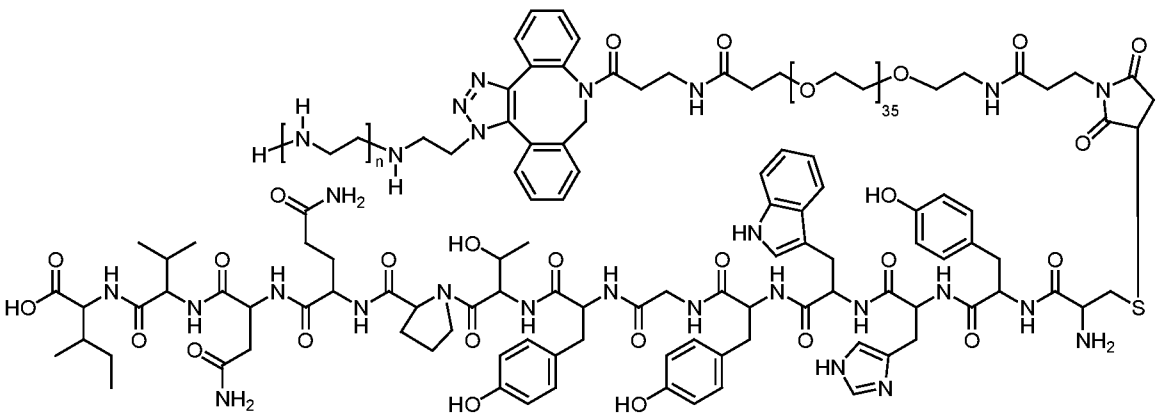
(77)



NaOAc (aq)



(75a)



(75b)

LPEI-N_3 solution (3.0 mL, 2.5 µmol, 1.0 eq, 0.84 mM) in 50 mM acetate buffer pH 4.0 was slowly added to 4.0 mL of a solution of DBCO-PEG_{36}-[MAL-S]-CysGE11 (Compound

77; 4.3 µmol, 1.7 eq, 1.1 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 16 hours, acetonitrile (0.78 mL) and TFA (78 µL) were added to the reaction mixture which was purified using RP-C_{18} preparative HPLC. Pooled fractions were lyophilized to yield LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-CysGE11 (60 mg) as a mixture of regioisomers 75a and 75b, which were characterized by RP-C_8-HPLC, copper assay and spectrophotometry at 280 nm to determination the peptide content. The lyophilizate had a weight percentage in LPEI of 27% w/w and a LPEI to CysGE11 ratio of 1/1.1.

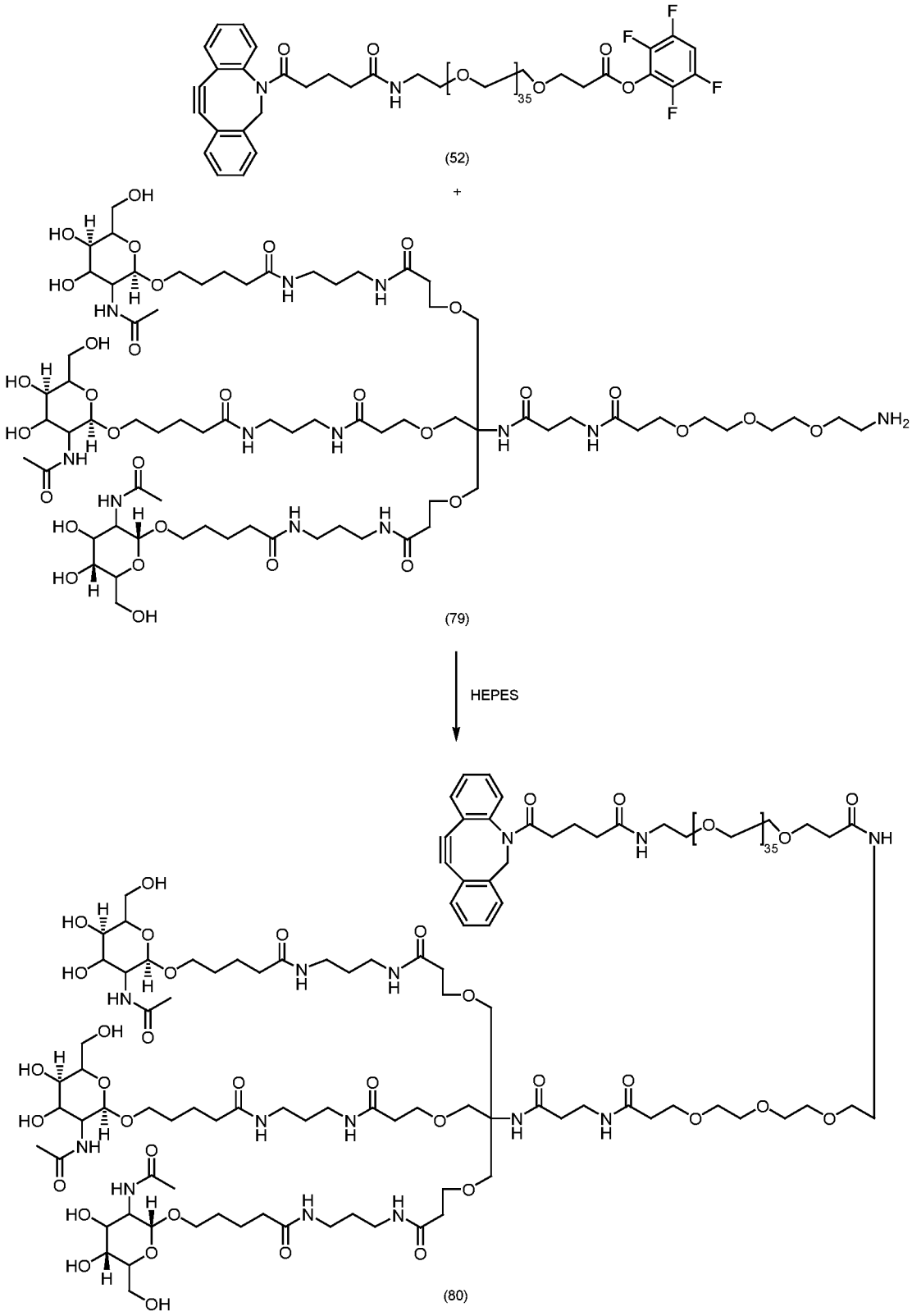
Step 3. Preparation of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-CysGE11 (Compounds 75a and 75b) HEPES salt

LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-CysGE11 (Compounds 75a and 75b) TFA salt (27.3 mg, w_{LPEI} = 27%, 7.4 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 each of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-CysGE11 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 245 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-CysGE11-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 (2.6 mg/mL in total LPEI and a LPEI to CysGE11 ratio of 1/1.1).

EXAMPLE 20

SYNTHESIS OF LPEI-*l*-[N_3:DBCO]-PEG_{36}-$(GalNAc)_3$ (COMPOUNDS 78a AND 78b)

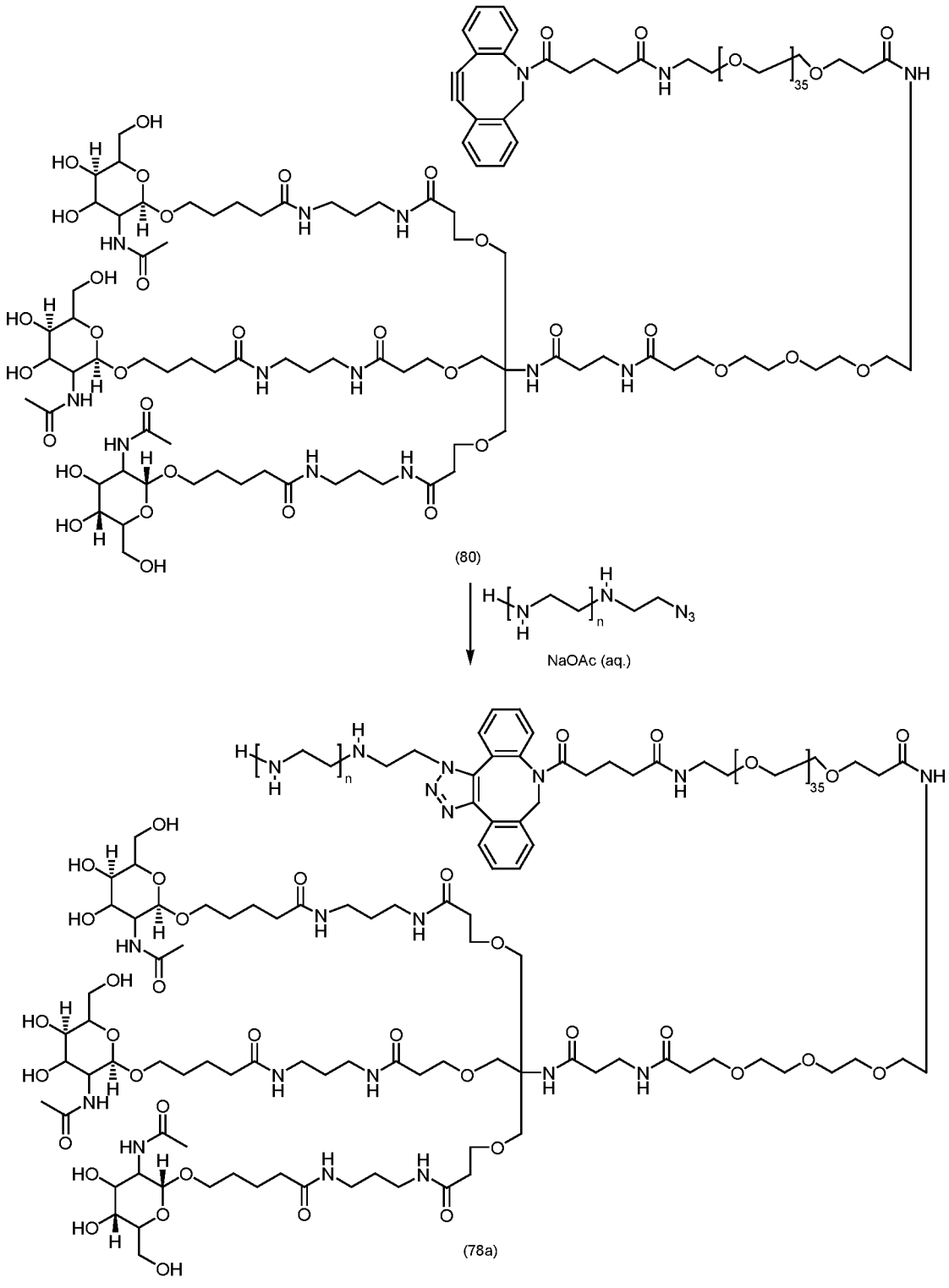
Step 1. Synthesis of DBCO-PEG_{36}-$(GalNAc)_3$ (Compound 80)

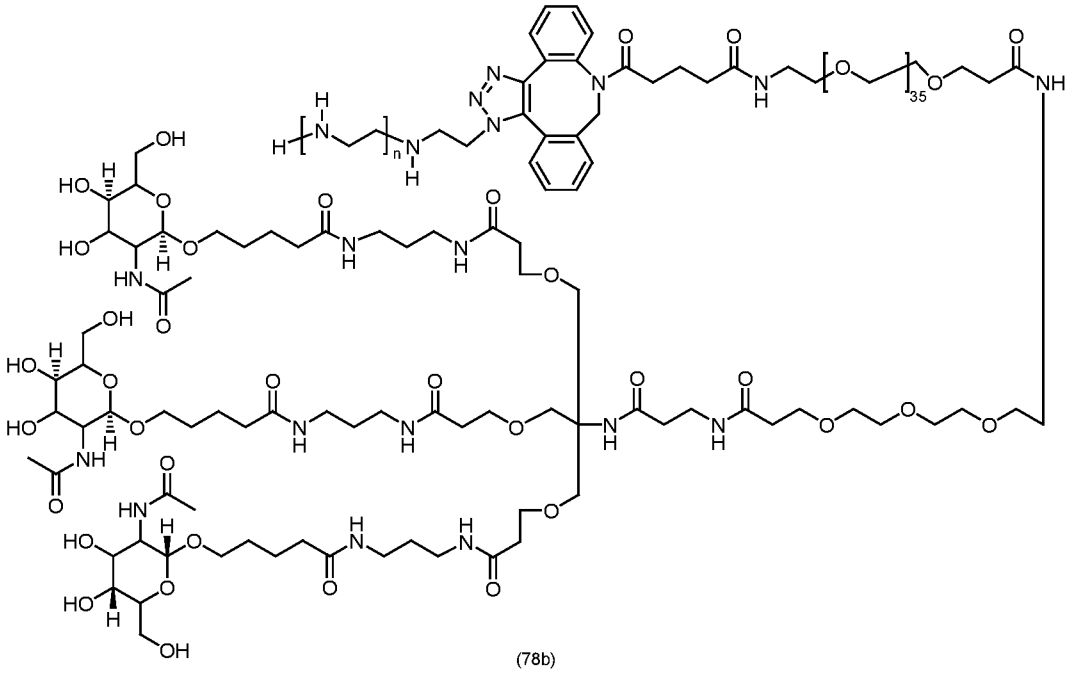


A solution of $(GalNAc)_3$-PEG_3-NH_2 (Compound 79; 5.6 µmol, 1.0 eq, 7.2 mM) in 20 mM HEPES pH 7.4 (0.5 mL) was added to a solution of DBCO-PEG_{36}-TFP (Compound 52; 7.5 µmol, 1.3 eq, 48 mM) in DMSO (0.155 mL). The reaction mixture was placed on a Stuart rotator at room temperature and the reaction was monitored by RP-C_8-HPLC. After 2 hours an

additional 3.0 mL of 20 mM HEPES buffer pH 7.4 was added. After a total of 20 hours, milliQ water (3.4 mL) and acetonitrile (0.78 mL) were added to the reaction mixture, which was purified using RP-C_{18} preparative HPLC. Pooled fractions containing purified DBCO-PEG_{36}-$(GalNAc)_3$ (Compound 80) were lyophilized to yield a solid (10 mg; ESI^+ qTOF mass spectrometry, calculated monoisotopic: mass: 3646.00 Da; measured: 3646.02 Da).

Step 2. Synthesis of LPEI-*l*-[N_3:DBCO]- PEG_{36}-$GalNAc)_3$ (Compounds 78a and 78b)





(78b)

LPEI-N_3 solution (3.0 mL, 2.5 µmol, 1.0 eq, 0.84 mM) in 50 mM acetate buffer pH 4.0 was slowly added to 4.0 mL of a solution of DBCO-PEG_{36}-$(GalNAc)_3$ (Compound 80; 3.0 µmol, 1.2 eq, 0.76 mM) in 50 mM acetate buffer pH 4.0. The mixture was placed on a Stuart rotator and protected from light. After 16 hours, acetonitrile (0.78 mL) and TFA (79 µL) were added to the reaction mixture for preparative chromatography. LPEI-*l*-[N_3:DBCO]-PEG_{36}-$(GalNAc)_3$ (Compounds 78a and 78b) was isolated as a mixture of regioisomers 78a and 78b using RP-C_{18} preparative HPLC and characterized by RP-C_8-HPLC and copper assay. Lyophilisate (63 mg) had a weight percentage in LPEI of 27% w/w.

Step 3. Preparation of LPEI-*l*-[N_3:DBCO]-PEG_{36}-GalNAc)$_3$-HEPES salt

LPEI-*l*-[N_3:DBCO]-PEG_{36}-$(GalNac)_3$ (Compounds 78a and 78b) TFA salt (42 mg, w_{LPEI} = 27%, 11.3 mg in total LPEI) was solubilized in 1.6 mL 20 mM HEPES pH 7.2. Four centrifugal filters (Amicon Ultra – 0.5 mL, 10kDa MWCO) were filled with 400 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-$(GalNac)_3$ 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 418 µL of LPEI-*l*-[N_3:DBCO]-PEG_{36}-$(GalNac)_3$-HEPES salt solution were recovered and supplemented with 4.0 mL 20 mM HEPES, pH 7.2. The concentration of the solution (4.4 mL) was determined by copper assay (2.2 mg/mL in total LPEI).

EXAMPLE 21

POLYPLEX SIZING AND ZETA POTENTIAL MEASUREMENTS

General Procedure for Polyplex Formation. For the preparation of preferred polyplexes,

triconjugates were complexed with nucleic acids such as poly(IC) at various N/P ratios in HBG buffer (20 mM HEPES, pH 7.2, 5% glucose, wt/vol). Nitrogen to phosphorous (N/P) ratios were calculated based on the nitrogen content in the LPEI portion of the used triconjugates and the phosphorous content in the nucleic acid such as poly(IC). Hereby, stock solutions of triconjugates such as LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF (also abbreviated to LPEI-*l*-PEG_{24}-hEGF in the Description) and nucleic acids such as poly(IC) were diluted with HBG to the appropriate concentrations for the selected N/P ratio. The diluted triconjugate solution was added to an equal volume of nucleic acid solution to a final concentration of 0.1-0.2 mg/mL of nucleic acid in the polyplex preparation and mixed by vigorously pipetting. The mixture was left at RT for 30 min for polyplex formation. In an analogous manner, polyplexes with polyanions such as poly(Glu) were prepared. The polyplexes were typically further characterized with respect to size and surface charge.

FIGs 1A, 1B and 1C show a comparison of physico chemical characterization for LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) polyplexes as a function of N/P ratio. FIG. 1A shows triplicate DLS backscatter measurements of z-average diameter and dispersity for a 100-mL sample of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) at an N/P ratio of 2.4. Three measurements were taken of the same sample. The concentration of the complexes in HBG is 0.125 mg/mL (pH 7.2). FIG. 1B shows triplicate DLS backscatter measurements of z-average diameter and dispersity for a 100-mL sample of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) at an N/P ratio of 4.0. Three measurements were taken of the same sample. The concentration of the complexes in HBG is 0.125 mg/mL (pH 7.2). FIG. 1C shows triplicate DLS backscatter measurements of size, z-average diameter and dispersity for a 100-mL sample of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) at an N/P ratio of 5.6. Three measurements were taken of the same sample. The concentration of the complexes in HBG is 0.125 mg/mL (pH 7.2). As shown in FIGs 1A, 1B and 1C, polyplexes with an N/P ratio of 4 and 5.6 had average diameters of 116 and 107 nm, and PDIs of 0.08 and 0.11, respectively. Polyplexes with an N/P ratio of 2.4 had an average diameter of 306 nm and a PDI of 0.35.

Analogously, FIG. 2 and FIG. 3 show triplicate DLS backscatter measurements of z-average diameter and dispersity for LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu) and LPEI-*l*-[N_3:DBCO]-PEG_{23}-OMe:poly(IC) polyplexes, being non-cytotoxic or non-targeted polyplexes, respectively, as described herein and used as control polyplexes in the cell survey experiments. FIG. 2 is a DLS back scatter plot of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu) polyplexes in HBG buffer at pH 7.2, 0.1 mg/mL, 1 mL volume, N/P ratio of 4. The z-average diameter was

121 nm with a polydispersity index (PDI) of 0.087. The ζ-potential was 28.7 mV. FIG. 3 is a DLS back scatter plot of LPEI-*l*-[N_3:DBCO]-PEG_{23}-OMe:poly(IC) polyplexes in 50 mM acetate buffer, 5% glucose at pH 4.3, 0.1875 mg/mL, 1 mL volume, N/P ratio of 4. The z-average diameter was 107 nm with a polydispersity index (PDI) of 0.139. The ζ-potential was 31.4 mV.

The data shown in FIGs 1A, 1B and 1C is summarized in Table 7, below. A larger sample volume of 900 mL was required for ζ-potential measurements.

Table 7. Physicochemical Characterization of polyplex LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) at N/P ratios of 2.4, 4.0, and 5.6

N/P	Sample vol (mL)	z-average diameter (nm)	PDI	ζ-potential (mV)
2.4	100 900	306 285	0.35 0.33	n.d. 21.9
4.0	100 900	116 105	0.08 0.18	n.d. 30.8
5.6	100 900	107 99.6	0.11 0.17	n.d. 33.4

In an analogous manner and as analogously determined, FIG. 4 and FIG. 5 show physico-chemical characterization for LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) polyplexes, both at an N/P ratio of 4. FIG. 4 is a DLS back scatter plot taken in triplicate of LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC) polyplexes measuring z-average diameter and dispersity in 50 mM acetate buffer, 5% glucose at pH 4.3, 0.1875 mg/mL, 1 mL volume, N/P ratio of 4. The z-average diameter was 156 nm with a polydispersity index (PDI) of 0.144. The ζ-potential was 38.3 mV. FIG. 5 is a DLS back scatter plot taken in triplicate of a LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA:poly(IC) polyplex measuring z-average diameter and dispersity at 0.1875 mg/mL, 1 mL volume, N/P 4. The z-average diameter was 120 nm with a polydispersity index (PDI) of 0.125. The ζ-potential was 31.1 mV.

Physico-chemical characterization of additional polyplexes prepared in the Examples above by DLS is shown in Tables 8-10 below.

Table 8. Physicochemical Characterization data for Triconjugate LPEI-*l*-PEG-hEGF/GE11:poly(IC) polyplex targeting EGFR in HBG (5% Glc), pH 7.2, N/P =4 ratio

Triconjugate	Polyplex conc. (mg/mL)	Diameter (nm)	Polydispersity Index (PDI)	ζ-potential (mV)

LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF (Compounds 7a and 7b)	0.1875	132	0.097	30.2
LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF (Compounds 4a and 4b)	0.1875	156	0.144	38.3
LPEI-*l*-[N_3:BCN]-PEG_{12}-hEGF (Compound 14)	0.1875	150	0.196	24.1
LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF (Compounds 1a and 1b)	0.1875	116	0.075	33.1
LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF (Compounds 70a and 70b)	0.1875	207	0.195	24.1
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-hEGF (Compounds 72a and 72b)	0.1875	149	0.140	26.3
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-CysGE11(Compounds 75a and 75b)	0.1875	155	0.147	23.2

Table 9. Physicochemical Characterization data for Triconjugate LPEI-*l*-PEG-DUPA:poly(IC) polyplex at 0.1875 mg/mL, in HBG (5% Glc), pH 7.2, N/P =4 ratio

Triconjugate	ZEN2112 (Quartz)		DTS1070 DLS*		
	DLS (nm)	**PDI**	**DLS (nm)**	**PDI**	**ζ-pot (mV)**
LPEI-*l*-[N_3:DBCO]-PEG_{24}-DUPA (Compounds 10a and 10b)	-	-	120	0.125	31.1
LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA (Compounds 31a and 31b)	140	0.120	123	0.138	35.2
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA (Compounds 38a and 38b)	154	0.151	148	0.153	34.5

LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA (Compound 43)	145	0.166	144	0.214	33.9
LPEI-*l*-[N_3:SCO]-PEG_{36}-DUPA (Compounds 47a and 47b)	143	0.156	132	0.153	34.0
LPEI-*l*-[N_3:DBCO]CONH-PEG_{36}-DUPA (Compounds 51a and 51b)	140	0.120	133	0.147	34.5
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA (Comounds 56a and 56b)	nd	nd	149	0.153	24.7

*for DLS and ζ-potential measured in DTS1070 cuvette samples were 2x diluted due to insufficient amount of the sample.

Table 10. Physicochemical Characterization data for Triconjugate:pIC polyplex targeting Folate, HER2 and ASGP receptors in HBG (5% Glc), pH 7.2, N/P =4 ratio

Triconjugate	Polyplex conc.	Size diam.(nm)	Polydispersity Index (PDI)	ζ-potential (mV)
LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate (Compounds 22a and 22b)	0.1875	131	0.125	33.5
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[MAL-S]-MTX (Compounds 62a and 62b)	0.1875	172	0.196	23.5
LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody (Compounds 28a and 28b)	0.1875	149	0.177	27.0
LPEI-*l*-[N_3:DBCO]-PEG_{36}-$(GalNAc)_3$ (Compounds 78a and 78b)	0.1875	149	0.213	18.9

EXAMPLE 22

CYTOTOXIC ACTIVITY OF INVENTIVE POLYPLEXES TRAGETING EGFR-EXPRESSING CELLS

Cell survival experiments examined the potency and selectivity of triconjugate LPEI-*l*-PEG-targeting fragment:nucleic acid polyplexes in various cancer cell lines with differential cell surface expression of receptor proteins. Triconjugate LPEI-*l*-PEG-targeting fragment:poly(Glu) polyplexes served as a control to demonstrate that the decrease in survival is mediated primarily by the targeted delivery of poly(IC) by the polyplexes. Moreover, a comparison with respect to prior art polyplexes was carried out to demonstrate the enhanced activity of the inventive polyplexes.

Cell Survival Assays of EGFR-Targeted Polyplexes in Cells with High and Low EGFR Expression. These assays examined the potency and selectivity of LPEI-PEG-hEGF:poly(IC) polyplexes in two cancer cell lines with differential cell surface expression levels of EGFR: A431 (high EGFR; see Phillips *et al.*, *Mol. Cancer. Ther*. 2016; **15**(4) 661-669) and MCF7 (low EGFR; see EP3098239B1) as shown in Table 11, below. A431 cells and MCF7 cells were obtained from ATCC. Cell-surface density of EGFR for both cell lines is given below in Table 11.

Table 11: Cell lines used and their cell surface density of EGFR.

Cell Line	**EGFR molecules/cell**
A431	$0.5\text{-}2 \times 10^6$
MCF7	$0.8\text{-}5 \times 10^3$

FIGs 6A-9B show cell survival experiments performed analogously in said two cancer cell lines with differential expression of EGFR: MCF7 (low EGFR) and A431 (high EGFR). Thus, cancer cell lines were treated with LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(IC), LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC), LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC), LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(IC), and their respective control polyplexes LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(Glu), LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu), LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(Glu) and LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(Glu) (FIGs 6A to 9B).

Polyplex samples comprising LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(IC), LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC), LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC), LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(Glu), LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(Glu), and LPEI-*l*-

[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu) were prepared in 50 mM acetate, pH 4.3 containing 5% glucose at an N/P ratio of 4. Cancer cells (3000 cell/well) with differential EGFR expression levels were treated with polyplexes for 72 h. Cell survival was analyzed using CellTiter-Glo (Promega). The concentrations shown as Log(polyplex) in FIGs 6A-9B reflect the concentrations of poly(Glu) or poly(IC) in the respective polyplexes.

Polyplex samples comprising LPEI-*l*-PEG_{36}-hEGF:poly(IC) or LPEI-*l*-PEG_{36}-hEGF:poly(Glu) were formulated in HBG buffer, 5% glucose, pH 7.2 at a N/P ratio of 4. Cancer cell lines (3000 cells/well) with differential expression of EGFR (MCF7: low EGFR expression; and A431: high EGFR expression) were treated with LPEI-*l*-PEG_{36}-hEGF:poly(IC) or LPEI-*l*-PEG_{36}-hEGF:poly(Glu) polyplexes for 72 h. 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.

FIG 6A shows the percent survival of MCF7 cells treated with LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(Glu), i.e., polyplexes comprising Compounds 70a and 70b. As shown in FIG 6A, LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(Glu) were inactive at concentrations as high as 0.625 µg/mL (i.e., no significant cell death was observed for either polyplex at concentrations as high as 0.625 µg/mL).

FIG 6B shows the percent survival of A431 cells treated with LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(Glu), i.e., polyplexes comprising Compounds 70a and 70b. As shown in FIG 6B, LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(IC) gave an IC_{50} of 0.0056 µg/mL. LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(Glu) was inactive at concentrations as high as 0.625 µg/mL (i.e., no significant cell death was observed for either polyplex at concentrations as high as 0.625 µg/mL).

FIG 7A shows the percent survival of MCF7 cells treated with LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu), i.e., polyplexes comprising Compounds 1a and 1b. As shown in FIG 7A, LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu) were inactive at concentrations as high as 0.625 µg/mL (i.e., no significant cell death was observed for either polyplex at concentrations as high as 0.625 µg/mL).

FIG 7B shows the percent survival of A431 cells treated with LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu), i.e., polyplexes comprising Compounds 1a and 1b. As shown in FIG 7B, LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) gave

an IC_{50} of 0.005 µg/mL. LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu) gave an IC_{50} value of 1.432 µg/mL.

FIG 8A shows the percent survival of MCF7 cells treated with LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(Glu), i.e., polyplexes comprising Compounds 4a and 4b. As shown in FIG 7A, LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(Glu) were inactive at concentrations as high as 0.625 µg/mL (i.e., no significant cell death was observed for either polyplex at concentrations as high as 0.625 µg/mL).

FIG 8B shows the percent survival of A431 cells treated with LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(Glu), i.e., polyplexes comprising Compounds 4a and 4b. As shown in FIG 7B, LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC) gave an IC_{50} of 0.003 µg/mL. LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu) gave an IC_{50} value of 1.020 µg/mL.

FIG 9A shows the percent survival of MCF7 cells treated with LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(Glu), i.e., polyplexes comprising Compounds 7a and 7b. As shown in FIG 9A, LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(Glu) were inactive at concentrations as high as 0.625 µg/mL (i.e., no significant cell death was observed for either polyplex at concentrations as high as 0.625 µg/mL).

FIG 9B shows the percent survival of A431 cells treated with LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(Glu), i.e., polyplexes comprising Compounds 7a and 7b. As shown in FIG 9B, LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(IC) gave an IC_{50} of 0.002 µg/mL. LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(Glu) gave an IC_{50} value of 1.026 µg/mL.

Table 12 provides the cell survival measured in MCF7 (low EGFR) cells as well as in A431 (high EGFR) cells as a function of treatment with linear LPEI-*l*-PEG_4-hEGF:poly(IC), LPEI-*l*-PEG_{12}-hEGF:poly(IC), LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) and LPEI-*l*-PEG_{36}-hEGF:poly(IC) polyplexes, as described above. Moreover, the cell survival data, measured in an analogous manner as described above, of branched, random LPEI-*r*-PEG_{2KDa}-hEGF:poly(IC) polyplexes taught in WO 2015/173824 is provided. The data shows that the linear polyplexes in accordance with the present invention are significantly more potent than the prior art random, branched polyplexes taught in WO 2015/173824, and demonstrated substantially higher cytotoxic potency and selectivity for the EGFR overexpressing cell line

A431.

Table 12: Cell survival data of linear and random, branched polyplexes.

polyplex	IC_{50} (µg/mL)	
	MCF7 cells (low EGFR)	A431 cells (high EGFR)
LPEI-*l*-[N_3:DBCO]-PEG_4-hEGF:poly(IC)	>0.625	0.002
LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC)	>0.625	0.003
LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC)	>0.625	0.005
LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF:poly(IC);	>0.625	0.0056
LPEI-*r*-PEG_{2KDa}-hEGF:poly(IC)*	2.049	0.178

*randomly (*r*)substituted analog: WO2015/173824

As shown in FIG. 7A treatment with both polyplexes [LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu)] did not have any effect on cell survival in MCF7 cells at concentrations as high as 1 µg/mL, while, as shown in FIG. 7B, LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) induced cell death at an IC_{50} of 0.005 µg/mL in A431 cells as compared to an IC_{50} of 1.432 µg/mL induced by the control polyanion LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(Glu) polyplex. Analogously, and as shown in FIG. 8A, treatment with both polyplexes [LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(Glu)] did not have any effect on cell survival, in MCF7 cells at concentrations as high as 1 µg/mL, while, as shown in FIG. 8B LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(IC) induced cell death at an IC_{50} of 0.003 µg/mL in A431 cells as compared to an IC_{50} of 1.020 µg/mL induced by the control polyanion LPEI-*l*-[N_3:DBCO]-PEG_{12}-hEGF:poly(Glu) polyplex. Similar results are shown in FIGs 6A and 6B and 9A and 9B.

In preferred embodiments, the inventive polyplexes comprising poly(IC) show high biological potency as evidenced by the high cytotoxicity of the inventive triconjugate:nucleic acid polyplexes. In preferred embodiments, the high cytotoxicity of the polyplexes is believed to be caused by poly(IC). Accordingly, in some embodiments the inventive polyplexes comprise poly(IC).

Moreover, the Examples herein demonstrate that the inventive polyplexes were

significantly more cytotoxic in A431 cells that expressed hEGFR at high (i.e., 10^6 molecules/cell) levels than in cells that expressed hEGFR at low (i.e., 10^3 molecules/cell) 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.

EXAMPLE 23

EFFECT OF TARGETING ON CYTOTOXIC ACTIVITY

Triconjugates of LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3 (Compounds 17a and 17b) were used to prepare polyplexes comprising poly(IC) or poly(Glu). LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3 (Compounds 17a and 17b) and poly(IC) or poly(glu) were dissolved in HEPES buffer, pH 7.2, containing 5% glucose. The solution comprising LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3 was added to an equal volume of poly(IC) or poly(Glu) solution to give a final concentration of 0.1 mg/mL of nucleic acid in the polyplex preparation. The combined solution of LPEI-*l*-]N_3:DBCO]-PEG_{23}-OCH_3 and nucleic acid was mixed by vigorously pipetting. The mixtures LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(Glu) were left at room temperature for 30 minutes to allow polyplex formation. The final N/P ratio of the complexes was 4.

A431 and MCF7 cells (see Table 11 above) were grown to a density of 3,000 cells/well. The cells were treated at increasing concentrations with polyplexes LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(Glu). Cell survival was analyzed using CellTiter-Glo (Promega). The results are shown in FIGs 10A and 10B.

FIG 10A shows the percent survival of MCF7 cells treated with LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(Glu). As shown in FIG 10A, LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(Glu) were inactive at concentrations as high as 0.625 µg/mL (i.e., no significant cell death was observed for either polyplex at concentrations as high as 0.625 µg/mL).

FIG 10B shows the percent survival of A431 cells treated with LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(IC) or LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(Glu). As shown in FIG 10B, LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(IC) gave an IC_{50} of 0.313 µg/mL. LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(Glu) was inactive at concentrations as high as 0.625 µg/mL (i.e., no significant cell death was observed for either polyplex at concentrations as high as 0.625

µg/mL).

FIGs 10A and 10B shows treatment with non-targeted polyplexes LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(Glu) to measure cell survival in MCF7 cells as well as in A431 cells. As shown in FIG. 10A treatment with both polyplexes did not have any effect on cell survival in MCF7 cells, while treatment with non-targeted polyplex LPEI-*l*-[N_3:DBCO]-PEG_{23}-OCH_3:poly(IC) induced cell death at an IC_{50} of 0.313 µg/mL in A431 cells and control polyanion LPEI-*l*-[N_3:DBCO]-PEG_{23}-OMe:poly(Glu) polyplex did not have any effect on cell survival in said cells at concentrations as high as 1 µg/mL (FIG. 10B). Accordingly, in preferred embodiments, cytotoxicity of the inventive triconjugate:nucleic acid polyplexes is due to primarily the delivery of the selected nucleic acid (e.g., poly(IC)). In preferred embodiments, the cytotoxicity of the inventive polyplexes can be increased by adding a targeting fragment to the inventive triconjugates.

EXAMPLE 24

SELECTIVE DELIVERY OF INVENTIVE POLYPLEXES DECREASES SURVIVAL OF PSMA-OVEREXPRESSING CELLS

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_{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}-DUPA:poly(IC); LPEI-*l*-[N_3:SCO]-PEG_{36}-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 were formulated in 20 mM HEPES with 5% glucose, pH 7.2 at a N/P ratio of 4.

Cancer cell lines (3000 cells/well) with differential expression of PSMA (PC3: low PSMA expression; DU145 low PSMA expression; and LNCaP: high PSMA expression) were treated with 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_{36}-DUPA:poly(IC); or LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(Glu) polyplexes for 72 h. 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.

Table 13 provides 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 more potent than the prior art random, branched polpylexes, and show a higher (i.e., about 10x) selectivity for the PSMA overexpressing cell line.

Table 13: PSMA expressing cell survival data of 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(pIC)	0.24	>0.625	0.020
LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(pIC)	0.22	>0.625	0.020
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 11A 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 for either polyplex 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 11B 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 for either polyplex at concentrations as high as 0.625 μg/mL), whereas 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 11C 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).

FIG 12A 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 12B 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 for either polyplex 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 12C 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 for either polyplex at concentrations as high as 0.625 μg/mL).

FIG 13 is a poly 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}-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.

FIG 14 is a poly 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}-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.

Table 14 provides 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_{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}-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 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.

Table 14: PSMA expressing cell survival data of linear polyplexes

Polyplex	IC_{50} (µg/mL)	
	DU145 cells (low PSMA)	LNCaPcells (high PSMA)
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[(NH_2)MAL-S]-DUPA:poly(IC) (based on compounds 31a and 31b)	>0.625	0.014
LPEI-*l*-[N_3:BCN]-PEG_{36}-DUPA:poly(IC) (based on Compound 43)	>0.625	0.013
LPEI-*l*-[N_3:SCO]-PEG_{36}-DUPA:poly(IC) (based on Compounds 47a and 47b)	>0.625	0.012
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[CONH]-DUPA:poly(IC) (based on Compounds 51a and 51b	>0.625	0.016
LPEI-*l*-[N_3:DBCO]-PEG_{36}-[S-MAL]-DUPA:poly(IC) (based on Compounds 56a and 56b)	>0.625	0.019

EXAMPLE 25

SELECTIVE DELIVERY OF INVENTIVE POLYPLEXES DECREASES SURVIVAL OF FOLATE-OVEREXPRESSING CELLS

LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC) polyplexes were formulated in 20 mM HEPES with 5% glucose, pH 7.2 at a N/P ratio of 4.

Cancer cell lines (3000 cells/well) with differential expression of folate receptor (MCF7: low folate receptor expression; SKOV3: high folate receptor expression) were treated with LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC) polyplexes for 72 h. Cell survival was analyzed using Cell Titer-Glo (Promega).

Selective delivery of LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC) decreased the survival of Folate overexpressing cells (SKOV3) as shown in FIG 15. In contrast, delivery of LPEI-*l*-[N_3:DBCO]-PEG_{24}-Folate:poly(IC) did not have a significant effect on cell survival in MCF7 cells at concentrations as high as 0.625 µg/mL.

EXAMPLE 26

SELECTIVE DELIVERY OF INVENTIVE POLYPLEXES DECREASES SURVIVAL OF HER2-OVEREXPRESSING CELLS

LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(Glu) polyplexes were formulated in 20 mM HEPES with 5% glucose, pH 7.2 at a N/P ratio of 4.

Cancer cell lines (3000 cells/well) with differential expression of HER2 (MCF7: low HER2 expression; SKBR3 and BT474: high HER2 expression) were treated with LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(Glu) polyplexes for 72 h. Cell survival was analyzed using Cell Titer-Glo (Promega). Selective delivery of LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(IC) decreased the survival of HER2 overexpressing cells as shown in FIGs 16A, 16B and 16C.

FIG 16A is a plot of cell survival in MCF7 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(Glu). LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody: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}-HER2-Affibody:poly(IC) inhibited MCF7 cell survival with an IC_{50} value of 0.85 µg/mL.

FIG 16B is a plot of cell survival in SKBR3 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(Glu). LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody: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}-HER2-Affibody:poly(IC) inhibited MCF7 cell survival with an IC_{50} value of 0.25 µg/mL.

FIG 16C is a plot of cell survival in BT474 cells as a function of treatment with LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(IC) and LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody:poly(Glu). LPEI-*l*-[N_3:DBCO]-PEG_{24}-HER2-Affibody: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}-HER2-Affibody:poly(IC) inhibited BT474 cell survival with an IC_{50} value of 0.34 μg/mL.

EXAMPLE 27

IP-10 CYTOKINE SECRETION IN EGFR CANCER CELL LINES

IP-10 secretion experiments examined the selective cytokine release induced by LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) polyplexes in two cancer cell lines with differential surface expression of EGFR: A431 (high EGFR) and MCF7 (low EGFR).

LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF: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) were treated for 5 hours with LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) at various concentrations (0.125, 0.25, 0.5, 1.0 μg/ml as determined using extinction coefficient (EM260) of 22.2 L/(g·cm)). Medium from treated cells was collected and analyzed for Human IP-10 (CXCL10) utilizing ELISA assay (PeproTech) and detected using a Microplate Reader Synergy H1 (BioTek). FIG. 17 shows IP-10 secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) concentration in A431 cells and MCF7 cells.

EXAMPLE 28

EGFR PHOSPHORYLATION

EGFR target engagement of LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) polyplexes was examined and the level of phosphorylation of EGFR in NIH3T3 cells was analyzed by immunoblot analysis.

LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) polyplexes were formulated in 20 mM HEPES with 5% glucose, pH 7.2 at a N/P ratio of 4. Cells (400,000 cells/well in 6-well plate) were serum starved and treated with the carrier LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF (0.04 μg/mL, reflecting total LPEI concentration), and polyplex LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) (0.0615 μg/mL, reflecting poly(IC) concentrations) for 30 min. Protein lysates were generated, electrophoresed and subjected to phospho-EGFR immunoblot analysis (10 μg total protein lysates). Serum starved condition functioned as negative control and human EGF treatment as positive control for EGFR protein phosphorylation. Tubulin demonstrates equal loading of total protein. FIG 18 demonstrates that LPEI-*l*-[N_3:DBCO]-PEG_{24}-hEGF:poly(IC) robustly induced the phosphorylation of EGFR (P-EGFR) after 30 minutes as a result of the binding of the

polyplex targeting fragment, hEGF, to EGFR in comparison to serum starved cells.

EXAMPLE 29

CYTOKINE SECRETION IN PSMA 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 for Human IP-10 (CXCL10), RANTES (CCL5) or interferon beta (IFN-β) 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 IP10, RANTES, and IFNb cytokine release in PSMA overexpressing cells (LNCaP) as compared to low PSMA expressing cells (PC-3 and DU145). The results are shown in FIGs 19A-21C.

FIG 19A 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. 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 19B 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. 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 19C 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. 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 19B and 19C, treatment with polypexes 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 IP10 secretion in LNCaP cells is the same.

FIG 20A 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. 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 20B 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. 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 20C 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. 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 20B and 20C, treatment with polypexes 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 21A is a plot of IFN-ß 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-ß 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 21B is a plot of IFN-ß secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration in LNCaP cells and PC-3 cells. In LNCaP cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced IFN-ß 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-ß secretion of 44.17 pg/mL and 86.57 pg/mL at 0.0625 µg/mL and 0.625 µg/mL, respectively.

FIG 21C is a plot of IFN-ß secretion as a function of LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) concentration in LNCaP cells and DU145 cells. In LNCaP cells, LPEI-*l*-[N_3:DBCO]-PEG_{36}-DUPA:poly(IC) induced IFN-ß 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-ß secretion of 4.37 pg/mL and 5 pg/mL at 0.0625 µg/mL and 0.625 µg/mL, respectively.

For 21B and 21C, treatment with polypexes 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 in 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_{24}-DUPA:poly(IC) at two concentrations, 0.0625 µg/mL and 0.625 µg/mL, selectively induces A) IP10 B) RANTES) C) IFNb) cytokine release, in PSMA overexpressing cells (LNCaP) as compared to low PSMA expressing cells, PC-3 or DU145.

EXAMPLE 30

SIGNALING IN PSMA 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 followed by Western blot 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)) (10 μg total protein lysates/lane). GAPDH (Cell Signaling 2118) and beta-Actin (Sigma A5441) were used as protein loading controls.

FIG. 22 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 an increase in protein 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) selectively. 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 31

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 31a and 31b, 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 23) shows that polyplexes particles comprising compounds 31 and 31b, 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 32

SELECTIVE DELIVERY OF mRNA BY INVENTIVE POLYPLEXES

Materials and Methods: Firefly Luciferase (Fluc) mRNA was purchased fromTriLink Biotechnologies USA (cat#L-7602; 1.0 mg/mL in 1 mM Sodium Citrate, pH 6.4; mRNA Length: 1929 nucleotides). Lipofectamine messenger MAX was purchased from ThermoFisher, and jetPEI was purchased from Polyplus (Cat# 101000053). Cell culture reagents were purchased from Biological Industries, Bet Ha'emek, Israel. All reagents were used according to manufacturer's instructions at the indicated concentrations.

Polyplexes comprising Fluc mRNA and LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF (i.e., Compounds 70a and 70b) were generated by complexing the Fluc mRNA at N/P ratios of 4, 6, 12 (where N =nitrogen from LPEI and P = phosphate of mRNA) in HEPES-buffered saline (HBS: 20 mM HEPES, 150 mM NaCl, pH 7.2) with the triconjugate LPEI-*l*-[N_3:DBCO]-PEG_{36}-hEGF. To allow complete formation of the polyplex particles, i.e., LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA], the samples were incubated for 30 min at room temperature.

Renca parental cells (mouse renal carcinoma, no human EGFR); and RencaEGFR M1 H cells (derivate of Renca parental engineered to overexpress human EGFR) were obtained from ATCC and were cultured according to manufacturer's protocol. Renca (parental) cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 104 U/L penicillin, 10 mg/L streptomycin at 37 °C in 5% CO_2. 400 µg/ml of G418 were added to the medium of RencaEGFR M1 H cells. 15,000 cells/well RencaEGFR M1 H cells, 10,000 cells/well Renca parental cells were seeded in triplicates at 90 µl into 96 well white plates (Greiner) and 96 well transparent plates (Nunc). Cells were transfected with 0.125-1 mg/ml of LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA].

Luciferase activity was measured with OneGloX assay (Promega) at the indicated time after the treatment. Luminescence measurements were performed using a Luminoskan Ascent Microplate Luminometer (Thermo Scientific). Values, in Arbitrary Units (AU), are presented as the mean and standard deviation of luciferase activity from the triplicate samples.

Cell survival was measured by means of a colorimetric assay using methylene blue assay. Briefly, the cells were fixed with 2.5% glutaraldehyde in PBS (pH 7.4), washed with deuterium depleted water (DDW), and then stained with a 1% (wt/vol) solution of methylene blue in borate buffer for one hour. Thereafter, the stain was extracted with 0.1 M HCl and the optical density

of the stain solution was read at 630 nm on a microplate reader (Synergy H1, Biotek). Luminescence and cell survival were measured 24 hrs after the treatment.

Physicochemical characterization of the LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] polyplexes was measured using DLS in 50 mM acetate buffer, 5% glucose at pH 4.3, at N/P ratios of 3, 4, 5, and 6. A summary of physicochemical measurements is given in Table 15. The z-average diameter ranged between 95 nm and 127 nm with a polydispersity index (PDI) of 0.134-0.209. The ζ-potential range measured by ELS was 29.7-45.6 mV.

Table 15: Physicochemical Characterization of polyplex LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF: FLuc mRNA in in 50 mM acetate buffer, 5% glucose at pH 4.3 at N/P ratios 3, 4, 5, 6

N/P	6	5	4	3
Fluc mRNA (mg/mL)	0.1	0.1	0.1	0.1
Average diameter (nm)	125.1	95	119.2	127.9
PDI	0.207	0.209	0.196	0.134
ζ-potential	45.6	42.4	29.7	41

FIG 24A is a plot of luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] compared to the control delivery vehicle Messenger MAX. The luminescence was measured at N/P ratios of 4, 6 and 12, and at concentrations from 0.125 to 1.0 μg/mL of LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] and lipofectamine messenger MAX at 24 hours after treatment.

FIG 24B is a plot of luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] compared to the control delivery vehicle jetPEI. The luminescence was measured at N/P ratios of 4, 6 and 12, and at concentrations from 0.125 to 1.0 μg/mL of LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] and jetPEI at 24 hours after treatment.

FIG 24C is a plot of the ratio of luminescence (AU) between Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA]. The luminescence was measured at N/P ratios of 4, 6 and 12, and at concentrations from 0.125 to 1.0 μg/mL of LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] and lipofectamine messenger MAX at 24 hours after treatment, and the ratio was calculated by dividing the luminescence signal from RencaEGFR M1 H cells by the luminescence signal from Renca parental cells.

FIG 24D is a plot of percent survival in Renca parenteral cells and Renca EGFR M1 H

cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] compared to the control delivery vehicle jetPEI. The percent survival was measured at N/P ratios of 4, 6 and 12, and at concentrations from 0.125 to 1.0 μg/mL of LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] and jetPEI at 24 hours after treatment.

FIGs 24A-24D show that selective mRNA delivery to RencaEGFR M1 H cells over renca parental cells was achieved using LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] at N/P 4-12. In contrast, the non-targeted delivery vehicles Lipofectamine messenger MAX and jetPEI did not show selective mRNA delivery to either cell line. In both cases superiority over non-targeted delivery systems was demonstrated across all N/P ratios.

FIG 24E shows that the LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] polyplexes were not cytotoxic at N/P 4 and 6.

FIGs 25A-25D show relative luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] at 24 and 48 h after delivery. Selective delivery and expression were achieved at 6 hrs, with peak at 22 hrs. No toxicity was observed 6 hours after delivery.

FIG 25A shows relative luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] at 6 hrs after treatment at an N/P of 4.

FIG 25B shows relative luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] at 6 hrs after treatment at an N/P of 6.

FIG 25C shows relative luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] at 22 hrs after treatment at an N/P of 4.

FIG 25D shows relative luminescence (AU) in Renca parenteral cells and Renca EGFR M1 H cells treated with LPEI-*l*-[N_3:DBCO]PEG_{36}-hEGF:[Fluc mRNA] at 22 hrs after treatment at an N/P of 6.

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 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 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 capable of binding to a targeting fragment.

2. The composition of claim 1, 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 any integer between 1 and 200;

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

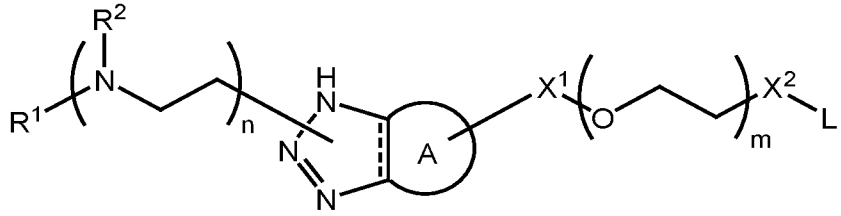
R^2 is independently -H or an organic residue, wherein at least 80%, preferably 90%, of said R^2 in said -(NR^2-CH_2-CH_2)$_n$- is H;

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 preferably said targeting fragment is capable of binding to a cell.

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 any integer between 1 and 200;

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 preferably said targeting fragment is capable of binding to a cell.

4. The composition of claim 2 or claim 3, wherein said R^1-$(NR^2-CH_2-CH_2)_n$-moiety is a disperse polymeric moiety with between about 115 and about 1150 repeating units n and a dispersity of about 5 or less, preferably between about 280 and about 700 repeating units n with a dispersity of about 3 or less, and further preferably between about 350 and about 630 repeating units n with a dispersity of about 2 or less, and wherein preferably R^1 is -H or $-CH_3$.

5. The composition of any one of the claims 2 to 4, wherein said $-(O-CH_2-CH_2)_m-$ is a disperse polymeric moiety with between about 2 and about 80 repeating units m and a dispersity of about 2 or less, preferably between about 2 and about 70 repeating units m with a dispersity

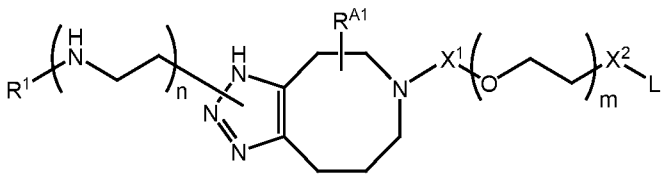
of about 1.8 or less; more preferably between about 2 and about 50 repeating units m with a dispersity of about 1.5.

6. The composition of any one of the claims 2 to 4, wherein said -(O-CH_2-CH_2)$_m$-moiety comprises, preferably consists of, a discrete number of repeating units m of 4 to 60, wherein preferably said -(O-CH_2-CH_2)$_m$-moiety comprises, preferably consists of, a discrete number of contiguous repeating units m of 4 to 60.

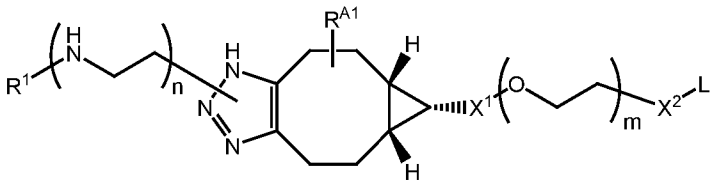
7. The composition of any one of the claims 3 to 6, wherein Ring A is an 8-membered cycloalkenyl, 5-membered heterocycloalkyl, or 7- to 8-membered heterocycloalkenyl, wherein each cycloalkenyl, heterocycloalkyl or heterocycloalkenyl is optionally substituted at any position with one or more R^{A1}.

8. The composition of any one of the claims 3 to 7, wherein Ring A is cyclooctene, succinimide, or 7- to 8-membered heterocycloalkenyl, wherein the heterocycloalkyl or heterocycloalkenyl comprises one or two heteroatoms selected from N, O and S, and wherein each cyclooctene, heterocycloalkyl 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.

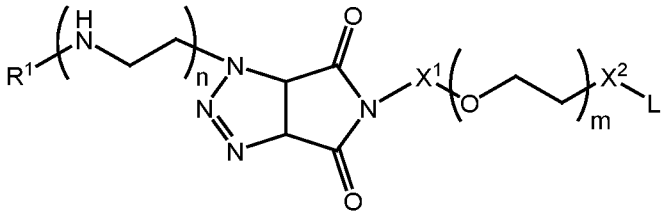
9. The composition of any one of the claims 3 to 8, wherein said conjugate of Formula I is selected from:



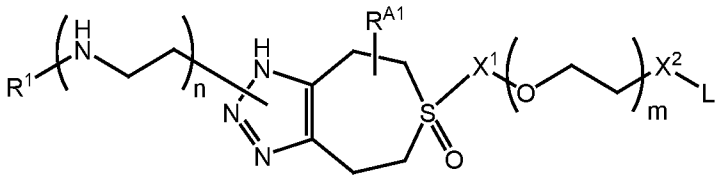
Formula IA,



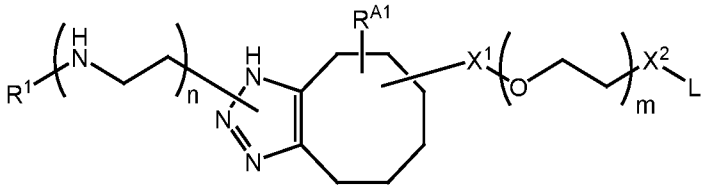
Formula IB,



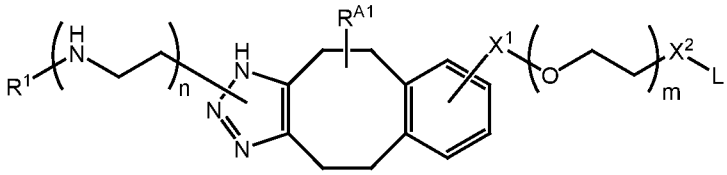
Formula IC,



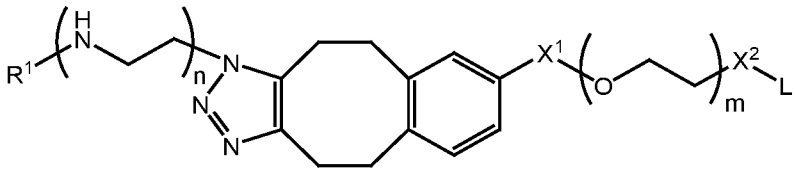
Formula ID,



Formula IE,

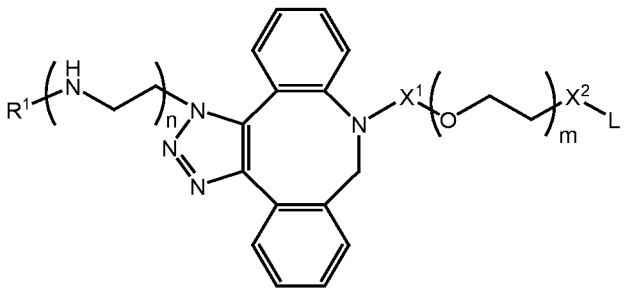


Formula IH, and

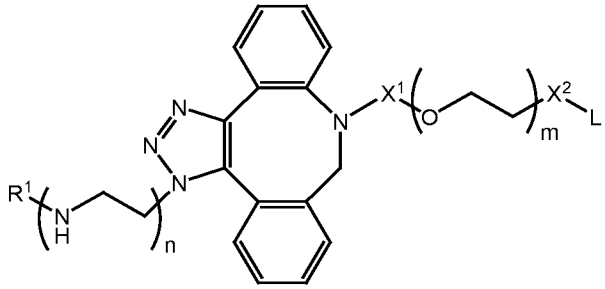


Formula IH-1.

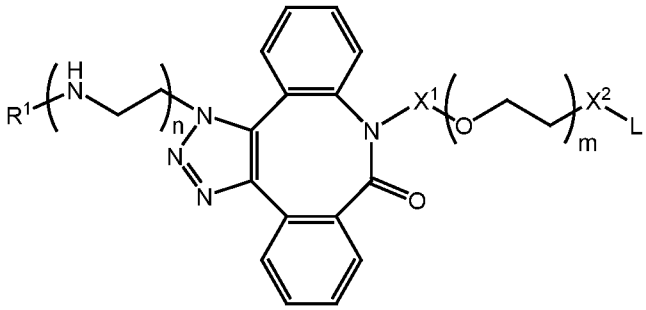
10. The composition of any one of the claims 3 to 9, wherein said conjugate of Formula I is selected from:



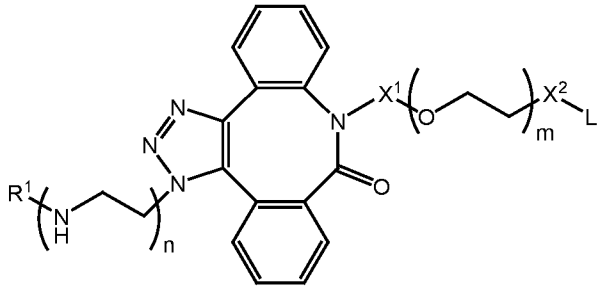
Formula IA-3,



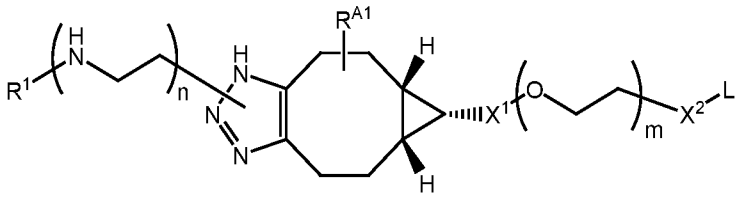
Formula IA-4,



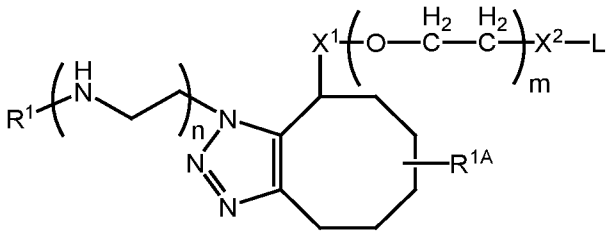
Formula IA-9,



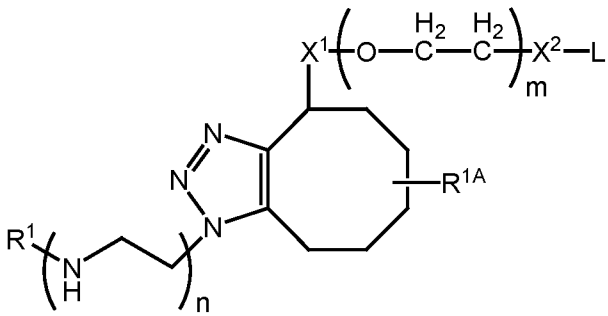
Formula IA-10,



Formula IB,

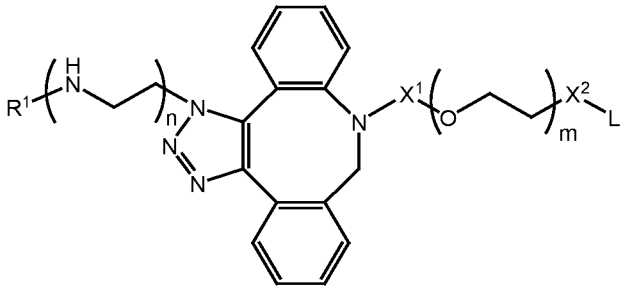


Formula IE-13, and



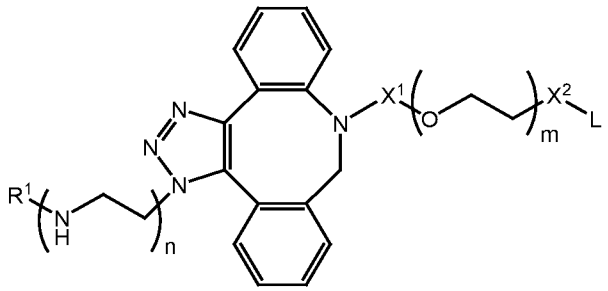
Formula IE-14.

11. The composition of any one of the claims 3 to 10, wherein said conjugate of Formula I is selected from:



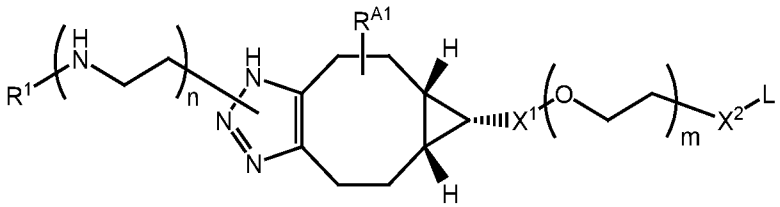
Formula IA-3,

and



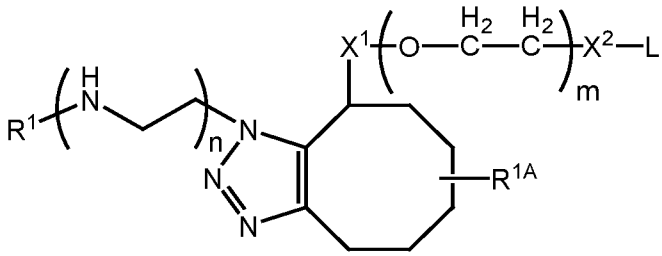
Formula IA-4.

12. The composition of any one of the claims 3 to 10, wherein said conjugate of Formula I is selected from:

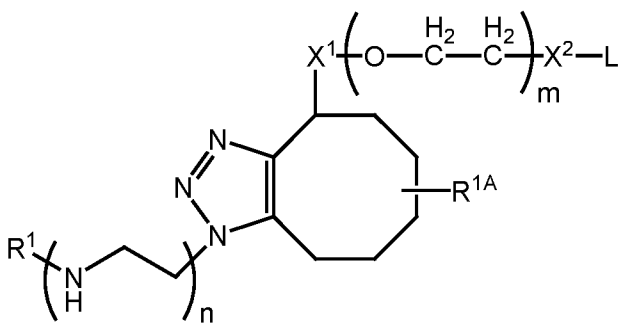


Formula IB.

13. The composition of any one of the claims 3 to 10, wherein said conjugate of Formula I is selected from:

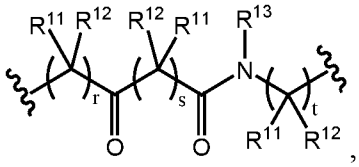
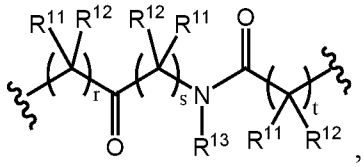
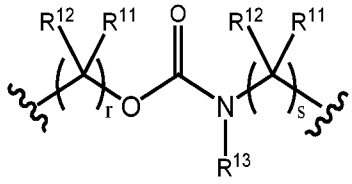


Formula IE-13, and



Formula IE-14.

14. The composition of any one of the claims 3-13, 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;

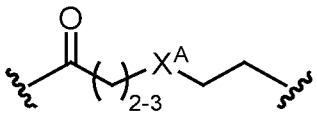
s is independently, at each occurrence, 0-6, preferably 0, 2, 3, or 4; more preferably 2 or 3;

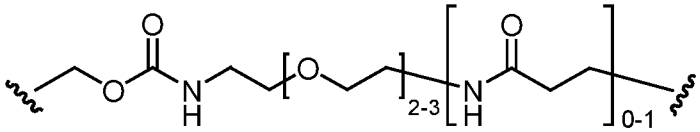
t is independently, at each occurrence, 0-6, preferably 0, 1, 2, 4; more preferably 2;

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

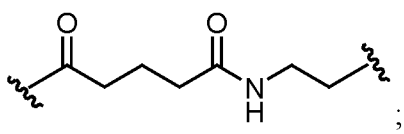
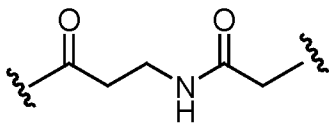
R^{13} is -H; preferably wherein the wavy line nearest to the integer "r" is a bond to Ring A and the wavy line nearest to the integer "s" or "t" is a bond to –$[OCH_2$-$CH_2]_m$–.

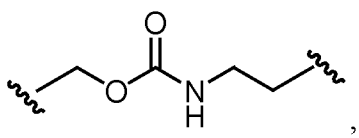
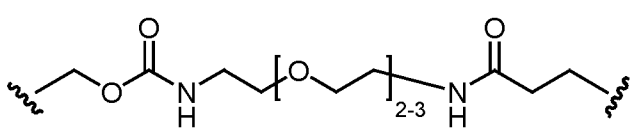
15. The composition of any one of the claims 3-13, wherein X^1 is selected from:

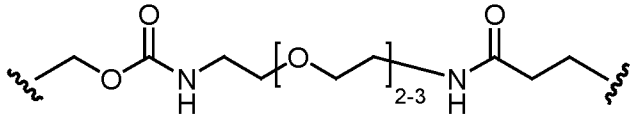

, wherein X^A is -NHC(O)- or -C(O)NH-; and


; 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 –$[OCH_2$-$CH_2]_m$–.

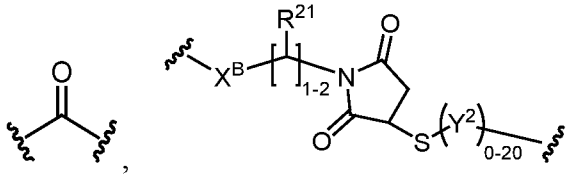
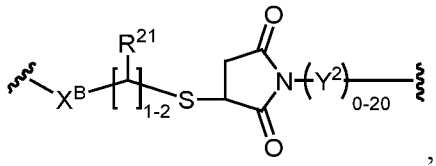
16. The composition of any one of claims 3-13, wherein X^1 is selected from:


;

;


, 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]$_m$–.

17. The composition of any one of claims 3-16, wherein X^2 is selected from:


, and 
,

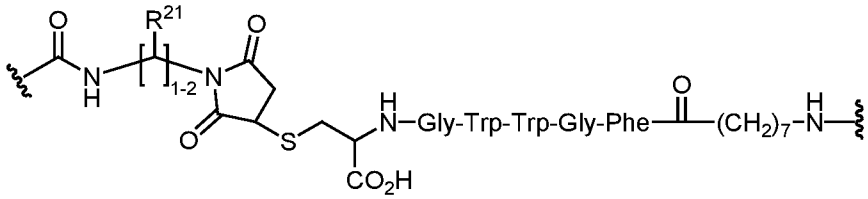
wherein X^B is -C(O)NH- or -NH-C(O)-;

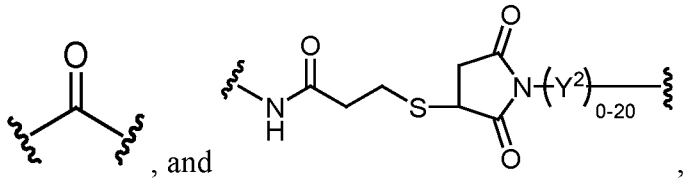
wherein each occurrence of Y^2 is independently selected from a chemical bond, -$CR^{21}R^{22}$-, NR^{23}-, -O-, -S-, -C(O)-, an amino acid residue, a divalent phenyl moiety, a divalent carbocyle moiety, a divalent heterocycle moiety, and a divalent heteroaryl moiety, wherein each divalent phenyl and 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.

18. The composition of any one of claims 3-16, wherein X^2 is selected from:


(SEQ ID NO: 10),


, and
,

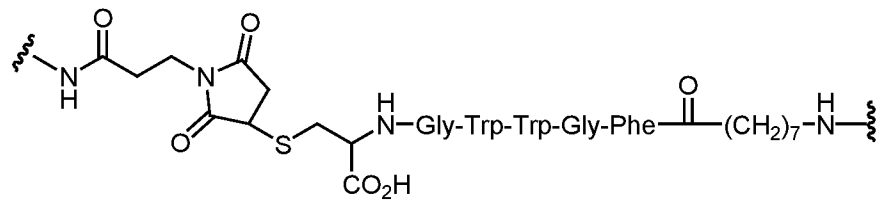
wherein each occurrence of Y^2 is independently selected from a chemical bond, -$CR^{21}R^{22}$-, NR^{23}-, -O-, -S-, -C(O)-, an amino acid residue, a divalent phenyl moiety, a divalent

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};

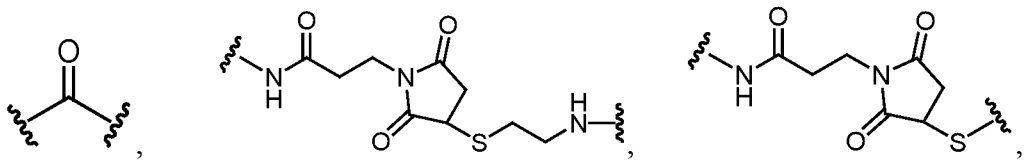
$R^{21,}$ $R^{22,}$ and R^{23} are each independently, at each occurrence, -H, $-SO_3H$, $-NH_2$, $-CO_2H$, or C_1-C_6 alkyl, wherein each C_1-C_6 alkyl is optionally substituted with one or more -OH, oxo, $-CO_2H$, $-NH_2$, C_6-C_{10} aryl, or 5 to 8-membered heteroaryl; and

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.

19. The composition of any of claims 3-16, wherein X^2 is selected from:



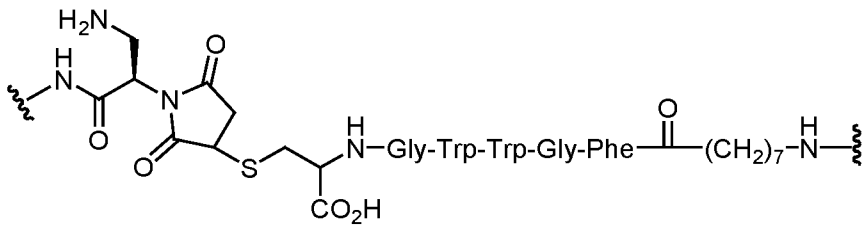
(SEQ ID NO: 11),



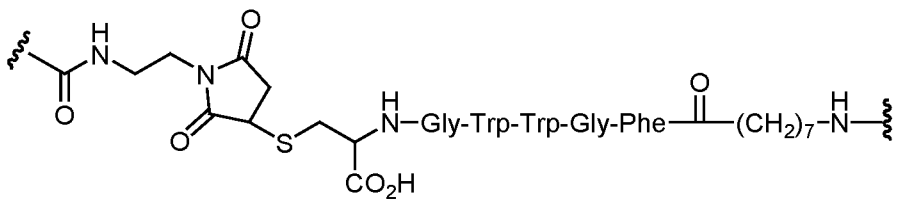
,

,

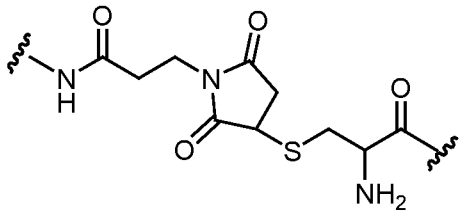
,



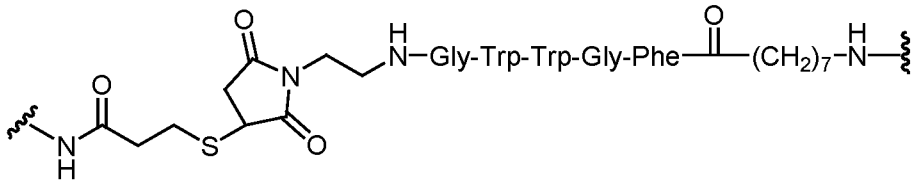
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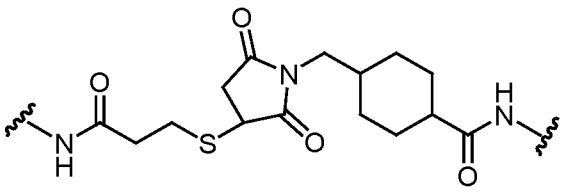
(SEQ ID NO: 13),



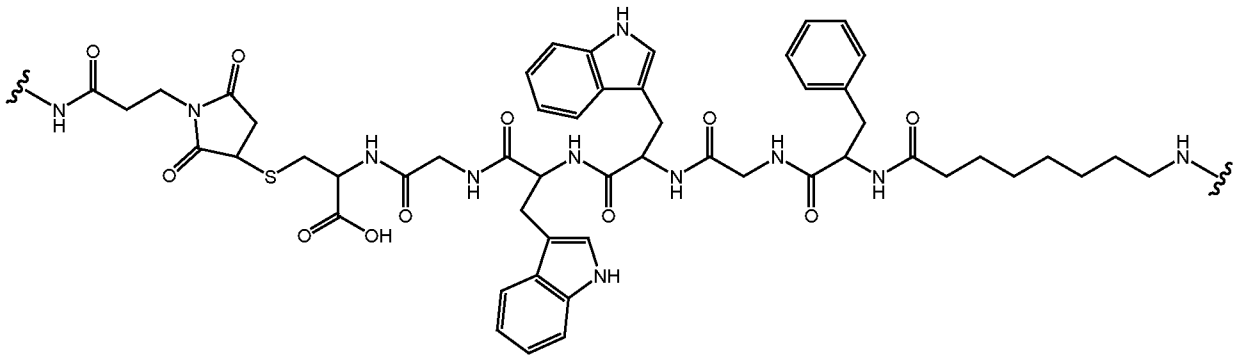
,



(SEQ ID NO: 14),

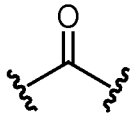
and  ; preferably wherein the wavy line on the left side is a bond to $–[OCH_2\text{-}CH_2]_m–$ and the wavy line on the right side is a bond to L.

20. The composition of any one of claims 3-16, 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.

21. The composition of any one of claims 3-16, wherein X^2 is



.

22. The composition of any one of the preceding claims, wherein said targeting fragment L is capable of binding to a cell surface receptor, wherein preferably said targeting fragment is capable of specifically binding to a cell surface receptor.

23. The composition of claim 22, wherein said cell surface receptor is selected a growth factor receptor, an extracellular matrix protein, a cytokine receptor, a hormone receptor, a glycosylphosphatidylinositol (GPI) anchored membrane protein, a carbohydrate-binding integral membrane protein, a lectin, an ion channel, a G-protein coupled receptor, and an enzyme-linked receptor such as a tyrosine kinase-coupled receptor.

24. The composition of claim 22 or claim 23, wherein said cell surface receptor is selected from an epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), prostate surface membrane antigen (PSMA), an insulin-like growth factor 1 receptor (IGF1R), a vascular endothelial growth factor receptor (VEGFR), a platelet-derived growth factor receptor (PDGFR), an asialoglycoprotein receptor (ASGPr) and a fibroblast growth factor receptor (FGFR).

25. The composition of any one of the preceding claims, wherein said targeting fragment L is capable of binding to a cell surface receptor, 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.

26. The composition of any one of the preceding claims, wherein said targeting fragment L is selected from an EGFR targeting fragment; a PSMA targeting fragment, preferably the DUPA residue; an anti-HER2 peptide, preferably an anti-HER2 antibody or affibody; folic acid; a somatostatin receptor-targeting fragment, preferably somatostatin and/or octreotide; an integrin-targeting fragment, preferably an arginine-glycine-aspartic acid (RGD)-containing fragment; a low pH insertion peptide; an ASGPr targeting fragment, preferably asialoorosomucoid; an insulin-receptor targeting fragment, preferably insulin; a mannose-6-phosphate receptor targeting fragment, preferably mannose-6-phosphate; a mannose-receptor targeting fragment, preferably mannose; a Sialyl Lewis^x antigen targeting fragments, preferably E-selectin; a sigma-2 receptor agonist, preferably N,N-dimethyltryptamine (DMT), sphingolipid-derived amine, and/or steroid, more preferably progesterone; a p32-targeting ligand, preferably anti-p32 antibody or p32-binding LyP-1 tumor-homing peptide; a Trop-2 targeting fragment, preferably an anti-Trop-2 antibody and/or antibody fragment; insulin-like growth factor 1; vascular endothelial growth factor; platelet-derived growth factor; and

fibroblast growth factor.

27. 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-).

28. The composition of any one of the preceding claims, wherein said conjugate is selected from Compound 1a, Compound 1b, Compound 4a, Compound 4b, Compound 7a, Compound 7b, Compound 10a, Compound 10b, Compound 14, Compound 17a, Compound 17b, Compound 18, Compound 19, Compound 22a, Compound 22b, Compound 28a, Compound 28b, Compound 31a, Compound 31b, Compound 38a, Compound 38b, Compound 43, Compound 47a, Compound 47b, Compound 51a, Compound 51b, Compound 56a, Compound 56b, Compound 62a, Compound 62b, Compound 70a, Compound 70b, Compound 72a, Compound 72b, Compound 75a, Compound 75b, Compound 78a and/or Compound 78b.

29. 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.

30. The composition of claim 29, wherein said polyanion is a nucleic acid, and wherein said nucleic acid is a dsRNA or a ssRNA.

31. The composition of claim 30, wherein said nucleic acid is a dsRNA.

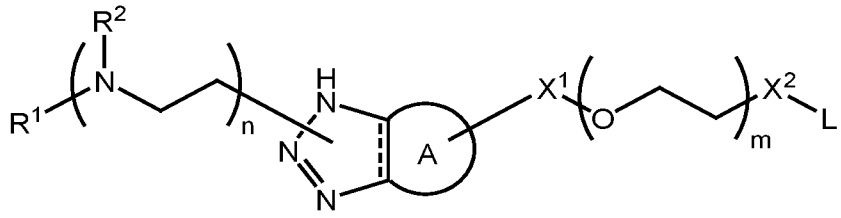
32. The composition of claim 31, wherein said dsRNA is polyinosinic:polycytidylic acid (poly(IC)).

33. The composition of claim 30, wherein said nucleic acid is a ssRNA.

34. The composition of claim 33, wherein said ssRNA is a mRNA.

35. 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.

36. 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 2 to 100, preferably of a discrete number of repeating units m of 4 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.

37. The polyplex of claim 35 or claim 36, wherein said polyanion is a nucleic acid, wherein said nucleic acis is a RNA.

38. The polyplex of claim 37, wherein said RNA is a dsRNA or a ssRNA.

39. The composition of claim 37, wherein said RNA is a dsRNA.

40. The composition of claim 39, wherein said dsRNA is polyinosinic:polycytidylic acid (poly(IC)).

41. The composition of claim 37, wherein said RNA is a ssRNA.

42. The composition of claim 41, wherein said ssRNA is a mRNA.

43. A pharmaceutical composition comprising a composition of any one of the claims 1 to 34 or a polyplex of any one of the claims 35 to 42, and optionally one or more pharmaceutically acceptable excipient(s) and/or carrier(s)..

44. A composition of any one of the claims 1 to 34 or a polyplex of any one of the claims 35 to 42 or a pharmaceutical composition according to claim 43, for use in the treatment of a cancer, preferably a cancer characterized by cells that overexpress EGFR; HER2; prostate-specific membrane antigen; folate receptor; an integrin, preferably an RGD integrin; an asialoglycoprotein receptor; an insulin receptor; a mannose-6-phosphate receptor; a mannose receptor; a glycosides, preferably a Sialyl Lewis^x antigen; a sigma-2 receptor; p32 protein; or Trop-2.

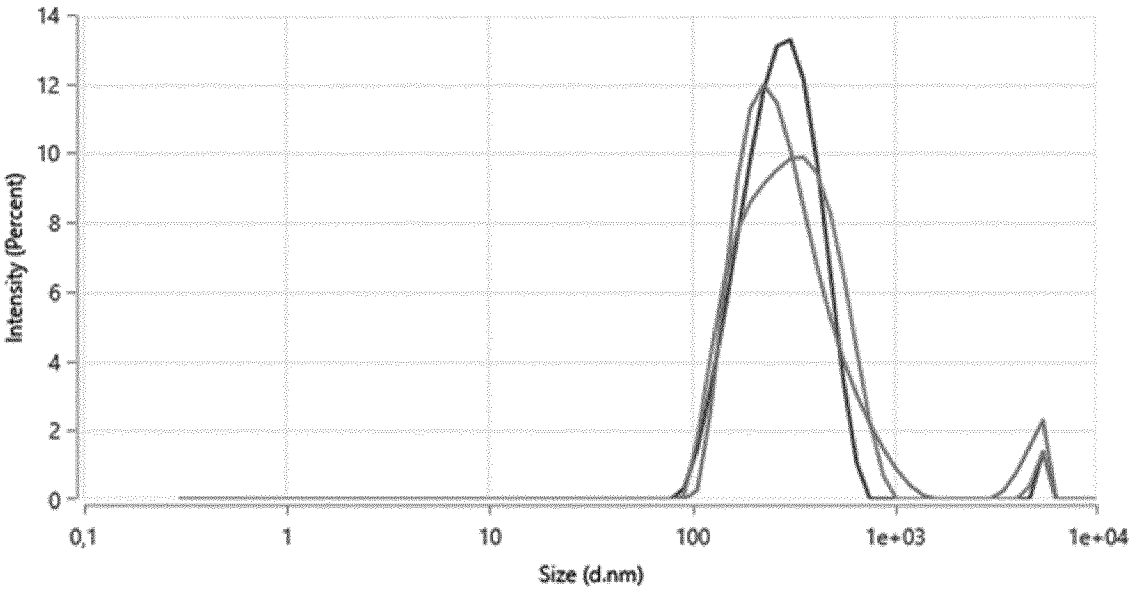

Intensity (Percent)
14
12
10
8
6
4
2
0
0,1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG 1A

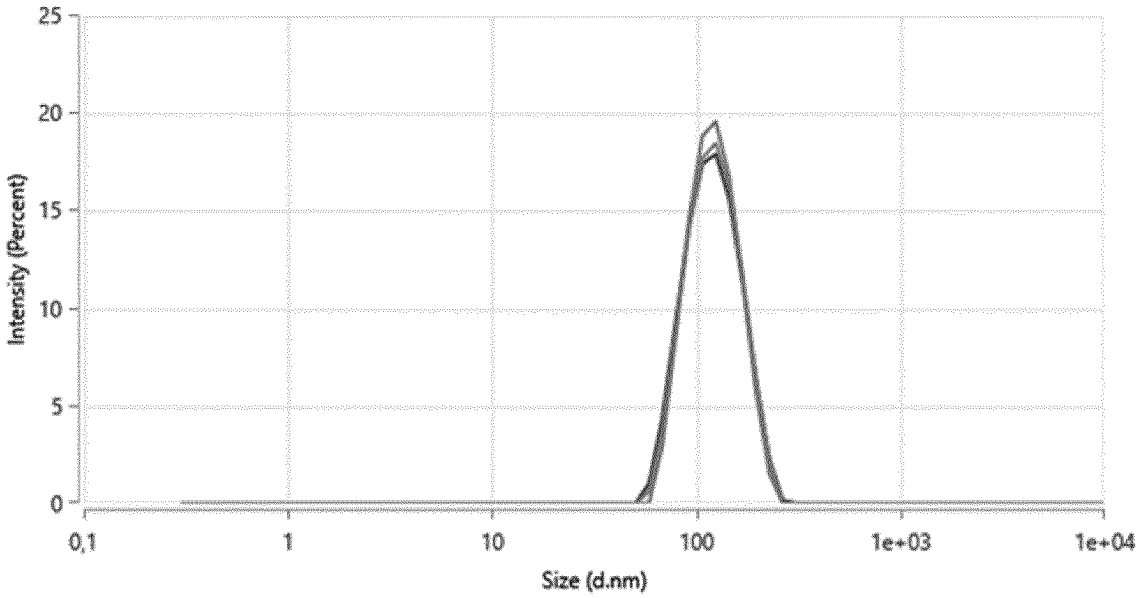

Intensity (Percent)
25
20
15
10
5
0
0,1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG 1B

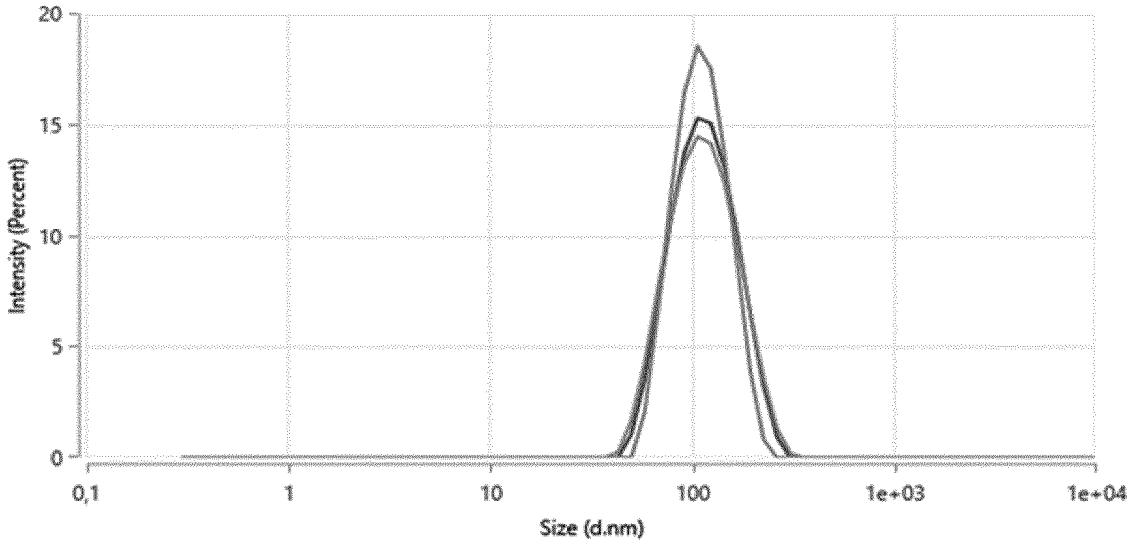

20
15
10
5
0
Intensity (Percent)
0,1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG 1C

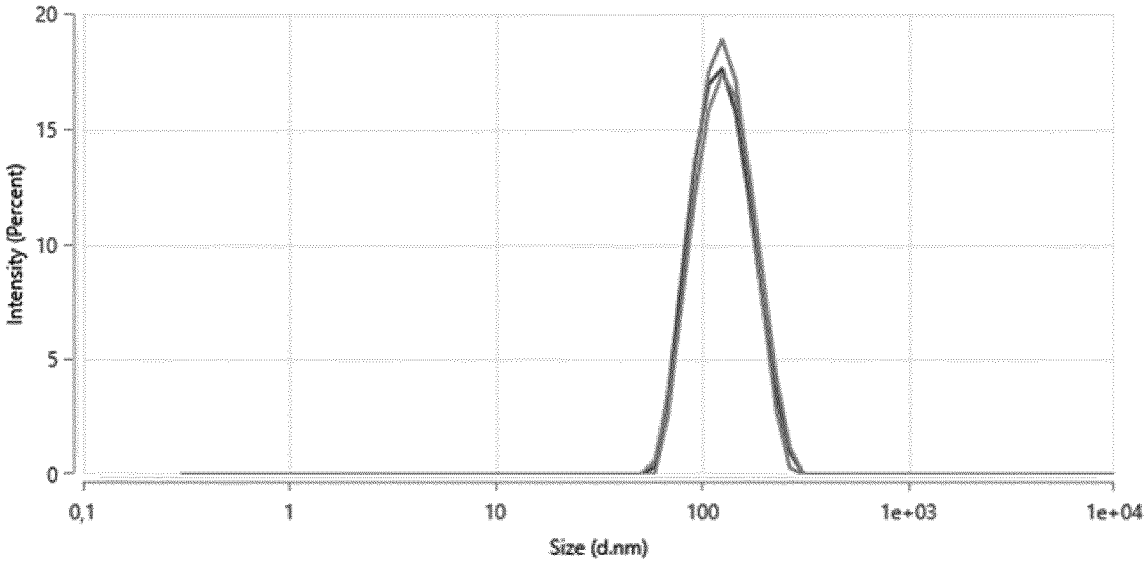

20
15
10
5
0
Intensity (Percent)
0,1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG 2

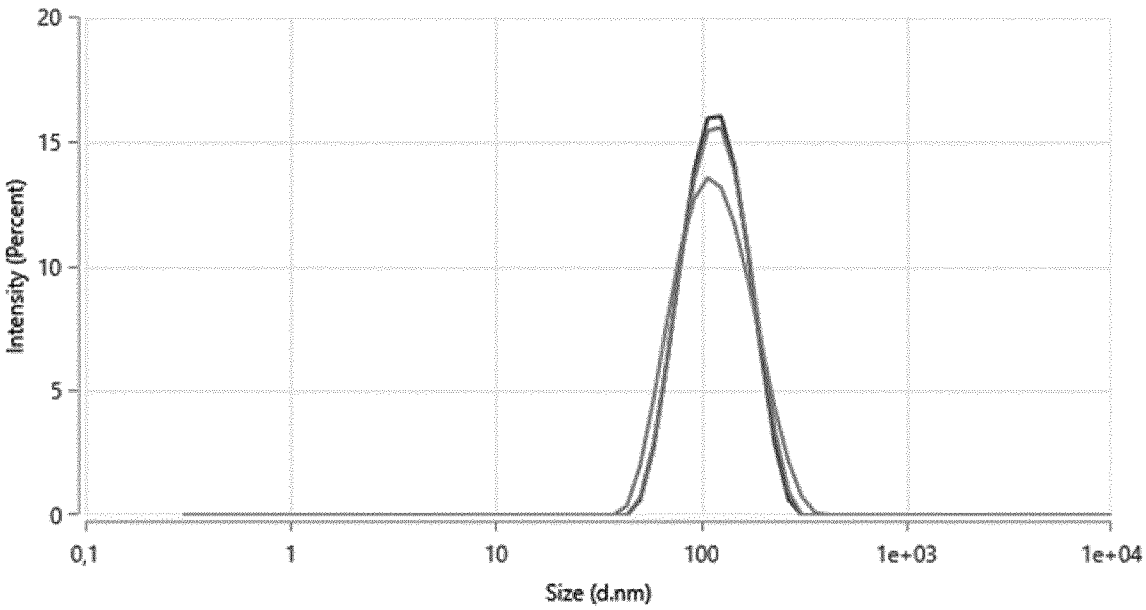

20
15
10
5
0
Intensity (Percent)
0,1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG 3

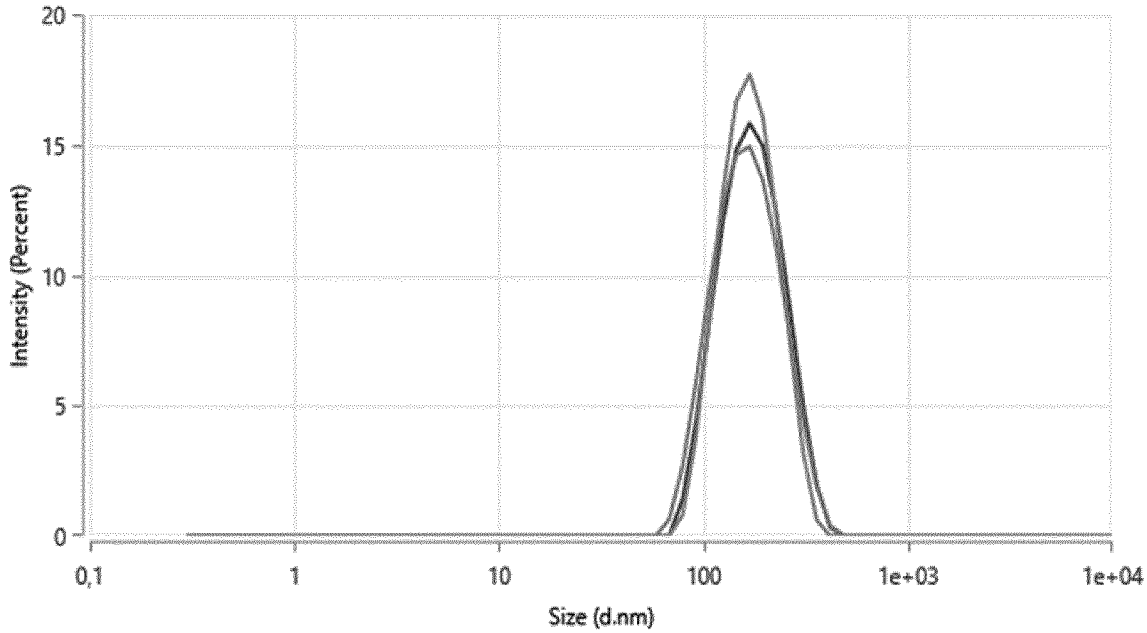

20
15
10
5
0
Intensity (Percent)
0,1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG 4

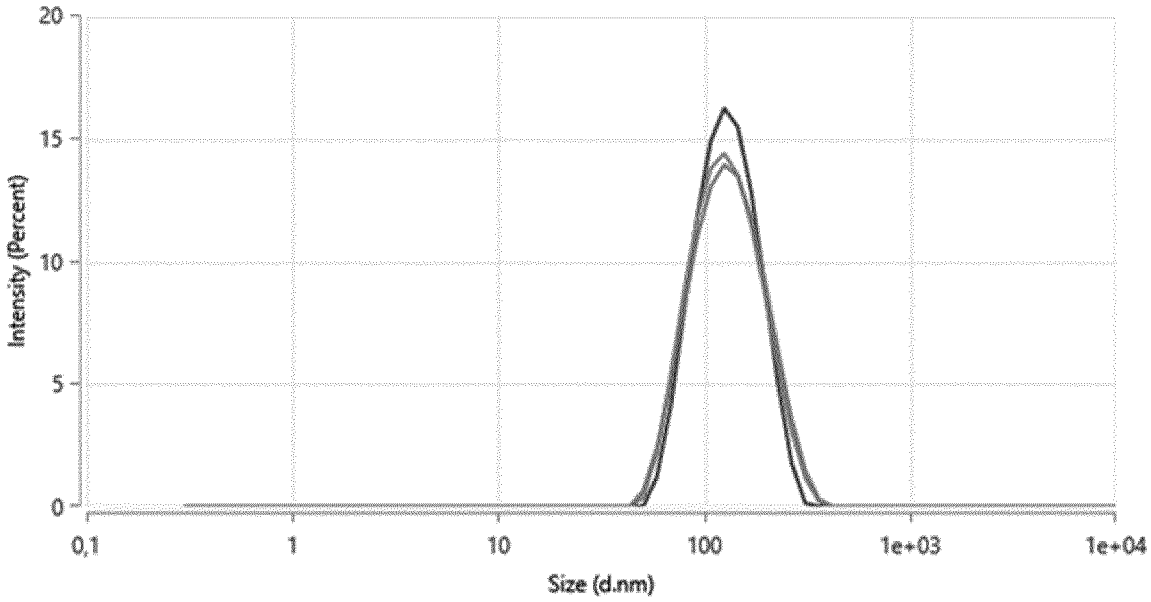

20
15
10
5
0
Intensity (Percent)
0,1
1
10
100
1e+03
1e+04
Size (d.nm)

FIG 5

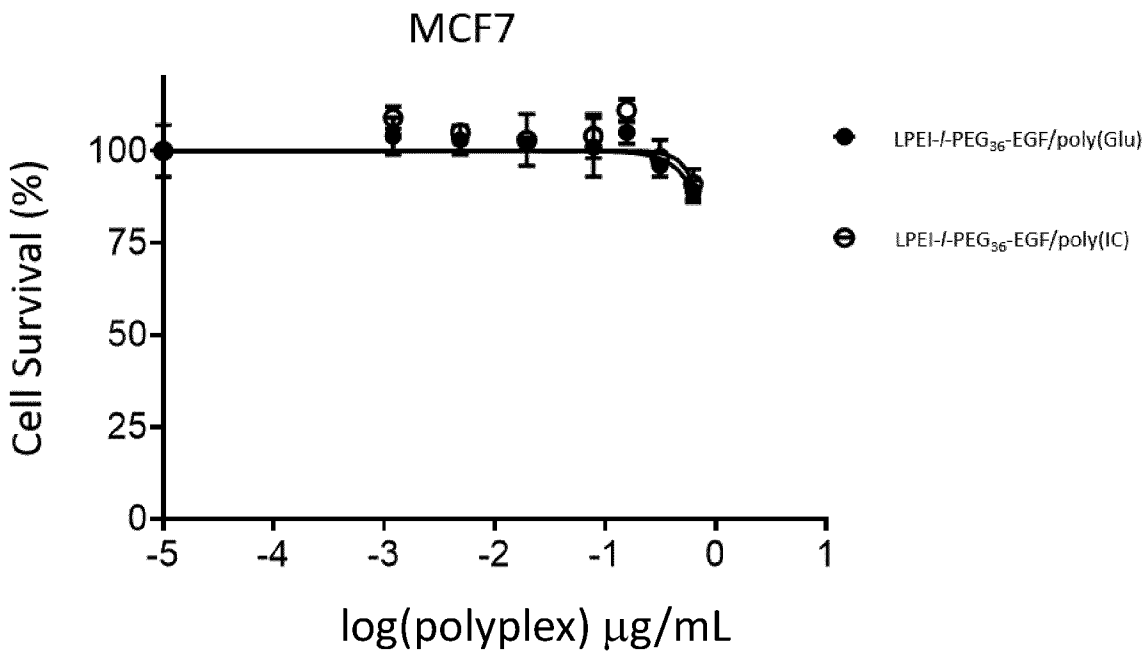

MCF7
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI-/-PEG36-EGF/poly(Glu)
LPEI-/-PEG36-EGF/poly(IC)

FIG 6A

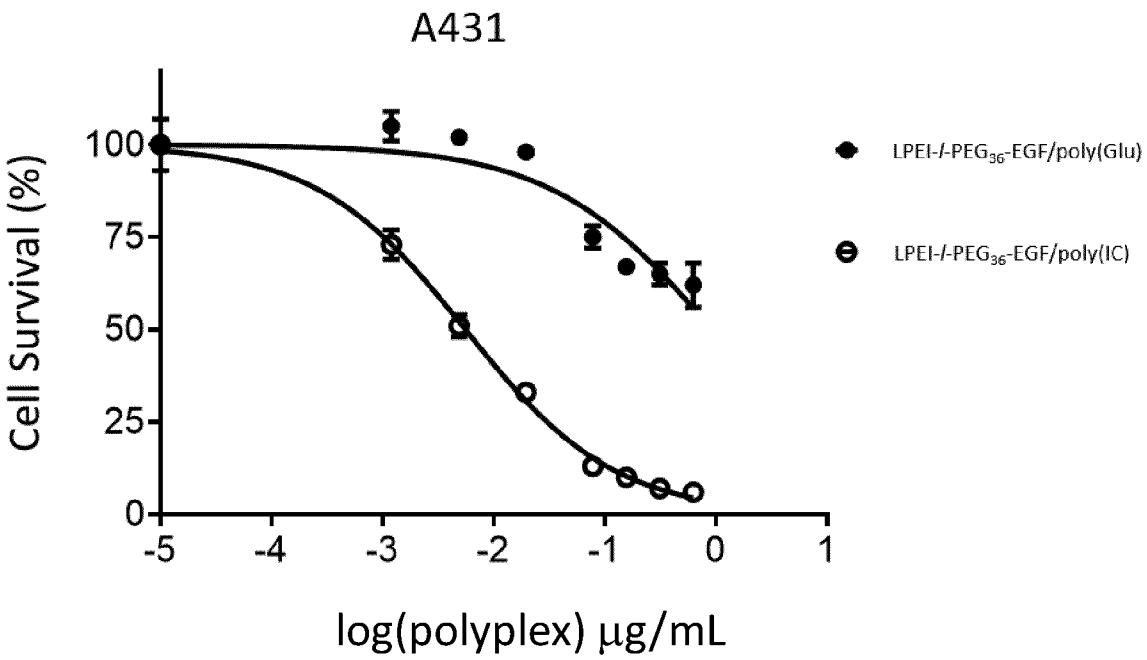

A431
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI-/-PEG36-EGF/poly(Glu)
LPEI-/-PEG36-EGF/poly(IC)

FIG 6B

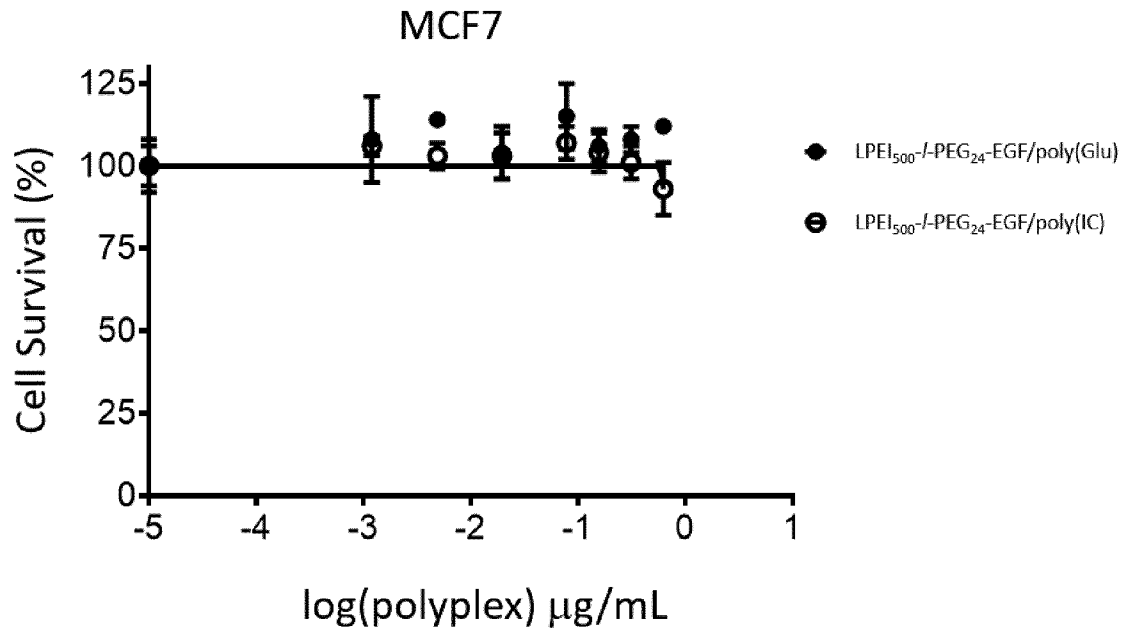

MCF7
Cell Survival (%)
125
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI500-/-PEG24-EGF/poly(Glu)
LPEI500-/-PEG24-EGF/poly(IC)

FIG 7A

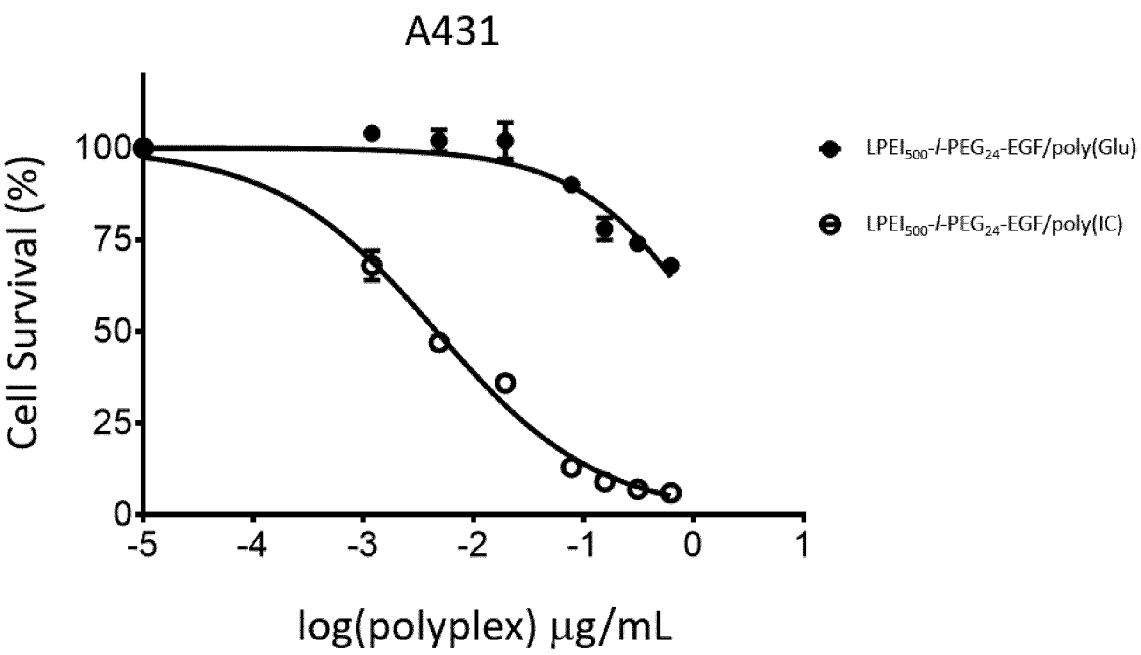

A431
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI500-l-PEG24-EGF/poly(Glu)
LPEI500-l-PEG24-EGF/poly(IC)

FIG 7B

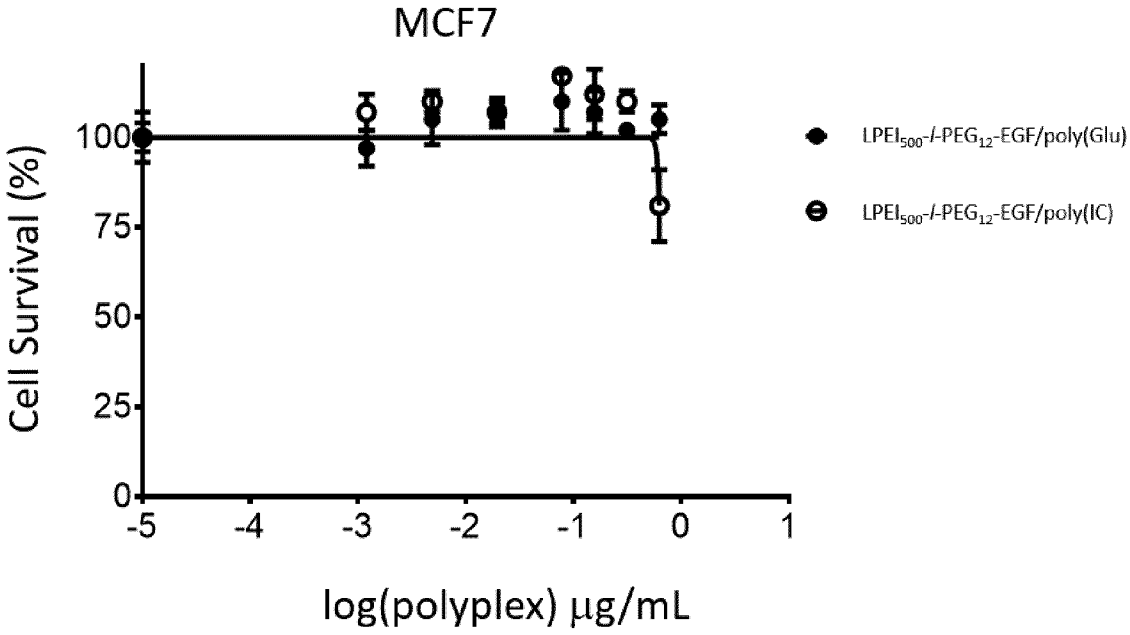

MCF7
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI500-l-PEG12-EGF/poly(Glu)
LPEI500-l-PEG12-EGF/poly(IC)

FIG 8A

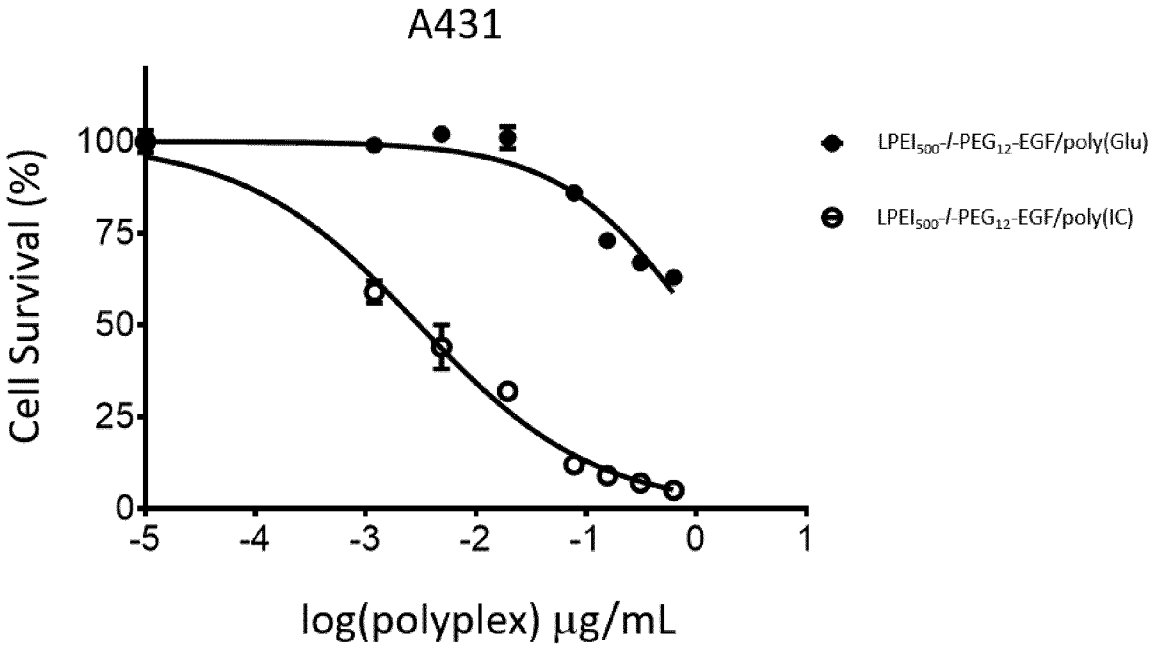

A431
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI500-/-PEG12-EGF/poly(Glu)
LPEI500-/-PEG12-EGF/poly(IC)

FIG 8B

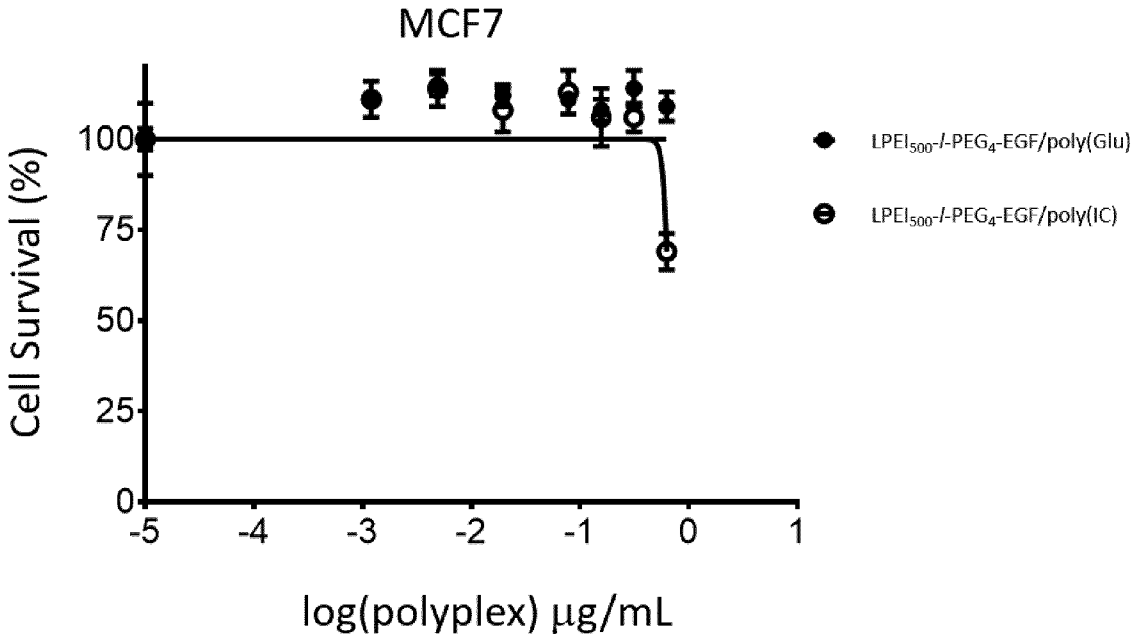

MCF7
Cell Survival (%)
100
75
50
25
0
-5
-4
-3
-2
-1
0
1
log(polyplex) µg/mL
LPEI500-/-PEG4-EGF/poly(Glu)
LPEI500-/-PEG4-EGF/poly(IC)

FIG 9A

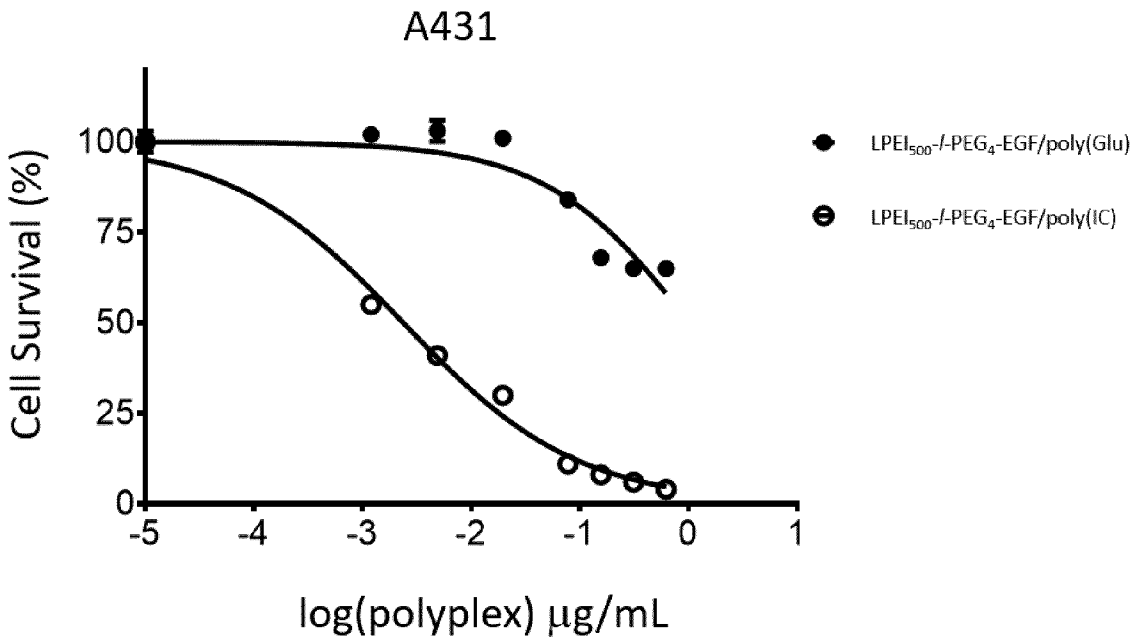


FIG 9B

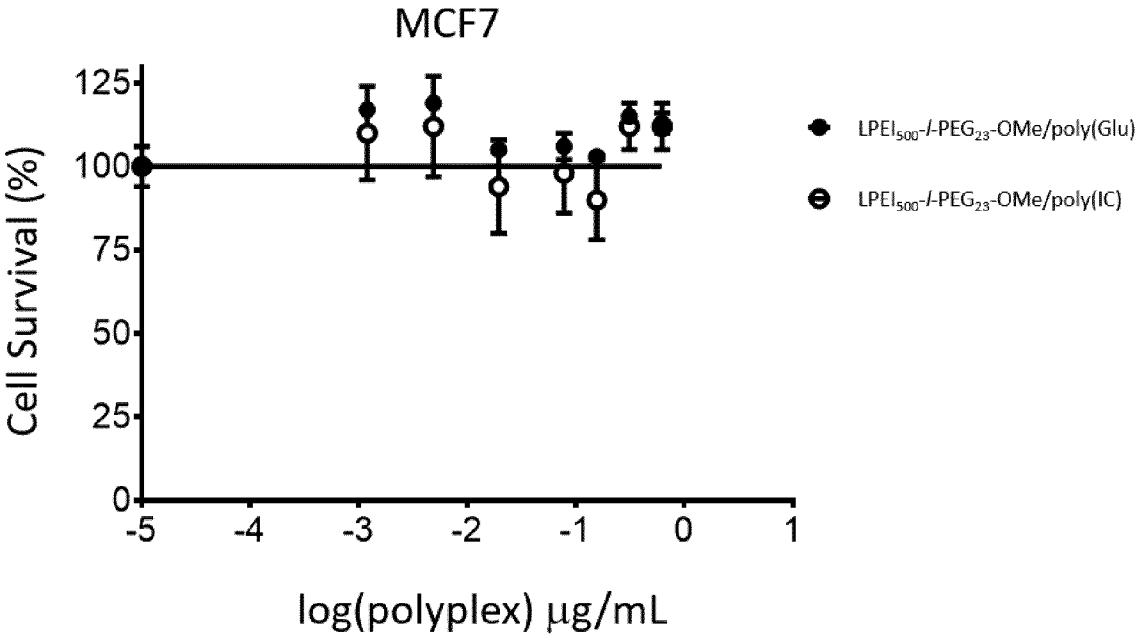


FIG 10A

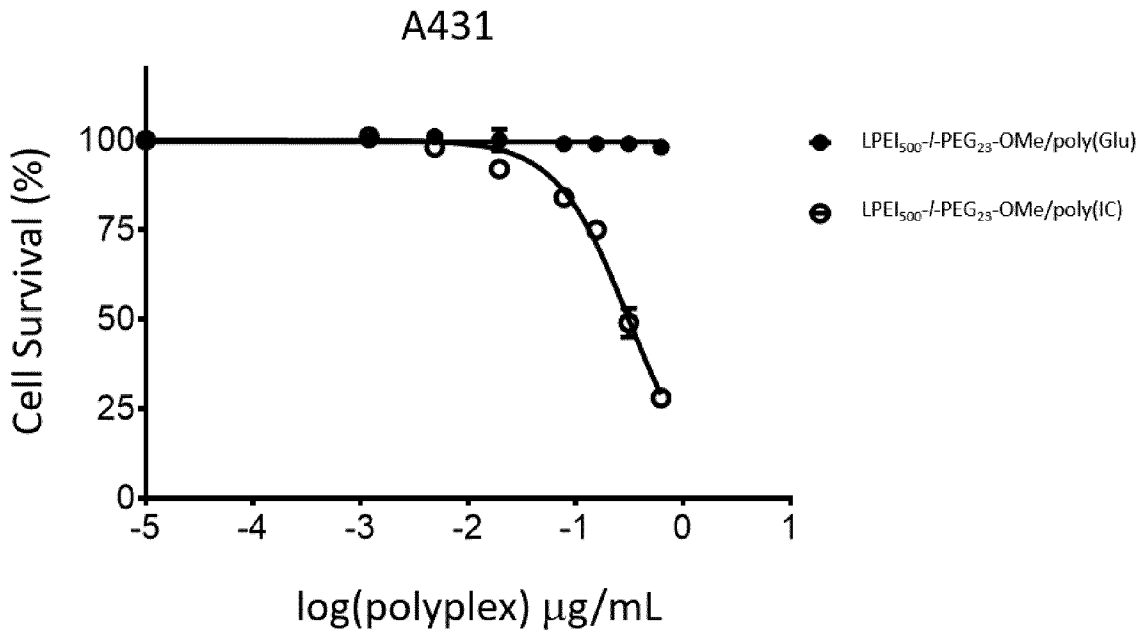


FIG 10B

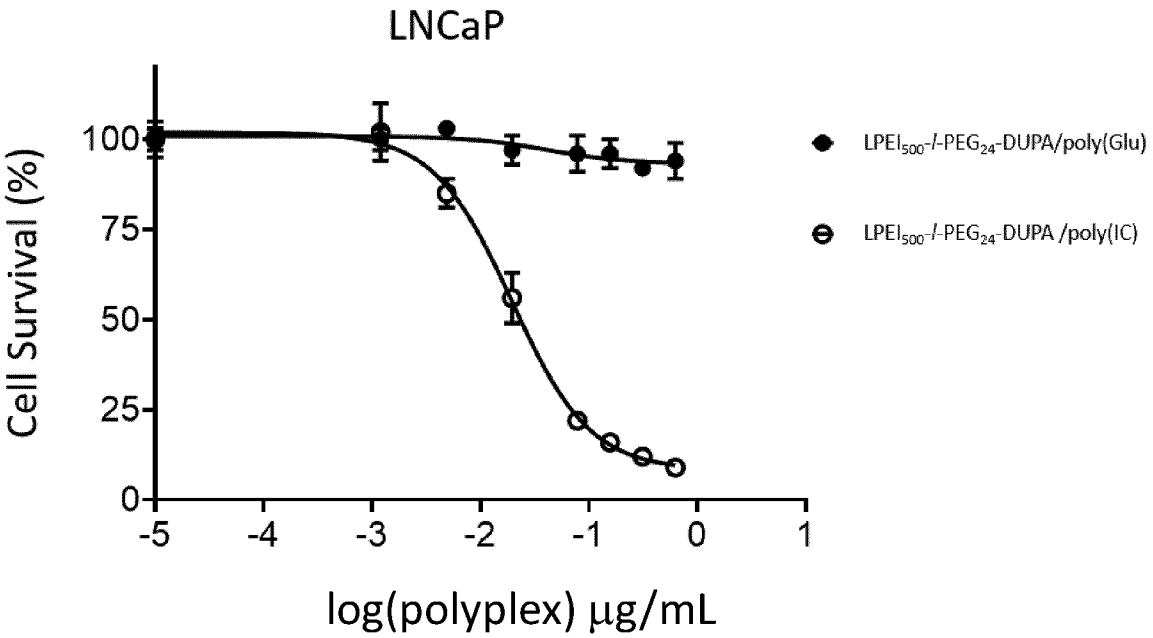


FIG 11A

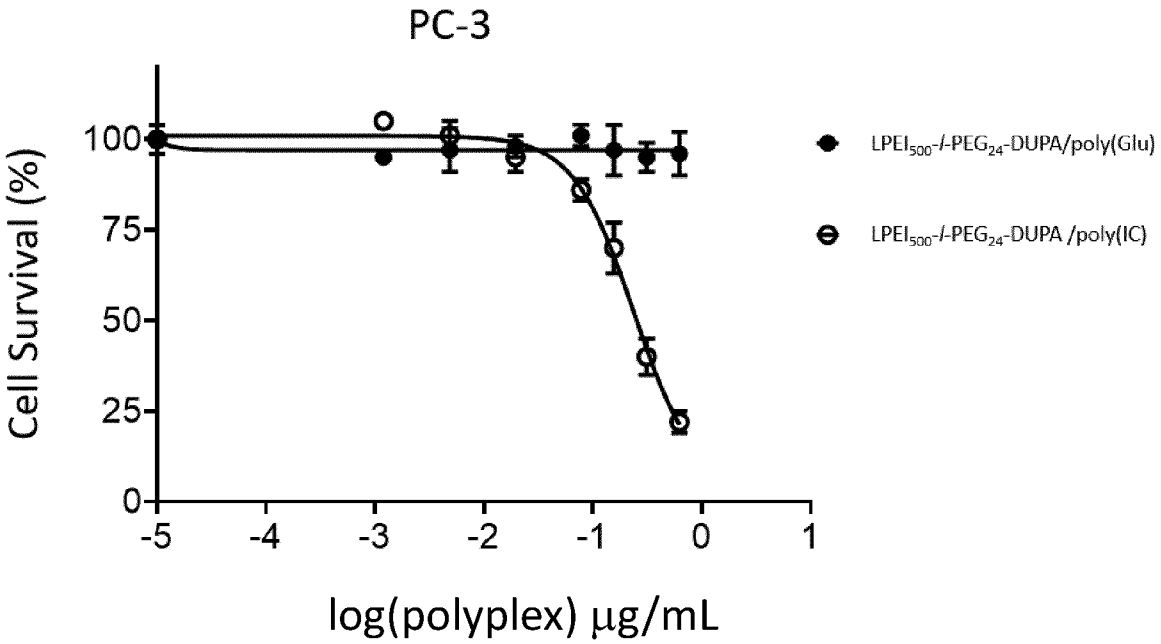


FIG 11B

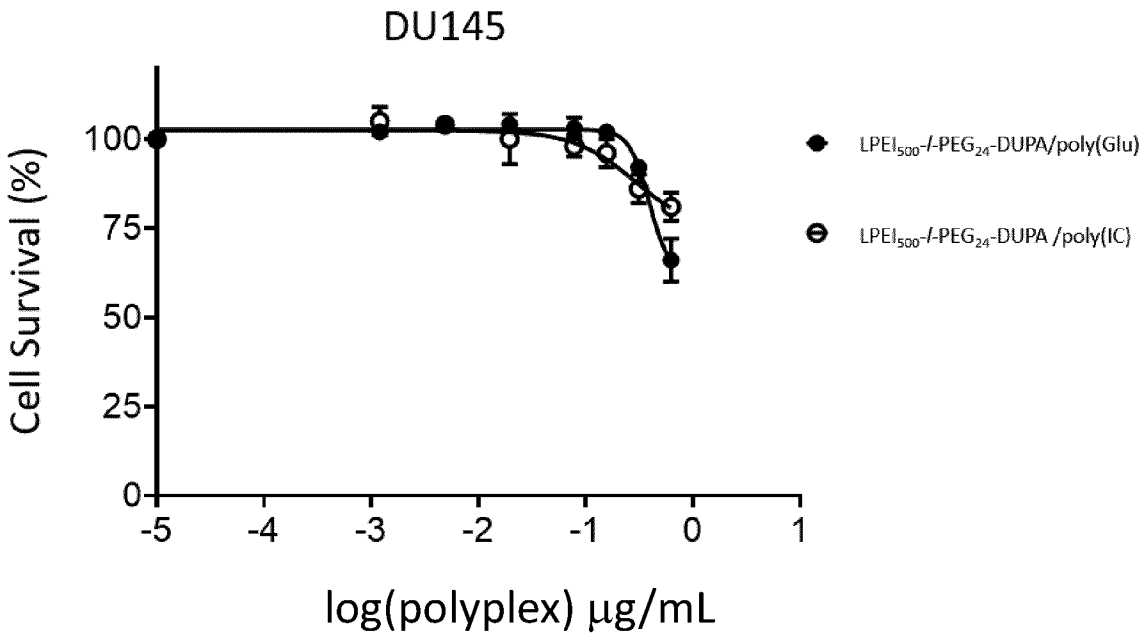


FIG 11C

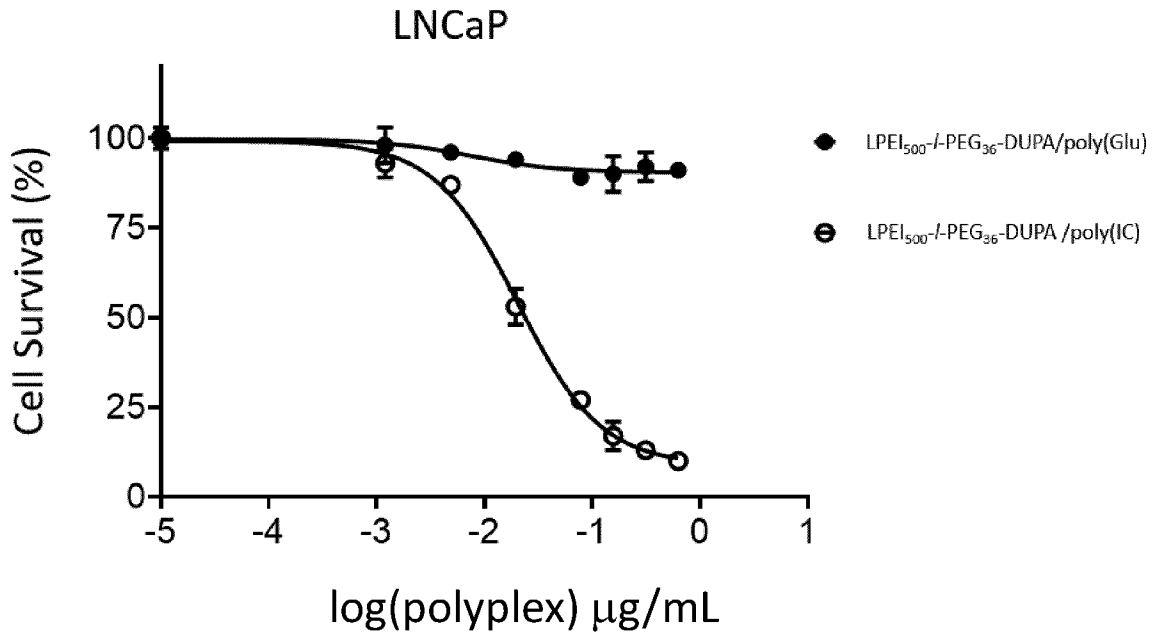


FIG 12A

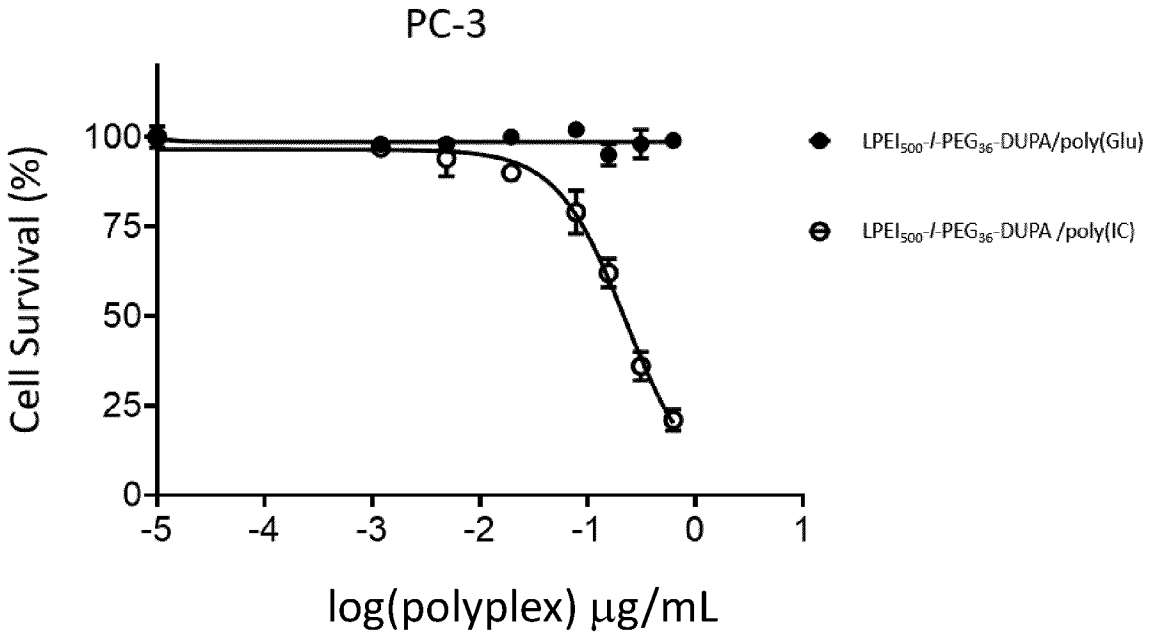


FIG 12B

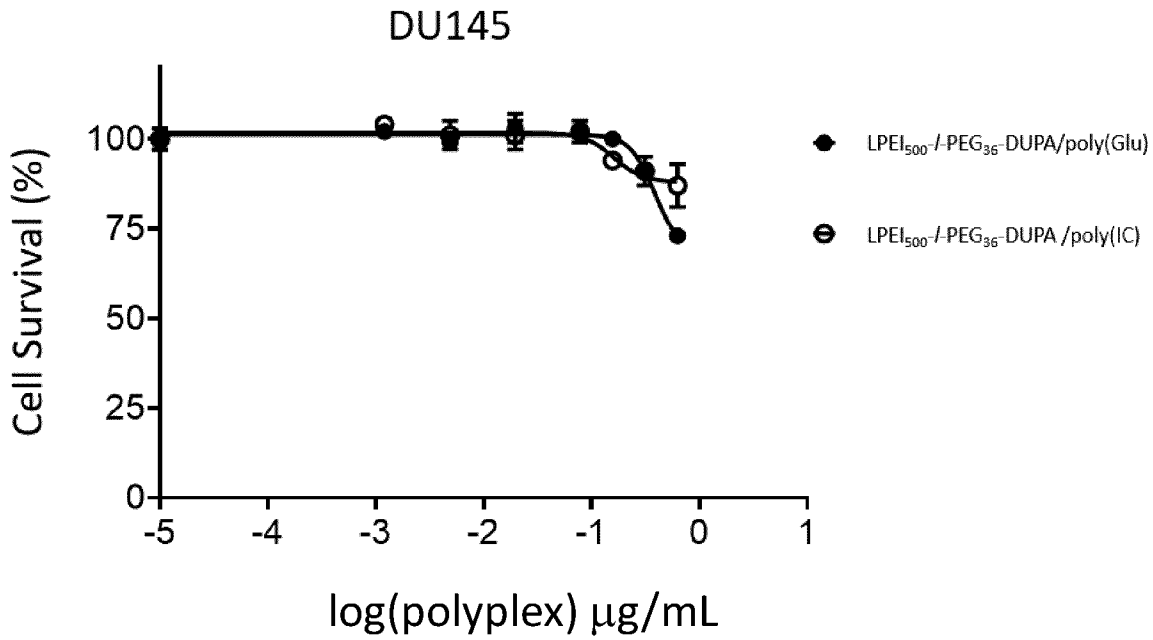


FIG 12C

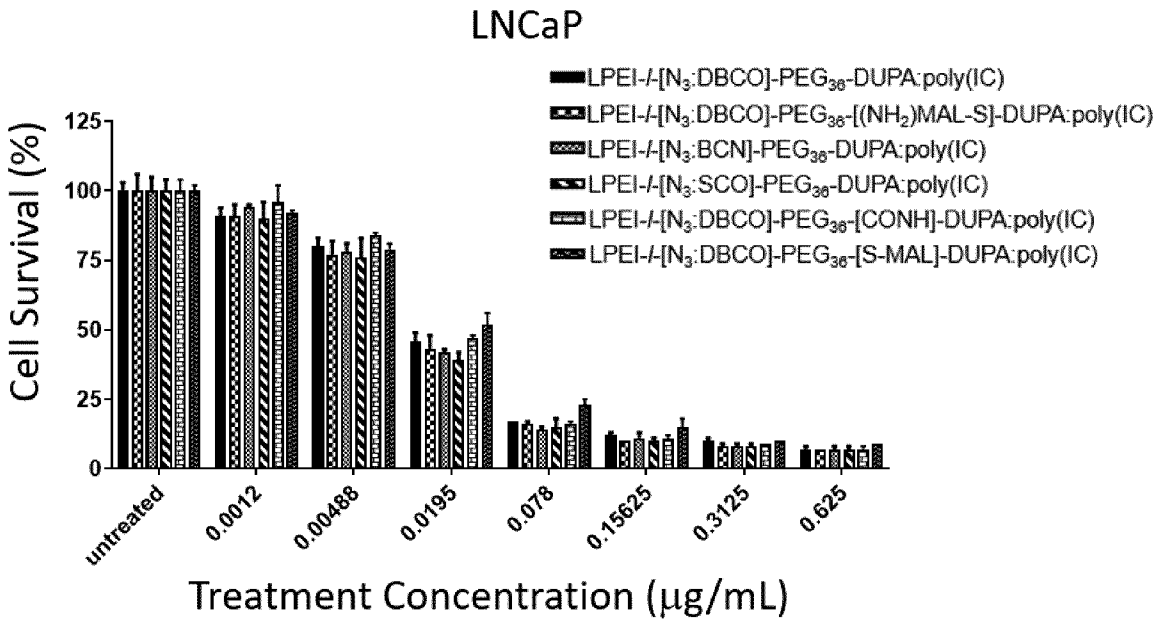


FIG 13

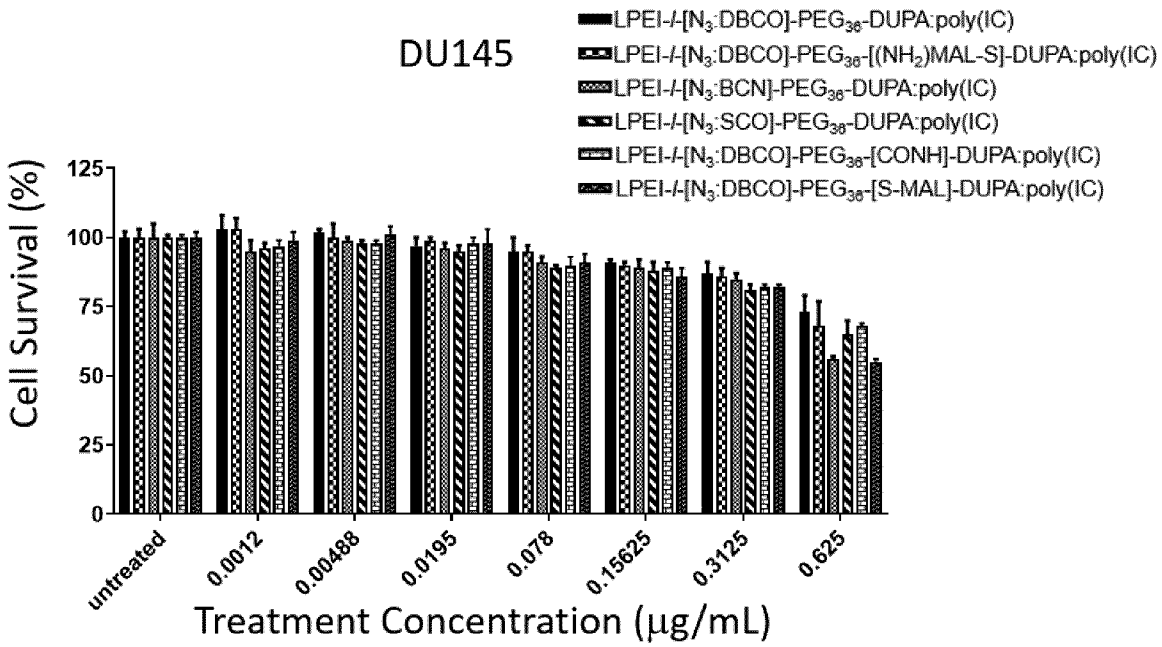

DU145
LPEI-/-[N3:DBCO]-PEG36-DUPA:poly(IC)
LPEI-/-[N3:DBCO]-PEG36-[(NH2)MAL-S]-DUPA:poly(IC)
LPEI-/-[N3:BCN]-PEG36-DUPA:poly(IC)
LPEI-/-[N3:SCO]-PEG36-DUPA:poly(IC)
LPEI-/-[N3:DBCO]-PEG36-[CONH]-DUPA:poly(IC)
LPEI-/-[N3:DBCO]-PEG36-[S-MAL]-DUPA:poly(IC)
Cell Survival (%)
125
100
75
50
25
0
untreated
0.0012
0.00488
0.0195
0.078
0.15625
0.3125
0.625
Treatment Concentration (µg/mL)

FIG 14

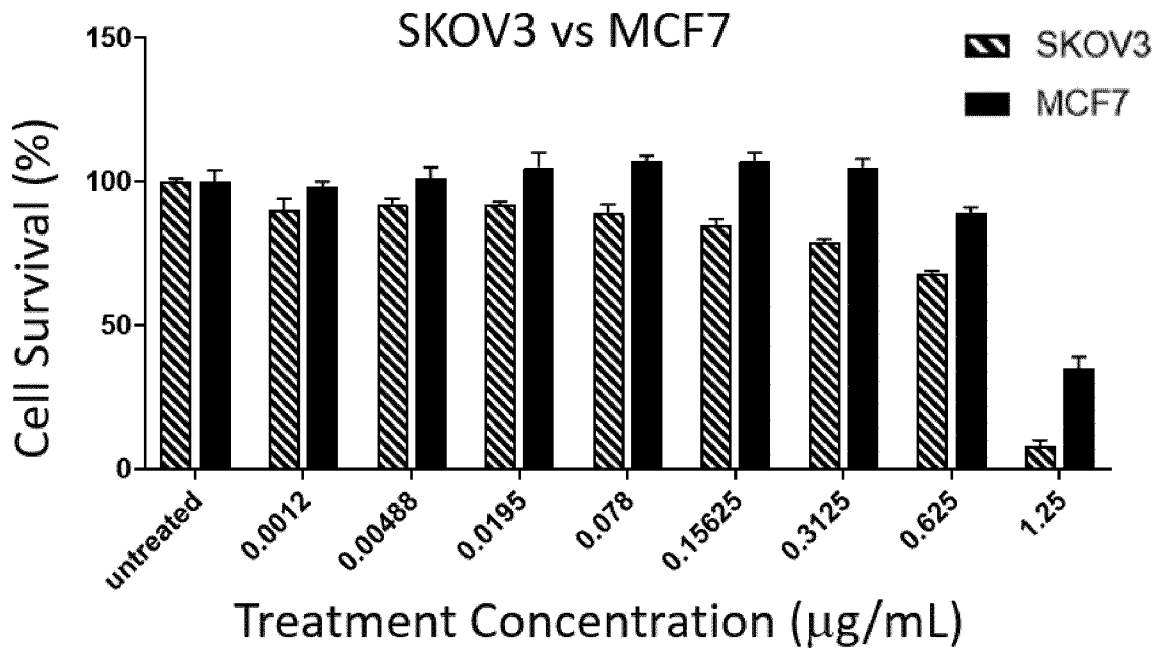

SKOV3 vs MCF7
SKOV3
MCF7
Cell Survival (%)
150
100
50
0
untreated
0.0012
0.00488
0.0195
0.078
0.15625
0.3125
0.625
1.25
Treatment Concentration (µg/mL)

FIG 15

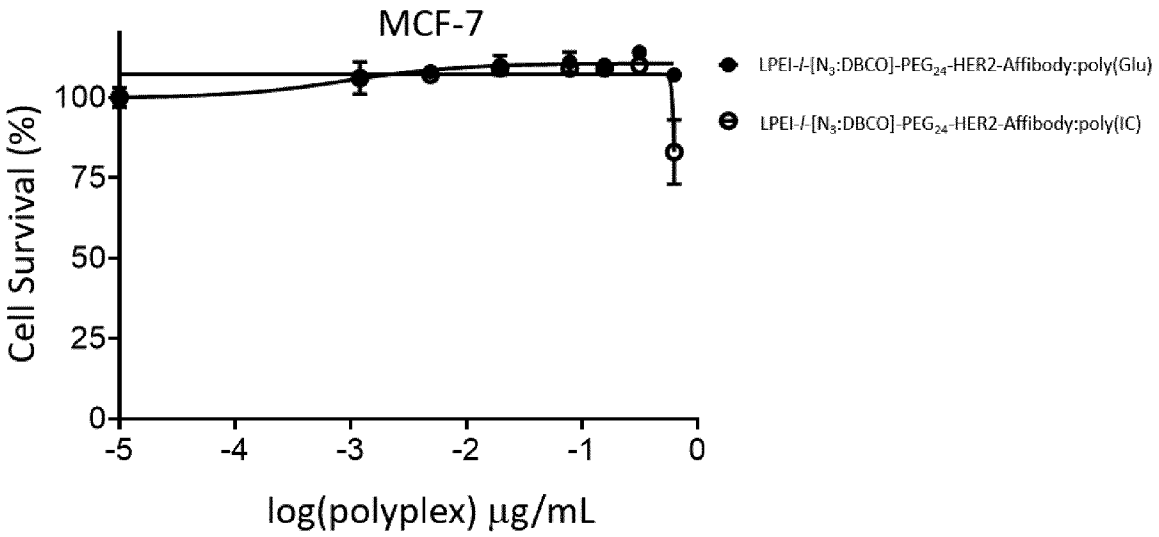


FIG 16A

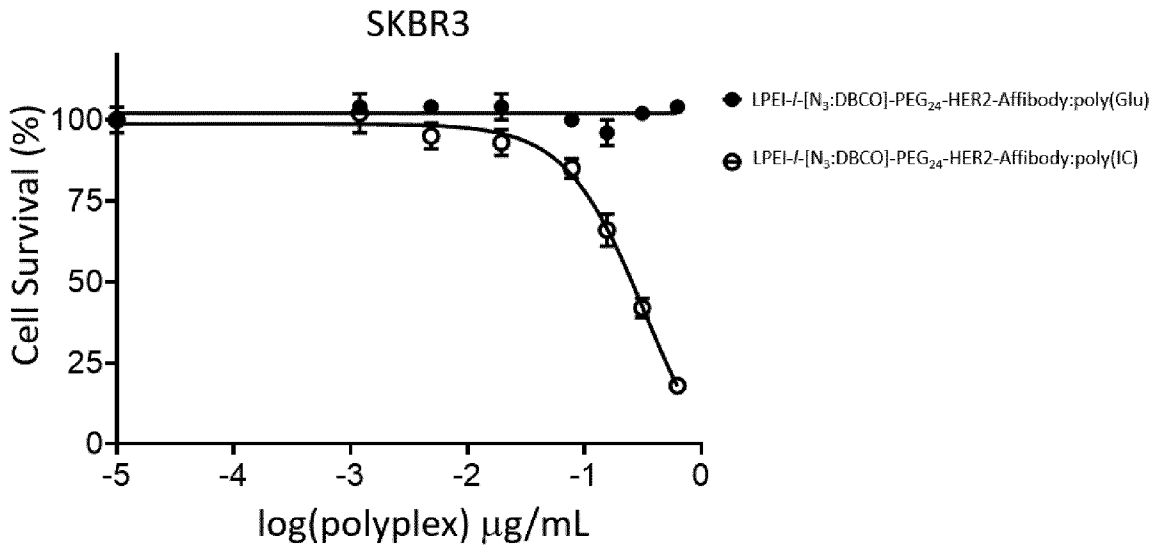


FIG 16B

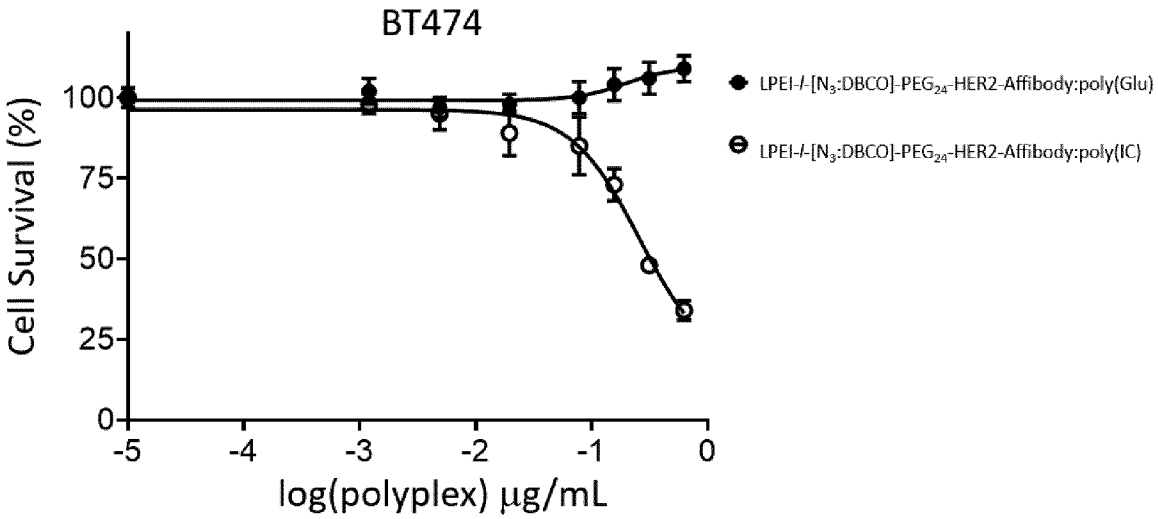


FIG 16C

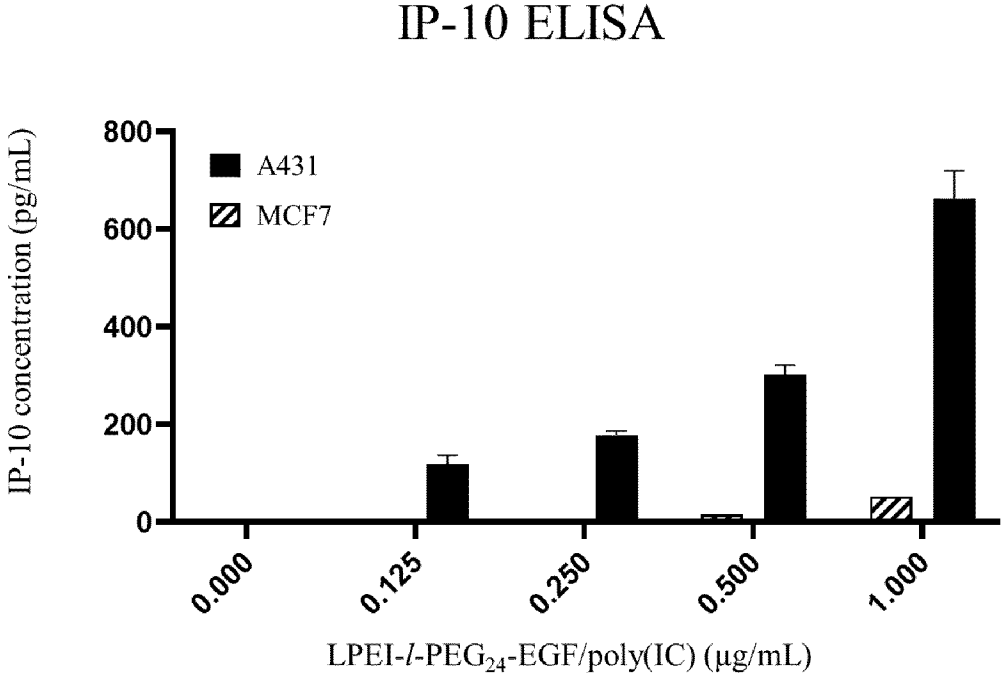


FIG 17

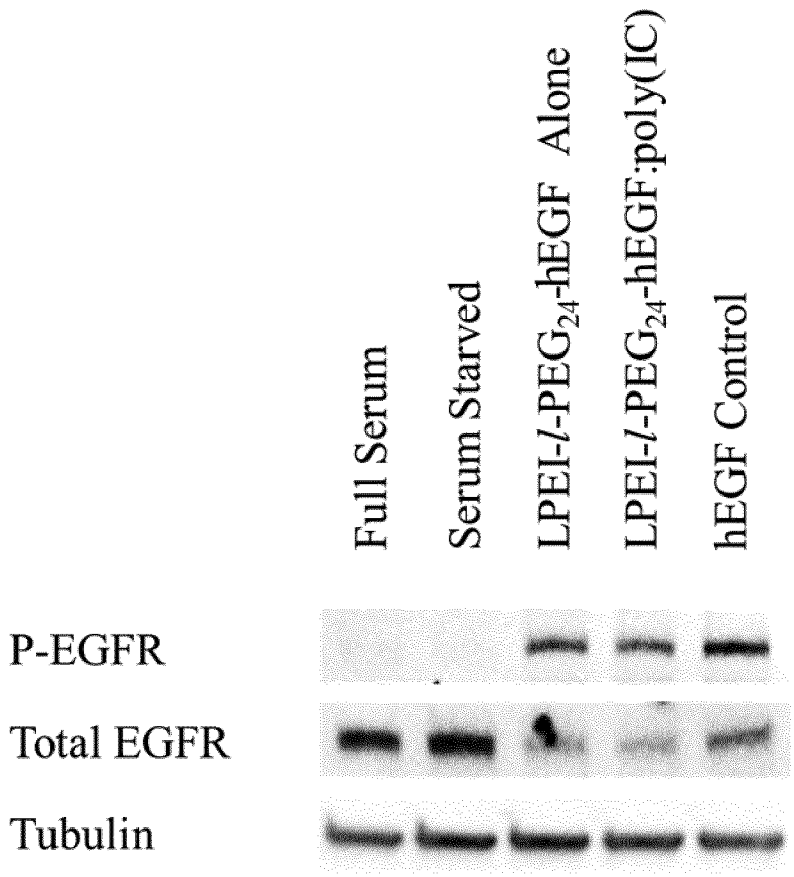

Full Serum
Serum Starved
LPEI-l-PEG24-hEGF Alone
LPEI-l-PEG24-hEGF:poly(IC)
hEGF Control
P-EGFR
Total EGFR
Tubulin

FIG 18

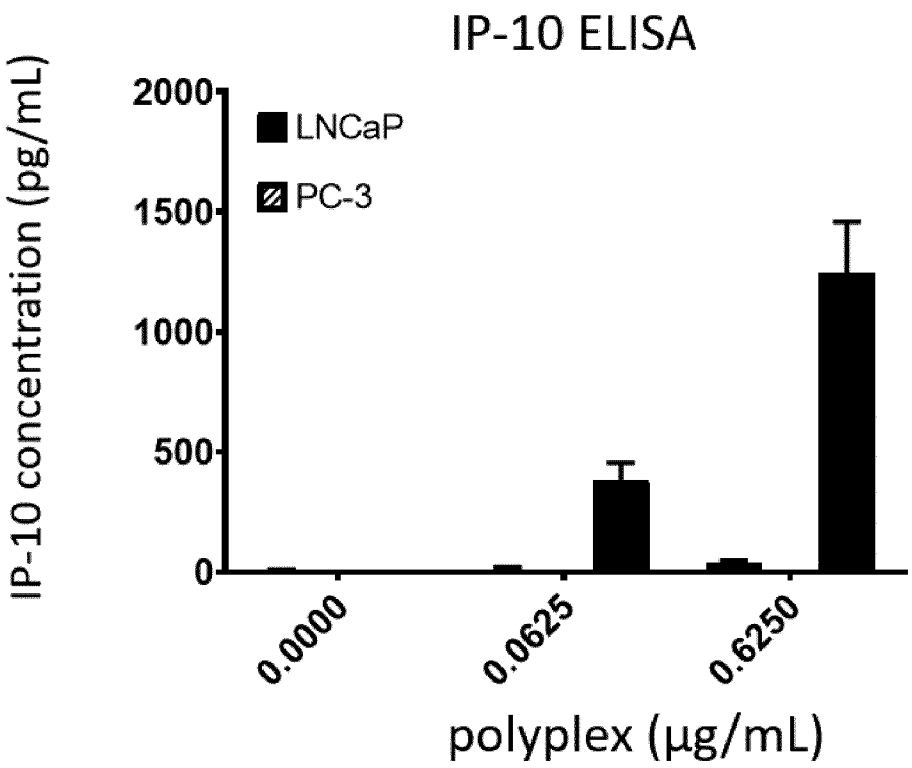


FIG 19A

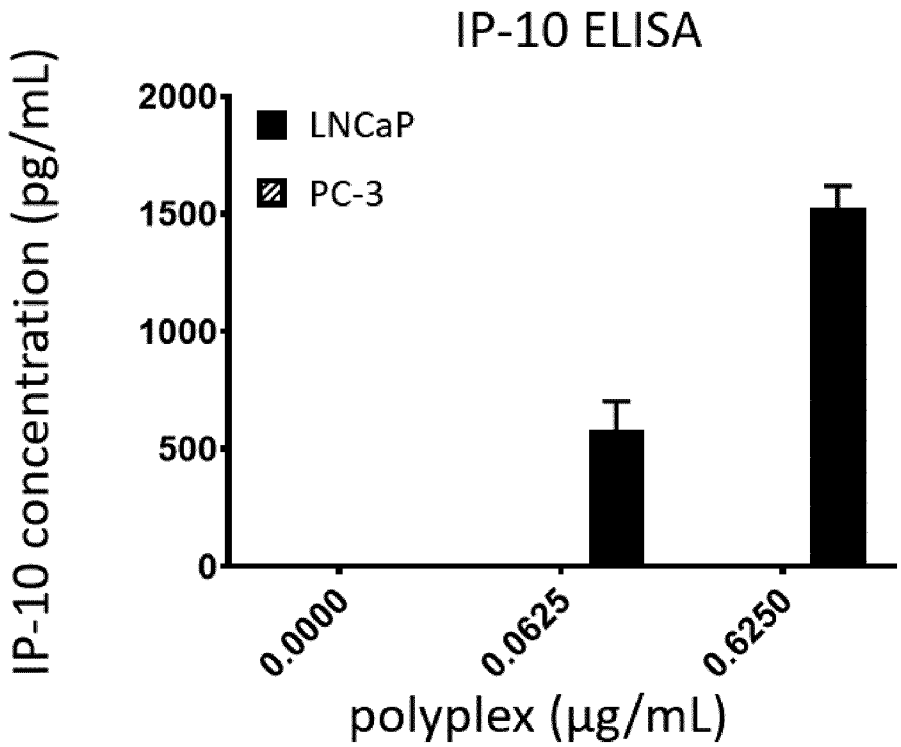


FIG 19B

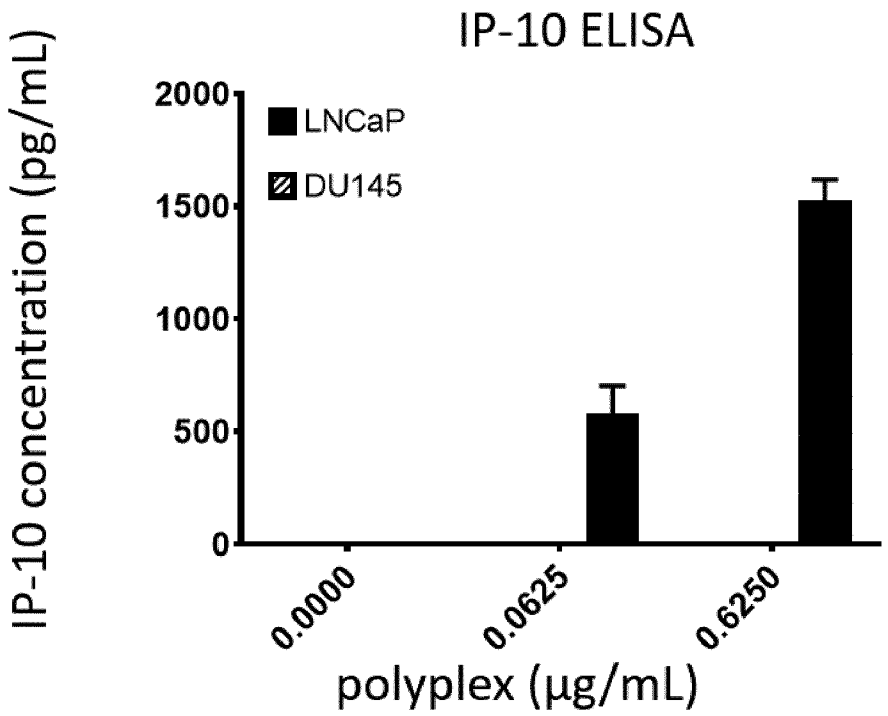


FIG 19C

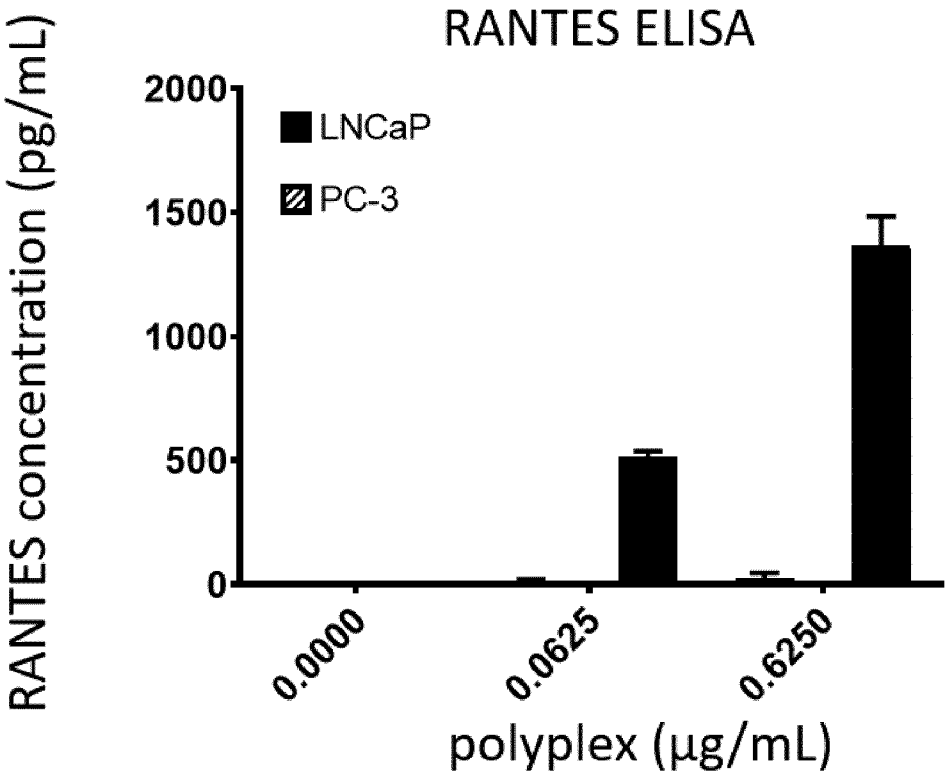


FIG 20A

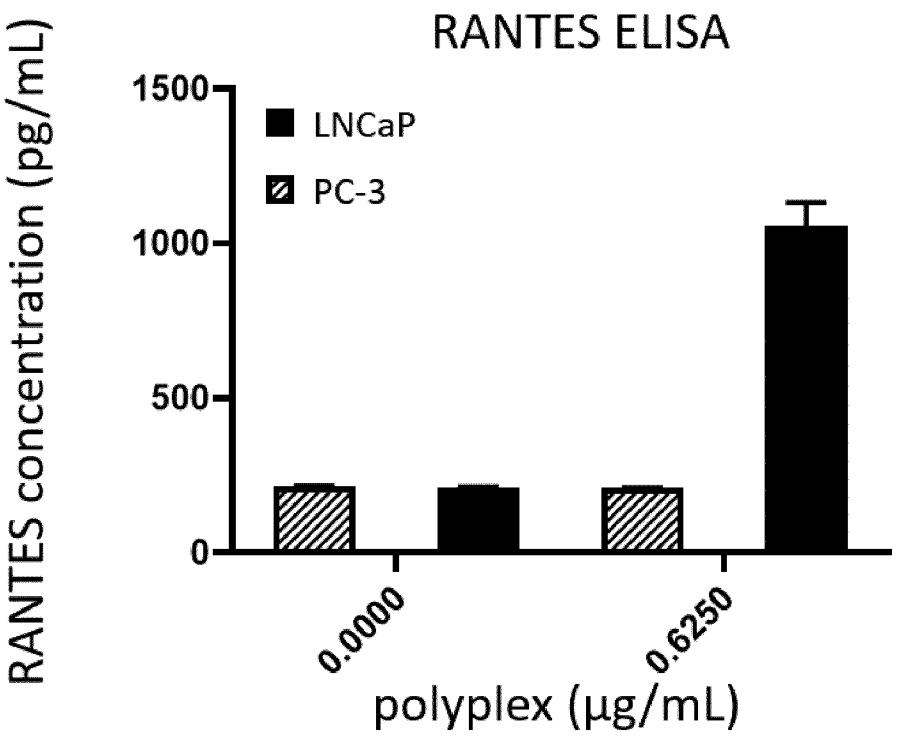


FIG 20B

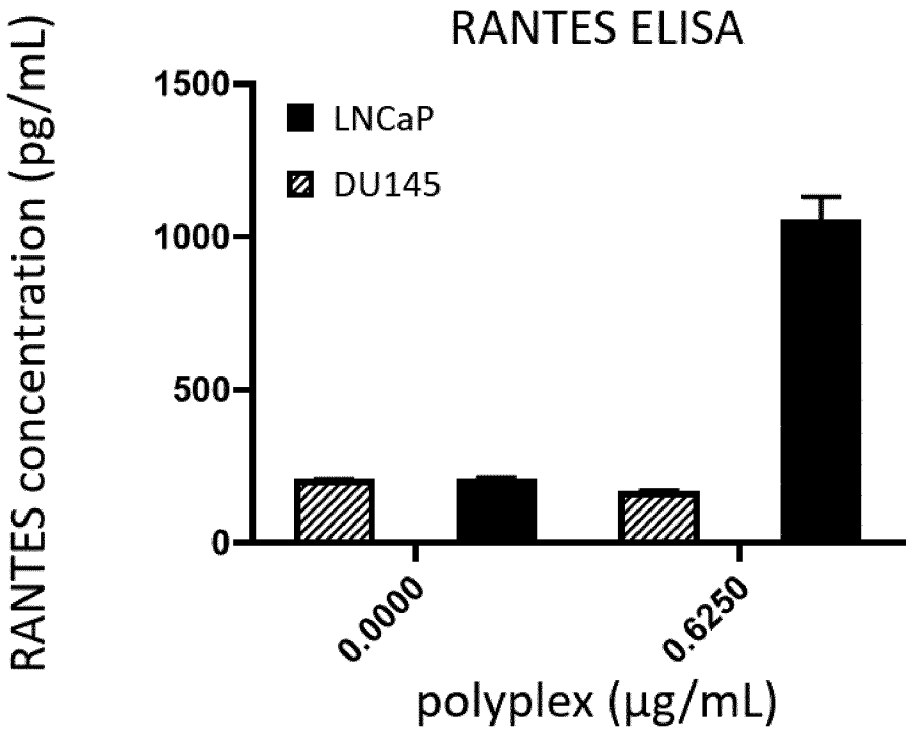


FIG 20C

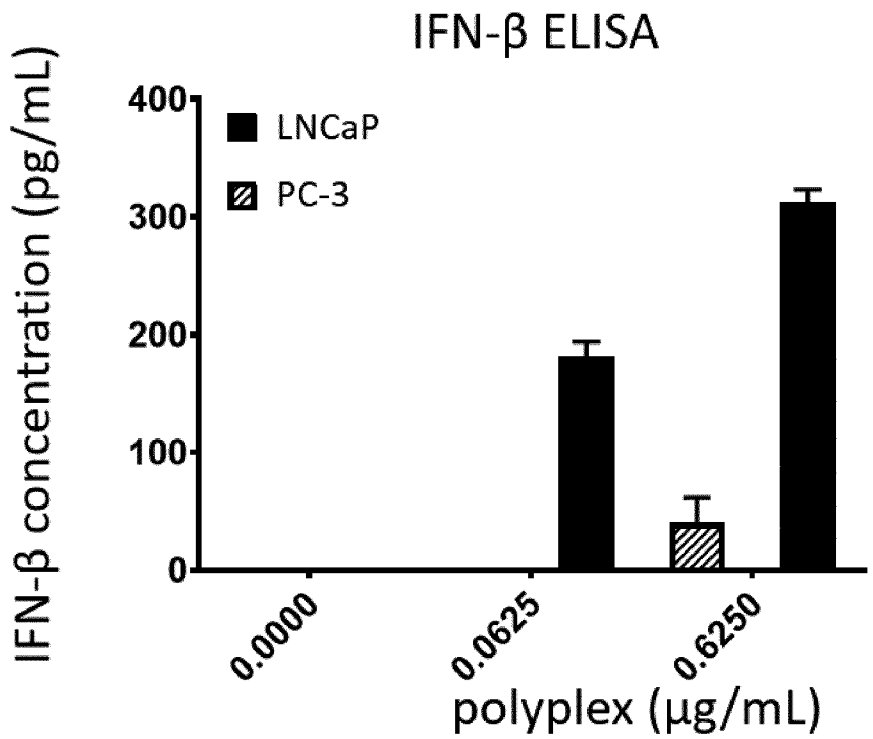


FIG 21A

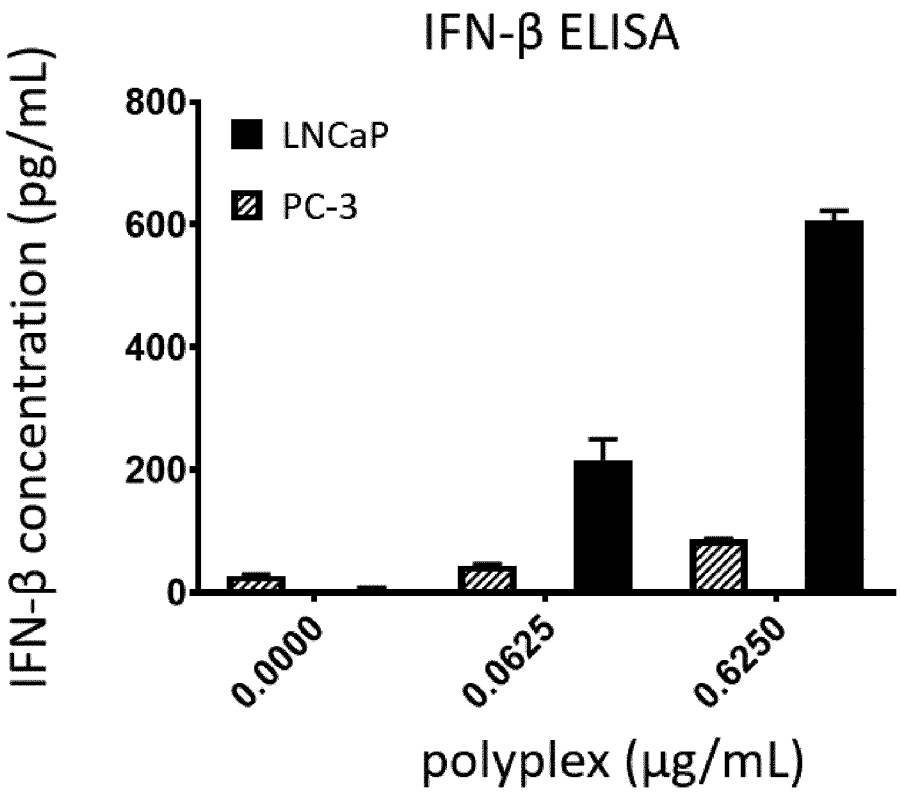


FIG 21B

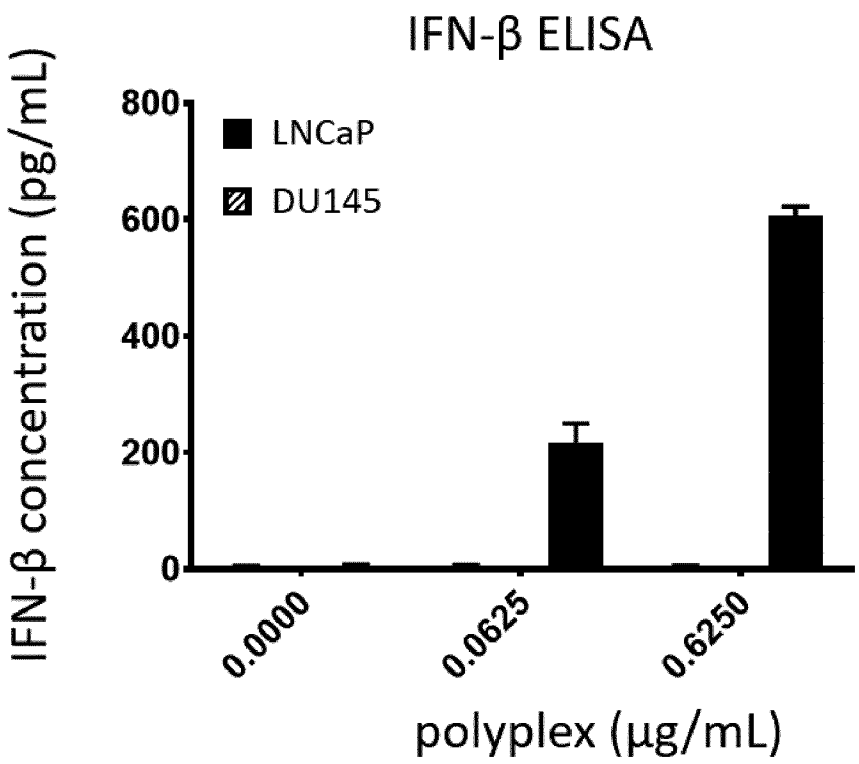

IFN-β ELISA
IFN-β concentration (pg/mL)
800
600
400
200
0
LNCaP
DU145
0.0000
0.0625
0.6250
polyplex (µg/mL)

FIG 21C

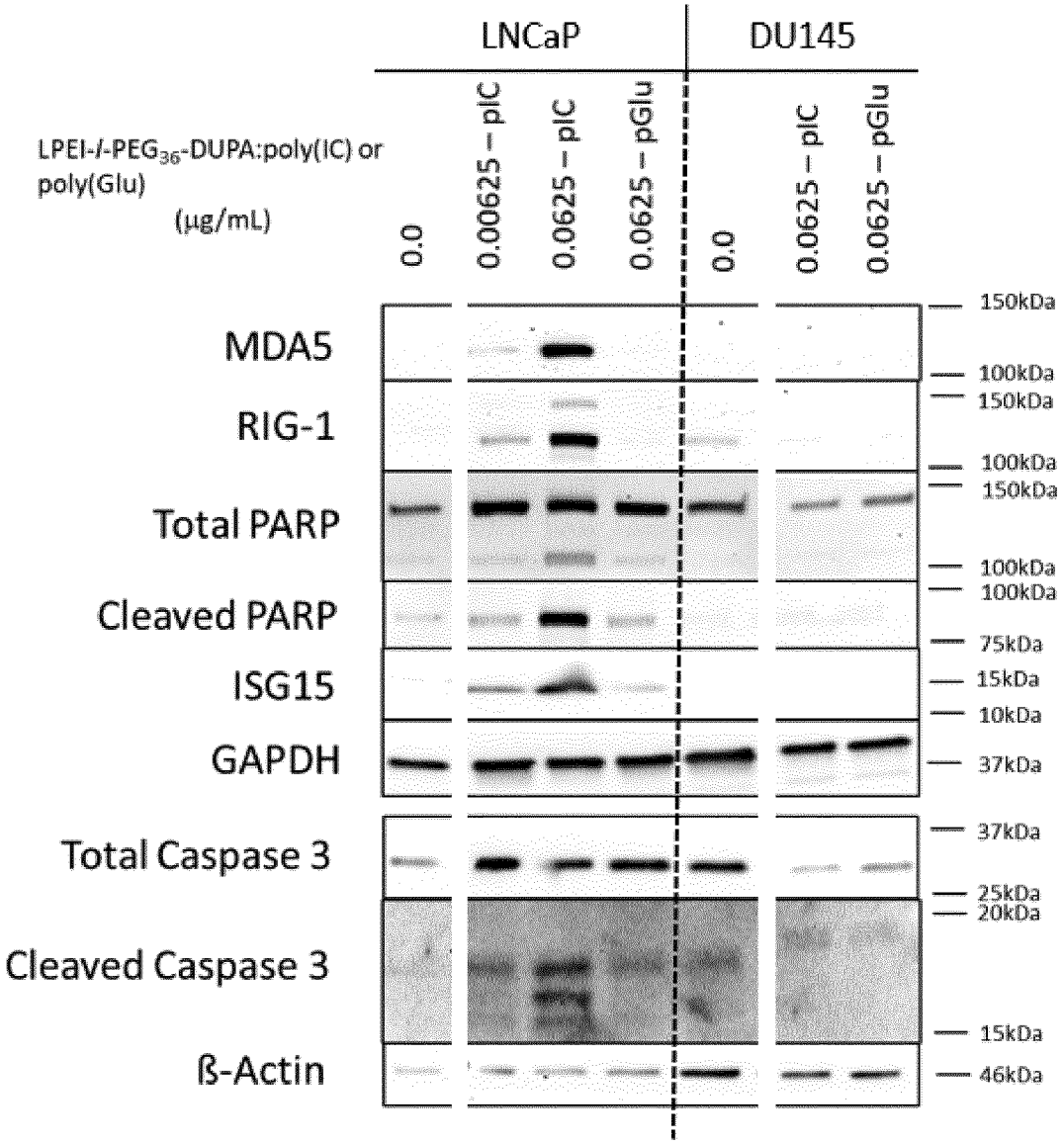

LNCaP
DU145
LPEI-/-PEG36-DUPA:poly(IC) or poly(Glu)
(µg/mL)
0.0
0.00625 – pIC
0.0625 – pIC
0.0625 – pGlu
0.0
0.0625 – pIC
0.0625 – pGlu
MDA5
RIG-1
Total PARP
Cleaved PARP
ISG15
GAPDH
Total Caspase 3
Cleaved Caspase 3
ß-Actin
150kDa
100kDa
150kDa
100kDa
150kDa
100kDa
100kDa
75kDa
15kDa
10kDa
37kDa
37kDa
25kDa
20kDa
15kDa
46kDa

FIG 22

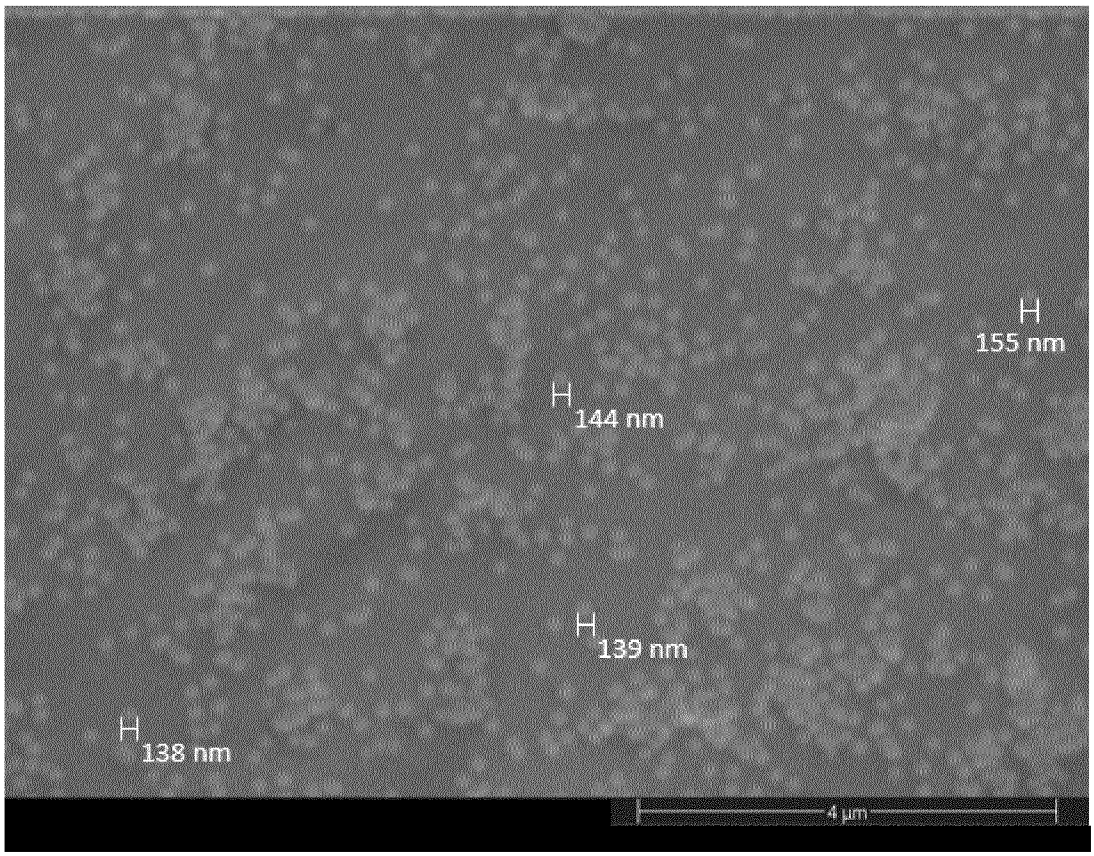
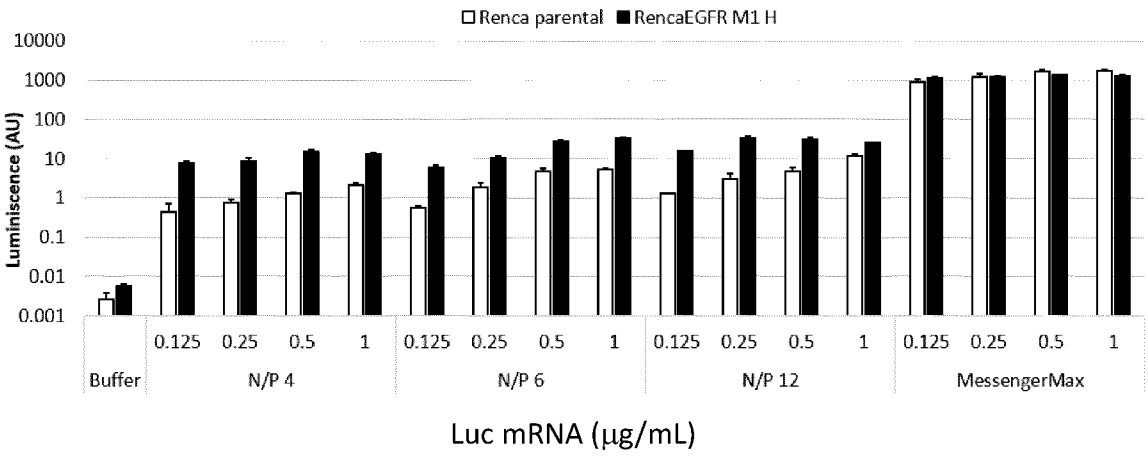


FIG 23



Luc mRNA (µg/mL)

FIG 24A

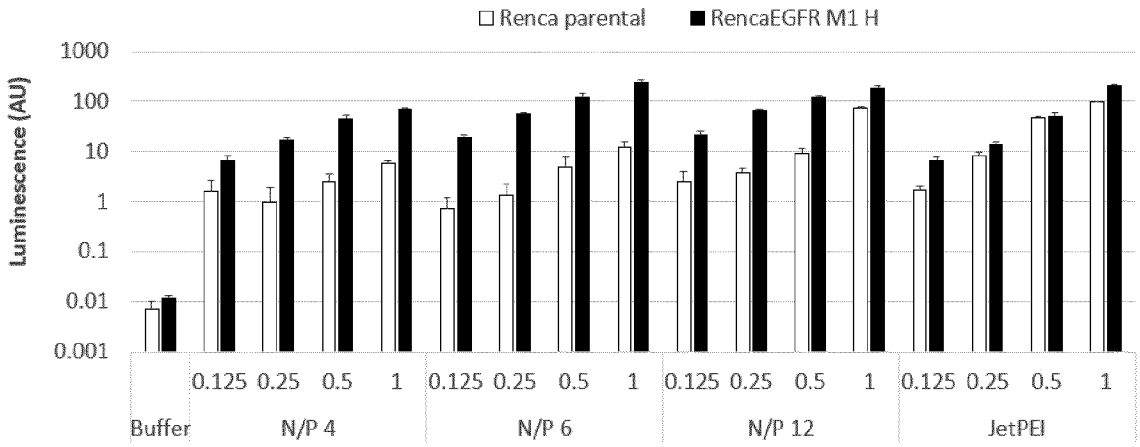


FIG 24B

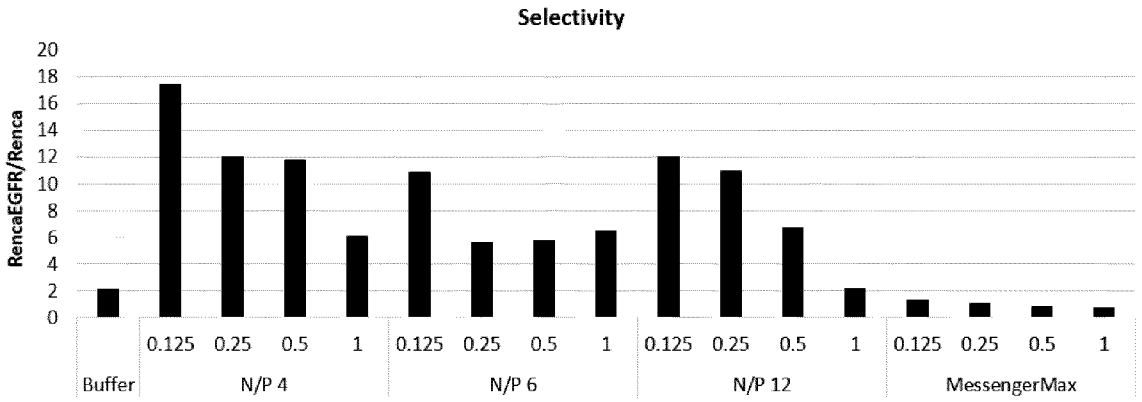


FIG 24C

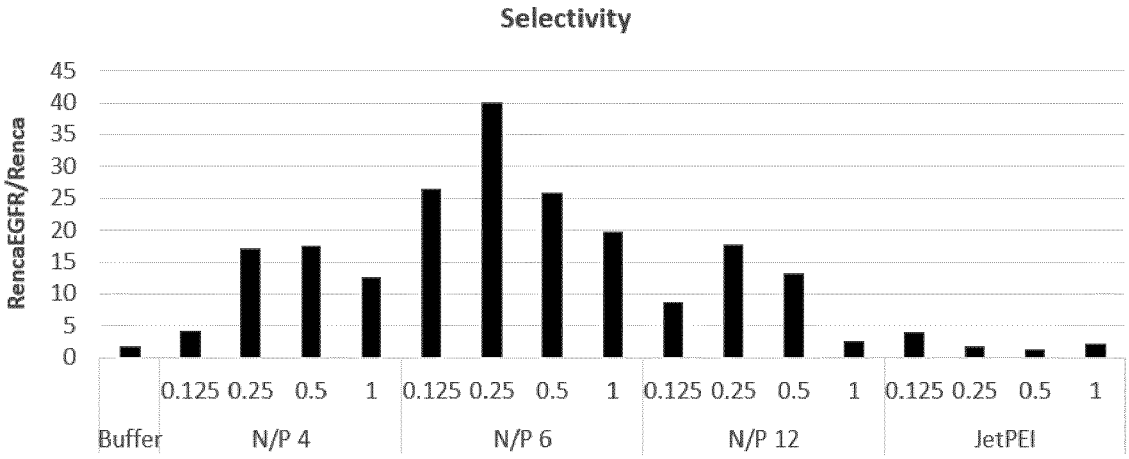


FIG 24D

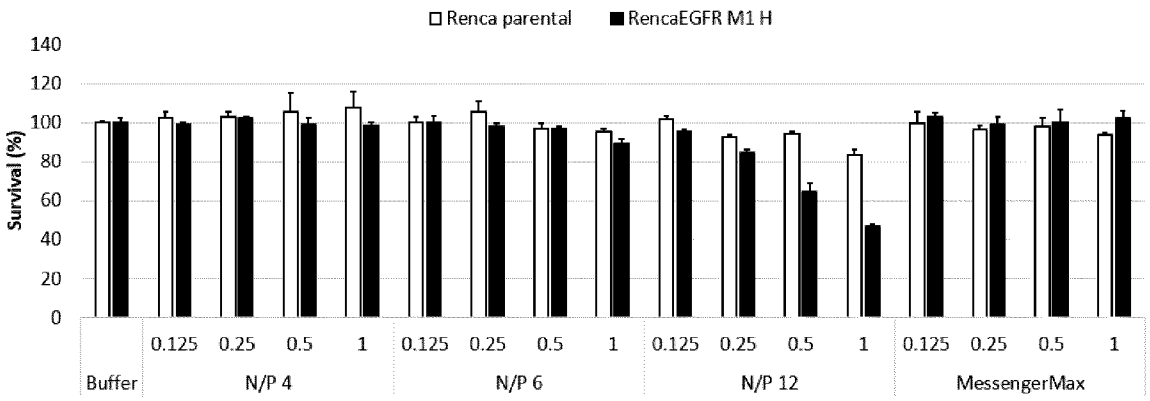


FIG 24E

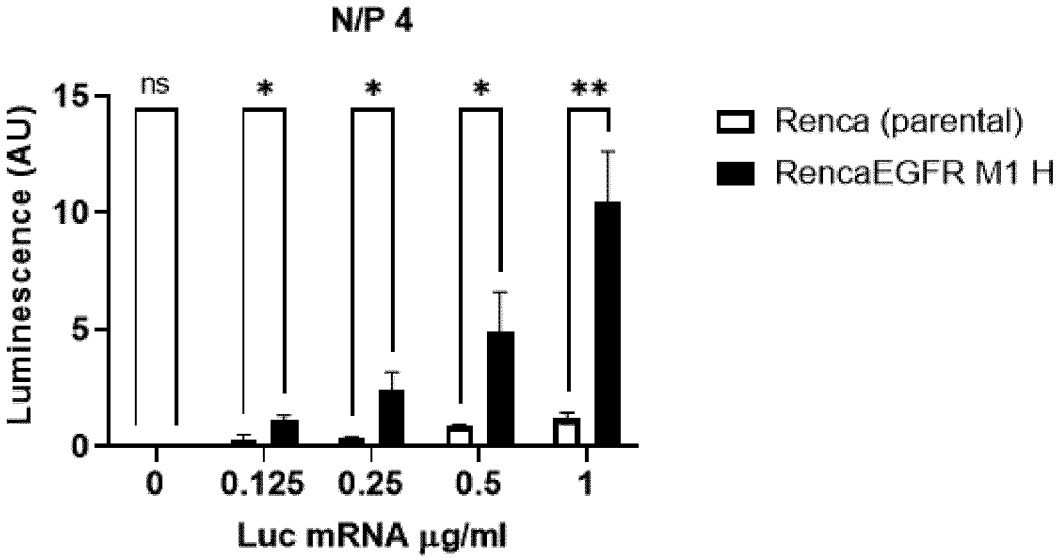


FIG 25A

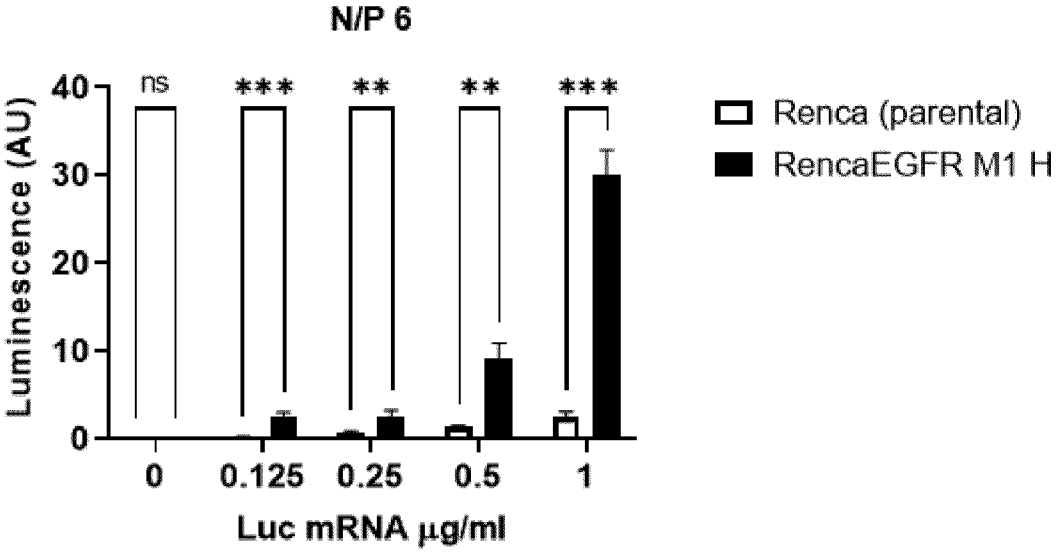


FIG 25B

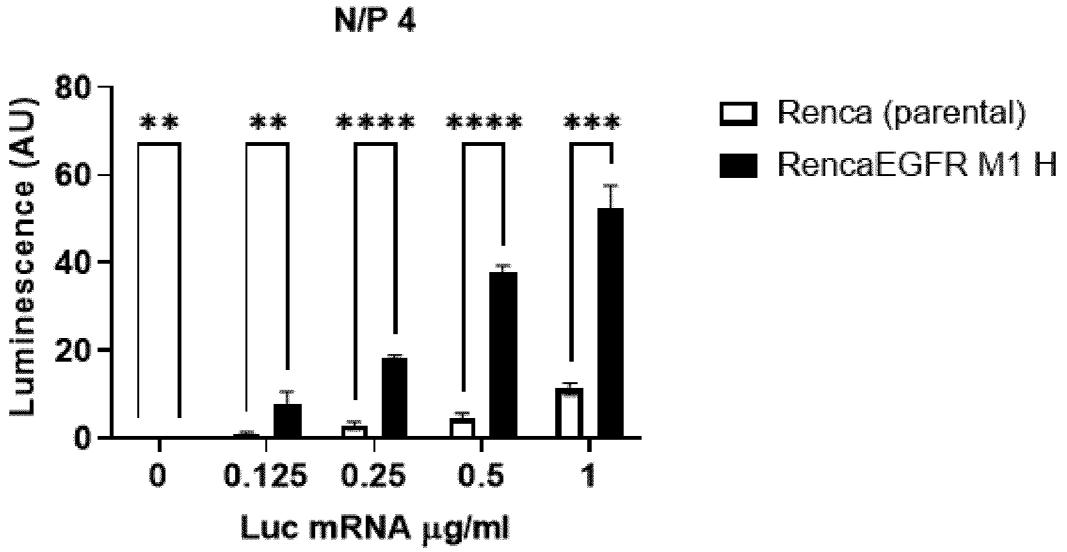


FIG 25C

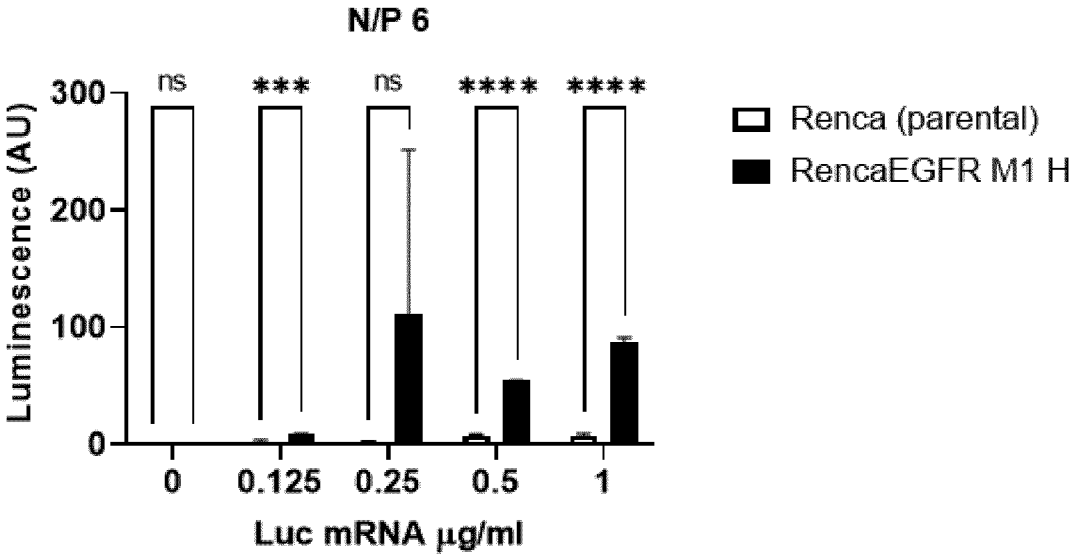


FIG 25D