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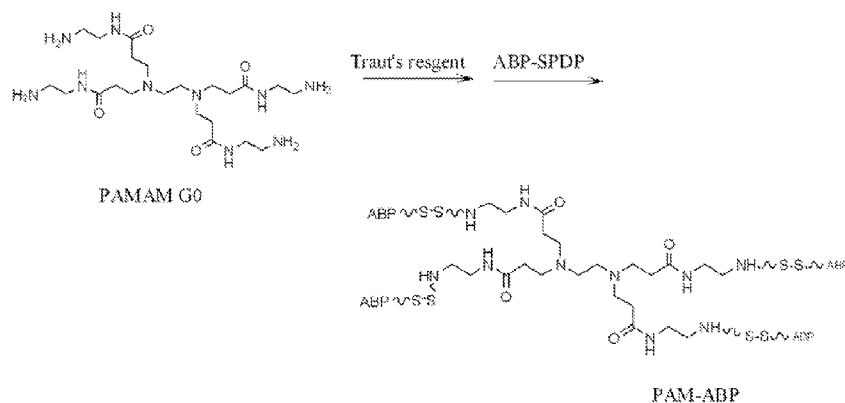
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(54) Title: HIGH MOLECULAR WIEGHT ARGININE-GRAFTED BIOREDUCIBLE POLYMERS



(57) Abstract: The polymeric carrier for delivering nucleic acid material to a cell is provided herein. The polymeric carrier can include a dendrimer group having 2 to 8 termini, each of the termini having an arginine-grafted bioreducible polymer attached thereto. In one embodiment, only a portion of the termini can have an arginine-grafted bioreducible polymer attached thereto.

HIGH MOLECULAR WEIGHT ARGININE-GRAFTED BIOREDUCTIBLE POLYMERS

PRIORITY CLAIM

This application claims the benefit of U.S. Provisional Patent Application No. 61/632,124, filed January 18, 2012 which is incorporated herein by reference.

5 BACKGROUND

This invention relates to non-viral gene delivery carriers. More particularly, this invention relates to high molecular weight arginine-conjugated bioreducible poly (disulfide amine) polymers (ABP) as gene delivery carriers.

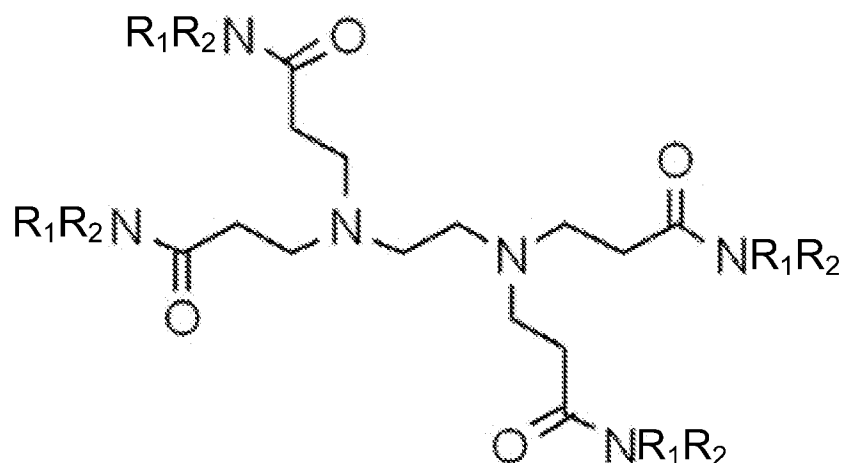
Gene therapy offers the potential to treat human congenital and acquired diseases
10 using therapeutic gene-based drugs. One of the requirements for successful gene therapy is the development of non-toxic and efficient carriers for gene delivery. Compared to viral vectors, non-viral gene carriers such as lipids, synthetic polymers and/or peptides offer a number of advantages including easy and large-scale production, non-immunogenicity, flexible DNA and RNA loading capacity and stability among others. Despite these
15 advantages, however, the widespread adoption of non-viral gene vectors has been limited by concerns related to cytotoxicity and decreased transfection efficiency. However, since the accumulation of non-degraded polymers inside cells is often the cause of cytotoxicity, the biodegradation of polymers after efficient transfection of DNA can reduce or eliminate this problem. Biodegradable polymers typically contain ester or disulfide-bonds. Ester bonds,
20 however, are easily hydrolyzed in the extracellular environment, while disulfide bonds are typically more stable, as they are not reduced until they are exposed to glutathione (GSH) in the intracellular cytoplasm. Based on these considerations, several types of bio-reducible polymers containing disulfide bonds have been developed.

25 SUMMARY OF THE INVENTION

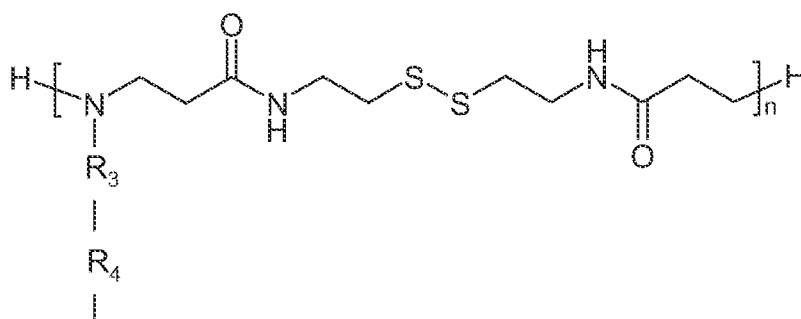
Arginine-grafted bioreducible polymers (ABP) were developed. Arginine modification of non-viral carriers is a promising area of investigation as the presence of arginine-rich peptides enhances the cell-penetrating ability of polymer carriers. Such ABP has been demonstrated *in vitro* to have a high transfection efficiency and low cytotoxicity. *In*
30 *vivo*, however, the use of ABP is limited by the fact that it has to be used at a weight ratio above 20 for optimal transfection efficiency, a level which is associated with increased

cytotoxicity. This requirement for an increased weight ratio *in vivo* has been attributed to the low molecular weight of ABP (approximately <5K). A polymeric gene carrier with a lower molecular weight may be less able to form compact polyplexes with the gene(s) to be delivered. The lack of formation of compact polyplexes can result in the formation of loose nanoparticles which are more susceptible to premature cleavage and gene release. This premature gene release decreases transfection efficiency compared to the polyplexes of longer and higher molecular weight polymers.

Accordingly, a polymeric carrier for delivering nucleic acid material to a cell and having low toxicity and a high transfection efficiency is provided herein. Such polymeric carrier can include a dendrimer group having 2 to 8 termini, with two or more of the termini having an arginine-grafted bioreducible polymer attached thereto. In one embodiment, only a portion of the termini have an arginine-grafted bioreducible polymer attached thereto. In another embodiment, all of the termini can have an arginine-grafted bioreducible polymer attached thereto. In a particular embodiment, the dendrimer group can have the general structure of:



wherein each R_1 is individually selected from H, or $(CH_2)_2-NH-(CH_2)_2-CO-NH-$
 $(CH_2)_2-NH-C(NH_2)-(CH_2)_3-S-S-(CH_2)_2-CO-NH-ABP$, and each R_2 is individually selected
 from either $(CH_2)_2-NH-C(NH_2)-(CH_2)_3-S-S-(CH_2)_2-CO-NH-ABP$, or $(CH_2)_2-NH-(CH_2)_2-CO-$
 $NH-(CH_2)_2-NH-C(NH_2)-(CH_2)_3-S-S-(CH_2)_2-CO-NH-ABP$, wherein ABP has the general
 5 structure:



wherein n is 1 to 10 and wherein R_3 is $(CH_2)_mNH$, wherein m is about 1 to about 18; and R_4
 is an arginine residue.

In another embodiment, a complex including a nucleic acid associated to a polymeric
 10 carrier for delivery of nucleic acid material to a cell is provided. The complex can include a
 polymeric carrier with a dendrimer group having 2 to 8 termini with all or a portion of the
 termini having an arginine-grafted bio reducible polymer attached thereto.

In another embodiment, a method for transfecting a cell is provided. The method
 includes providing any one of the complexes described herein and contacted a cell with the
 15 complex. The contacting can occur *in vitro* or *in vivo*.

BRIEF DESCRIPTION OF THE DRAWINGS

Additional features and advantages of the invention will be apparent from the detailed
 description which follows, taken in conjunction with the accompanying drawings, which
 20 together illustrate, by way of example, features of the invention.

FIG. 1 shows a scheme for synthesis of an illustrative embodiment of a high
 molecular weight ABP polymer according to an embodiment of the present invention.

FIG. 2 shows the results of agarose gel electrophoresis of ABP and PAM-ABP
 polyplexes at different weight ratios with and without 5.0 DTT according to an embodiment
 25 of the invention. The numbers refer to the mean weight ratios of the polyplexes.

FIG. 3A and 3B show the average size (3A) and zeta potential value (3B) of ABP
 polyplexes and PAM-ABP polyplexes in certain embodiments of the invention.

FIG. 4 shows the relative degradation of various polyplexes with 5.0 mM DTT using a picogreen assay according to one embodiment of the present invention.

FIG. 5A-D show the relative transfection efficiencies of various polymeric polyplexes including ABP, PEI, and PAM-ABP with different cell types with FIG. 5A being NIH 3T3 cells at various weight ratios, FIG 5B being NIH 3T3, FIG 5C with Hela cells, and FIG 5 D with C2C12 cells in DMEM media containing 10% fetal bovine serum (FBS) in accordance with embodiments of the present invention. The small numbers in the boxes of each figure refer to the mean weight ratios of the polyplexes. Branched PEI25 was used at a weight ratio of 1.

FIG. 6 shows microscopic images of ABP and PAM-ABP polyplex expression of pGFP transfection in C2C12 cells in accordance with one embodiment of the present invention. Branched PEI25k was used at a weight ratio of 1.

FIG. 7A-C shows the results of cytotoxicity assays for PEI, ABP, and PAM-ABP in HeLa cells (FIG. 7A), C2C12 cells at various concentration of polymers (FIG. 7B), and in C2C12 cells at various weight ratios of polyplexes by MTT assay, in accordance with embodiments of the present invention. The results are reported in relative cell viability (%).

FIG. 8A-D shows flow cytometric analyses of cellular uptake assay in C2C12 cells for some embodiments of the present invention. Specifically, FIG 8A shows histograms of ABP polyplexes at different weight ratios, FIG 8B shows histograms of PAM-ABP polyplexes at different weight ratios, FIG 8C shows representative histograms of ABP and PAM-ABP at weight ratios of 5 and 10, while FIG 8D shows a bar graph representing the mean percentages of cellular uptake with M region gating. ** $P < 0.01$.

FIG. 9A-B shows luciferase expression and cytotoxicity for exemplary embodiments of the present invention after treatment with BSO, an inhibitor of GSH synthetase, in (A) MCF-7 cells and (B) A549 cells.

FIG. 10 shows a general reaction scheme for the formation of G0 and G1 PAM-ABP polymeric carriers in accordance with one embodiment of the present invention.

FIG. 11 shows general structures for PAM-ABP G0 and G1 polymeric carriers in accordance with embodiments of the present invention.

FIG. 12 shows gel retardation for PEI, ABP, PAM-ABP G0, and PAM-ABP G1 complexes at various weight ratios. The PAM-ABP G1 forms more stable complexes than ABP and PAM-ABP G0.

FIG. 13 shows the Zeta potential and size for exemplary embodiments of ABP, PAM-ABP G0, and PAM-ABP G1 complexes.

FIG. 14 shows the transfection efficiency of various polymeric carriers, including embodiments of the dendrimer polymeric carriers described herein.

FIG. 15A-B show the GFP expression in A549 cells (15A) and C2C12 cells (15B) based on transfection using one embodiment of the complexes of the present invention. At a weight ratio of 5, PAM-ABP G0 and G1 showed higher cellular uptake than ABP and PEI complexes.

FIG. 16A-C shows the results of cytotoxicity assays for PEI, ABP, and PAM-ABP G0 and PAM-ABP G1 at various concentration of polymers in 293T cells (FIG. 16A), A549 cells (FIG. 16B), and in C2C12 cells (FIG. 16C). The results are reported in relative cell viability (%).

Reference will now be made to the exemplary embodiments illustrated, and specific language will be used herein to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended.

DETAILED DESCRIPTION OF EXAMPLE EMBODIMENT(S)

Before the present polymeric carriers, complexes, and methods are disclosed and described, it is to be understood that this invention is not limited to the particular process steps and materials disclosed herein, but is extended to equivalents thereof, as would be recognized by those ordinarily skilled in the relevant arts. It should also be understood that terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting.

It should be noted that, the singular forms “a,” “an,” and, “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a termini” includes reference to one or more of such termini’s, and reference to “the selected nucleic acid” includes reference to one or more of such nucleic acids.

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 invention belongs.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

As used herein, the term “about” is used when used in the context of a numerical range provides flexibility to the numerical range endpoint(s) by providing that a given value may be “a little above” or “a little below” the endpoint(s).

As used herein, "comprising," "including," "containing," "characterized by," and grammatical equivalents thereof are inclusive or open-ended terms that do not exclude additional, unrecited elements or method steps. "Comprising" is to be interpreted as including the more restrictive terms "consisting of" and "consisting essentially of." As used herein, "consisting of" and grammatical equivalents thereof exclude any element, step, or ingredient not specified in the claim. As used herein, "consisting essentially of" and grammatical equivalents thereof limit the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic or characteristics of the claimed invention.

As used herein, "poly(CBA-DAH)" means polymers formed between cystaminebisacrylamide ("CBA") and 1,6-diaminohexane ("DAH"). Similarly, "poly(CBA-DAB)" means polymers formed between CBA and 1,4-diaminobutane ("DAB"), and "poly(CBA-DAE)" means polymers formed between CBA and 1,2-diaminoethane ("DAE").

As used herein, "siRNA" means small interfering RNA, and "RNAi" means RNA interference.

As used herein, "PEI" means polyethylenimine, "PEI25k" means polyethylenimine having a nominal molecular weight of about 25,000, and "bPEI" means branched polyethylenimine.

As used herein, "administering" and similar terms mean delivering a complex to an individual being treated such that the complex can contact and be internalized in cells, such as cancer cells. Thus, in one embodiment the complex can be administered to the individual by systemic administration, such as by subcutaneous, intramuscular, or intravenous administration, or intraperitoneal administration. Injectables for such use can be prepared in conventional forms, either as a liquid solution or suspension or in a solid form suitable for preparation as a solution or suspension in a liquid prior to injection, or as an emulsion. Suitable excipients include, for example, water, saline, dextrose, glycerol, ethanol, and the like; and if desired, minor amounts of auxiliary substances such as wetting or emulsifying agents, buffers, and the like can be added. Other known modes of administration can also be used including, but not limited to oral administration and transdermal administration for either local or systemic delivery.

As used herein, a plurality of items, structural elements, compositional elements, and/or materials may be presented in a common list for convenience. However, these lists should be construed as though each member of the list is individually identified as a separate and unique member. Thus, no individual member of such list should be construed as a de

facto equivalent of any other member of the same list solely based on their presentation in a common group without indications to the contrary.

Concentrations, amounts, levels and other numerical data may be expressed or presented herein in a range format. It is to be understood that such a range format is used merely for convenience and brevity and thus should be interpreted flexibly to include not only the numerical values explicitly recited as the limits of the range, but also to include all the individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly recited. As an illustration, a numerical range of “about 1 to about 5” should be interpreted to include not only the explicitly recited values of about 1 to about 5, but also include individual values and sub-ranges within the indicated range. Thus, included in this numerical range are individual values such as 2, 3, and 4 and sub-ranges such as from 1-3, from 2-4, and from 3-5, etc., as well as 1, 2, 3, 4, and 5, individually. This same principle applies to ranges reciting only one numerical value as a minimum or a maximum. Furthermore, such an interpretation should apply regardless of the breadth of the range or the characteristics being described.

INVENTION

Reference will now be made in detail to preferred embodiments of the invention. While the invention will be described in conjunction with the preferred embodiments, it will be understood that it is not intended to limit the invention to those preferred embodiments. To the contrary, it is intended to cover alternatives, variants, modifications, and equivalents as may be included within the spirit and scope of the invention as defined by the appended claims.

It has been discovered that higher molecular weight ABP polymers are capable of providing higher transfection efficiency for nucleic acid materials than lower molecular weight ABP polymers. As used herein, the term “high molecular weight” or “higher molecular weight” when used to describe polymers or polymeric carriers refers to weights of 5 kDa or more. Accordingly, in one embodiment, high molecular weight ABP polymers are disclosed including a dendrimer type of ABP having a dendrimer backbone and ABP residues at the surface which can in some aspects, be used as a carrier for nucleic acid materials.

In another embodiment, a complex comprising selected nucleic acid material associated with a polymeric carrier for delivery thereof to a cell is provided. Without being limited by theory, the association between the nucleic acid material and the polymeric carrier is believed to be due to electrostatic interactions between the plasmid DNA and the polymeric

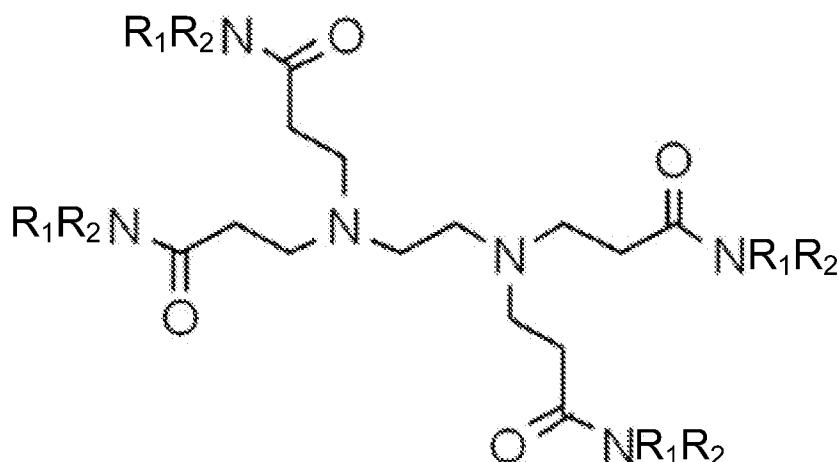
carrier. The polymer carrier can include a dendrimer group having 2 to 8 termini, with one or more of the termini having an arginine-grafted bioreducible polymer attached thereto. The selected nucleic acid material to be delivered by the polymeric carrier can generally be any type of nucleic acid material including, but not limited to oligonucleotides, plasmids, siRNA, and the like. One of the advantages of the polymeric carriers disclosed herein is their ability to efficiently delivery nucleic acid material to a cell at low ratios (weight to weight W/W) of polymeric carrier to nucleic acid material. In one embodiment, the polymeric carrier can be such that, when present in a complex with a nucleic acid material, the ratio (W/W) of polymeric carrier to selected nucleic acid material is about 5 or less. In another embodiment, the ratio (W/W) of polymeric carrier to selected nucleic acid material is about 3 or less. In still a further embodiment, the ratio (W/W) of polymeric carrier to selected nucleic acid material is about 2 or less.

In still another embodiment, a method for transfecting a cell is provided. The method includes providing any one of the complexes described herein and contacted a cell with the complex. The contacting can occur *in vitro* or *in vivo*. In one embodiment, the cell is a mammalian cell.

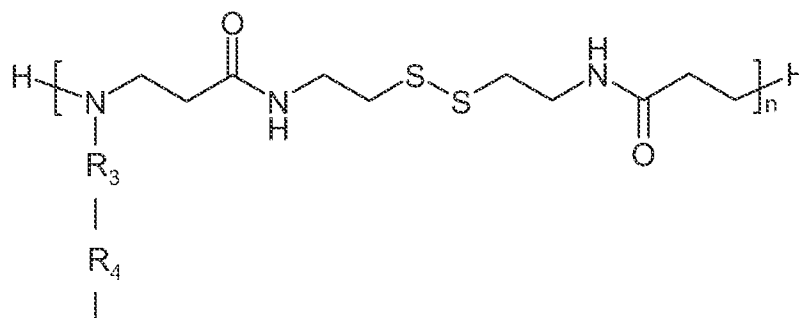
It is noted that when discussing a polymer carrier for delivery of nucleic acid material, a complex for delivering nucleic acid to a cell, or a method for transfecting a cell, each of these discussions can be considered applicable to the other embodiments, whether or not they are explicitly discussed in the context of that embodiment. Thus, for example, in discussing an dendrimer as a component of a polymer carrier for delivery of nucleic acid material, such dendrimer discussion can also be used in association with the complex for delivering nucleic acids to a cell and the method of transfecting a cell, and *vice versa*.

In one embodiment, a polymeric carrier for delivery of nucleic acid material to a cell is provided which includes a dendrimer group having 2 to 8 termini, at least one of the termini having an arginine-grafted bioreducible polymer attached thereto. Generally, any known dendrimer group having 2 to 8 termini can be used although it is preferable that the dendrimer be a biocompatible composition. In one embodiment, the dendrimer group of the polymeric carrier can have at least 4 termini. In another embodiment, the dendrimer group can have at least 8 termini. In another embodiment, at the dendrimer can have at least 4 termini and at least 4 of the termini have an ABP residue attached thereto. The high molecular weight polymeric carrier can have a molecular weight of about 5 kDa to about 50 kDa. In one embodiment, the polymeric carrier can have a molecular weight of about 9 kDa to about 50 kDa.

In one embodiment, the dendrimer group can have the general structure of:

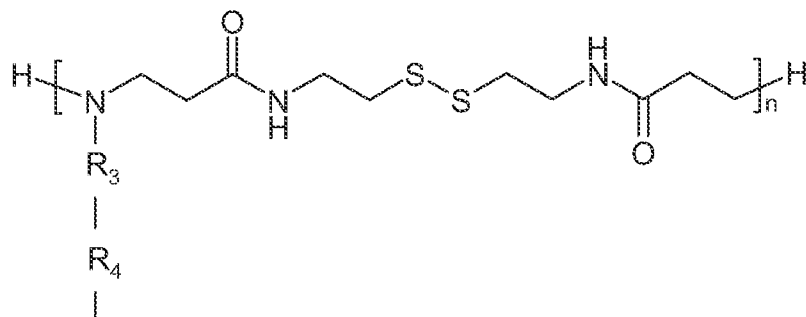


with each R_1 being individually selected from H, or $(\text{CH}_2)_2\text{-NH-(CH}_2)_2\text{-CO-NH-(CH}_2)_2\text{-NH-C(NH}_2\text{)-(CH}_2)_3\text{-S-S-(CH}_2)_2\text{-CO-NH-ABP}$, and each R_2 being individually selected from either $(\text{CH}_2)_2\text{-NH-C(NH}_2\text{)-(CH}_2)_3\text{-S-S-(CH}_2)_2\text{-CO-NH-ABP}$ or $(\text{CH}_2)_2\text{-NH-(CH}_2)_2\text{-CO-NH-(CH}_2)_2\text{-NH-C(NH}_2\text{)-(CH}_2)_3\text{-S-S-(CH}_2)_2\text{-CO-NH-ABP}$. It is noteworthy that the ABP component of the polymer can have the general structure:



wherein n is 1 to 10 and wherein R_3 is $(\text{CH}_2)_m\text{NH}$, wherein m is about 1 to about 18; and R_4 is an arginine residue. FIG. 11 shows additional embodiments of both generation 0 (G0) and generation 1 (G1) dendrimer structures that can be used as polymeric carriers and complexes in accordance with the present invention. FIG. 10 provides a generalized description of one embodiment of the reaction scheme for forming an embodiment of the disclosed polymeric carrier.

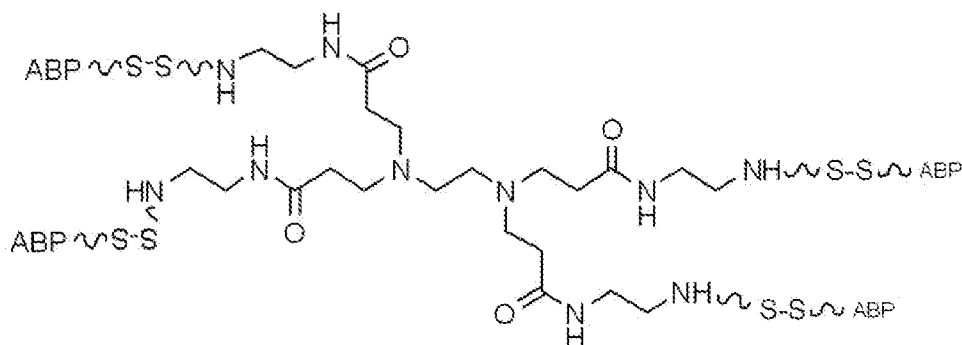
The arginine-grafted bioreducible polymer which forms a portion of the polymer carrier or complex can have the general structure set forth below:



wherein n is 1 to 10 and wherein R_3 is $(CH_2)_mNH$, wherein m is 1 to 18; and R_4 is an arginine residue. In one embodiment, m can be 2 to 8. In another embodiment, m can be 6. In still another embodiment, n can be about 4 to 8.

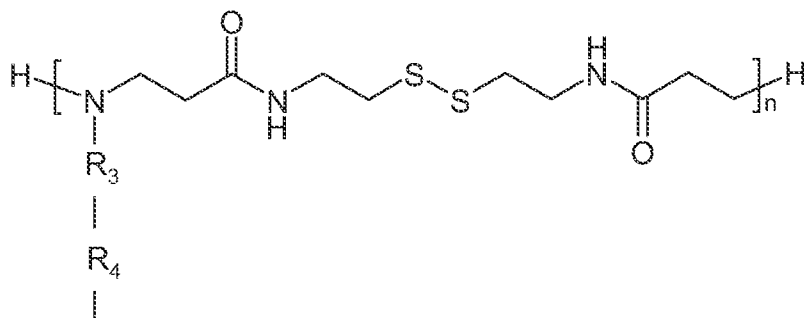
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In one embodiment, the polymeric carrier can have the structure:



wherein $-NH-S-S-$ is $\begin{array}{c} \text{NH}_2^+ \text{Cl}^- \\ | \\ \text{N} \\ | \\ \text{CH}_2 \\ | \\ \text{CH}_2 \\ | \\ \text{CH}_2 \\ | \\ \text{S} \\ | \\ \text{S} \\ | \\ \text{CH}_2 \\ | \\ \text{CH}_2 \\ | \\ \text{C=O} \\ | \\ \text{N} \end{array}$, and

wherein ABP has the general structure:



10

wherein n is 1 to 10 and wherein R_3 is $(CH_2)_mNH$, wherein m is about 1 to about 18; and R_4 is an arginine residue.

EXAMPLES

The following examples are provided to promote a more clear understanding of certain embodiments of the present invention, and are in no way meant as a limitation thereon.

MATERIALS

The following materials are used in one or more of the examples described herein. Specifically, hyperbranched poly(ethylenimine) (bPEI, Mw = 25 kDa), *tert*-butyl-*N*-(6-aminoethyl) carbamate (*N*-Boc-1,6-diaminohexane, *N*-Boc-DAH), trifluoroacetic acid (TFA), triisobutylsilane (TIS), DL-buthionine-sulfoxamine (BSO), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). *N,N'*-Cystaminebisacrylamide (CBA) was purchased from PolySciences, Inc. (Warrington, PA). Plasmid DNA encoding firefly luciferase (pLuc) or green fluorescent protein (pGFP) was purchased from Aldevron, Inc. (Fargo, ND). The Luciferase assay system and reporter lysis buffer were purchased from Promega (Madison, WI). Traut's reagent, SPDP and BCA protein assay kits were purchased from Pierce (Rocford, IL). All cell culture products including fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (PBS), antibiotics, trypsin-EDTA and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen (Gibco BRL, Carlsbad, CA). YOYO-1 iodide (1 mM solution in DMSO) and SYBR safe DNA gel stain were also purchased from Invitrogen (Carlsbad, CA).

Where results of an analysis are provided, the results are expressed as mean values $\pm$ standard deviation (SD). Differences between groups were assessed by one-way analysis of variance (ANOVA) using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by Tukey *post hoc* analysis was used to identify significance between groups.

EXAMPLE 1 – *Synthesis of PAM-ABP Polymers*

ABP was synthesized as described in U.S. Patent Nos. 8,153,154 and 8,153,155, each of which is incorporated herein by reference. The synthesized and purified ABP was dissolved in 0.1 M phosphate buffered saline (pH 7.2, 0.15 M NaCl). A 1.2 molar excess of SPDP dissolved in DMF (6.2 mg/mL) was added to the ABP solution. The mixture was stirred for one hour at room temperature, and then dialyzed against ultrapure water using a dialysis membrane (MWCO=1,000 Da, Spectrum Laboratories, Inc., Rancho Dominguez,

CA), followed by lyophilization. For the thiolation of PAMAM, PAMAM was dissolved in 0.1 M phosphate buffered saline (pH 8.0, 0.15 M NaCl, 2.0 mM EDTA). Eight equivalents of Traut's reagent per surface primary amines of PAMAM G0 were added to the PAMAM solution with stirring, and the mixture was further reacted for one hour. The product was dialyzed against ultrapure water using a dialysis membrane (dialysis membrane (MWCO=500 Da) and the product PAMAM-SH was then lyophilized.

To a prepared PAMAM-SH solution in 50 mM phosphate buffered saline (pH 7.2, 0.15 M NaCl, 10 mM EDTA), 4.4 equivalents of ABP-SPDP were added and the reaction mixture was stirred for four hours at room temperature. The reaction was monitored by

UV spectroscopy at 343 nm for the presence of released pyridine-2-thione. The mixture was dialyzed against pure water with a dialysis membrane (MWCO=3,500 Da), followed by lyophilization. The synthesis of PAM-ABP was confirmed by ¹H NMR (400 MHz, D₂O).

The general reaction scheme of the synthesis is shown in FIG. 1, with the four primary amines of PAMAM being modified with Traut's reagent, and the thiol groups of the modified PAMAM being reacted with SPDP linked ABP. The reaction was monitored by Thin-Layer Chromatography (TLC) with ninhydrin staining and UV spectroscopy for the presence of released pyridine-2-thion, which has a maximum extinction at 343 nm. The conjugation of ABP to the PAMAM was confirmed by proton NMR spectra, with four representative peaks for PAMAM (3.5, 3.1, 2.7, 2.4 ppm) as well as representative peaks for the arginine of ABP (1.6 ~ 1.2 ppm). Based on the results of the TLC staining, UVmeasurements, and NMR spectra, it was determined that four ABPs had been conjugated to the four primary amines of PAMAM (generation 0 (G0)). It is noted that this same technique can also be used for PAM-ABP polymers wherein the PAMAM is a generation 1 G1 dendrimer having eight termini with primary amines.

EXAMPLE 2 – Preparation, Gel Retardation Assay, and Zeta-potential values for polyplexes

Polyplexes were prepared by vortexing a pDNA solution with an equal volume of polymer solution in Hepes buffered saline (10 mM Hepes, 1 mM NaCl, pH 7.4) at various weight ratios, followed by incubation for 30 min. In order to examine the condensing ability of PAM-ABP with plasmid DNA at different weight ratios, a gel-retardation assay was performed. In order to compare the degree of DNA release, each polyplex was incubated in the presence of 5 mM DTT for 30 min at 37°C. The samples were then analyzed by gel electrophoresis as described below. An agarose gel (0.8%, w/v) containing an SYBR gel

staining solution was prepared in TAE (10 mM Tris/HCl, 1% (v/v) acetic acid, 1mM EDTA) buffer. Loading dye was added to each polyplex sample, and the mixtures were loaded onto an agarose gel and electrophoresed at 100 V for 30 min. The migration of DNA bands was visualized by a UV illuminator using a Gel Documentation System (Bio-Rad, Hercules, CA).

As shown in FIG. 2, the PAM-ABP completely retarded the electrophoretic mobility of the pDNA at a weight ratio of 2, while the ABP alone did not demonstrate complete retardation until a weight ratio above 5, confirming that the PAM-ABP condenses pDNA at a lower weight ratio and more effectively than ABP alone.

The particle size and zeta-potential values of the polyplexes were measured using a Nano ZS (ZEN3600, Malvern Instruments) with a He-Ne ion laser (633 nm). Fifty microliters of polyplex solution (0.5 μ g of pDNA) were prepared at weight ratios (polymer/pDNA) ranging from 1 to 40. After 30 min incubation, the polyplex solutions were diluted in filtered water to a final volume of 600 μ L before measurement. Zeta-potential values and particle sizes of the ABP and PAM-ABP polyplexes provide details of complex formation. As shown in FIG. 3, at a weight ratio of 5, the surface charge and average particle size of the ABP polyplexes were determined to be + 13.0 mV and 242 nm, respectively, while the PAM-ABP formed polyplexes with a zeta-potential value of + 11.0 mV and a particle size of 126 nm. These results indicate that the PAM-ABP has the physical characteristics to form more compact polyplexes than ABP alone.

EXAMPLE 3 – Degradation Study of PAM-ABP polyplexes

The degradation patterns of the PAM-ABP polyplex under reductive conditions were determined by picogreen assay. Each polyplex at a fixed weight ratio was incubated in the presence of 5 mM DTT at 37°C. The picogreen reagent was added at the indicated time intervals and further incubated for 2 min. Fluorescence was measured using a Qubit® 2.0 Fluorometer (Invitrogen). DTT is a well-known reducing agent that mimics the reductive environment of the intra-cellular cytoplasm where the disulfide bonds of bio-reducible polymers, such as ABP and PAM-ABP, are degraded. The rate of reduction of these disulfide bonds regulates the degree of DNA release from the polyplexes, and subsequent gene expression. As shown in FIG. 2, 70% of the pDNA was released from the ABP polyplex at a weight ratio of 20 in the presence of DTT. In the case of PAM-ABP, only 5% of the pDNA was released from the polyplexes at a weight ratio of 20 in the presence of DTT.

For more precise analysis of the DNA release from the polyplexes in the presence of a reducing agent, a picogreen assay was carried out at weight ratios of 5 and 10 (FIG. 4). At a

weight ratio of 5, the ABP formed a less compact polyplex than the PAM-ABP. Both polymers condensed pDNA successfully at a weight ratio of 10. With increasing incubation time with DTT, the ABP polyplexes displayed a more rapid increase in the release of pDNA than the PAM-ABP polyplexes. Summarizing these findings, the polyplexes of the low molecular weight ABP are more rapidly degraded in a reductive environment than the higher molecular weight PAM-ABP. The PAM-ABP forms compact and nanosized polyplexes with pDNA at a lower weight ratio and maintains more stable polyplexes in a reductive environment, allowing for more controlled carrier gene release.

EXAMPLE 4 – *In Vitro* transfection of polyplexes

Various types of cells were seeded in 24-well plates at a density of 5.0×10^4 cells/well and incubated in the absence or presence of buthionine-sulfoximine (BSO) for 24 h (around 70–80% confluence) in DMEM media containing 10% Fetal Bovine Serum (FBS) at 37°C. Plasmid DNA (0.5 µg/well) was complexed with polymer at different weight ratios and incubated for 30 min. The cells were then treated with the polyplexes for 4 h, after which the medium was exchanged with fresh medium containing 10% FBS and the cells incubated for 2 days before analysis. For the luciferase analysis, the cells were rinsed with DPBS and treated with 200 µL of reporter lysis buffer, followed by shaking for 30 min at room temperature. The luciferase activity of 25 µL cell lysate was measured by using 100 µL of luciferase assay reagent on a luminometer (Dynex Technologies Inc., Chantilly, CA). All experiments were performed in triplicate. The degree of GFP expression was measured using an EVOS microscope (AMG, Bothell, WA).

The transfection efficiency of the newly synthesized bio-reducible PAM-ABP was compared to the transfection efficiency of ABP in HeLa, C2C12 and NIH 3T3 cells in DMEM media containing 10% Fetal Bovine Serum (FBS) using firefly luciferase and green fluorescent protein (GFP) expression. The polymer carrier PEI was used as a control for these experiments. To determine the optimal weight ratio of PAM-ABP for gene delivery, the transfection experiments were carried out at a series of weight ratios in NIH 3T3 cells. Luciferase expression increased in a dose-dependent manner for both polymers in NIH 3T3 cells (FIG. 5A). While ABP demonstrated good transfection efficiency above a weight ratio of 20, the PAM-ABP demonstrated a similar transfection efficiency beginning at a significantly lower weight ratio of 5 and continuing on up to a weight ratio of 40. As shown in FIG. 5, at a weight ratio of 5, the transfection efficiency of PAM-ABP was almost 100 fold higher than ABP and PEI in all three cell types. Without wishing to be bound by theory, it is

speculated that at least one reason for such increase is likely due to the ability of PAM-ABP to form more compact polyplexes as described above. Similar results were seen with GFP expression in C2C12 cells when comparing gene delivery using PAM-ABP to delivery using ABP alone at weight ratios of 5 and 10 (FIG. 6). These results indicate that the dendrimer
5 ABP derivative, PAM-ABP, greatly enhances the transfection efficiency of pDNA at a lower weight ratio of polymer compared to ABP.

EXAMPLE 5 – *Cytotoxicity of polymers and polyplexes*

In order to evaluate the cytotoxicity of PAM-ABP and ABP, an MTT assay was
10 performed. PEI was used as a control. To assess the cytotoxicity of the polyplexes, the cells were seeded in 24-well plates at a density of 5.0×10^4 cells/well and incubated for 24 h in DMEM medium containing 10% FBS at 37°C. Polyplexes were prepared and treated using the same protocol as the transfection experiments. After 48 h incubation, 50 µL of stock solution of MTT (2 mg/mL in PBS) was added into each well and incubated for 2 h at 37°C.
15 The medium was then removed and 200 µL DMSO was added to dissolve the formazan crystal formed by viable cells. Similarly, to assess the cytotoxicity of the polymers, the cells were seeded in a 96-well culture plate at 1.0×10^4 cells/well in 90 µL DMEM medium containing 10% FBS. After 24 h incubation, cells were treated with 10 µL of the polymer solutions at different concentrations for 4 h in a DMEM medium without serum. After
20 exchange of medium with fresh DMEM with 10% serum, the cells were further maintained for 48 h. Then, 25 µL of stock solution of MTT (2 mg/ml in PBS) were added to each well. After 2 h of incubation at 37°C, the medium was removed carefully and 150 µL of DMSO was added to each well to dissolve the formazan crystal. The absorption was measured at 570 nm using a microplate reader (Model 680, Bio-Rad Laboratory, Hercules, CA), and the
25 cell viability was calculated as a percentage relative to untreated control cells.

As shown in FIGs. 7A and 7B, the relative viability of HeLa and C2C12 cells treated with PEI was less than 20%, even at very low polymer concentrations (20 µg/mL). Both PAM-ABP and ABP demonstrated low levels of cytotoxicity, with approximately 80% of cells being viable up to a polymer concentration of 60 µg/mL. Approximately 60% of cells
30 remained viable up to an even higher polymer concentration of 100 µg/mL. The cytotoxicity of the polyplexes based on weight ratio was examined by an MTT assay in C2C12 cells. Both the PAM-ABP and ABP polyplexes were consistently associated with cell viabilities above 80% from weight ratios as low as 5 to weight ratios as high as 40 (FIG. 7C). The optimal polycationic polymer for gene delivery carrier should combine high transfection efficiency

with low cytotoxicity. By this measure and the fact that PAM-ABP is efficacious at lower doses, PAM-ABP appears to be a superior carrier for gene delivery compared to ABP.

EXAMPLE 6 – Cellular uptake using flow cytometry

5 The cellular uptake of the PAM-ABP polyplexes with YOYO-1 intercalated plasmid DNA was determined by flow cytometry using PEI as a control. C2C12 cells were seeded at a density of 1.0×10^5 cells/well in a 12-well plate in DMEM medium containing 10% FBS and grown for 24 h. pDNA was labeled with YOYO-1 iodide (1 molecule of the dye per 50 base pairs of nucleotide) for 30 min before use. The polyplexes were prepared with YOYO-1
10 labeled plasmid DNA (pDNA 1.0 μ g) and the polymers at the designated weight ratios and incubated for 30 min. The polyplexes were added to the cells and incubated for 4 h at 37°C in serum-free medium. After removing the medium, the cells were washed with cold PBS, trypsinized and collected by centrifugation. The collected cells were suspended in 500 μ L of cold PBS, and the degree of cellular uptake was examined using a BD FACScan analyzer.

15 As shown in FIG. 8, the cellular uptake of both the PAM-ABP and ABP polyplexes increased as the weight ratio increased. While there was a slow increase in uptake of the ABP polyplexes with increasing weight ratio, the uptake of the PAM-ABP polyplexes increased sharply at a weight ratio of 5. In order to compare the relative uptake of DNA by ABP and PAM-ABP, the quantitative cellular uptake of the polyplexes was calculated as a percentage
20 of cell counts in the M gated region (Fig. 8D). Both polyplexes exhibited similar gating values and greater cellular uptake than PEI at a weight ratio of 20. Notably, at a weight ratio of 5, there was significantly higher uptake of the PAM-ABP polyplexes (~80%) than the ABP polyplexes (below 20%). These results suggest that the ability of PAM-ABP to form compact polyplexes enhances its cellular uptake, resulting in an increase in transfection efficiency
25 compared to ABP.

EXAMPLE 7 – Investigation of cellular gene delivery

 PAM-ABP and ABP are bio-reducible polymers with an internal disulfide bond, which is degraded in the reductive environment of the intracellular cytoplasm. The
30 concentration of intracellular glutathione (GSH) determines the degree of reduction of the disulfide bonds in bio-reducible polymers such as PAM-ABP and ABP. To determine the impact of the reductive environment on the transfection efficiency of the polymers, the ability of the polymers to transfect cells was measured after treatment with DL-buthionine-sulfoxamine (BSO), a glutathione-depleting agent. As depletion of GSH can lead to cell

death, toxicity testing was performed. Treatment with BSO was toxic to C2C12 cells, resulting in 80% cell viability at 50 μ M of BSO and 40% cell viability at 100 μ M of BSO (data not shown). BSO, however, was much less toxic to a breast cancer cell line (MCF-7) and human lung adenocarcinoma epithelial cell line (A549), with greater than 95% of cells remaining viable, even at a concentration of BSO as high as 200 μ M. As shown in FIG. 9, the luciferase expression for both the PAM-ABP and ABP polyplexes was decreased in a BSO dose-dependent manner. ABP showed a significantly higher susceptibility to treatment with BSO, indicating that the reduction of the internal disulfide bonds of the ABP polyplex was abruptly decreased with the inhibition of GSH. These results indicate that the regulated release of pDNA through controlled polymer degradation is a critical step for efficient gene delivery.

EXAMPLE 8 – Stability of PEI, ABP, PAM-ABP G0 and PAM-ABP G1 Complexes

The stability of PEI, ABP, PAM-ABP G0 and PAM-ABP complexes were tested utilizing agarose gel retardation at various weight ratios of polymeric carrier to nucleic acid. bPEI 25kDa is a non-degradable polymer and PAM-ABP is a degradable polymer. The PAM-ABP formed complexes with siRNA that were approximately 200 nm in diameter (surface charge : 20.5 ± 4.89 mV). However, the size of complexes increased after DTT treatment for 2h (surface charge : -36.3 ± 16.8 mV). A reductive environment caused complete siRNA release from the PAM-ABP polyplexes while the PEI polyplexes was not affected (5.0 mM DTT condition. As can be seen in FIG. 12, the PAM-ABP G1 formed more stable polyplexes than ABP and PAM-ABP G0.

EXAMPLE 9 – In Vitro transfection of polyplexes

Various types of cells were seeded and treated with polyplexes/complexes containing luciferase in a similar manner as described in Example 4. The cells were then analyzed in a manner similar to that described in Example 4.

The transfection efficiency of the PEI, ABP, PAM-ABP G0, and PAM-ABP G1 were analyzed for each of the cell types of A549 (Human adenocarcinoma epithelial cell line) 293T (human embryonic kidney epithelial cells) and C2C12 (mouse myoblast cells). Various weight ratios of the polymeric carriers were analyzed. Luciferase expression tended to increase a dose-dependent manner for the ABP, PAM-ABP G0, and the PAM-ABP G1 complexes. FIG. 14 shows the transfection efficiency of various polymeric carriers, including embodiments of the dendrimer polymeric carriers described herein. FIG. 15A and

15B show the GFP expression in A549 cells (15A) and C2C12 cells (15B). At a weight ratio of 5, PAM-ABP G0 and G1 showed higher cellular uptake than ABP and PEI complexes.

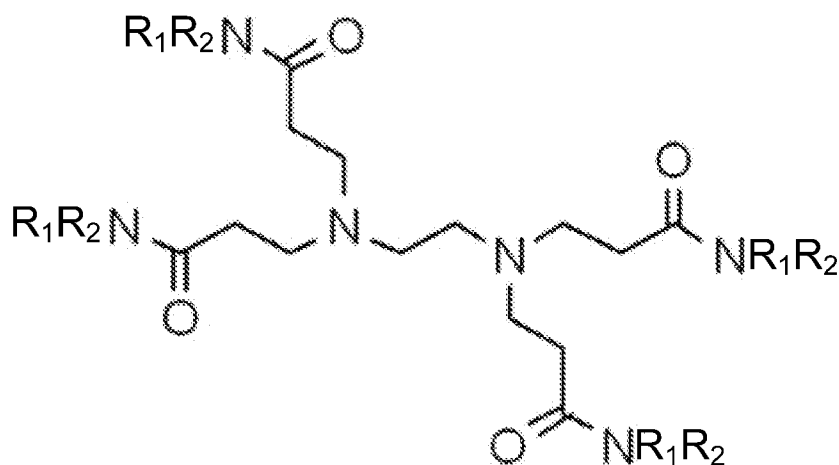
EXAMPLE 10 – *Cytotoxicity of polymers and polyplexes*

5 In order to evaluate the cytotoxicity of PEI, ABP, PAM-ABP G0, and PAM-ABP G1, an MTT assay was performed in a similar manner as described in Example 5. FIG. 16A-C shows the results of cytotoxicity assays for the various complexes at various concentration of polymers in 293T cells (FIG. 16A), A549 cells (FIG. 16B), and in C2C12 (FIG. 16C) cells. The results are reported in relative cell viability (%). As can be seen from the FIG 16, the
10 cytotoxicity of the ABP, PAM-ABP G0 and PAM-ABP G1 was similar.

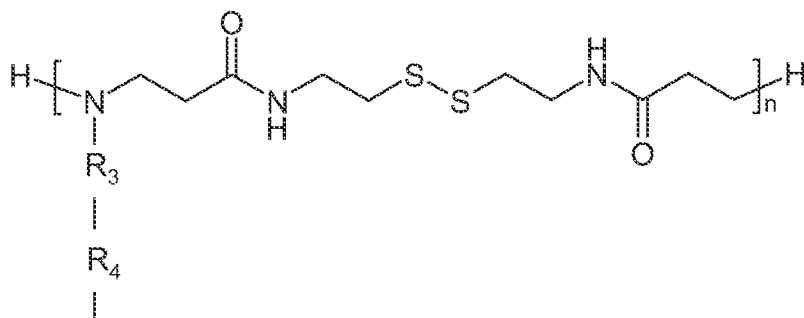
 While the forgoing examples are illustrative of the principles of the present invention in one or more particular applications, it will be apparent to those of ordinary skill in the art that numerous modifications in form, usage and details of implementation can be made
15 without the exercise of inventive faculty, and without departing from the principles and concepts of the invention. Accordingly, it is not intended that the invention be limited, except as by the claims set forth below.

CLAIMS

1. A polymeric carrier for delivery of nucleic acid material to a cell, comprising: a dendrimer group having 2 to 8 termini, each of the termini having an arginine-grafted bioreducible polymer attached thereto.
2. The polymeric carrier of claim 1, wherein the dendrimer group has the general structure of:

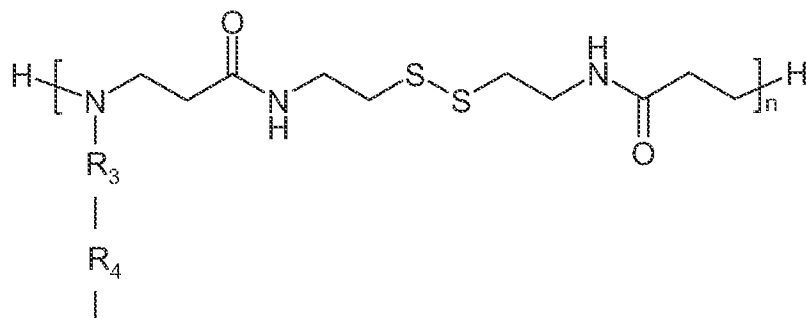


wherein each R_1 is individually selected from H, or $(CH_2)_2-NH-(CH_2)_2-CO-NH-(CH_2)_2-NH-C(NH_2)-(CH_2)_3-S-S-(CH_2)_2-CO-NH-ABP$, and each R_2 is individually selected from either $(CH_2)_2-NH-C(NH_2)-(CH_2)_3-S-S-(CH_2)_2-CO-NH-ABP$, or $(CH_2)_2-NH-(CH_2)_2-CO-NH-(CH_2)_2-NH-C(NH_2)-(CH_2)_3-S-S-(CH_2)_2-CO-NH-ABP$, wherein ABP has the general structure:



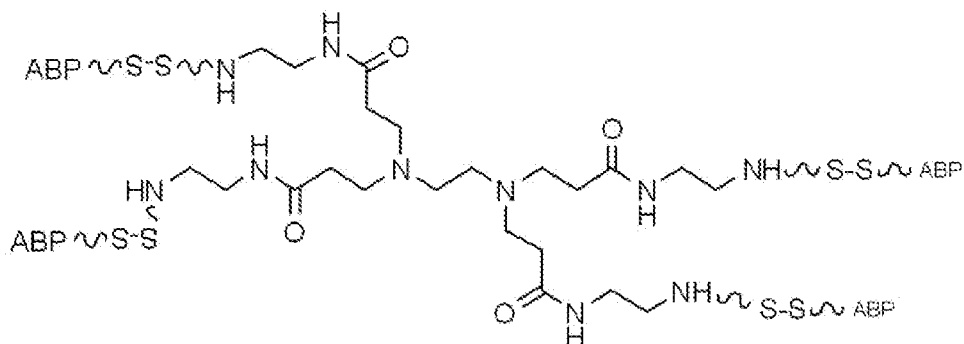
wherein n is 1 to 10 and wherein R_3 is $(CH_2)_mNH$, wherein m is about 1 to about 18; and R_4 is an arginine residue.

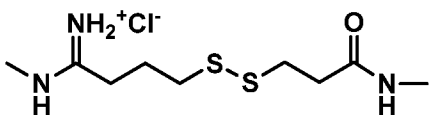
3. The polymeric carrier of claim 1, wherein the dendrimer group has at least 4 termini.
4. The polymeric carrier of claim 1, wherein the dendrimer group has 8 termini.
5. The polymer carrier of claim 1, wherein the arginine-grafted bio-reducible polymer has the general structure of:

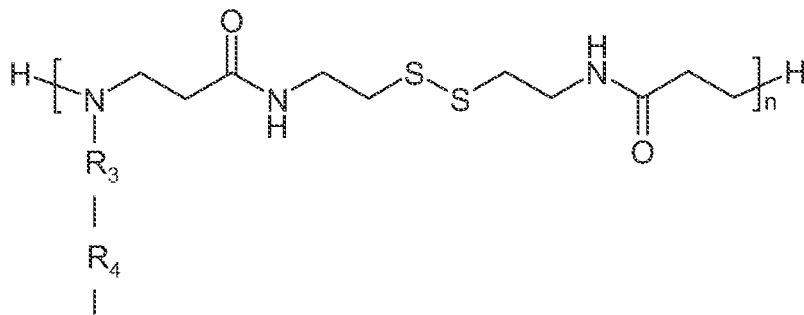


wherein n is 1 to 10 and wherein R_3 is $(CH_2)_mNH$, wherein m is about 1 to about 18; and R_4 is an arginine residue.

6. The polymeric carrier of claim 5, wherein m is 6.
7. The polymeric carrier of claim 5, wherein n is 4 to 8.
8. The polymeric carrier of claim 1, wherein the polymeric carrier has the structure:

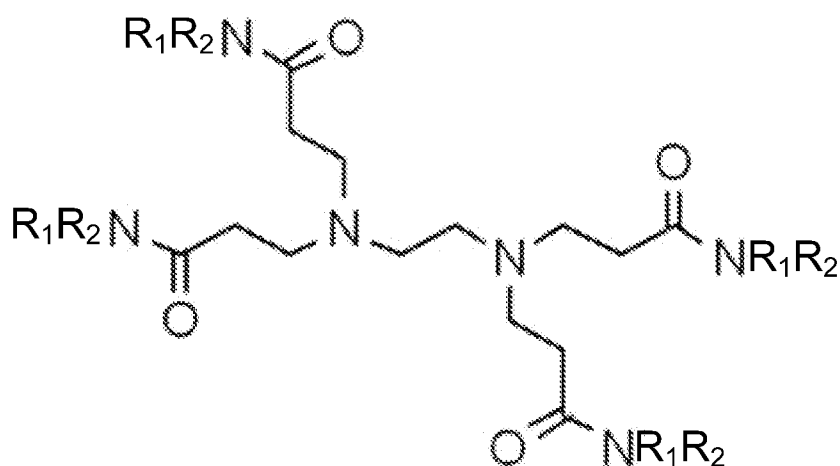


wherein —NH—S—S— is , and wherein ABP has the general structure:

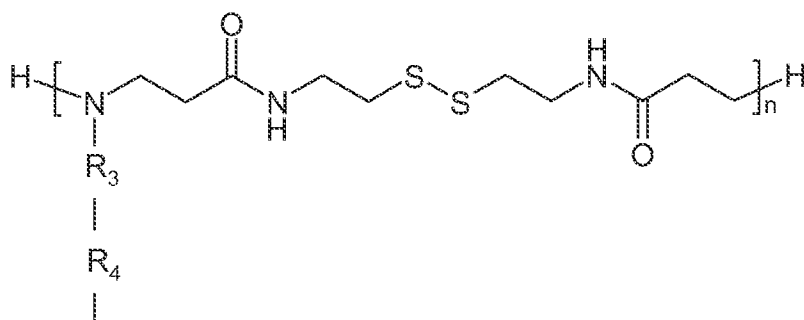


wherein n is 1 to 10 and wherein R_3 is $(\text{CH}_2)_m\text{NH}$, wherein m is about 1 to about 18; and R_4 is an arginine residue.

9. The polymeric carrier of claim 1, wherein the polymeric carrier has a molecular weight of 5 kDa to about 50 kDa.
10. The polymeric carrier of claim 1, wherein the polymeric carrier has a molecular weight 9 kDa to about 50 kDa.
11. A complex comprising a selected nucleic acid associated to a polymeric carrier for delivery of nucleic acid material to a cell, said polymer carrier comprising a dendrimer group having 2 to 8 termini, each of the termini having an arginine-grafted bioreducible polymer attached thereto.
12. The complex of claim 11, wherein the dendrimer group has the general structure of:



wherein each R_1 is individually selected from H, or $(CH_2)_2-NH-(CH_2)_2-CO-NH-$
 $(CH_2)_2-NH-C(NH_2)-(CH_2)_3-S-S-(CH_2)_2-CO-NH-ABP$, and each R_2 is individually
 selected from either $(CH_2)_2-NH-C(NH_2)-(CH_2)_3-S-S-(CH_2)_2-CO-NH-ABP$, or $(CH_2)_2-$
 5 $NH-(CH_2)_2-CO-NH-(CH_2)_2-NH-C(NH_2)-(CH_2)_3-S-S-(CH_2)_2-CO-NH-ABP$, wherein
 ABP has the general structure:

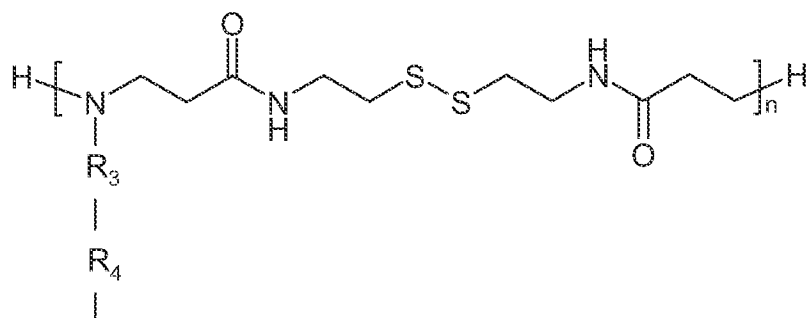


wherein n is 1 to 10 and wherein R_3 is $(CH_2)_mNH$, wherein m is about 1 to about 18;
 and R_4 is an arginine residue.

13. The complex of claim 11, wherein the dendrimer group has at least 4 termini.

14. The complex of claim 11, wherein the dendrimer group has 8 termini.

15. The complex of claim 11, wherein the arginine-grafted bioreducible polymer has the general structure of:

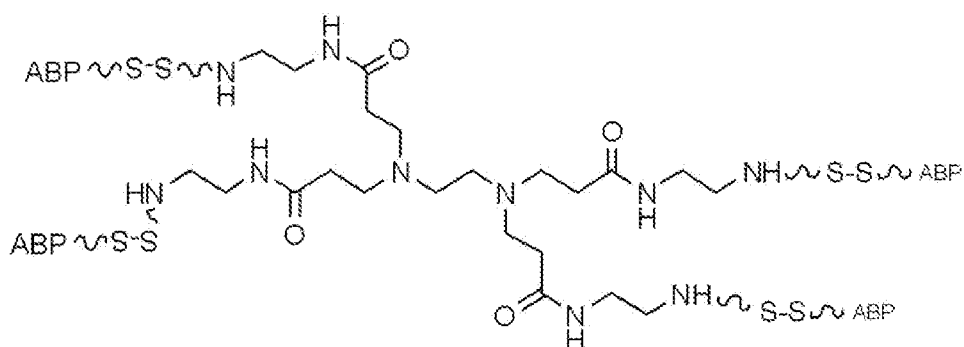


wherein n is 1 to 10 and wherein R_3 is $(\text{CH}_2)_m\text{NH}$, wherein m is about 1 to about 18; and R_4 is an arginine residue.

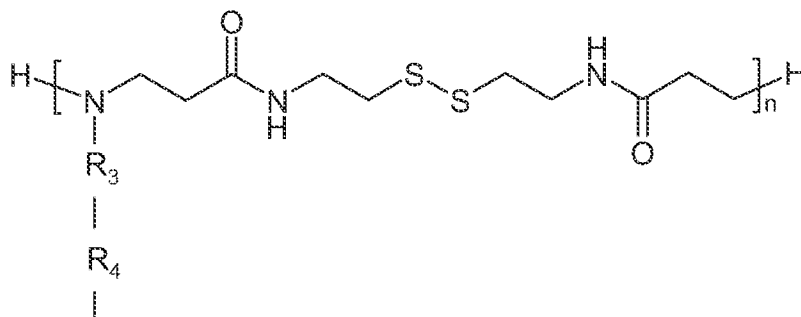
16. The complex of claim 15, wherein m is 6.

17. The complex of claim 15, wherein n is 4 to 8.

18. The complex of claim 11, wherein the polymeric carrier has the structure:



wherein —NH—S—S— is $\text{—NH—CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{S—S—CH}_2\text{CH}_2\text{C}(=\text{O})\text{NH—}$, and wherein ABP has the general structure:



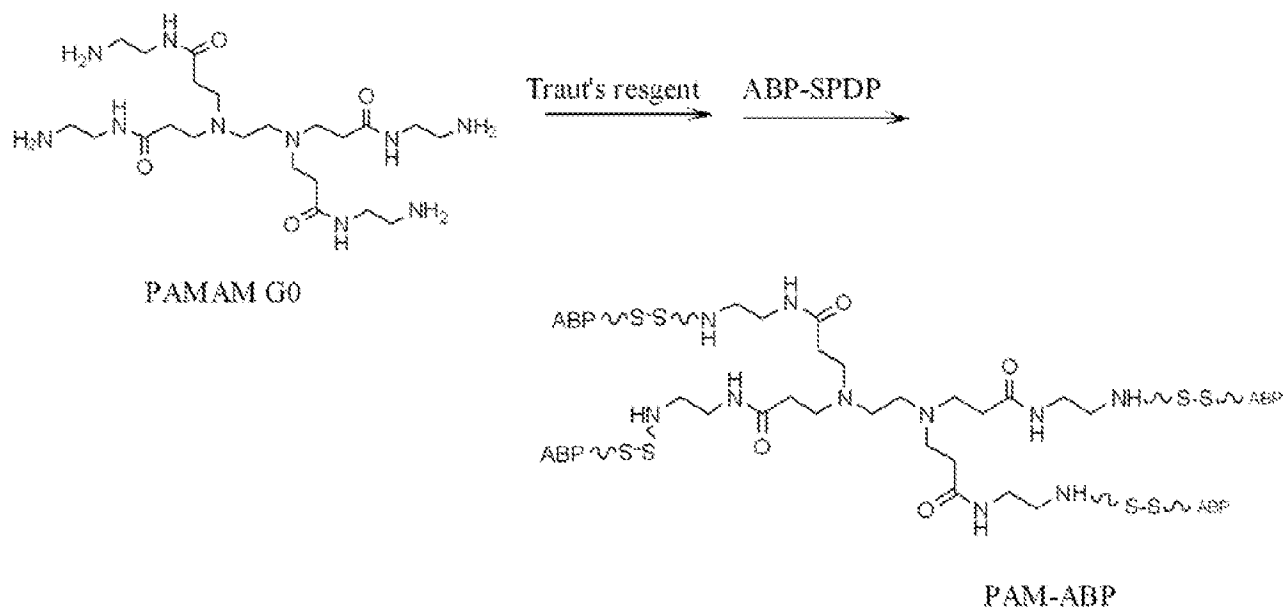
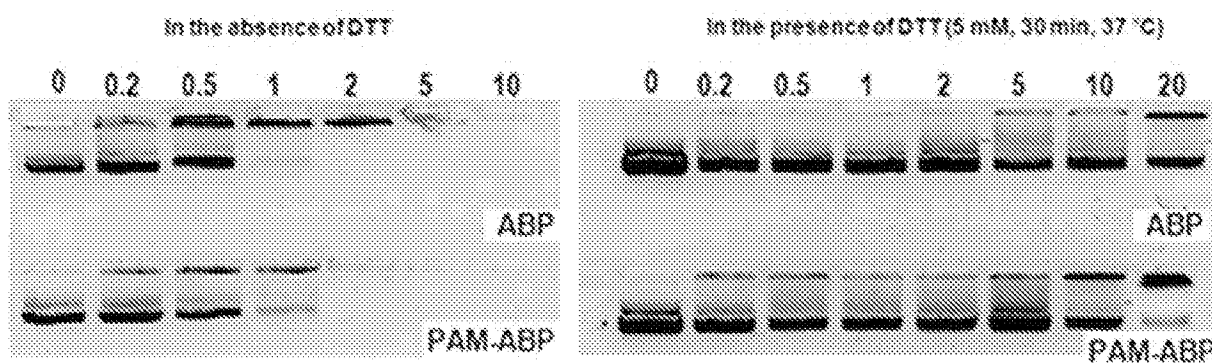
wherein n is 1 to 10 and wherein R_3 is $(CH_2)_mNH$, wherein m is about 1 to about 18; and R_4 is an arginine residue.

19. The complex of claim 11, wherein the polymeric carrier has a molecular weight of 5 kDa to about 50 kDa.
20. The complex of claim 11, wherein the polymeric carrier has a molecular weight 9 kDa to about 50 kDa.
21. The complex of claim 11, wherein the selected nucleic acid is an oligonucleotide.
22. The complex of claim 11, wherein the selected nucleic acid comprises a plasmid.
23. The complex of claim 11, wherein the selected nucleic acid comprises siRNA.
24. The complex of claim 11, wherein the complex has a ratio (W/W) of polymeric carrier to selected nucleic acid material of less than about 5.
25. The complex of claim 11, wherein the complex has a ratio (W/W) of polymeric carrier to selected nucleic acid material of less than about 3.
26. The complex of claim 11, wherein the complex has a ratio (W/W) of polymeric carrier to selected nucleic acid material of about 2.
27. A method for transfecting a cell, comprising:
providing a complex as set forth in claim 11, and
contacting a cell with the complex.
28. The method of claim 27, wherein the contacting occurs *in vitro*.

29. The method of claim 27, wherein the contacting occurs *in vivo*.

30. The method of claim 27, wherein the cell is a mammalian cell.

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**FIG. 1****FIG. 2**

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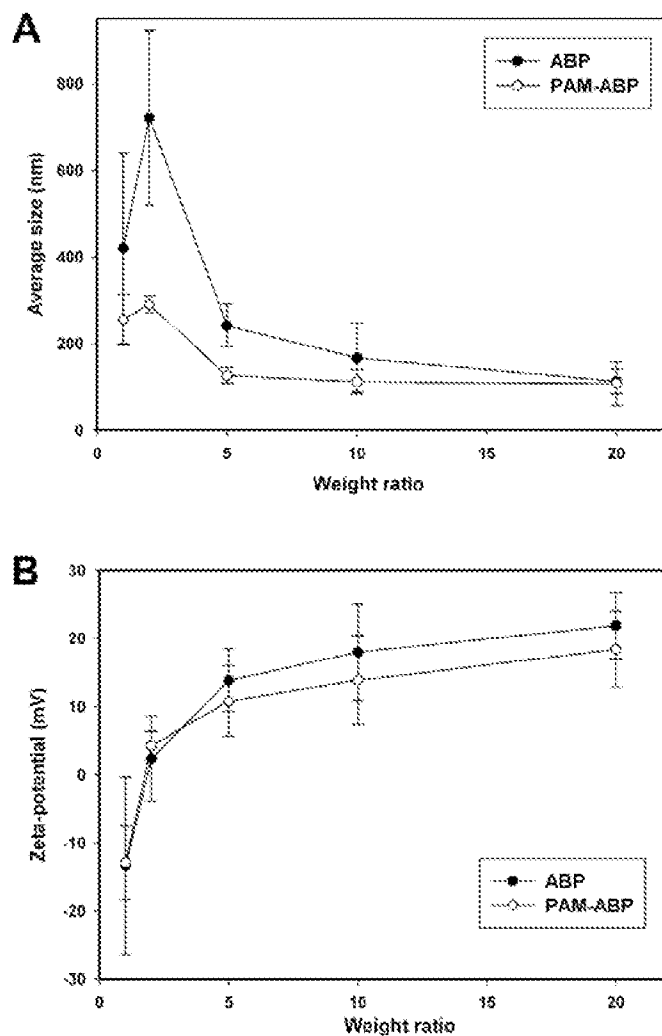


FIG. 3

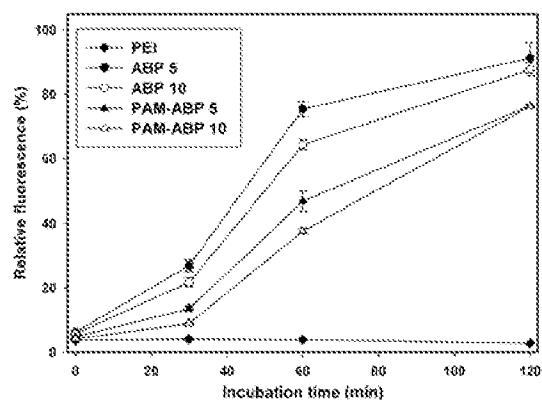


FIG. 4

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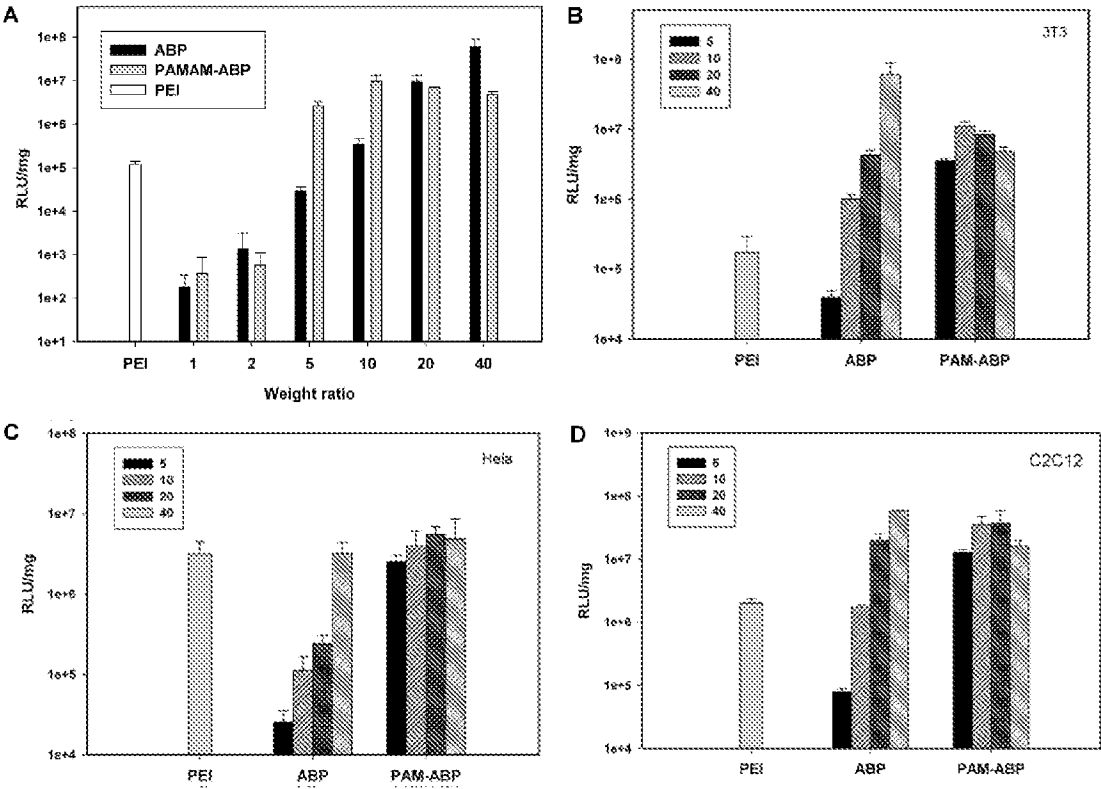


FIG. 5

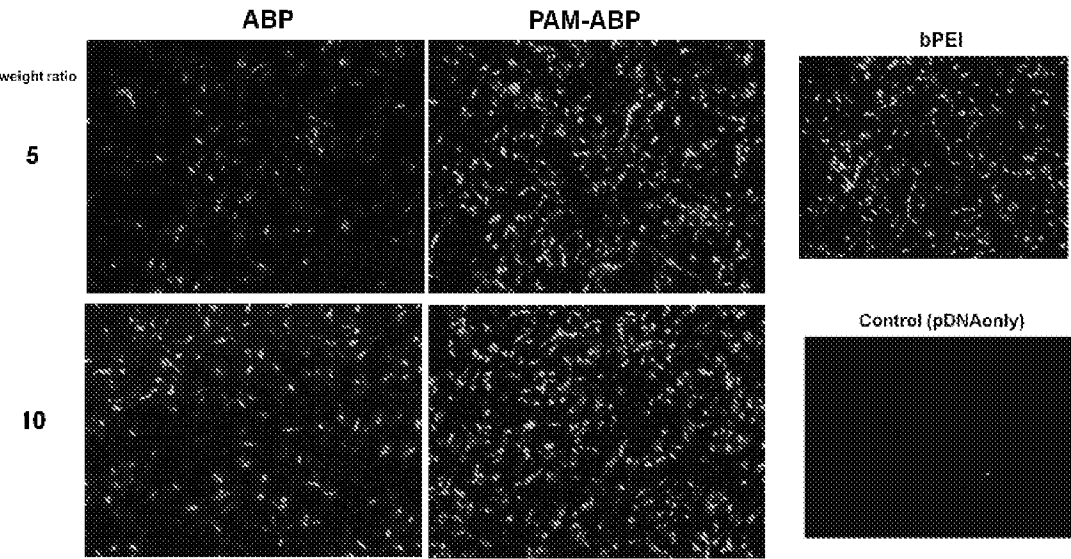


FIG. 6

4/12

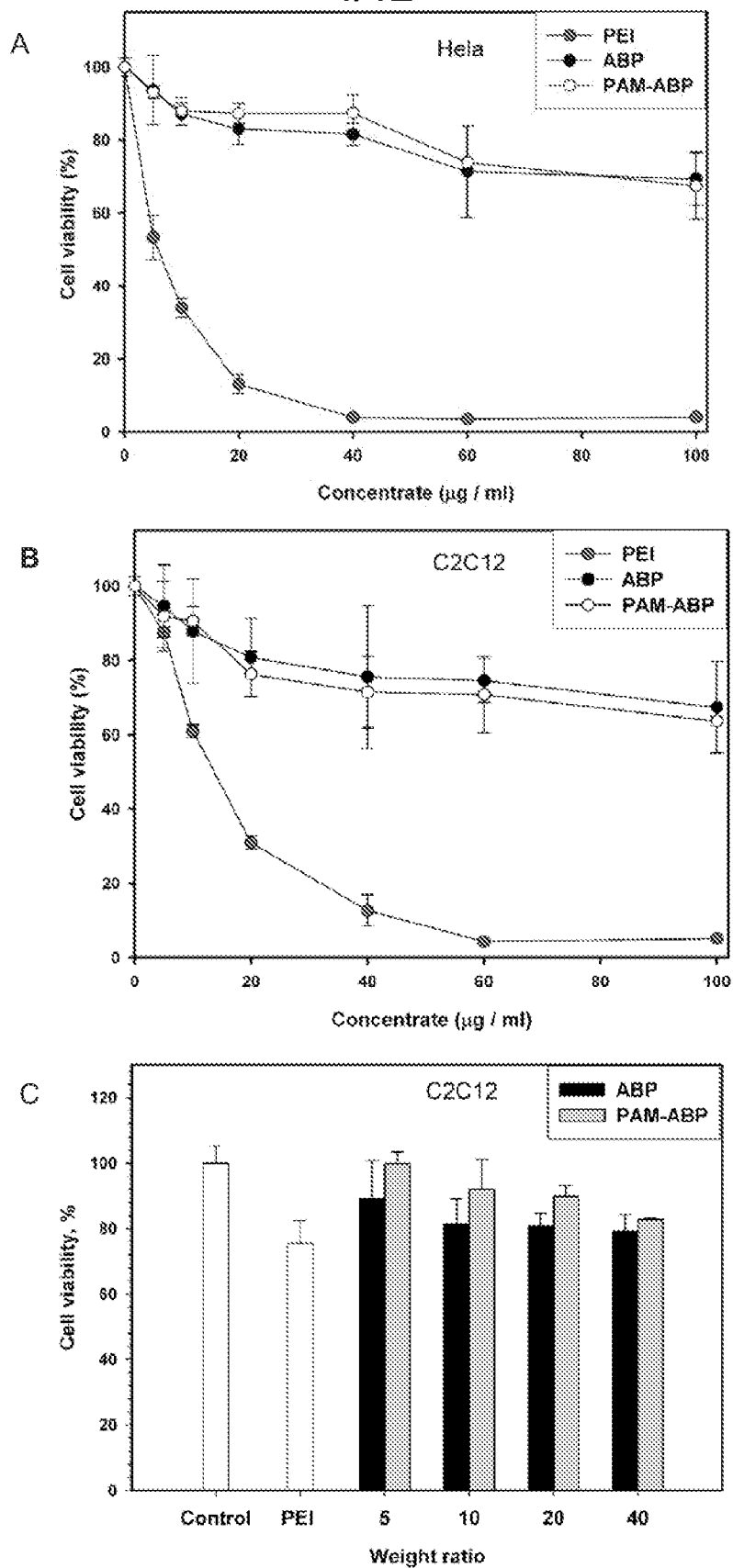


FIG. 7

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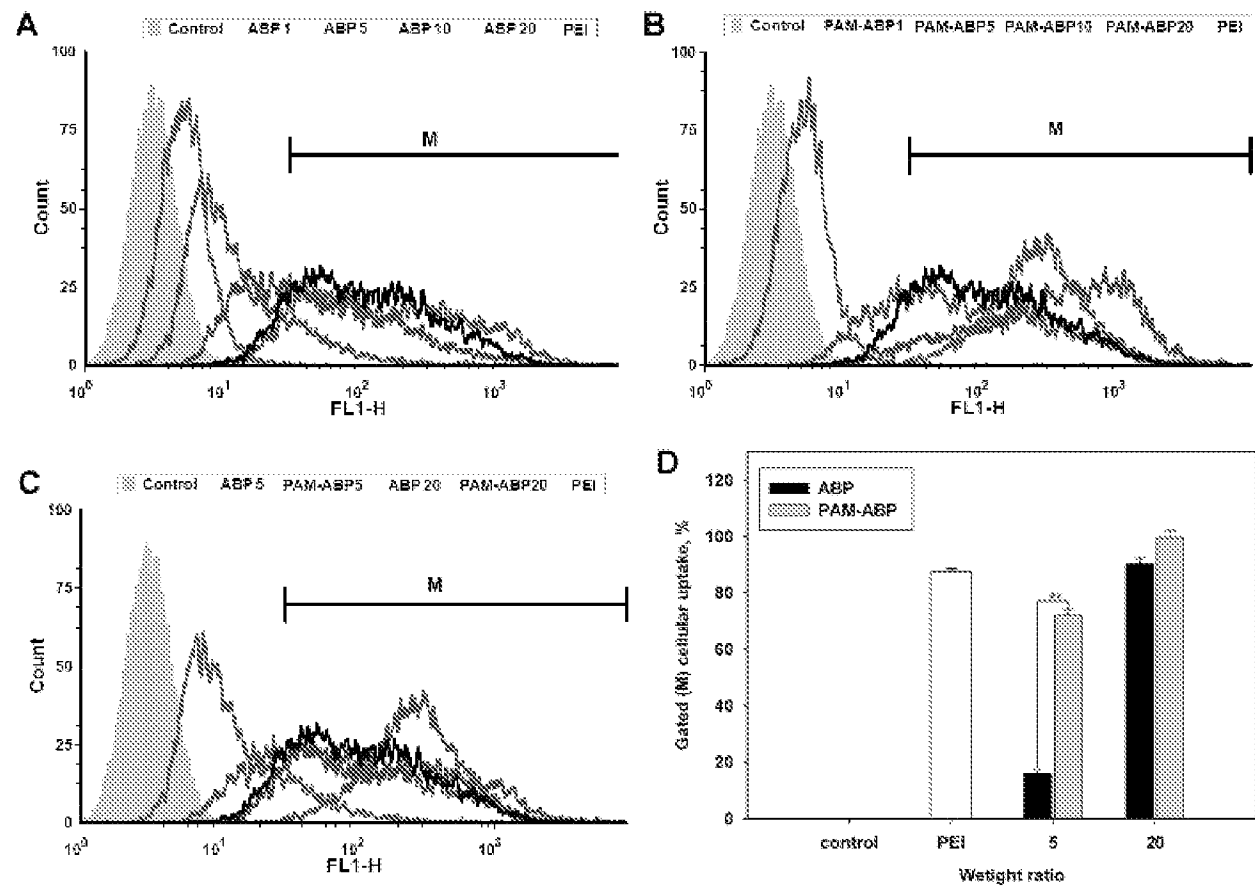


FIG. 8

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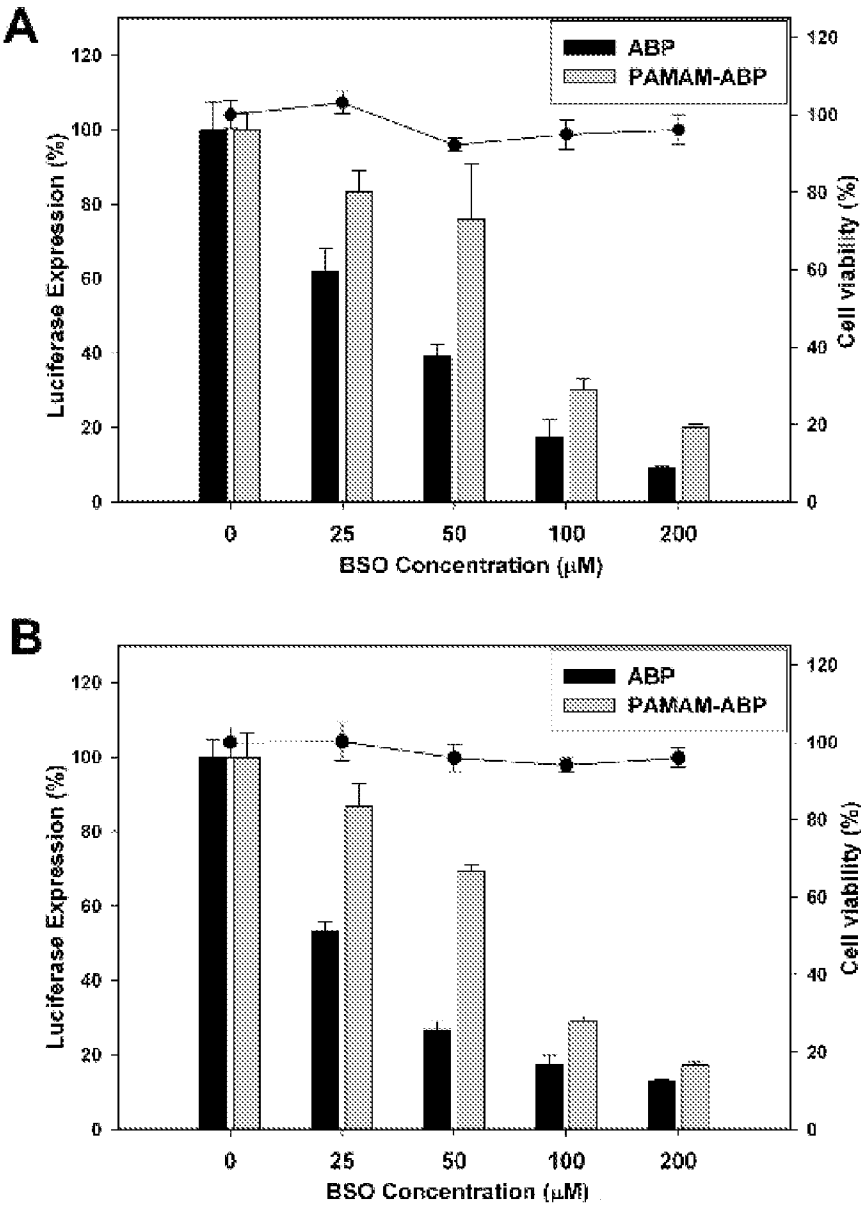


FIG. 9

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PAMAM G0

PAMAM G0-SH

+ Traut's reagent

PAMAM G1

PAMAM G1-SH

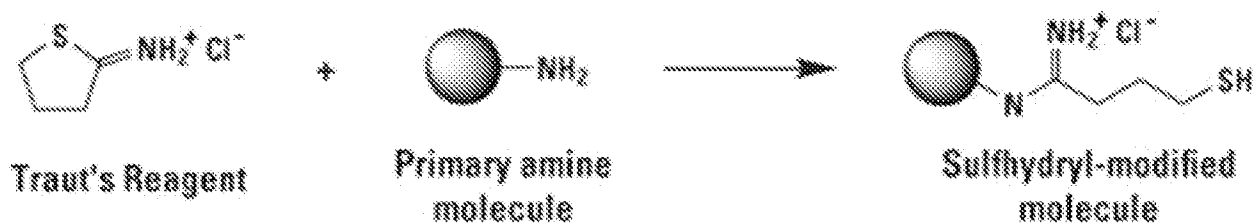


FIG. 10

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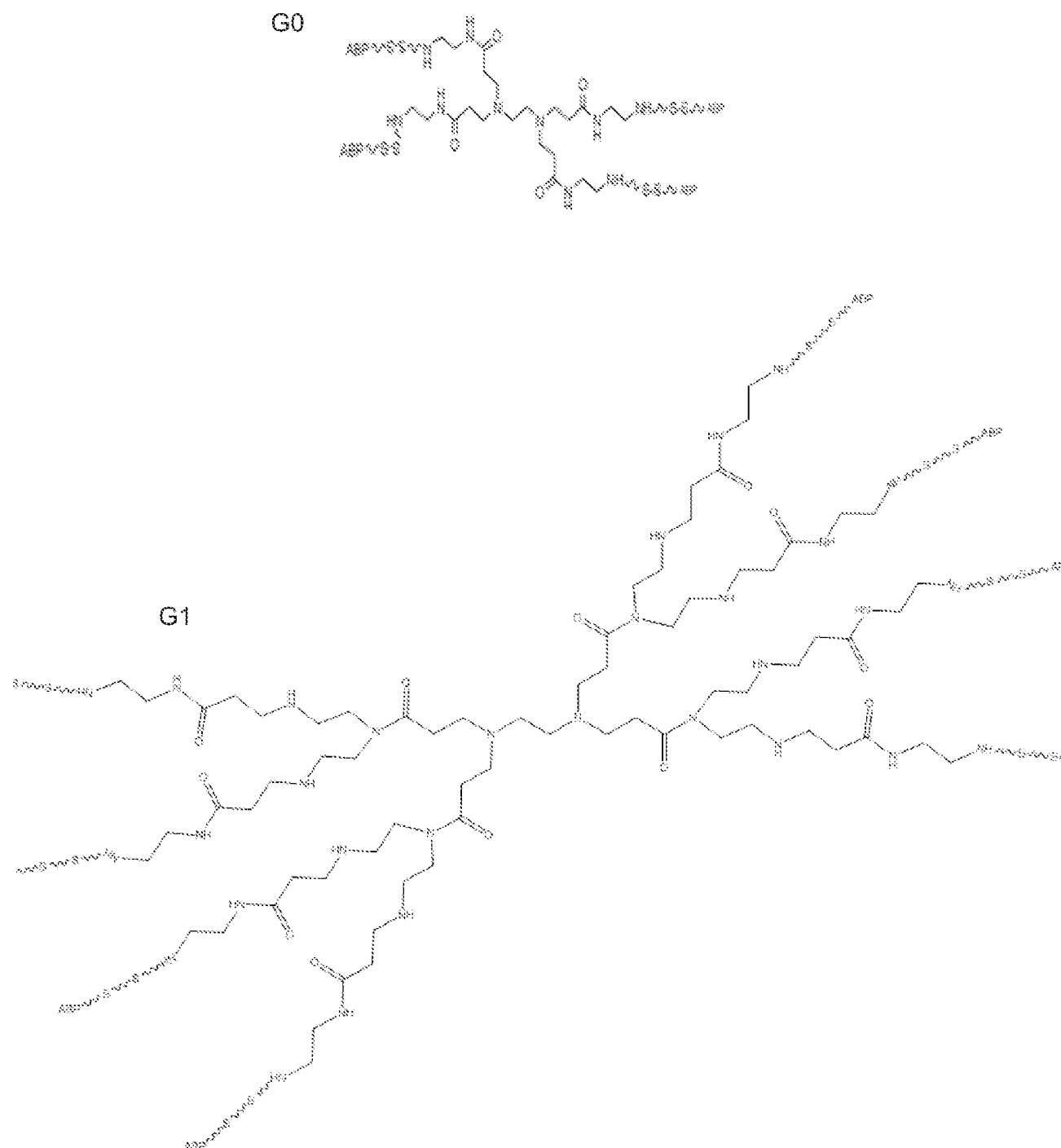


FIG. 11

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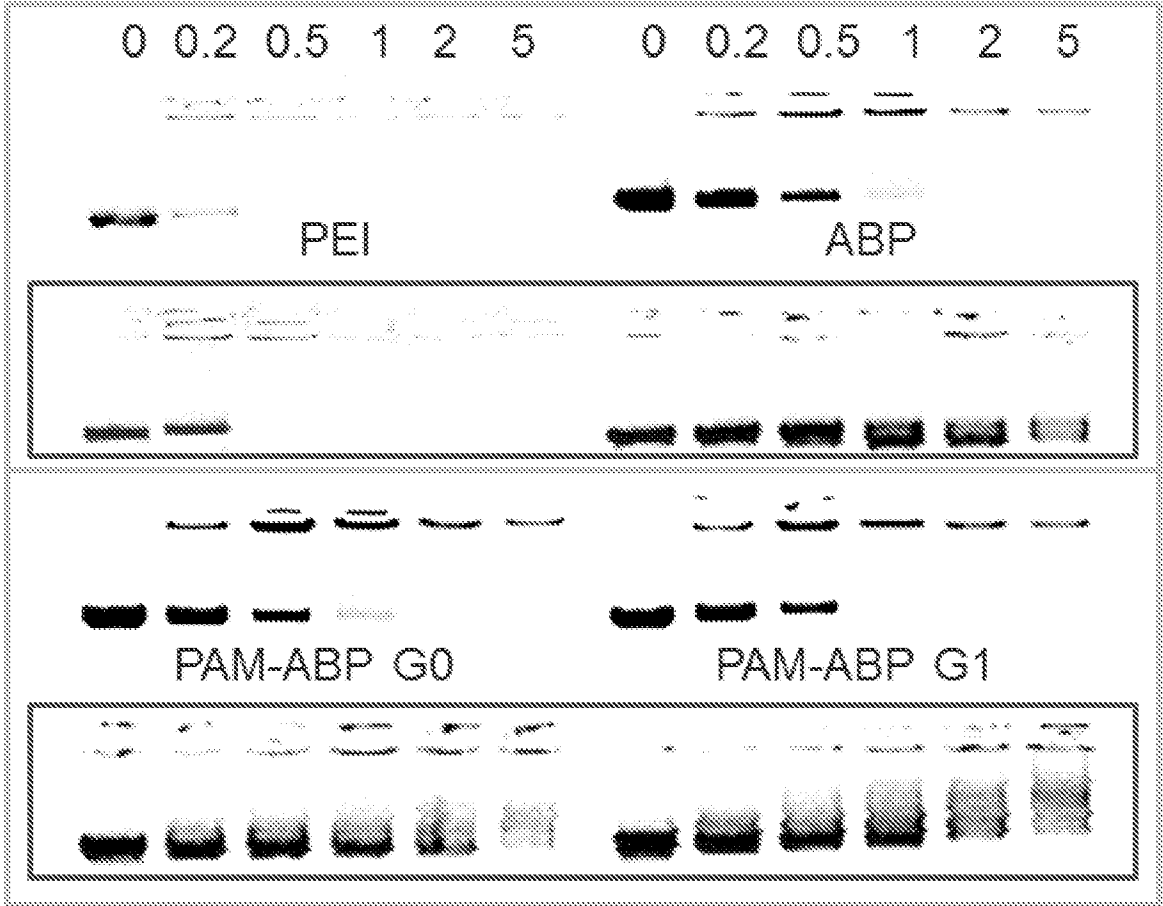


FIG. 12

	Size	Zeta potential
ABP	132.9 nm	25.3 mV
PAM-ABP G0	116.1 nm	18.1 mV
PAM-ABP G1	110.6 nm	24.6 mV

At weight ratio of 5

FIG. 13

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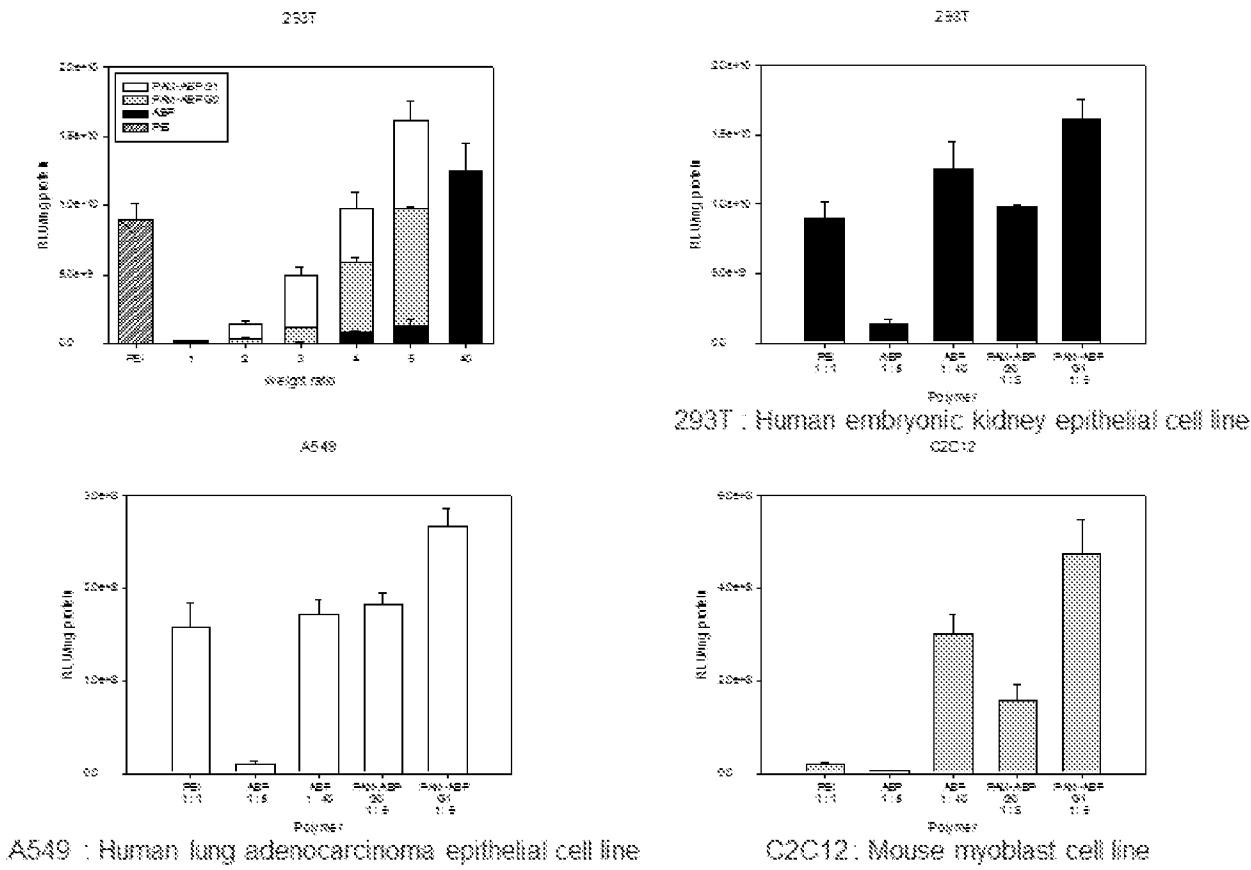
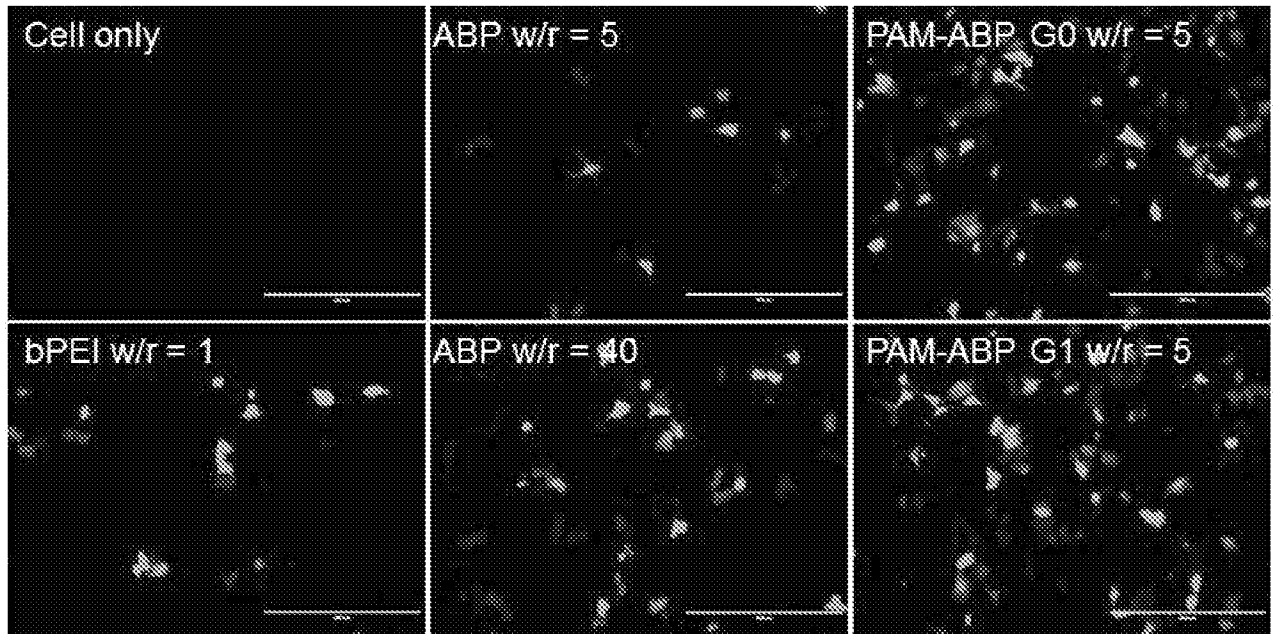
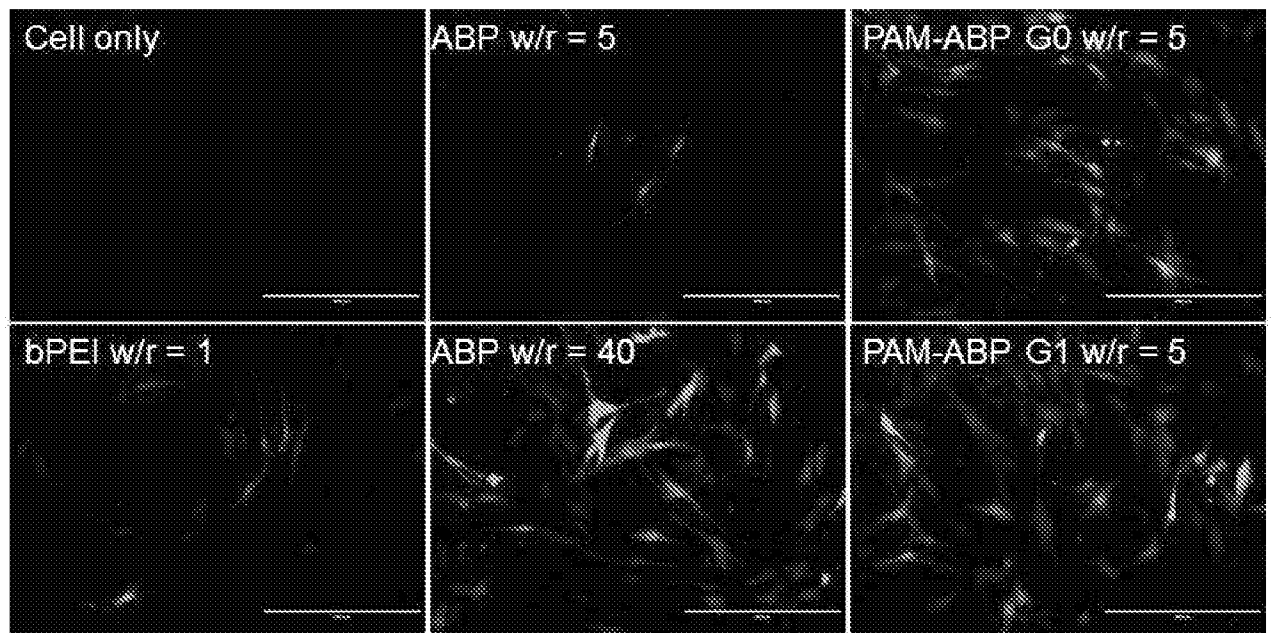


FIG. 14

11/12

**FIG. 15A****FIG. 15B**

C2C12

12/12

FIG. 16A

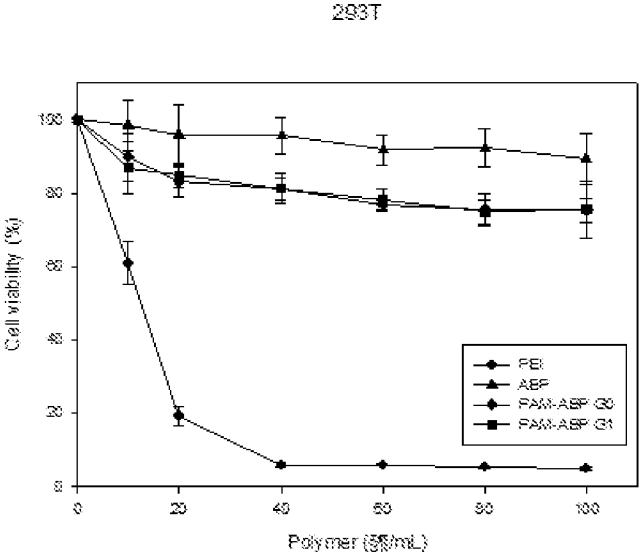


FIG. 16B

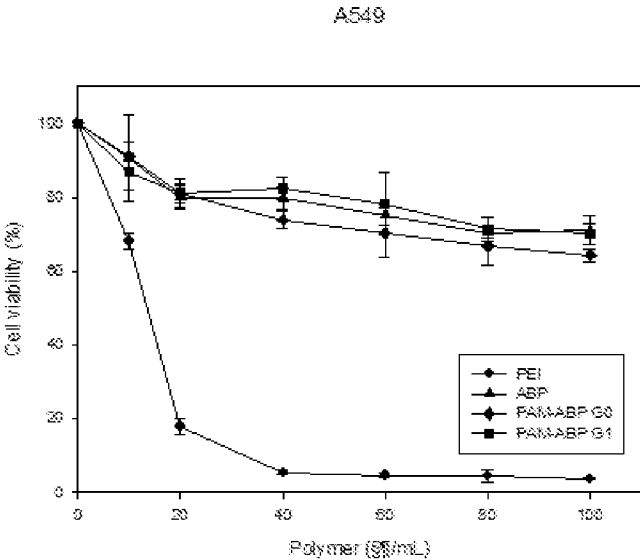
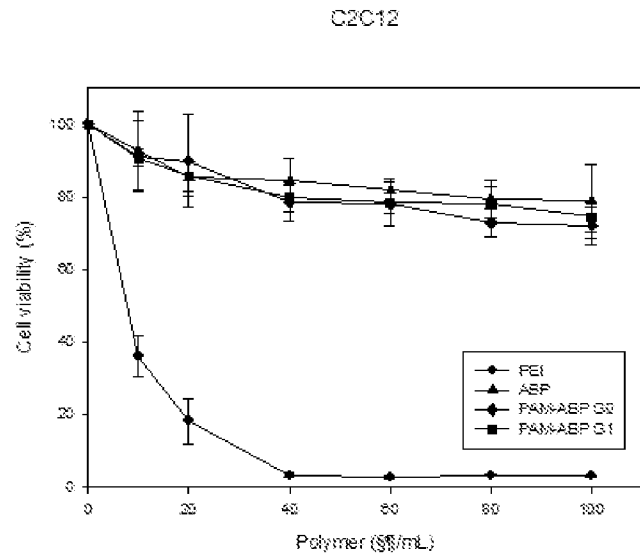


FIG. 16C



A. CLASSIFICATION OF SUBJECT MATTER**A61K 47/48(2006.01)i, A61K 47/30(2006.01)i, A61K 48/00(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 47/48, A61K 47/30, A61K 48/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: polymeric carrier, dendrimer, arginine-grafted bioreducible polymer, nucleic acid

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2009-0233365 A1 (KIM, S. W. et al.) 17 September 2009 See claims 1, 3-4, 7, 9-10 and 13-16.	1-26,28
A	CHOI, J. S. et al., "Enhanced transfection efficiency of PAMAM dendrimer by surface modification with L-arginine", Journal of Controlled Release, 2004, Volume 99, Pages 445-456. See abstract and fig. 1.	1-26,28
A	US 2009-0297487 A1 (BOE, S. et al.) 03 December 2009 See abstract; and claims 1-41 and 45-47.	1-26,28
A	EICHMAN, J. D. et al., "The use of PAMAM dendrimers in the efficient transfer of genetic material into cells", Pharmaceutical Science & Technology Today, 2000, Volume 3, Issue 7, Pages 232-245. See the whole document.	1-26,28
A	US 2006-0204472 A1 (PALEOS, C. et al.) 14 September 2006 See abstract and claims 1-27.	1-26,28



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 June 2013 (19.06.2013)

Date of mailing of the international search report

19 June 2013 (19.06.2013)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City,
302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

CHOI, Sung Hee

Telephone No. 82-42-481-8740



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/022294

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 27, 29, 30
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 27, 29 and 30 pertain to method for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2013/022294

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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