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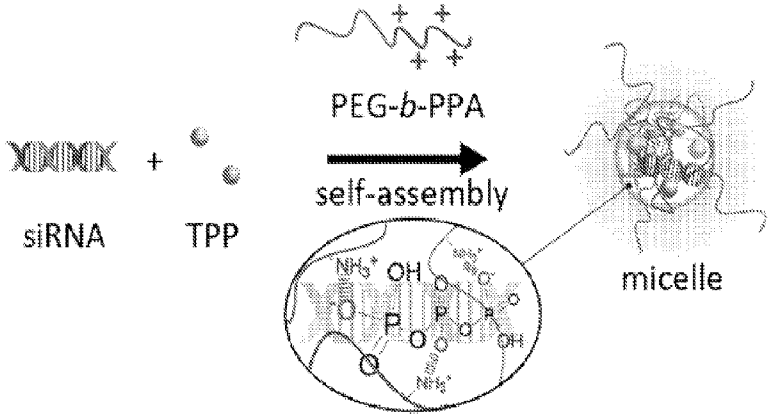
(54) **Title**: STABILIZED POLYRIBONUCLEOTIDE NANOPARTICLES



(57) **Abstract**: One or more stabilized polymeric nanoparticles comprising: (a) polycationic polymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent are provided. The stabilized polymeric nanoparticles exhibited high uniformity with small size, and showed increased stability in physiological ionic strength and serum containing medium. The transfection and gene silencing efficiency of the stabilized polymeric nanoparticles was markedly improved over nanoparticles which did not contain the stabilization reagent in serum-containing medium. Methods of making the stabilized polymeric nanoparticles, pharmaceutical compositions comprising same, and methods for their use are also provided.

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STABILIZED POLYRIBONUCLEOTIDE NANOPARTICLES

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 61/430,242, filed on January 6, 2011, which is hereby incorporated by reference for all purposes as if fully set forth herein.

STATEMENT OF GOVERNMENTAL INTEREST

[0002] This invention was made with U.S. government support under grant no. W81XWH-10-2-0053. The U.S. government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Small interfering RNA (siRNA) has been recognized as a powerful therapeutic agent for effectively silencing a specific gene on a post-transcriptional level. Many siRNA targets and RNA interference (RNAi) strategies have been devised as a therapeutic approach in the treatment of diseases such as macular degeneration, hepatitis C infection, and cancer. However, therapeutic application of siRNA has been hampered by its limited stability within physiological fluids, and its inefficient cell membrane permeation due to the high density of negative charge in naked siRNA. Recent studies have successfully demonstrated that siRNA carriers based on various cationic polymers, lipids, and peptides, have been used to form nanosized complexes with siRNA. However, these polycation/siRNA nanoparticles still exhibit poor stability in buffers at physiological ionic strength, or in serum-containing media, due to the small molecular weight of siRNA chains.

[0004] The inventors previously reported that the condensation of DNA by the polycationic block copolymer, poly(ethylene glycol)-*b*-polyphosphoroamidate (PEG-*b*-PPA), produced self-assembled micellar nanoparticles with a complex core surrounded by a PEG corona. The advantages of these micellar nanoparticles include smaller and more uniform size, improved colloidal stability in serum-containing media, higher protection of incorporated DNA against enzymatic degradation, and prolonged blood circulation. Despite the success of mediating DNA delivery, PEG-*b*-PPA condensed micelles with siRNA suffered from low stability in salt solution, which may be due to the short and rigid structure of siRNA in contrast to plasmid

DNA. Accordingly, there is still a need in the art for nanoparticles which have improved stability in salt and serum-containing solutions, and that have the ability to protect incorporated nucleic acids such as siRNA against enzymatic degradation in blood and other bodily fluids, while also having high efficiency in delivering the nucleic acids to the target cytosol.

BRIEF SUMMARY OF THE INVENTION

[0005] In an embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent.

[0006] In another embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers selected from the group consisting of linear or branched polycationic homopolymers, block polycationic copolymers and graft polycationic copolymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent.

[0007] In a further embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers selected from the group consisting of linear and/or branched polyethyleneimines (PEI), polyphosphoroamidates (PPA), block polycationic copolymers comprising PEG and PEI or PPA, and graft polycationic copolymers comprising PEG and PEI or PPA, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent.

[0008] In still another embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more block copolymers of polyethylene glycol-*b*-polyphophoramidate (PEG-*b*-PPA), (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent.

[0009] In another embodiment, present invention provides one or more polymeric nanoparticles comprising: (a) one or more graft copolymers of polyethyleneimine-*g*-polyethylene glycol (PEI-*g*-PEG), (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent.

[0010] In a further embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers, (b) at least one or more

polyribonucleotide molecules, and (c) an anionic stabilization reagent, wherein the polyribonucleotide molecule is a single stranded RNA molecule, a double stranded RNA molecule, a micro-RNA (miRNA) molecule, a short-hairpin RNA (shRNA) molecule, or a siRNA molecule.

[0011] In an embodiment, the present invention provides a method for making one or more polymeric nanoparticles having (a) one or more polycationic polymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, said method comprising: 1) obtaining a solution comprising at least one or more polyribonucleotide molecules, 2) adding to the solution of (1), a sufficient quantity of a solution containing an anionic stabilization reagent, and mixing the resulting solution; and 3) adding to the solution of (2), a sufficient quantity of a solution containing one or more polycationic polymers, mixing the solution, and incubating the resulting solution for a sufficient time to allow a complexation reaction to occur and the nanoparticles to assemble.

[0012] In another embodiment, the present invention provides a method for making one or more polymeric nanoparticles having (a) one or more polycationic polymers selected from the group consisting of linear or branched polycationic homopolymers, block polycationic copolymers and graft polycationic copolymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, said method comprising: 1) obtaining a solution comprising at least one or more polyribonucleotide molecules, 2) adding to the solution of (1), a sufficient quantity of a solution containing an anionic stabilization reagent, and mixing the resulting solution; and 3) adding to the solution of (2), a sufficient quantity of a solution containing one or more polycationic polymers, mixing the solution, and incubating the resulting solution for a sufficient time to allow a complexation reaction to occur and the nanoparticles to assemble.

[0013] In a further embodiment, the present invention provides a method for making one or more polymeric nanoparticles having (a) one or more block copolymers of polyethylene glycol-*b*-polyphophoramidate (PEG-*b*-PPA), (b) at least one polynucleotide molecule; and (c) an anionic stabilization reagent, said method comprising: 1) obtaining a solution comprising at least one or more polyribonucleotide molecules, 2) adding to the solution of (1), a sufficient quantity of a solution containing an anionic stabilization reagent, and mixing the resulting solution; and 3) adding to the solution of (2), a sufficient quantity of a solution containing PEG-*b*-PPA, mixing

the solution, and incubating the resulting solution for a sufficient time to allow a complexation reaction to occur and the nanoparticles to assemble.

[0014] In an embodiment, the present invention provides a method of modulating expression of a target gene in a cell, or population of cells, comprising administering to the cell or population of cells, one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, in an amount sufficient to modulate target gene expression with the cell or population of cells. In certain embodiments, the cells are mammalian cells.

[0015] In another embodiment, the present invention provides a method of modulating expression of a target gene in a cell or population of cells comprising administering to the cell, or population of cells, one or more polymeric nanoparticles having: (a) one or more polycationic polymers selected from the group consisting of linear or branched polycationic homopolymers, block polycationic copolymers and graft polycationic copolymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, in an amount sufficient to modulate target gene expression with the cell, or population of cells.

[0016] In another embodiment, the present invention provides a method of modulating expression of a target gene in a cell, or population of cells, comprising administering to the cell, or population of cells, one or more polymeric nanoparticles having: (a) one or more block copolymers of PEG-*b*-PPA, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, in an amount sufficient to modulate target gene expression with the cell or population of cells.

[0017] In another embodiment, the present invention provides a method of modulating expression of a target gene in a cell, or population of cells, comprising administering to the cell, or population of cells, one or more polymeric nanoparticles having: (a) one or more block copolymers of PEI-*g*-PEG, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, in an amount sufficient to modulate target gene expression with the cell or population of cells.

[0018] In an embodiment, the present invention provides a use of a composition of one or more polymeric nanoparticle comprising: (a) one or more polycationic polymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, in an effective amount, wherein the composition includes a pharmaceutically and physiologically acceptable

carrier, to prepare a medicament, preferably for use as a medicament for treating a disease in a subject.

[0019] In an embodiment, the present invention provides a use of a composition of one or more polymeric nanoparticle comprising: (a) one or more polycationic polymers selected from the group consisting of linear or branched polycationic homopolymers, block polycationic copolymers and graft polycationic copolymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, in an effective amount, wherein the composition includes a pharmaceutically and physiologically acceptable carrier, to prepare a medicament, preferably for use as a medicament for treating a disease in a subject.

[0020] In an embodiment, the present invention provides a use of a composition of one or more polymeric nanoparticle comprising: (a) one or more polycationic polymers selected from the group consisting of linear or branched polycationic homopolymers, block polycationic copolymers and graft polycationic copolymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, in an effective amount, wherein the composition includes a pharmaceutically and physiologically acceptable carrier, to prepare a medicament, preferably for use as a medicament for treating a disease in a subject.

[0021] In an embodiment, the present invention provides a use of a composition of one or more polymeric nanoparticle comprising: (a) one or more block copolymers of polyethylene glycol-*b*-polyphophoramidate (PEG-*b*-PPA), (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, in an effective amount, wherein the composition includes a pharmaceutically and physiologically acceptable carrier, to prepare a medicament, preferably for use as a medicament for treating a disease in a subject.

[0022] In an embodiment, the present invention provides a method of modulating expression of a target gene in a mammalian cell or population of cells comprising administering to the cell, or population of cells, one or more polymeric nanoparticles having: (a) one or more polycationic polymers selected from the group consisting of linear polycationic polymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, in an amount sufficient to modulate target gene expression with the cell, or population of cells and diagnose the role of the target gene in a clinical condition or disease.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

[0023] FIG. 1 depicts a schematic illustrating the synthesis of the polycationic block copolymer PEG-*b*-PPA in accordance with an embodiment of the present invention.

[0024] FIG. 2 depicts a schematic illustrating the synthesis of the self-assembling polymeric PEG-*b*-PPA/siRNA/TPP ternary nanoparticles formed through ionic crosslinking with the anionic stabilization reagent, sodium triphosphate (TPP), in accordance with an embodiment of the present invention.

[0025] FIG. 3 depicts a graph showing the mean particle hydrodynamic diameter (z-average) (left ordinate) and polydispersity index (PDI) (right ordinate) measured by dynamic light scattering (DLS) analysis of nanoparticles of the present invention formed at various P'/N ratios at a N/P ratio of 4 before (●, ○) and after (■, □) incubation with 150 mM NaCl for 24 hours.

[0026] FIG. 4 depicts a graph showing the z-average (left ordinate) and PDI (right ordinate) measured by dynamic light scattering analysis of an embodiment of the nanoparticles of the present invention formed at various P'/N ratios at a N/P ratio of 8 before (●, ○) and after (■, □) incubation with 150 mM NaCl for 24 hours.

[0027] FIG. 5 is a series of transmission electron microscopy (TEM) images of an embodiment of the nanoparticles of the present invention formed at P'/N ratios of 0 and 0.5 for N/P ratios of 4 and 8, respectively. Scale bar = 100 nm.

[0028] FIG. 6 is a graph depicting the zeta-potential of an embodiment of the nanoparticles of an embodiment of the present invention prepared at various P'/N ratios for N/P ratios of 4 (■) and 8 (□), respectively. Values represent Mean ± SEM (n = 3).

[0029] FIG. 7A is a graph depicting gene silencing efficiency mediated by PEG-PPA/siRNA/TPP ternary nanoparticles of an embodiment of the present invention at various P'/N ratios for N/P ratios of 4 and 8 in HeLa and D407 cells in the absence of 10% serum. An siRNA dose of 100 nM was used in all transfection groups. Values are expressed as Mean ± SEM (n = 4).

[0030] FIG 7B is a graph depicting gene silencing efficiency mediated by PEG-PPA/siRNA/TPP ternary nanoparticles of an embodiment of the present invention at various P'/N ratios for N/P ratios of 4 and 8 in HeLa and D407 cells in the presence of 10% serum. A

siRNA dose of 100 nM was used in all transfection groups. Values are expressed as Mean ± SEM ($n = 4$).

[0031] FIG. 8 is a graph depicting the viability of HeLa and D407 cells transfected with PEG-PPA/siRNA/TPP ternary nanoparticles of an embodiment of the present invention in 10% serum-containing medium, under the conditions described in Figure 7. Values are expressed as Mean ± SEM ($n = 4$).

[0032] Fig. 9 is a series of transmission electron micrographs of (a) branched PEI/siRNA nanoparticles, (b) hyaluronic acid (HA)-coated branched PEI/siRNA nanoparticles, showing severe aggregation, (c) TPP stabilized branched PEI/siRNA nanoparticles, (d) HA-coated branched PEI/siRNA/TPP nanoparticles, (e) TPP stabilized linear PEI/siRNA nanoparticles, and (f) HA-coated linear PEI/siRNA/TPP nanoparticles.

[0033] Fig. 10 depicts a pair of bar graphs showing the gene knockdown efficiency (a), and cell viability (b) after treatment with PEI/siRNA nanoparticles of an embodiment of the present invention in HCE-2 cells (human corneal epithelial cell).

DETAILED DESCRIPTION OF THE INVENTION

[0034] In accordance with an embodiment, the present invention provides one or more self-assembling polymeric nanoparticles comprising a polynucleotide molecule, which has improved stability in salt and serum-containing solutions, and has the ability to protect incorporated polynucleotide molecules, such as siRNA, against enzymatic degradation in blood and other bodily fluids, while also having high efficiency in delivering the polynucleotide molecules to the target cytosol, and therefore can modulate the expression of a target gene of interest. Methods of making these self-assembling polymeric nanoparticles, as well as their use in treating populations of cells and /or for use in diagnosing or treating a disease in a subject, are also provided.

[0035] In an embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic homopolymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent.

[0036] As used herein, the term "polycationic homopolymers" means polymer chains of repeating subunits that have cationic residues. Polycationic polymers are polymers that contain net positively charged atom(s) or associated group(s) of atoms covalently linked to the polymer molecules. This definition includes, but is not limited to phosphonium, sulfonium, and ammonium cations. Other examples of cationic groups that can be covalently linked include, but are not limited to, amines (primary, secondary, tertiary, and aromatic) isocyanates, polyacrylamides, polyisobutylene, poly(N-vinylcarbazole), and polyquaternium polymers.

[0037] Polycationic homopolymers useful in accordance with the present invention include, for example, polymers such as linear or branched homopolymers, including, for example, linear and/or branched polyethyleneimines and derivatives thereof, and polyphosphoroamidates and derivatives thereof.

[0038] In an embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers selected from the group consisting of linear or branched PEI, PPA, block polycationic copolymers comprising PEG and PEI or PPA and derivatives thereof, and graft polycationic copolymers comprising PEG and PEI or PPA and derivatives thereof, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent.

[0039] As used herein, the term "derivatives" will be understood by those of ordinary skill in the art. Polycationic block and graft copolymers and their derivatives, can also be used in the nanoparticles, and include, for example, polyethylene glycol polymers. Examples of block copolymers useful in the present invention include, PEG-*b*-PPA, and derivatives thereof, and examples of polycationic graft copolymers useful in the present invention include, PEI-*g*-PEG and derivatives thereof.

[0040] Furthermore, it is understood that various embodiments comprising two or more different polycationic polymers can be used to produce the nanoparticles of the present invention. Therefore, in accordance with the present invention, in an embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, wherein when there are two or more polycationic polymers.

[0041] In a further embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more linear and or branched PEI polymers or derivatives

thereof, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent.

[0042] In still another embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more linear and/or branched PPA polymers or derivatives thereof, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent.

[0043] As used herein, the polycationic polymers, including linear and branched polymers, as well as the block and graft copolymers used in various embodiments of the present invention, are derivatives of polycationic polymers that include biocompatible polymers (that is, polymers that do not cause significant undesired physiological reactions), that can be either biodegradable or non-biodegradable polymers or blends or copolymers thereof.

[0044] In accordance with an embodiment, examples of such biocompatible polymers include, but are not limited to, polycationic biodegradable PPA. In a preferred embodiment, the block copolymer of the present invention is the PEG-*b*-PPA copolymer. The synthesis of an embodiment of the PEG-*b*-PPA copolymer is shown in Fig. 1.

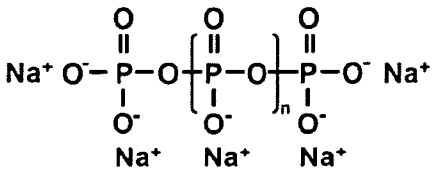
[0045] In accordance with the present invention, in another embodiment, polycationic graft copolymers can be used. For example, in a preferred embodiment, the polycationic graft copolymer PEI-*g*-PEG is suitable for use with the nanoparticles of the present invention. As such, in an embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic graft copolymers of PEI-*g*-PEG, or derivatives thereof, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent.

[0046] The term "biodegradable" as used herein, refers to degradation in a biological system, for example enzymatic degradation or hydrolytic degradation.

[0047] The term "anionic stabilization reagent," as used herein, means an inorganic or organic polyanionic molecule having a high charge density, and is capable of physically cross-linking the positively charged amino groups of the polycationic polymer chains of the nanoparticles, with the negatively charged polyanions of the anionic stabilization reagent.

[0048] The term "charge density" is defined herein as a ratio of a charge unit per unit of molecular weight. A "charge unit" is defined as a molecular moiety having a positive or negative charge when in a solution at a pH range of between about 4 to about 12. Molecules having high charge density have a greater number of charge units per unit of molecular weight.

In accordance with an embodiment of the present invention, the molecule TPP has five negatively charged moieties in solution, and the anionic form ($[O_3POP(O)_2OPO_3]^{-5}$), and has a molecular weight of approximately 368 Daltons, thus TPP has a charge density of 1 anionic charge unit/74 Daltons. Polyanionic molecules with high charge densities, (i.e, 1 charge unit/150 Daltons or less) generally have the capability of having a high condensation capacity with polycations. In a preferred embodiment, the anionic stabilization reagent is sodium triphosphate (TPP). Other possible examples of the anionic stabilization reagent known to those of skill in the art, including, for example, similar molecules (e.g., tetrasodium pyrophosphate and hexasodium metaphosphate), or other inorganic oligophosphates, in linear or cyclic chains ($n = 1 - 4$). One of ordinary skill in the art would understand that these molecules are also referred to as inorganic polyphosphates. When $n = 1$, it is TPP. This is perhaps the most stable. The stability is lower when n goes higher.



Oligophosphate ($n = 1 - 4$)

[0049] It is also understood that in certain embodiments, there can be more than one anionic stabilization reagent present. Thus, in an embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers, (b) at least one or more polynucleotide molecules, and (c) one or more anionic stabilization reagents.

[0050] Methods for making the nanoparticles are also disclosed herein. In an embodiment, the present invention provides a method for making one or more polymeric nanoparticles having (a) one or more polycationic polymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, said method comprising: 1) obtaining a solution comprising at least one or more polyribonucleotide molecules, 2) adding to the solution of (1), a sufficient quantity of a solution containing an anionic stabilization reagent, and mixing the resulting solution; and 3) adding to the solution of (2), a sufficient quantity of a solution containing one or more polycationic polymers of (a), mixing the solution, and incubating the resulting solution for a sufficient time to allow a complexation reaction to occur and the nanoparticles to assemble.

[0051] In another embodiment, the present invention provides a method for making one or more polymeric nanoparticles having (a) one or more polycationic polymers selected from the group consisting of linear or branched polycationic homopolymers, block polycationic copolymers and graft polycationic copolymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, said method comprising: 1) obtaining a solution comprising at least one or more polyribonucleotide molecules, 2) adding to the solution of (1), a sufficient quantity of a solution containing an anionic stabilization reagent, and mixing the resulting solution; and 3) adding to the solution of (2), a sufficient quantity of a solution containing one or more polycationic polymers of (a), mixing the solution, and incubating the resulting solution for a sufficient time to allow a complexation reaction to occur and the nanoparticles to assemble.

[0052] In a further embodiment, the present invention provides a method for making one or more polymeric nanoparticles having (a) one or more polycationic polymers selected from the group consisting of linear or branched polyethyleneimines (PEI), polyphosphoroamidates (PPA), block polycationic copolymers comprising PEG and PEI or PPA, and graft polycationic copolymers comprising PEG and PEI or PPA, and derivatives thereof, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, said method comprising: 1) obtaining a solution comprising at least one or more polyribonucleotide molecules, 2) adding to the solution of (1), a sufficient quantity of a solution containing an anionic stabilization reagent, and mixing the resulting solution; and 3) adding to the solution of (2), a sufficient quantity of a solution containing one or more polycationic polymers of (a), mixing the solution, and incubating the resulting solution for a sufficient time to allow a complexation reaction to occur and the nanoparticles to assemble.

[0053] In still another embodiment, the present invention provides a method for making one or more polymeric nanoparticles having (a) one or more PEG-*b*-PPA polycationic block copolymers or derivatives thereof, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, said method comprising: 1) obtaining a solution comprising at least one or more polyribonucleotide molecules, 2) adding to the solution of (1), a sufficient quantity of a solution containing an anionic stabilization reagent, and mixing the resulting solution; and 3) adding to the solution of (2), a sufficient quantity of a solution containing one or more PEG-*b*-PPA polycationic block copolymers or derivatives thereof, mixing the solution, and

incubating the resulting solution for a sufficient time to allow a complexation reaction to occur and the nanoparticles to assemble.

[0054] In yet a further embodiment, the present invention provides a method for making one or more polymeric nanoparticles having (a) one or more polycationic graft copolymers of PEI-*g*-PEG or derivatives thereof, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, said method comprising: 1) obtaining a solution comprising at least one or more polyribonucleotide molecules, 2) adding to the solution of (1), a sufficient quantity of a solution containing an anionic stabilization reagent, and mixing the resulting solution; and 3) adding to the solution of (2), a sufficient quantity of a solution containing one or more polycationic graft copolymers of PEI-*g*-PEG or derivatives thereof, mixing the solution, and incubating the resulting solution for a sufficient time to allow a complexation reaction to occur and the nanoparticles to assemble.

[0055] As used herein, the term "sufficient time to allow a complexation reaction to occur and the nanoparticles to assemble," means the time necessary for the one or more polyribonucleotide molecules, and an anionic stabilization reagent, to be mixed with the one or more polycationic polymers and have the self-assembly, or complexation to occur, and the nanoparticles to form. While not being limited to any particular time period, in accordance with the present invention, the complexation reaction can occur within 5 minutes to an hour or more. Preferably, the complexation reaction occurs in less than an hour. More preferably, the complexation reaction occurs within about 10 minutes to about 30 minutes, and still more preferably, the complexation reaction occurs in about 15 minutes. The time for complexation to occur can vary according to temperature, concentration of the reagents, and the type of polycationic polymers, anionic stabilization reagent and polyribonucleotide used.

[0056] In accordance with the invention, in an embodiment, the polymeric nanoparticles have a diameter between 30 nm and 300 nm, preferably between 40 nm and 150 nm, more preferably between 50 nm and 120 nm. The diameter of the nanoparticles of the present invention can be measured by any means technically feasible that is known in the art. Preferred methods include photon correlation spectroscopy, doppler anemometry, light scattering measurement, and transmission electron microscopy.

[0057] In yet a further embodiment, the present invention provides that the one or more polymeric nanoparticles comprise at least one or more polynucleotide molecules which are

between 10 bp and 10,000 bp in length, preferably between 20 bp and 50 bp in length, more preferably between 30 bp and 40 bp in length.

[0058] The term "polynucleotide," as used herein, includes and/or is synonymous with "nucleic acid," "oligonucleotide," and "nucleic acid molecule," and generally means a polymer of DNA or RNA, which can be single-stranded or double-stranded, synthesized or obtained (e.g., isolated and/or purified) from natural sources, which can contain natural, non-natural or altered nucleotides, and which can contain a natural, non-natural or altered internucleotide linkage, such as a phosphoroamidate linkage or a phosphorothioate linkage, instead of the phosphodiester found between the nucleotides of an unmodified oligonucleotide.

[0059] The term "polyribonucleotide," as used herein, includes "ribonucleic acid," "oligoribonucleotide," and "ribonucleic acid molecule," and generally means a polymer of RNA which can be single-stranded or double-stranded, synthesized or obtained (e.g., isolated and/or purified) from natural sources, which can contain natural, non-natural or altered nucleotides, and which can contain a natural, non-natural or altered internucleotide linkage, such as a phosphoroamidate linkage or a phosphorothioate linkage, instead of the phosphodiester found between the nucleotides of an unmodified oligonucleotide. It may be suitable in some instances, in an embodiment, for the nucleic acids to comprise one or more insertions, deletions, inversions, and/or substitutions.

[0060] Preferably, the nucleic acids of the invention are recombinant. As used herein, the term "recombinant" refers to (i) molecules that are constructed outside living cells by joining natural or synthetic nucleic acid segments to nucleic acid molecules that can replicate in a living cell, or (ii) molecules that result from the replication of those described in (i) above. For purposes herein, the replication can be *in vitro* replication or *in vivo* replication.

[0061] The nucleic acids can be constructed based on chemical synthesis and/or enzymatic ligation reactions using procedures known in the art. For example, a nucleic acid can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed upon hybridization (e.g., phosphorothioate derivatives and acridine substituted nucleotides). Examples of modified nucleotides that can be used to generate the nucleic acids include, but are not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-

carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N^6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N^6-substituted adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N^6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine. Alternatively, one or more of the nucleic acids of the invention can be purchased from companies, such as Macromolecular Resources (Fort Collins, CO) and Synthegen (Houston, TX).

[0062] In another embodiment, the present invention provides one or more polymeric nanoparticles, wherein the polyribonucleotide molecule is selected from the group consisting of single stranded RNA, double stranded RNA, micro-RNA (miRNA), short-hairpin RNA (shRNA), and/or analogs thereof.

[0063] Therefore, in accordance with the present invention, in an embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, wherein the polyribonucleotide molecule is a single stranded RNA molecule, a double stranded RNA molecule, a miRNA molecule, a shRNA molecule, or a siRNA molecule.

[0064] In another embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers selected from the group consisting of linear polycationic homopolymers, block polycationic copolymers and graft polycationic copolymers, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, wherein the polyribonucleotide molecule is a single stranded RNA molecule, a double stranded RNA molecule, a miRNA molecule, shRNA molecule, or a siRNA molecule.

[0065] In a further embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers selected from the group consisting of linear and/or branched PEI, PPA, block polycationic copolymers comprising PEG

and PEI or PPA, and graft polycationic copolymers comprising PEG and PEI or PPA, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, wherein the polyribonucleotide molecule is a single stranded RNA molecule, a double stranded RNA molecule, a miRNA molecule, a shRNA molecule, or a siRNA molecule.

[0066] In still a further embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) PEG-*b*-PPA, and derivatives thereof, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, wherein the polyribonucleotide molecule is a single stranded RNA molecule, a double stranded RNA molecule, a miRNA molecule, a shRNA molecule, or a siRNA molecule.

[0067] In yet another embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) PEI-*g*-PEG, and derivatives thereof, (b) at least one or more polyribonucleotide molecules, and (c) an anionic stabilization reagent, wherein the polyribonucleotide molecule is a single stranded RNA molecule, a double stranded RNA molecule, a miRNA molecule, a shRNA molecule, or a siRNA molecule.

[0068] The polyribonucleotides incorporated within the nanoparticles of the present invention can comprise any nucleotide sequence that encodes for a target gene of interest. In an embodiment, the present invention provides that the polynucleotide encodes for a complementary sequence to a target mRNA sequence of a target gene of interest in a cell or population of cells, either *in vitro,* or *in vivo* in a host. In another embodiment, the polynucleotide is an isolated or purified nucleic acid comprising a nucleotide sequence which is complementary to the nucleotide sequence of any target nucleotide sequence or a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of any of the nucleic acids described herein.

[0069] The present invention also relates to compounds, compositions, and methods useful for modulating the expression and activity of a target gene of interest, or expression and/or activity by RNAi using small nucleic acid molecules. As used herein, the instant invention features small nucleic acid molecules, or polyribonucleotides, and includes terms such as such as siRNA, siNA, dsRNA, miRNA, and shRNA molecules and methods used to modulate the expression of target genes of interest.

[0070] A polyribonucleotide of the invention can be unmodified or chemically modified. A polyribonucleotide of the instant invention can be chemically synthesized, expressed from a

vector or enzymatically synthesized. The instant invention also features various chemically modified polyribonucleotides, including, for example, siRNA molecules capable of modulating repeat expansion gene expression or activity in cells by RNAi. The use of chemically modified siRNA improves various properties of native siRNA molecules through increased resistance to nuclease degradation *in vivo* and/or through improved cellular uptake.

[0071] In one embodiment, the polyribonucleotide molecule of the present invention comprises modified nucleotides while maintaining the ability to mediate RNAi. The modified nucleotides can be used to improve *in vitro* or *in vivo* characteristics, such as stability, activity, and/or bioavailability. For example, when the polyribonucleotide molecule is a siRNA molecule, the invention can comprise modified nucleotides as a percentage of the total number of nucleotides present in the siRNA molecule. As such, an siRNA molecule of the invention can generally comprise about 5% to about 100% modified nucleotides (e.g., 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides). The actual percentage of modified nucleotides present in a given siRNA molecule will depend on the total number of nucleotides present in the siRNA. If the siRNA molecule is single-stranded, the percent modification can be based upon the total number of nucleotides present in the single-stranded siRNA molecules. Likewise, if the siRNA molecule is double-stranded, the percent modification can be based upon the total number of nucleotides present in the sense strand, antisense strand, or both the sense and antisense strands.

[0072] The term "modulate," as used herein means that the expression of the target gene, or level of RNA molecule or equivalent RNA molecules encoding one or more target proteins or protein subunits, or activity of one or more proteins or protein subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

[0073] The terms "inhibit," "down-regulate," "reduce," or "knockdown," as used herein, means that the expression of the target gene, or level of RNA molecules or equivalent RNA molecules encoding one or more target proteins or protein subunits, or activity of one or more target proteins or protein subunits, is reduced below that observed in the absence of the polyribonucleotide molecules (e.g., siRNA) of the invention. In an embodiment, inhibition, down-regulation or reduction with a siRNA molecule is below that level observed in the

presence of an inactive or attenuated molecule. In another embodiment, inhibition, down-regulation, or reduction with siRNA molecules is below that level observed in the presence of, for example, a siRNA molecule with scrambled sequence or with mismatches. In another embodiment, inhibition, down-regulation, or reduction of target gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence.

[0074] In accordance with an embodiment of the present invention, the amount of time of exposure of the nanoparticles to the host cells, population of cells or subject should be sufficiently long to effect gene "knockdown" or modulation of the expression of the target gene in the host cell, population of cells or in the subject. The time for the desired effect varies with dosage, target, age and other factors known to those of skill in the art. Generally, the time of exposure of the nanoparticles to the host cells, population of cells or subject should range from about 1 hour to about 120 hours, preferably from about 1 hour to about 48 hours, more preferably from about 1 hour to about 24 hours.

[0075] By "gene", or "target gene", is meant, a nucleic acid that encodes a RNA, for example, nucleic acid sequences including, but not limited to, structural genes encoding a polypeptide. A gene or target gene can also encode a functional RNA (fRNA) or non-coding RNA (ncRNA), such as small temporal RNA (stRNA), miRNA, small nuclear RNA (snRNA), siRNA, small nucleolar RNA (snRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and precursor RNAs thereof. Such non-coding RNAs can serve as target nucleic acid molecules for siRNA mediated RNA interference in modulating the activity of fRNA or ncRNA involved in functional or regulatory cellular processes. Aberrant fRNA or ncRNA activity leading to disease can therefore be modulated by polyribonucleotide molecules of the invention. Polyribonucleotide molecules targeting fRNA and ncRNA can also be used to manipulate or alter the genotype or phenotype of an organism or cell, by intervening in cellular processes such as genetic imprinting, transcription, translation, or nucleic acid processing (e.g., transamination, methylation etc.). The target gene can be a gene derived from a cell, an endogenous gene, a transgene, or exogenous genes such as genes of a pathogen, for example a virus, which is present in the cell after infection thereof.

[0076] As used herein, the term "complementarity" or "complementary" means that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. In reference to the polyribonucleotide molecules of the

present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner et al., 1987, *CSH Symp. Quant. Biol.* LII pp. 123-133; Frier et al., 1986, *Proc. Nat. Acad. Sci.* USA 83:9373-9377; Turner et al., 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, or 10 nucleotides out of a total of 10 nucleotides in the first oligonucleotide being based paired to a second nucleic acid sequence having 10 nucleotides represents 50%, 60%, 70%, 80%, 90%, and 100% complementary respectively).

[0077] As used herein, the term "RNA" means a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2′ position of a β-D-ribo-furanose moiety. The terms "RNA," "ribonucleotides" and "polyribonucleotide," also include double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the 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 the siRNA, or internally, for example, at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

[0078] In accordance with the present invention, as used herein, the term "one or more polyribonucleotide molecules" means that the nanoparticles of the present invention can comprise more than one polyribonucleotide molecule. Furthermore, the more than one polyribonucleotide molecules can include molecules having different nucleotide sequences directed to more than one mRNA nucleotide sequences. For example, in an embodiment, the nanoparticles of the present invention can comprise polyribonucleotide molecules having, two, three or four distinct nucleotide sequences specific for different target genes or different sequences of the same target gene.

[0079] In a further embodiment, the present invention provides one or more polymeric nanoparticles wherein the polyribonucleotide molecule is a double stranded RNA molecule or siRNA. The length of the siRNA molecule can be any length greater than about 10 bp, which is capable of binding its complementary sequence on the mRNA of the target gene of interest in the cytosol of a cell or population of cells. The length of the siRNA can be about 20 to about 50 bp, including, for example, 20 bp, 25 bp, 30 bp, 35 bp, 40 bp, 45 bp, up to and including 50 bp.

[0080] It is contemplated that any of the nanoparticle embodiments of the present invention described above can also encompass a pharmaceutical composition comprising the polymeric nanoparticles and a pharmaceutically acceptable carrier. For example, in an embodiment, the present invention provides a pharmaceutical composition comprising one or more polymeric nanoparticles and a pharmaceutically acceptable carrier, wherein the nanoparticles comprise: (a) one or more polycationic homopolymers, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent.

[0081] In another embodiment, the present invention provides a pharmaceutical composition comprising one or more polymeric nanoparticles and a pharmaceutically acceptable carrier, wherein the nanoparticles comprise: (a) one or more polycationic polymers selected from the group consisting of linear and/or branched polyethyleneimines (PEI), polyphosphoroamidates (PPA), block polycationic copolymers comprising PEG and PEI or PPA, and graft polycationic copolymers comprising PEG and PEI or PPA, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent.

[0082] In a further embodiment, the present invention provides a pharmaceutical composition comprising one or more polymeric nanoparticles and a pharmaceutically acceptable carrier, wherein the nanoparticles comprise: (a) one or more polycationic block copolymers of PEG-*b*-PPA or derivatives thereof, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent.

[0083] In yet another embodiment, the present invention provides a pharmaceutical composition comprising one or more polymeric nanoparticles and a pharmaceutically acceptable carrier, wherein the nanoparticles comprise: (a) one or more polycationic graft copolymers of (PEI-*g*-PEG) or derivatives thereof, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent.

[0084] With respect to nanoparticle compositions described herein, the carrier can be any of those conventionally used, and is limited only by physico-chemical considerations, such as solubility and lack of reactivity with the active compound(s), and by the route of administration. The carriers described herein, for example, vehicles, adjuvants, excipients, and diluents, are well-known to those skilled in the art and are readily available to the public. It is preferred that the carrier be one which is chemically inert to the active agent(s), and one which has little or no detrimental side effects or toxicity under the conditions of use. Examples of the carriers include soluble carriers such as known buffers which can be physiologically acceptable (e.g., phosphate buffer) as well as solid compositions such as solid-state carriers or latex beads.

[0085] The carriers or diluents used herein may be solid carriers or diluents for solid formulations, liquid carriers or diluents for liquid formulations, or mixtures thereof.

[0086] Solid carriers or diluents include, but are not limited to, gums, starches (e.g., corn starch, pregelatinized starch), sugars (e.g., lactose, mannitol, sucrose, dextrose), cellulosic materials (e.g., microcrystalline cellulose), acrylates (e.g., polymethylacrylate), calcium carbonate, magnesium oxide, talc, or mixtures thereof.

[0087] For liquid formulations, pharmaceutically acceptable carriers may be, for example, aqueous or non-aqueous solutions, or suspensions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, and injectable organic esters such as ethyl oleate. Aqueous carriers include, for example, water, alcoholic/aqueous solutions, cyclodextrins, emulsions or suspensions, including saline and buffered media.

[0088] Parenteral vehicles (for subcutaneous, intravenous, intraarterial, or intramuscular injection) include, for example, sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Formulations suitable for parenteral administration include, for example, aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives.

[0089] Intravenous vehicles include, for example, fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Examples are sterile liquids such as water and oils, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants. In general, water, saline, aqueous dextrose and related sugar solutions, and

glycols such as propylene glycols or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions.

[0090] The choice of carrier will be determined, in part, by the particular nanoparticle containing composition, as well as by the particular method used to administer the composition. Accordingly, there are a variety of suitable formulations of the pharmaceutical composition of the invention. The following formulations for parenteral, subcutaneous, intravenous, intramuscular, intraarterial, intrathecal and interperitoneal administration are exemplary, and are in no way limiting. More than one route can be used to administer the compositions of the present invention, and in certain instances, a particular route can provide a more immediate and more effective response than another route.

[0091] Injectable formulations are in accordance with the invention. The requirements for effective pharmaceutical carriers for injectable compositions are well-known to those of ordinary skill in the art (see, e.g., *Pharmaceutics and Pharmacy Practice*, J.B. Lippincott Company, Philadelphia, PA, Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Trissel, 15th ed., pages 622-630 (2009)).

[0092] In accordance with the invention, in an embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic homopolymers, (b) at least one or more polynucleotide molecules, (c) an anionic stabilization reagent, and (d) one or more pharmaceutically active compounds.

[0093] In another embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers selected from the group consisting of linear and/or branched PEI, PPA, block polycationic copolymers comprising PEG and PEI or PPA, and graft polycationic copolymers comprising PEG and PEI or PPA, (b) at least one or more polynucleotide molecules, (c) an anionic stabilization reagent, and (d) one or more pharmaceutically active compounds.

[0094] In still another embodiment, the present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic block copolymers of PEG-*b*-PPA or derivatives thereof, (b) at least one or more polynucleotide molecules, (c) an anionic stabilization reagent, and (d) one or more pharmaceutically active compounds.

[0095] In a further embodiment, present invention provides one or more polymeric nanoparticles comprising: (a) one or more polycationic graft copolymers of PEI-*g*-PEG or

derivatives thereof, (b) at least one or more polynucleotide molecules, (c) an anionic stabilization reagent, and (d) one or more pharmaceutically active compounds.

[0096] As used herein the term "pharmaceutically active compound" or "therapeutically active compound" means a compound useful for the treatment or modulation of a disease or condition in a subject suffering therefrom. Examples of pharmaceutically active compounds can include any drugs known in the art for treatment of disease indications. A particular example of a pharmaceutically active compound is a chemotherapeutic agent.

[0097] The term "chemotherapeutic agent" as well as words stemming therefrom, as used herein, generally includes pharmaceutically or therapeutically active compounds that work by interfering with DNA synthesis or function in cancer cells. Based on their chemical action at a cellular level, chemotherapeutic agents can be classified as cell-cycle specific agents (effective during certain phases of cell cycle) and cell-cycle nonspecific agents (effective during all phases of cell cycle). Without being limited to any particular example, examples of chemotherapeutic agents can include alkylating agents, angiogenesis inhibitors, aromatase inhibitors, antimetabolites, anthracyclines, antitumor antibiotics, monoclonal antibodies, platinums, topoisomerase inhibitors, and plant alkaloids.

[0098] For purposes of the invention, the amount or dose of the nanoparticles of the present invention that is administered should be sufficient to effectively target the cell, or population of cells *in vivo*, such that the modulation of the expression of the target gene of interest can be detected, in the subject over a reasonable time frame. The dose will be determined by the efficacy of the particular nanoparticle formulation and the location of the target population of cells in the subject, as well as the body weight of the subject to be treated.

[0099] The dose of the nanoparticles of the present invention also will be determined by the existence, nature and extent of any adverse side effects that might accompany the administration of a particular nanoparticle. Typically, an attending physician will decide the dosage of the nanoparticle with which to treat each individual subject, taking into consideration a variety of factors, such as age, body weight, general health, diet, sex, compound to be administered, route of administration, and the severity of the condition being treated. By way of example, and not intending to limit the invention, the dose of the nanoparticles of the present invention can be about 0.001 to about 1000 mg/kg body weight of the subject being treated, from about 0.01 to about 100 mg/kg body weight, from about 0.1 mg/kg to about 10 mg/kg, and from about 0.5 mg

to about 5 mg/kg body weight. In another embodiment, the dose of the nanoparticles of the present invention can be at a concentration from about 1 nM to about 10,000 nM, preferably from about 10 nM to about 5,000 nM, more preferably from about 100 nM to about 500 nM.

[0100] In accordance with an embodiment, the N/P ratio of the nanoparticles of the present invention can vary in accordance with the amount the polycationic polymer added. In an embodiment, the amount of polycationic polymer added to the polynucleotide containing mixture can vary from none, to an amount that results in a ratio of 80, and any amount in between, for example, a N/P ratio of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, to 20, can be achieved using the methods of the present invention.

[0101] In accordance with an embodiment, the present invention provides one or more polymeric nanoparticles wherein the ratio of the number of primary amino groups of the polycationic polymer, for example, the PPA moiety to the number of phosphate groups in the polynucleotide molecule (N/P ratio) is between 1 to 20, preferably between about 5 to about 15, more preferably between about 8 to 10.

[0102] In accordance with an embodiment, the ratio of the number of phosphate groups of TPP to the number of primary amino groups in the polycationic polymer (PPA moiety) (P'/N ratio) of the nanoparticles of the present invention can vary in accordance with the amount the anionic stabilization reagent added. In an embodiment, the anionic stabilization reagent is TPP, and the amount of TPP added can vary from none, to an amount that results in a ratio of 1, and any amount in between, for example, P'/N = 0 to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, to 1.0.

[0103] In accordance with another embodiment, the present invention provides one or more polymeric nanoparticles wherein the ratio of the number of phosphate groups of TPP to the number of primary amino groups in the polycationic polymer, for example, the PPA moiety (P'/N ratio) is between about 0.1 to about 1.0, preferably between about 0.2 to about 0.8, more preferably about 0.5.

[0104] In a further embodiment, the present invention provides a method for making one or more polymeric nanoparticles, wherein the quantity of solution containing the polycationic polymer added is such that the ratio of the number of primary amino groups of the PPA moiety to the number of phosphate groups in the polynucleotide molecule (N/P ratio) is between 1 to 20, preferably between about 5 to about 15, more preferably between about 8 to 10.

[0105] In another embodiment, the present invention provides a method for making one or more polymeric nanoparticles, wherein the quantity of solution containing the anionic stabilization reagent added is such that the ratio of the number of negatively charged groups of the anionic stabilization reagent to the number of positively charged groups in the polycationic polymer (P'/N ratio) is between about 0 to about 1.0, preferably between about 0.2 to about 0.8, more preferably about 0.5. In a preferred embodiment, the polycationic polymer is PPA, and the positively charged moieties are the amino groups. In another preferred embodiment, the anionic stabilization reagent is TPP, and the negatively charged groups are phosphate groups.

[0106] In an embodiment, the present invention provides a method of modulating the expression of a target gene in a mammalian cell or population of cells comprising administering to the cell or population of cells, one or more polymeric nanoparticles, wherein the polynucleotide molecule of the nanoparticles is a double stranded RNA molecule or siRNA, in an amount sufficient to modulating the target gene expression with the cell or population of cells.

[0107] In a further embodiment, the present invention provides a method of modulating the expression of a target gene in a host cell or population of cells comprising administering to the cell or population of cells, one or more polymeric nanoparticles, wherein the polynucleotide molecule of the nanoparticles is a double stranded RNA molecule or siRNA, in an amount sufficient to modulating the target gene expression with the cell or population of cells, wherein the target gene is a gene that when expression is increased, is associated with a disease. As such, in an embodiment, the administering to a host cell or a population of cells, one or more polymeric nanoparticles of the present invention, can be used to treat a disease in those cells. Examples of such diseases include, but are not limited to, neurological diseases, cardiovascular diseases, endocrine diseases, autoimmune diseases, gastrointestinal diseases, musculoskeletal diseases and cancer.

[0108] The terms "treat," and "prevent" as well as words stemming therefrom, as used herein, do not necessarily imply 100% or complete treatment or prevention. Rather, there are varying degrees of treatment or prevention of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. In this respect, the inventive methods can provide any amount of any level of treatment or prevention of cancer in a mammal. Furthermore, the treatment or prevention provided by the inventive method can include treatment or prevention of one or more conditions or symptoms of the disease, e.g., cancer, being treated or

prevented. Also, for purposes herein, "prevention" can encompass delaying the onset of the disease, or a symptom or condition thereof.

[0109] In accordance with an embodiment, the nanoparticles of the present invention can be designed to down regulate or inhibit target gene expression through RNAi targeting of a variety of RNA molecules. In one embodiment, the nanoparticles of the invention comprising siRNA molecules are used to target various RNAs corresponding to a target gene. Non-limiting examples of such RNAs include messenger RNA (mRNA), alternate RNA splice variants of target gene(s), post-transcriptionally modified RNA of target gene(s), pre-mRNA of target gene(s), and/or RNA templates. If alternate splicing produces a family of transcripts that are distinguished by usage of appropriate exons, the instant invention can be used to inhibit gene expression through the appropriate exons to specifically inhibit or to distinguish among the functions of gene family members. For example, a protein that contains an alternatively spliced transmembrane domain can be expressed in both membrane bound and secreted forms. Use of the invention to target the exon containing the transmembrane domain can be used to determine the functional consequences of pharmaceutical targeting of membrane bound as opposed to the secreted form of the protein. Non-limiting examples of applications of the invention relating to targeting these RNA molecules include therapeutic pharmaceutical applications, pharmaceutical discovery applications, molecular diagnostic and gene function applications, and gene mapping.

[0110] The invention further provides a host cell comprising any of the nanoparticles described herein. As used herein, the term "host cell" refers to any type of cell that can contain the inventive nanoparticles. The host cell can be a eukaryotic cell, e.g., plant, animal, fungi, or algae. The host cell can be a cultured cell or a primary cell, i.e., isolated directly from an organism, e.g., a human. The host cell can be an adherent cell or a suspended cell, i.e., a cell that grows in suspension. Suitable host cells are known in the art and include, for instance, HeLa cells (human epithelial cervical cancer cell line), D407 cells (human retinal pigmented epithelial cell line), Chinese hamster ovarian cells, monkey VERO cells, COS cells, HEK293 cells, and the like. For purposes of modulating the expression of a target gene of interest in a cell, the host cell is preferably a mammalian cell. Most preferably, the host cell is a human cell. Examples of suitable human host cells can include, but are not limited to, cells of the major organs of the body, including, for example, cells of the lung, including hepatocytes and hepatic stellate cells, cells of the breast, cells of the prostate, cells of the cornea, including corneal epithelial cells, cells

of the lung, including lung epithelial cells, and cells of the brain, such as neurons. While the host cell can be of any cell type, can originate from any type of tissue, and can be of any developmental stage, the host cell preferably is a cancer cell.

[0111] The population of cells can be a heterogeneous population comprising the host cell comprising any of the nanoparticles described, in addition to at least one other cell, e.g., a host cell (e.g., a epithelial cell), which does not comprise any of the nanoparticles, or a cell other than a epithelial cell, e.g., a macrophage, a neutrophil, an erythrocyte, a hepatocyte, a hepatic stellate cell, an endothelial cell, an epithelial cell, a muscle cell, a brain cell, etc. Alternatively, the population of cells can be a substantially homogeneous population, in which the population comprises mainly of host cells (e.g., consisting essentially of) comprising the nanoparticles.

[0112] In an embodiment, the present invention provides a use of a composition comprising one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent, in an effective amount, wherein the composition includes a pharmaceutically and physiologically acceptable carrier, to prepare a medicament, preferably for use as a medicament for treating a disease in a subject.

[0113] In another embodiment, the present invention provides a use of a composition comprising one or more polymeric nanoparticles comprising: (a) one or more polycationic homopolymers, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent, in an effective amount, wherein the composition includes a pharmaceutically and physiologically acceptable carrier, to prepare a medicament, preferably for use as a medicament for treating a disease in a subject.

[0114] In a further embodiment, the present invention provides a use of a composition comprising one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers selected from the group consisting of linear and/or branched PEI, PPA, block polycationic copolymers comprising PEG and PEI or PPA, and graft polycationic copolymers comprising PEG and PEI or PPA, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent, in an effective amount, wherein the composition includes a pharmaceutically and physiologically acceptable carrier, to prepare a medicament, preferably for use as a medicament for treating a disease in a subject.

[0115] In still another embodiment, the present invention provides a use of a composition comprising one or more polymeric nanoparticles comprising: (a) one or more block copolymers of PEG-*b*-PPA, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent, in an effective amount, wherein the composition includes a pharmaceutically and physiologically acceptable carrier, to prepare a medicament, preferably for use as a medicament for treating a disease in a subject.

[0116] In a further embodiment, the present invention provides a use of a composition comprising one or more polymeric nanoparticles comprising: (a) one or more graft copolymers of PEI-*g*-PEG, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent, in an effective amount, wherein the composition includes a pharmaceutically and physiologically acceptable carrier, to prepare a medicament, preferably for use as a medicament for treating a disease in a subject.

[0117] In accordance with an embodiment of the present invention, the medicament for treating a disease in a subject can encompass many different formulations known in the pharmaceutical arts, including, for example, intravenous and sustained release formulations. With respect to the inventive methods, the disease can include cancer. Cancer can be any cancer, including any of acute lymphocytic cancer, acute myeloid leukemia, alveolar rhabdomyosarcoma, bone cancer, brain cancer, breast cancer, cancer of the anus, anal canal, or anorectum, cancer of the eye, cancer of the intrahepatic bile duct, cancer of the joints, cancer of the neck, gallbladder, or pleura, cancer of the nose, nasal cavity, or middle ear, cancer of the oral cavity, cancer of the vulva, chronic lymphocytic leukemia, chronic myeloid cancer, colon cancer, esophageal cancer, cervical cancer, gastrointestinal carcinoid tumor. Hodgkin lymphoma, hypopharynx cancer, kidney cancer, larynx cancer, liver cancer, lung cancer, malignant mesothelioma, melanoma, multiple myeloma, nasopharynx cancer, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, peritoneum, omentum, and mesentery cancer, pharynx cancer, prostate cancer, rectal cancer, renal cancer (e.g., renal cell carcinoma (RCC)), small intestine cancer, soft tissue cancer, stomach cancer, testicular cancer, thyroid cancer, ureter cancer, and urinary bladder cancer. Preferably, the cancer is breast cancer and/or prostate cancer.

[0118] The nanoparticles of the present invention are useful in preparation of a medicament for treating cancers selected from the group consisting of melanoma, skin cancer, lung cancer,

kidney cancer, stomach cancer, colon cancer, prostate cancer, breast cancer, ovarian cancer, and lymphoid cancer.

[0119] In an embodiment, the present invention provides a composition comprising one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent, in an amount effective for use in a medicament, and most preferably for use as a medicament for treating cancer, or inhibiting the growth of a tumor, or neoplasm in a subject, when administered to the subject in a therapeutically effective amount.

[0120] In another embodiment, the present invention provides a composition comprising one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers selected from the group consisting of linear and/or branched PEI, PPA, block polycationic copolymers comprising PEG and PEI or PPA, and graft polycationic copolymers comprising PEG and PEI or PPA, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent, in an amount effective for use in a medicament, and most preferably for use as a medicament for treating cancer, or inhibiting the growth of a tumor, or neoplasm in a subject, when administered to the subject in a therapeutically effective amount.

[0121] In a further embodiment, the present invention provides a composition comprising one or more polymeric nanoparticles comprising: (a) one or more polycationic homopolymers, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent, in an amount effective for use in a medicament, and most preferably for use as a medicament for treating cancer, or inhibiting the growth of a tumor, or neoplasm in a subject, when administered to the subject in a therapeutically effective amount.

[0122] In yet another embodiment, the present invention provides a composition comprising one or more polymeric nanoparticles comprising: (a) one or more polycationic homopolymers, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent, in an amount effective for use in a medicament, and most preferably for use as a medicament for treating cancer, or inhibiting the growth of a tumor, or neoplasm in a subject, when administered to the subject in a therapeutically effective amount.

[0123] In still a further embodiment, the present invention provides a composition comprising one or more polymeric nanoparticles comprising: (a) one or more polycationic block copolymers of PEG-*b*-PPA or derivatives thereof, (b) at least one or more polynucleotide

molecules, (c) an anionic stabilization reagent, and (d) one or more pharmaceutically active compounds, in an amount effective for use in a medicament, and most preferably for use as a medicament for treating cancer, or inhibiting the growth of a tumor, or neoplasm in a subject, when administered to the subject in a therapeutically effective amount.

[0124] In another embodiment, the present invention provides a composition comprising one or more polymeric nanoparticles comprising: (a) one or more polycationic graft copolymers of polyethyleneimine-g-polyethylene glycol (PEI-*g*-PEG) or derivatives thereof, (b) at least one or more polynucleotide molecules, (c) an anionic stabilization reagent, and (d) one or more pharmaceutically active compounds, in an amount effective for use in a medicament, and most preferably for use as a medicament for treating cancer, or inhibiting the growth of a tumor, or neoplasm in a subject, when administered to the subject in a therapeutically effective amount.

[0125] As defined herein, in one or more embodiments, "administering" means that the one or more nanoparticles of the present invention are introduced into a sample having at least one cell, or population of cells, having a target gene of interest, and appropriate enzymes or reagents, in a test tube, flask, tissue culture, chip, array, plate, microplate, capillary, or the like, and incubated at a temperature and time sufficient to permit uptake of the at least one nanoparticles of the present invention into the cytosol, where it will bind to the mRNA of the target gene of interest and attenuate the expression of the target gene in the at least one cell or population of cells.

[0126] In another embodiment, the term "administering" means that at least one or more nanoparticles of the present invention are introduced into a subject, preferably a subject receiving treatment for a disease, and the at least one or more nanoparticles are allowed to come in contact with the one or more disease related cells or population of cells having the target gene of interest *in vivo*.

[0127] As used herein, the term "treat," as well as words stemming therefrom, includes diagnostic and preventative as well as disorder remitative treatment.

[0128] As used herein, the term "subject" refers to any mammal, including, but not limited to, mammals of the order Rodentia, such as mice and hamsters, and mammals of the order Logomorpha, such as rabbits. It is preferred that the mammals are from the order Carnivora, including Felines (cats) and Canines (dogs). It is more preferred that the mammals are from the order Artiodactyla, including Bovines (cows) and Swines (pigs) or of the order Perssodactyla,

including Equines (horses). It is most preferred that the mammals are of the order Primates, Ceboids, or Simoids (monkeys) or of the order Anthropoids (humans and apes). An especially preferred mammal is the human.

[0129] In a further embodiment, the nanoparticles of the present invention can be used in combination with one or more additional therapeutically active agents which are known to be capable of treating conditions or diseases discussed above. For example, the described nanoparticles of the present invention could be used in combination with one or more known therapeutically active agents, to treat a disease or condition. Non-limiting examples of other therapeutically active agents that can be readily combined in a pharmaceutical composition with the nanoparticles of the present invention are enzymatic nucleic acid molecules, allosteric nucleic acid molecules, antisense, decoy, or aptamer nucleic acid molecules, antibodies such as monoclonal antibodies, small molecules, and other organic and/or inorganic compounds including metals, salts and ions.

[0130] In an embodiment, the present invention provides a pharmaceutical composition comprising, in an effective amount, one or more polymeric nanoparticles, and pharmaceutically and physiologically acceptable carrier, wherein the nanoparticles comprise (a) one or more polycationic polymers, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent.

[0131] Therefore, in accordance with an embodiment, the present invention provides a use of the pharmaceutical composition comprising, in an effective amount, one or more polymeric nanoparticles, and pharmaceutically and physiologically acceptable carrier, wherein the nanoparticles comprise (a) one or more polycationic polymers, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent, to prepare a medicament, preferably for use as a medicament for treating a disease in a subject.

[0132] The nanoparticles of the present invention can be used in a variety of diagnostic applications, such as in the identification of molecular targets (e.g., RNA) in a variety of applications, for example, in clinical, industrial, environmental, agricultural and/or research settings. Such diagnostic use of siRNA containing nanoparticles involves utilizing reconstituted RNAi systems, for example, using cellular lysates or partially purified cellular lysates. siRNA in nanoparticles of the present invention can be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of endogenous or exogenous, for

example viral, RNA in a cell. The close relationship between siRNA activity and the structure of the target RNA allows the detection of mutations in any region of the molecule, which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple siRNA molecules in the nanoparticles of the present invention, one can map nucleotide changes, which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with siRNA molecules can be used to inhibit gene expression and define the role of specified gene products in the progression of disease or infection, or other clinical condition. In this manner, other genetic targets can be defined as important mediators of the disease.

[0133] Therefore, in accordance with an embodiment, the present invention provides a use of the composition comprising one or more polymeric nanoparticles comprising: (a) one or more polycationic polymers, (b) at least one or more polynucleotide molecules, and (c) an anionic stabilization reagent, in an effective amount, for use in diagnosing a disease or condition in a subject.

[0134] These experiments will lead to better treatment of the disease progression by affording the possibility of combination therapies (e.g., multiple siRNA molecule containing nanoparticles targeted to different genes, siRNA molecule containing nanoparticles coupled with known small molecule inhibitors, or intermittent treatment with combinations siRNA molecules and/or other chemical or biological molecules). Other *in vitro* uses of siRNA containing nanoparticles of the present invention are well known in the art, and include detection of the presence of mRNAs associated with a disease, infection, or related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a siRNA using standard methodologies, for example, fluorescence resonance emission transfer (FRET).

EXAMPLES

[0135] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

[0136] **Reagents**. Sodium triphosphate (TPP) and poly(vinyl sulfate) potassium salt (PVSK) were purchased from Sigma-Aldrich Chemical Co. Ltd (Milwaukee, WI, USA). WST-1 was purchased from Roche (Penzberg, Germany). UltraPure™ DNase/RNase-Free distilled water, Dulbecco's Modified Eagle's Medium (DMEM), Opti-MEM™ and Lipofectamine2000™ were purchased from Invitrogen (Carlsbad, CA, USA). The Dual-luciferase reporter assay system and

pGL3-control and pRL-CMV vectors were purchased from Promega (Madison, WI, USA). The siRNA was purchased from Ambion (Austin, TX, USA). Formvar-coated carbon grid was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). The sequences of siRNA against *Photinus pyralis* luciferase were as follows: sense 5'-CUUACGCUGAGUACUUCGAdTdT-3' (SEQ ID NO: 1), antisense 5'-UCGAAGUACUCAGCGUAAGdTdT-3' (SEQ ID NO: 2). The cyclic monomer 4-methyl-2-oxo-2-hydro-1,3,2-dioxaphospholane was prepared as reported previously in *J. Am. Chem. Soc.*, 1950, 72:5491–5497. N^1,N^9-bis(trifluoroacetyl) dipropyltriamine was synthesized following the procedure reported by O'Sullivan et al. at *Tetrahedron Lett.*, 1995, 36:3451–3452.

[0137] Preparation of PEG-*b*-PPA block copolymer. The polycationic PEG-*b*-PPA block copolymer solutions were prepared as described in *J. Control Release*, 2007, 122(3): 297-304. Briefly, the synthesis of PEG-*b*-PPA is summarized in Fig. 1. PEG-O^-K^+ macroinitiator (**1**) was prepared by reacting about 1.0 g of methoxy PEG with potassium granules (over stoichiometry) in 50 ml of anhydrous THF for 8 hours under refluxing. The concentration of PEG-O^-K^+ was determined by titration using 50 mM HCl. The polymerization of 4-alkyl-2-oxo-2-hydro-1,3,2-dioxaphospholane was initiated by adding PEG-O^-K^+ solution into the reaction vessel at a molar ratio of 1:500. The mixture was stirred at room temperature for about 48 hours. The precursor polymer (**2**) was obtained by precipitation into anhydrous toluene followed by vacuum drying. The precursor polymer (**2**) (3.0 g) was then dissolved in 20 ml of anhydrous DMF under argon. To this solution was added 27.2 g of N^1,N^9-bis(trifluoroacetyl) dipropyltriamine, followed by addition of 10 ml of anhydrous triethylamine and 10 ml of anhydrous CCl_4. The mixture was stirred at 0 °C for 30 minutes, then at room temperature for 24 hours. The reaction mixture was then precipitated into ether and dried under vacuum to yield polymer (**3**). The resulting residue was suspended in 25% ammonia solution and stirred at 60 °C for 16 hours. The solution was concentrated and dialyzed in dialysis tubing (MWCO 3500, Spectrapor, Spectrum Labs, CA) against distilled water for 2 days with frequent water change. The PEG-*b*-PPA (**4**) was obtained after lyophilization (0.6 g, yield 12%).

[0138] Preparation of self-assembling nanoparticle with TPP-crosslinked core. PEG-*b*-PPA (molecular weights: PEG, 12 kDa, PPA, 38 kDa) was prepared as described above. About 10 μl of 4 μM siRNA solution (10 mM Tris-HCl, pH 7.4) was mixed with 10 μl of TPP solution (10 mM Tris-HCl, pH 7.4), followed by the addition of 20 μl of PEG-*b*-PPA solution (10 mM

Tris-HCl, pH 7.4), into the previous mixture of siRNA and TPP solutions, at different mixing ratios. For the various experiments, the volume and concentration of siRNA solution were fixed: 10 μL of 4 μM siRNA dissolved in 10 mM Tris-HCl buffer (pH 7.4), as shown in Table 1.

TABLE 1

N/P ratio	PEG-*b*-PPA solution* (N/P ratio = 0 – 8)		TPP solution* (P'/N ratio = 0 – 1.0)	
	Volume (μL)	Concentration (μM)	Volume (μL)	Concentration (μM)
0	20	0 (buffer only)	10	0 (buffer only)
4	20	1.68	10	0 – 672
8	20	3.36	10	0 – 1,344

*All solutions were made in 10 mM Tris-HCl buffer (pH 7.4).

[0139] The solutions were mixed by pipetting, followed by gentle vortex and spin-down. The resulting nanoparticles were then incubated at room temperature for 1 hour before use or further analysis. The concentration of PEG-*b*-PPA solution (20 μL) was varied from about 0.42 μM to about 13.44 μM for the N/P ratios used for these experiments. The mixing ratio for each formulation was determined by N/P and P'/N: [primary amino group of PPA]/[phosphate group of siRNA] and [phosphate group of TPP]/[primary amino group of PPA], respectively. The negative charge number of TPP is defined as 3 in this study according to the methods described in *J. Drug Target.*, 2000, 8:267–279.

[0140] **Measurement of size and ζ-potential of nanoparticles.** The z-average and ζ-potential of the nanoparticles were determined by photon correlation spectroscopy and laser Doppler anemometry, respectively, using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK) equipped with a He-Ne laser (λ = 633 nm) as the incident beam. Size distributions were determined by cumulate and histogram analysis, and results are shown as the

z-averaged size (cumulate mean) with PDI (defined in the ISO standard document 13 321:1996). All samples were equilibrated to the defined temperature for 1 hour prior to measurement. The ζ -potential values of the complexes were measured in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl at 37 °C. All samples were equilibrated to the defined temperature for 1 hour prior to measurement.

[0141] **Stability of nanoparticles in the physiological ionic strength**. The effect of crosslinking on nanoparticle stability in buffers with the physiological ionic strength was determined using the Zetasizer Nano ZS90 (Malvern Instruments). The assay was performed by measuring the size and scattering light intensity (SLI) of nanoparticles after 24 hours of incubation at 37 °C, in solutions containing 150 mM of NaCl.

[0142] **Transmission electron microscopy**. An aliquot of 10 µl of the nanoparticle solution of the present invention was added to a formvar carbon TEM grid and incubated for 5 minutes at room temperature, followed by washing with deionized water. The grid was further stained with 2 % of a uranyl acetate solution and washed twice with deionized water. Transmission electron microscopy was carried out on a Tecnai(TM) 12 (FEI Company, OR, USA) run at 100 kV.

[0143] **Gel retardation assay**. The incorporation of siRNA into the nanoparticles of the present invention was determined by electrophoresis on a 0.8% agarose gel. Electrophoresis was carried out at a constant voltage of 90 V for 0.5 hour in TAE buffer (4.45 mM Tris-acetic acid containing 1.7 mM sodium acetate, pH 8.3). The band of migrated siRNA was visualized under a UV transilluminator (UVP, Upland, CA), at a wavelength of 365 nm, after soaking the gel in distilled water containing ethidium bromide (EtBr) (0.5 µg/ml).

[0144] **Stability of encapsulated siRNA in serum-containing medium**. The siRNA-incorporated nanoparticles of an embodiment of the present invention were incubated at 37 °C with 50% final concentration of fetal bovine serum (FBS) for 1 hour, and 4 hours, respectively. Samples were then incubated in 10 µl of 50 mM EDTA for 5 minutes, and 10 µl of 10 mM PVSK was added to displace the siRNA from the nanoparticles. The released siRNA was analyzed by electrophoresis on a 20% polyacrylamide gel prepared in 7 M urea and TBE buffer (0.089 M Tris base, 0.089 M boric acid, and 2 mM sodium EDTA, pH 8.3). Polyacrylamide–urea gel (20%) was used due to its high efficiency in separating small fragments of possibly degraded siRNA. Electrophoresis was then carried out with 1X TBE buffer at a constant voltage of 100 V for 1 hour. The siRNA bands were visualized under a UV transilluminator after

staining for 40 minutes with a 1:10,000 dilution of SYBR-Green II RNA gel stain (Molecular Probes) in RNase-free water.

[0145] Polyanion-exchange analysis. The self-assembled nanoparticles of the present invention, prepared above, were incubated with in 10 mM Tris-HCl, pH 7.4 at [sulfonate of PVSK]/[phosphate of siRNA] ratio of 5 at 37 °C for 5 hours. The released siRNA from nanoparticles was analyzed at 1 hour and 5 hours by the gel retardation assay under the same conditions as described above.

[0146] Knockdown efficiency by nanoparticles and cell viability. HeLa cells (human epithelial cervical cancer cell line) and D407 cells (human retinal pigment epithelial cell line) were seeded onto 24-well culture plates at a density of 5×10^4 cells per well in 500 µl of medium, followed by 20 hours of incubation in DMEM containing 10% FBS without antibiotics. Then 720 ng/well pGL3-control plasmid encoding *Photinus pyralis* luciferase (P-Luc) and 80 ng/well pRL-CMV plasmid encoding *Renilla reniformis* luciferase (R-Luc) were co-transfected to the cells with Lipofectamine2000 (TM), according to the manufacturer's instructions, and cells were further incubated for an additional 4 hours. For nanoparticle-transfection groups, the medium was replaced with fresh serum-free medium or medium with 10% FBS and nanoparticles containing siRNA (100 nM) against P-Luc with, or without TPP crosslinking, were applied to each well and incubated for 4 hours. The medium was then replaced with complete media containing 10% serum, followed by incubation at 37 °C with a 5% of CO_2 atmosphere. After 44 hours, cells were rinsed with PBS and subjected to a luciferase expression assay using the Dual-Luciferase Reporter Assay System. For each assay, P-Luc and R-Luc luminescence was measured using FLUOstar OPTIMA plate reader (BMG LABTECH, Germany) after the addition of appropriate substrates. P-Luc activities were normalized by R-Luc activities, and values are expressed as a ratio to the control value (mean ± SD, $n = 4$). Cell viability was determined using WST-1 according to the manufacture's protocol. Incubation conditions were identical to those used in the transfection protocol.

EXAMPLE 1

[0147] Preparation of PEG-*b*-PPA/siRNA nanoparticles and stabilization with an anionic stabilization reagent. This example describes the method for determining how the nanoparticles of the present invention showed enhanced stabilization.

[0148] In order to test whether the co-condensation of the polynucleotide with an anionic stabilization reagent enhances the nanoparticle formation, PEG-*b*-PPA/siRNA nanoparticles were prepared with the stabilization reagent, TPP, added to the siRNA solution. PEG-*b*-PPA solutions were incubated with a mixture of siRNA and TPP at N/P ratios of 4 and 8 and a variety of P'/N ratios of 0 to 1 to form polyelectrolyte complexes. In the absence of TPP (P'/N = 0), there is no distinct particle formation for N/P ratios of both 4 and 8, where the measured z-average size was less than 10 nm with large PDI (Figs. 3 and 4). On the other hand, when TPP was used during the condensation, nanoparticle formations were observed at P'/N ratio of 0.1 or higher. The mean particle size of the assembled nanoparticles with TPP-co-condensation increased to about 80 to 100 nm as measured by DLS (Figs. 3 and 4). Of interest, PDI of nanoparticles prepared with TPP was less than 0.1 and the histogram analysis showed unimodal size distribution. These data indicated that TPP was highly effective in facilitating siRNA condensation and nanoparticle formation.

[0149] Significant differences in particle morphology were also observed in TEM analysis between nanoparticle preparations in the presence and absence of TPP. TEM images of nanoparticles prepared with TPP showed that these particles were mostly spherical with diameters ranging from about 80 to 100 nm (Fig. 5), and which corroborated well with the average size (98.2 ± 10.2 nm) measured by DLS method. On the other hand, the TEM images of particles formed without TPP, revealed aggregates with irregular shape, and ranged in size from about 20 to 100 nm, correlated with higher PDI. Nanoparticles with TPP showed much higher contrast than that without TPP in TEM images, indicating that the TPP-condensed nanoparticles have a more compact polyplex core than that without TPP.

[0150] As shown in Fig. 6, the ζ-potential values of nanoparticles prepared at different P'/N ratios varied significantly and appeared to be highly dependent on P/N ratio. The ζ-potential values decreased sharply from +15 to -1.5 mV when the P'/N ratio increased from 0.1 to 1.0. The near electrostatic neutrality of the particle surface at P'/N ratio of 0.5 to 1.0, with a PEG

corona, may be advantageous in preventing nanoparticle aggregation when applied in physiological media.

EXAMPLE 2

[0151] Complex stability of nanoparticles in salt solution with physiological ionic strength. This example illustrates how the complex stability of an embodiment of the nanoparticles of the present invention was assessed.

[0152] The stability of nanoparticles of the present invention was assessed by monitoring changes in size and PDI in 0.15 M of NaCl solution. No dynamic scattering signals were detected for nanoparticle embodiments prepared without TPP (P'/N = 0) at N/P ratio of 4, or 8, after they were incubated with 0.15 M NaCl solution, due to the charge screening effect. Nanoparticle embodiments prepared with lower P'/N ratios (0.1 and 0.2) showed increased sizes after incubating in 0.15 M NaCl. In contrast, nanoparticles prepared at higher P'/N ratios only showed slightly increased particle sizes (average size of 110 and 100 nm for N/P of 4 and 8, respectively) and low PDIs (Figs. 3 and 4). These results indicate that the anionic stabilization reagent TPP crosslinked the nanoparticles of the present invention, and stabilized the siRNA containing nanoparticles in a solution having ionic strengths within acceptable physiological ranges.

EXAMPLE 3

[0153] Stability of nanoparticles in the presence of serum. This example shows how the nanoparticle embodiments of the present invention continue to be stable, even in the presence of serum proteins.

[0154] To show that the encapsulated siRNA of the nanoparticles of the present invention have improved stability in serum containing medium, a sample of nanoparticles of the present invention were incubated with medium containing 50% FBS at 37 °C, followed by gel electrophoresis, in order to analyze the integrity of siRNA within the nanoparticles. It was determined that siRNA without nanoparticles, that was incubated with 50% FBS, was significantly degraded. In contrast, siRNA recovered from the nanoparticles of the present

invention showed only trace amounts of degradation for all N/P and P'/N ratios. The siRNA recovered from nanoparticles both with, and without TPP, were still more intact for 4 hours at all the P'/N ratios (data not shown).

EXAMPLE 4

[0155] **Complex stability of nanoparticles against challenges with polyanions.** This example describes the encapsulation efficiency of the nanoparticles and their testing in various polyanionic environments.

[0156] The encapsulation efficiency of siRNA into nanoparticles was confirmed by agarose gel electrophoresis (data not shown). Free siRNA was not observed for all samples including the complexes prepared both with, and without TPP, highlighting the good siRNA condensation ability of PEG-*b*-PPA. More importantly, the complexation ability was not compromised by the addition of TPP at all P'/N ratios tested. In combining with the DLS analysis (Figs. 3 and 4), these data indicated that complexation between PEG-*b*-PPA and siRNA was strong enough to inhibit the gel migration ability of siRNA at both N/P ratios of 4 and 8, but distinct particle formation was only observed only with TPP-assisted condensation.

[0157] The stability of the nanoparticles was also analyzed by characterizing the release profile of incorporated siRNA using a polyanion-exchange reaction (data not shown). There exist various types of anionic polymers including anionic proteins, sulfated polysaccharides, nuclear chromatin and messenger RNA (mRNA) as essential cellular components. Exchange reaction of polycations with these negatively-charged polymers take place in a biological environment. As a result, siRNA is released from nanoparticles through the intermolecular exchange and facilitates a series of subsequent RNAi processes for gene silencing. On the other hand, nanoparticles should be sufficiently stable to resist decomplexation and release of siRNA before they reach the cytosol of target cells. Therefore, maintaining a balanced complex stability is important to successful transfection and subsequent knockdown of the desired target.

[0158] Gel electrophoresis confirmed that nanoparticle stability increased with increasing P'/N ratio and N/P ratio. Incubation of nanoparticle with PVSK for 1 hour resulted in minimal release of siRNA from particles formed with N/P ratios of 4 and 8 at various P'/N ratios. However, the differences began to emerge at later time point (5 hours after incubation). The

intensity of released siRNA bands were easily detected and their intensity decreased with increasing P'/N ratio. Moreover, the observed intensity of migratory siRNA band at N/P of 8 was slightly weaker than that at N/P of 4. These data gave comparative stabilities of various nanoparticles prepared with different N/P and P'/N ratios, confirming that both PEG-*b*-PPA and TPP contributed to the stability of nanoparticles (data not shown).

EXAMPLE 5

[0159] Transfection and knockdown efficiency of PPA-*b*-PPA/siRNA nanoparticles. In this example, the knockdown efficiency of PPA-*b*-PPA/siRNA nanoparticles *in vitro* was assessed in two different types of cells.

[0160] Knockdown efficiency (i.e., the ability of the siRNA incorporated within the nanoparticles of the present invention to attenuate the expression of a target gene of interest) was tested in two different cell types, HeLa, and D407, in the absence (Fig. 7A) and presence of serum (Fig. 7B). In this experiment, HeLa and D407 cells were transiently transfected, respectively, with both reporter genes P-Luc and R-Luc, followed by treatment with the nanoparticles of the present invention, prepared at different P'/N ratios (0 to 1.0) with siRNA against P-Luc. The expression level of R-Luc was used as an internal reference for the initial transgene expression level. After a treatment (transfection) period of 44 hours, the inhibition of P-Luc expression was evaluated by measuring the relative expression ratio of P-Luc/R-Luc at a concentration of 100 nM siRNA.

[0161] PEG-*b*-PPA/siRNA complexes without TPP failed to show distinct nanoparticles in DLS, although they did produce an average knockdown efficiency of 24% (P-Luc) and 58% (R-Luc) in HeLa cells, as well as a knockdown efficiency of 40% (P-Luc) and 56% (R-Luc) in D407 cells, in serum-free medium, at N/P ratios of 4 and 8, respectively. TPP-crosslinked nanoparticles of an embodiment of the present invention resulted in significantly higher knockdown efficiencies at both N/P ratios in both cell lines. As the P'/N ratio increases, the gene knockdown efficiency increased gradually. At N/P ratio of 4, the knockdown efficiency reached the plateau of about 60% at P'/N ratio of 0.5 in HeLa cells, and the highest knockdown efficiency of about 60% at P'/N ratio of 1.0 in D407 cells (Fig. 7A). The knockdown efficiency was higher at N/P of 8, and reached about 75 % of knockdown efficiency in both types of cells.

[0162] More importantly, when transfections were conducted in 10% serum-containing medium, the knockdown efficiency of PEG-*b*-PPA/siRNA complexes without TPP stabilization was significantly reduced compared to that obtained in serum-free condition (Fig. 7B). The knockdown efficiency for N/P ratio of 4 was about 10-12% (P-Luc), and 23-34% (R-Luc) at N/P ratio of 8 in both cell lines. In contrast, the knockdown efficiency of TPP-crosslinked nanoparticles of the present invention was maintained, or slightly enhanced, as compared to that obtained in serum-free medium transfection. As the P'/N ratio increased, the gene knockdown efficiency maintained similar level in HeLa cells and moderately increased in D407 cells. At N/P ratio of 4, the knockdown efficiency reached the plateau of about 50% in Hela cells, and about a maximum of 70% in D407 cells. Higher knockdown efficiency was obtained at N/P of 8 for both cell lines, with 75% to 80% knockdown efficiency obtained in HeLa cells and D407 cells, respectively, at P'/N ratio of 1.

EXAMPLE 6

[0163] **Cytotoxicity of PEG-*b*-PPA/siRNA nanoparticles.** This example describes how the nanoparticles of the present invention were tested for cytotoxicity.

[0164] The potential cytotoxicity of the nanoparticles of the present invention was assessed in both HeLa cells and D407 cells under the same transfection conditions. The cell viability was determined by WST assay (Roche Applied Science, Indianapolis, IN)using water-soluble tetrazolium salt (Fig. 8). Nearly 100% of cell viability was observed in all transfection conditions in HeLa cells, and a cell viability of over 90% was observed in D407 cells, under identical conditions. There was no significant difference in cell viability observed between nanoparticles prepared with and without TPP crosslinking. Similar cytotoxicity results were obtained under serum-free transfection condition (data not shown). These results demonstrate that the addition of TPP into nanoparticle assemblies does not influence their cytotoxicity.

EXAMPLE 7

[0165] **Preparation of Linear and/or Branched PEI/siRNA Nanoparticles.** This example describes for preparation of linear or branched PEI/siRNA nanoparticles with TPP as the stabilizing agent.

[0166] Linear PEI (17 kDa) and branched PEI (25 kDa) were used. About 10 µl of 4 µM siRNA solution (10 mM Tris-HCl, pH 7.4) was mixed with 10 µl of TPP solution (10 mM Tris-HCl, pH 7.4), followed by the addition of 20 µl of 1.12 µM linear PEI solution or 0.637 µM linear PEI solution (10 mM Tris-HCl, pH 7.4), into the previous mixture of siRNA and TPP solutions, at different mixing ratios. The mixing ratio for each formulation was determined by N/P and P'/N: [primary amino group of PEI]/[phosphate group of siRNA] and [phosphate group of TPP]/[primary amino group of PEI], respectively. The concentration of TPP solution (10 µL) varied from 0 µM to 220 µM for the mixing ratio used for this experiment.

[0167] In the absence of TPP, linear PEI and siRNA did not form nanoparticles; branched PEI was able to form particles with siRNA alone. Nanoparticles prepared with TPP as the stabilizing agent were uniform spherical particles with an average size of 107 nm and 84 nm for linear PEI/siRNA/TPP and branched PEI/siRNA/TPP, respectively (data not shown).

EXAMPLE 8

[0168] **Effect of TPP on Functionalization (via surface coating) of PEI/siRNA nanoparticles**. This example describes the effect of different nanoparticle surface coatings of the present invention in different environments.

[0169] Small compact nanoparticles are usually formed at high charge ratios, rendering nanoparticles highly positively charged. These positively charged nanoparticles will severely aggregate in the presence of serum. One simple method to convert the surface charge is through surface coating of polyanion, e.g. hyaluronic acid (HA). In principle, HA-coating will render the nanoparticle surface negatively charged, thus increase the serum stability of these nanoparticles.

[0170] Linear PEI (17 kDa) and branched PEI (25 kDa) were used. About 10 µl of 4 µM siRNA solution (10 mM Tris-HCl, pH 7.4) was mixed with 10 µl of 220 µM TPP solution (10 mM Tris-HCl, pH 7.4), followed by the addition of 20 µl of 1.12 µM linear PEI solution (10 mM Tris-HCl, pH 7.4), into the previous mixture of siRNA and TPP solutions, at different mixing ratios. For branched PEI-based nanoparticle formation, 10 µl of 110 µM TPP solution (10 mM Tris-HCl, pH 7.4), followed by the addition of 20 µl of 0.637 µM linear PEI solution (10 mM Tris-HCl, pH 7.4) were used. The solutions were mixed by pipetting, followed by gentle vortex and spin-down. The resulting nanoparticles were then incubated at room temperature for 1 hour

before HA-coating. The nanoparticle solution prepared by the method described above was added into 10 μl of 1.47 μM HA solution (10 mM Tris-HCl, pH 7.4) at different mixing ratios. The solutions were mixed by pipetting, followed by gentle vortex and spin-down. The resulting HA-coated siRNA nanoparticles were then incubated at room temperature for 1 hour before use or further analysis. The mixing ratio for each formulation was determined by C/N/P'/P: [carboxylic acid of HA]/[primary amino group of PEI]/[phosphate group of TPP]/[phosphate group of siRNA], respectively.

[0171] As shown in Fig. 9, in the absence of TPP, HA-coating onto branched PEI/siRNA nanoparticles caused aggregation, generating large irregular particles (a-b). In contrast, both linear and branched PEI/siRNA/TPP nanoparticles could allow for HA surface coating, and maintain uniform and stable nanoparticle size and spherical morphology (c-e).

EXAMPLE 9

[0172] **Effect of TPP stabilization on gene knockdown efficiency of PEI/siRNA nanoparticles**. This example shows how the PEI/siRNA nanoparticles of an embodiment of the present invention were able to inhibit target gene expression.

[0173] HCE-2 cells (human corneal epithelial cell) were seeded onto 24-well culture plates at a density of 50,000 cells per well in 500 μl of medium, followed by 20 hours of incubation in serum-free keratinocyte medium(KSF) for HCE-2 and KSF/Ham's F12/DMEM (2:1:1). Then, 720 ng/well pGL3-control plasmid encoding P-Luc and 80 ng/well pRL-CMV plasmid encoding R-Luc were co-transfected to the cells with Lipofectamine2000™ according to the manufacturer's instructions, and the cells were further incubated for 4 hours. For nanoparticle-transfection groups, the medium was replaced with fresh medium, and nanoparticles containing siRNA (100 nM) against P-Luc were applied to each well and incubated for 4 hours. The medium was then replaced with complete media, followed by incubation at 37°C with a 5 % of carbon dioxide atm. After 44 hours, cells were rinsed with PBS and subjected to a luciferase expression assay using the Dual-Luciferase Reporter Assay System. For each assay, P-Luc and R-Luc luminescence was measured using FLUOstar OPTIMA plate reader after the addition of appropriate substrates. P-Luc activities were normalized by R-Luc activities, and values are expressed as a ratio to the control value (mean ± SD, n=4). Cell viability was determined using

WST-1 according to the manufacture's protocol. Incubation conditions were identical to those used in the transfection protocol.

[0174] The data provided in Fig. 10 show that HA-coating on the nanoparticle surface significantly increased the transfection and gene knockdown efficiency (Fig 10 (a)), and reduced the cytotoxicity of the nanoparticles (Fig 10 (b)). In the absence of TPP, HA coating resulted in aggregation, which likely is the cause of low gene knockdown activity.

[0175] All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

[0176] One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

CLAIM(S):

1. One or more polymeric nanoparticles comprising: (a) one or more polycationic polymers; (b) at least one or more polyribonucleotide molecules; and (c) an anionic stabilization reagent.

2. The polymeric nanoparticles of claim 1, wherein the polycationic polymers are selected from the group consisting of linear or branched polycationic homopolymers, polycationic block copolymers, polycationic graft copolymers, and combinations thereof.

3. The polymeric nanoparticles of claims 1 or 2, wherein the linear or branched polycationic homopolymers are selected from the group consisting of linear and/or branched polyethyleneimines (PEI), and polyphosphoroamidates (PPA).

4. The polymeric nanoparticles of claims 1 or 2, wherein the polycationic block copolymers are selected from the group consisting of block polycationic copolymers comprising PEG and PEI or PPA.

5. The polymeric nanoparticles of claims 1 or 2, wherein the polycationic graft copolymers are selected from the group consisting of graft polycationic copolymers comprising PEG and PEI or PPA.

6. The polymeric nanoparticles of any of claims 1-5, wherein the at least one or more polyribonucleotide molecules are between 10 bp and 100 bp in length, preferably between 20 bp and 50 bp in length, more preferably between 30 bp and 40 bp in length.

7. The polymeric nanoparticles of any of claims 1-6, wherein the one or more polynucleotide molecules are selected from the group consisting of: single stranded RNA; double stranded RNA; micro-RNA (miRNA); short-hairpin RNA (shRNA); siRNA; and/or RNA analogs thereof.

8. A method for making one or more polymeric nanoparticles of any of claims 1-7, the method comprising:

1) obtaining a solution comprising at least one or more polyribonucleotide molecules of claim 7;

2) adding to the solution of (1), a sufficient quantity of a solution containing an anionic stabilization reagent, and mixing; and

3) adding to the solution of (2), a sufficient quantity of a solution containing the polycationic polymers of any of claims 1-5, mixing the solution, and incubating the resulting solution for a sufficient time to allow a complexation reaction to occur and the nanoparticles to assemble.

9. The method of claim 8, wherein the sufficient time to allow a complexation reaction to occur is between 5 minutes to 60 minutes, preferably, within 10 minutes to 30 minutes, and more preferably, within 15 minutes.

10. The polymeric nanoparticles of any of claims 1-7, or the method of claims 8 or 9, wherein the anionic stabilization reagent is an inorganic or organic polyanionic molecule having a high charge density, and is capable of physically cross-linking the positively charged amino groups of the polymer chains of the nanoparticles, with the negatively charged polyanions of the anionic stabilization reagent.

11. The anionic stabilization reagent of claim 10, wherein the reagent is selected from the group consisting of: trisodium phosphate, tetrasodium pyrophosphate and hexasodium metaphosphate.

12. A pharmaceutical composition comprising one or more polymeric nanoparticles of any of claims 1 to 11, and a pharmaceutically acceptable carrier.

13. A pharmaceutical composition comprising one or more polymeric nanoparticles of any of claims 1 to 11, a second therapeutic agent, and a pharmaceutically acceptable carrier.

14. A method of modulating expression of a target gene in a host cell or population of cells comprising administering to the cell or population of cells the polymeric nanoparticles of

any of claims 1-7, or the pharmaceutical composition of claims 12 or 13, in an amount sufficient to modulate target gene expression with the host cell or population of cells.

15. The method of claim 14, wherein the target gene is a gene that when expression is increased, is associated with a disease.

16. The method of claim 15, wherein the disease is cancer.

17. A method of modulating expression of a target gene in a host cell or population of cells comprising administering to the cell or population of cells the polymeric nanoparticles of any of claims 1-7, or the pharmaceutical composition of claims 12 or 13, in an amount sufficient to modulate target gene expression with the host cell or population of cells and diagnose the role of the target gene in a clinical condition or disease.

18. Use of the polymeric nanoparticles of any of claims 1-7, made using the method of any of claims 8 or 9, in an effective amount, to prepare a medicament, preferably for use as a medicament for treating a disease in a subject.

19. The use of claim 18, wherein the medicament further comprises a pharmaceutically acceptable carrier.

20. The use of claims 18 or 19, wherein the medicament further comprises a second therapeutic agent.

FIGURE 1 1/10

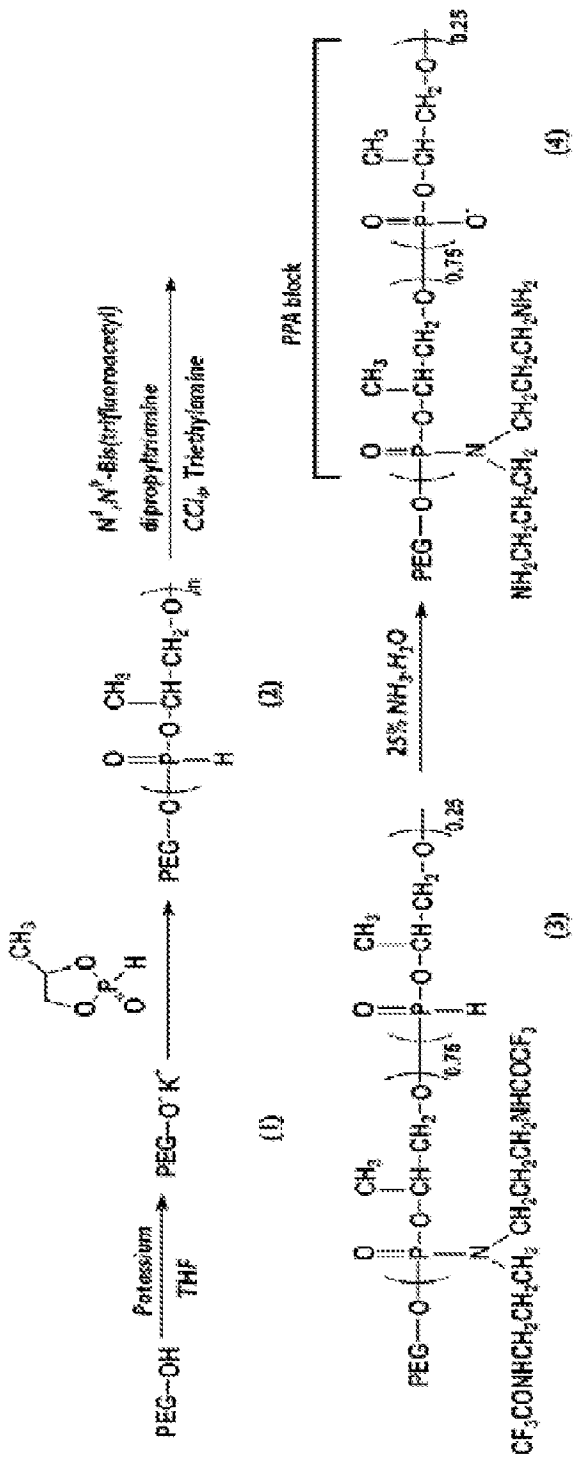

PEG—OH
Potassium
THF
PEG—O⁻K⁺
(1)
N¹,N⁶-Bis(trifluoroacetyl)
diprophylamine
CCl₄, Triethylamine
(2)
PPA block
25% NH₃.H₂O
NH₂CH₂CH₂CH₂
CH₂CH₂CH₂NH₂
(4)
CF₃CONHCH₂CH₂CH₂
CH₂CH₂CH₂NHCOCF₃
(3)

FIGURE 2 2/10

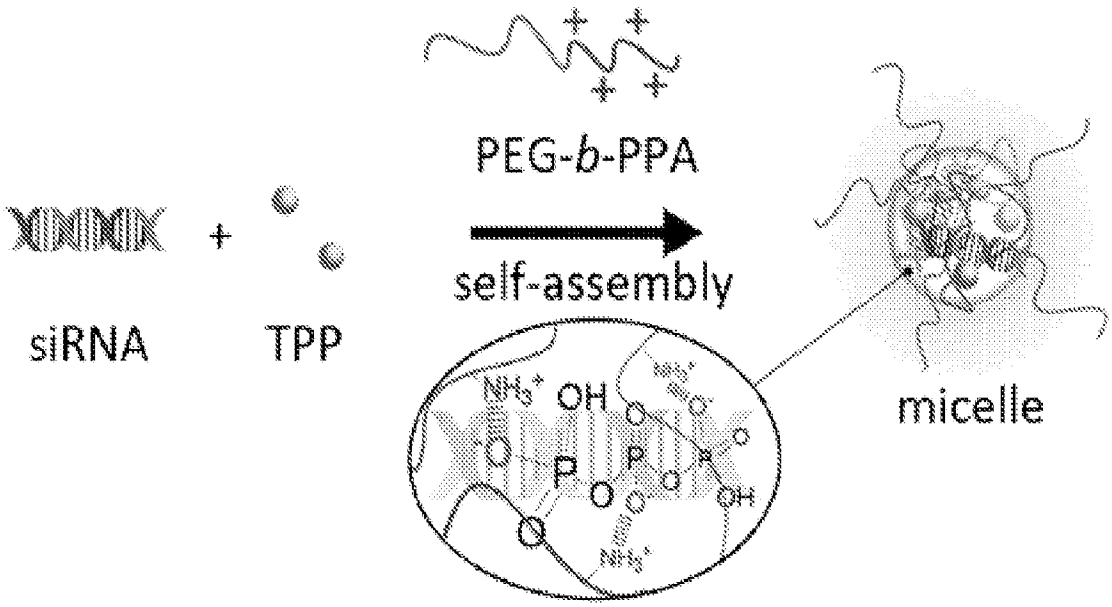


FIGURE 3 3/10

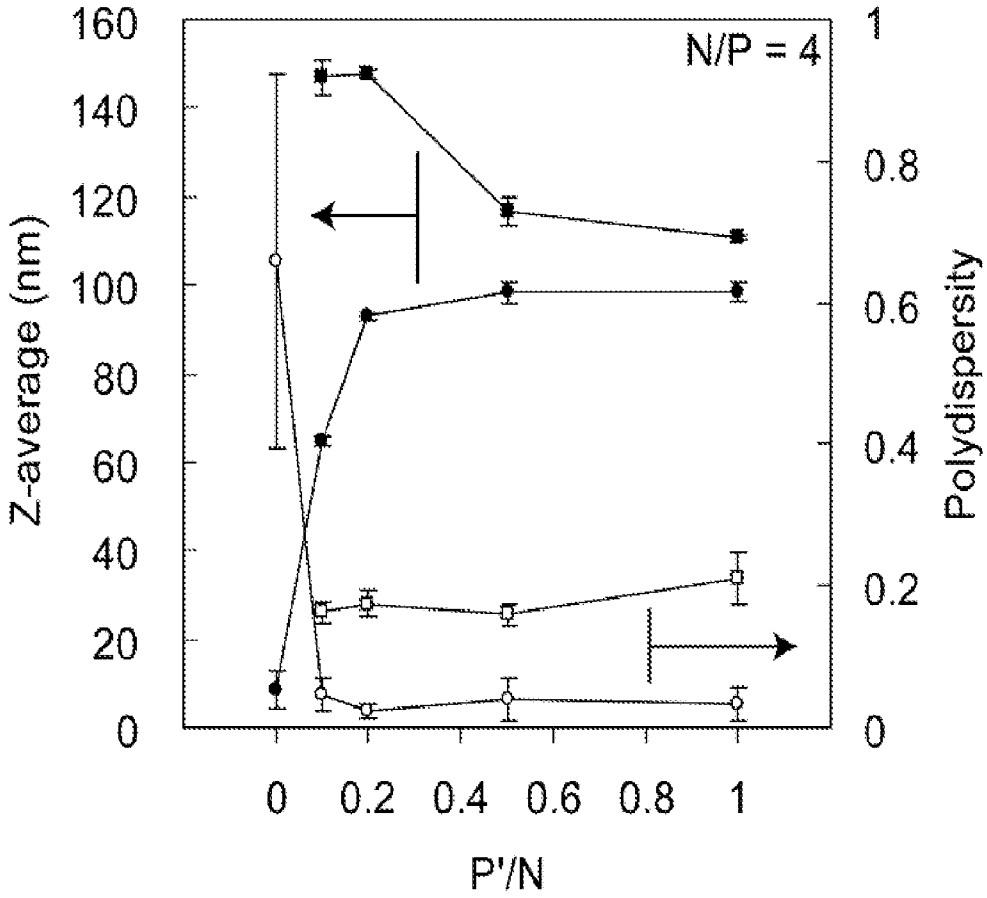

N/P = 4
Z-average (nm)
Polydispersity
P'/N
160
140
120
100
80
60
40
20
0
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1

FIGURE 4 4/10

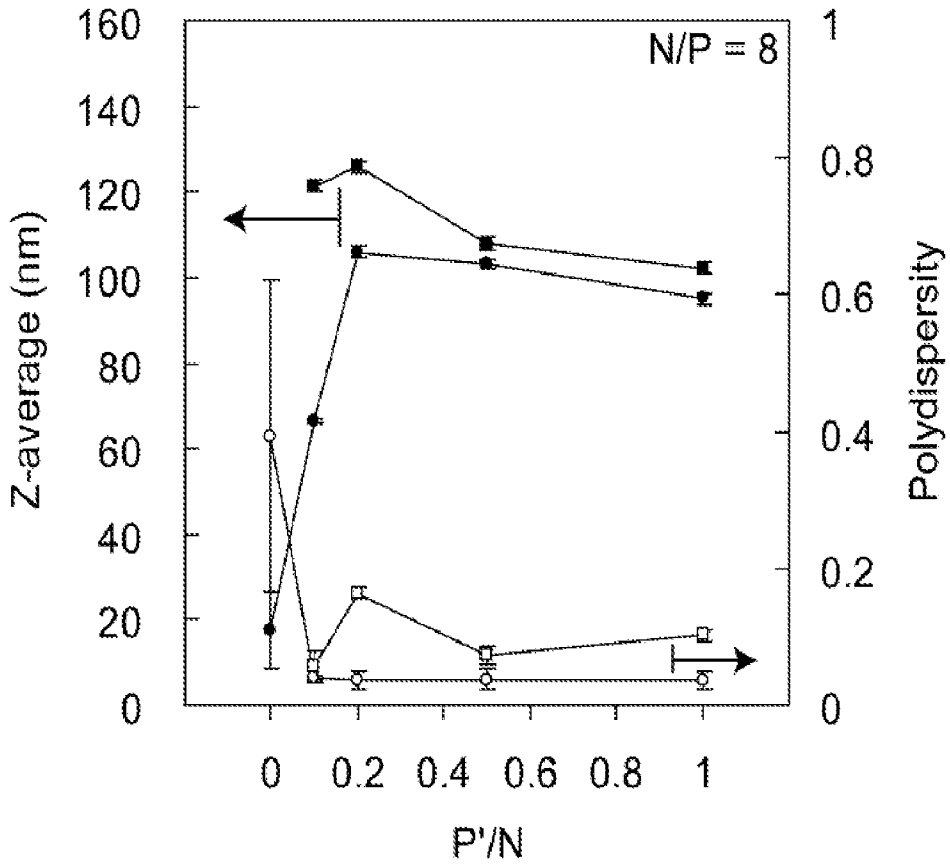

N/P = 8
Z-average (nm)
Polydispersity
P'/N
160
140
120
100
80
60
40
20
0
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1

FIGURE 5 5/10

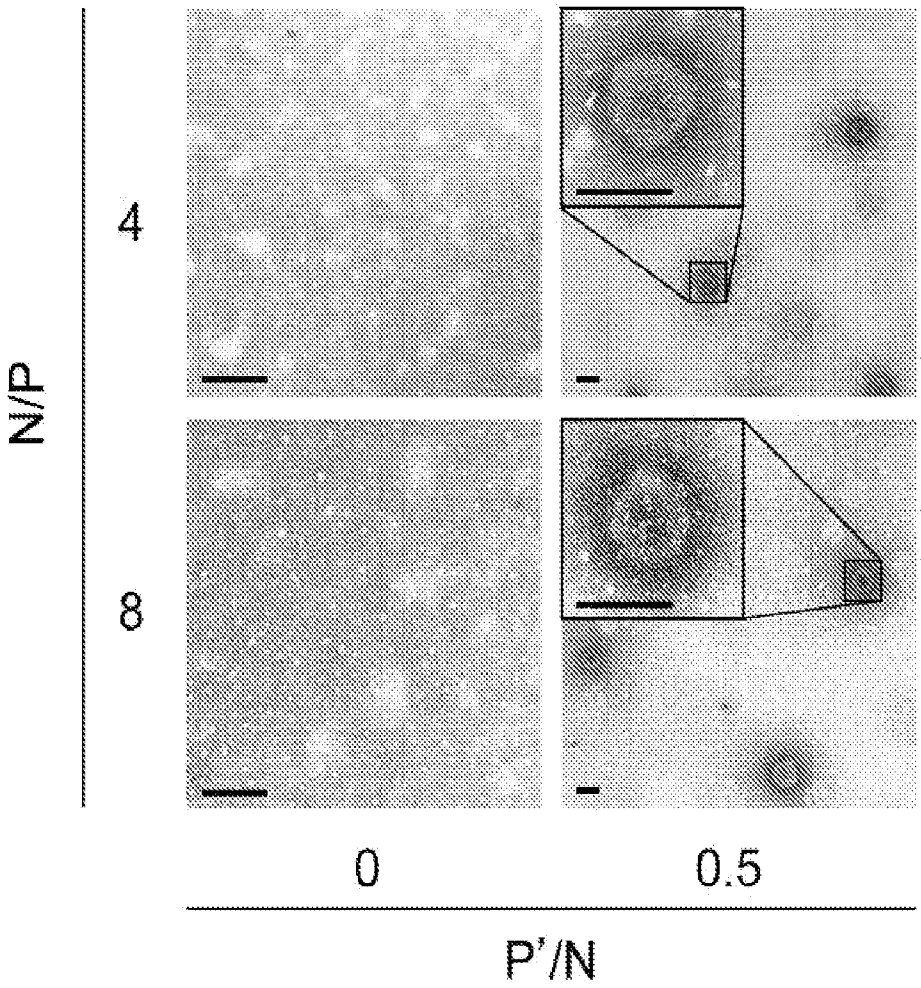
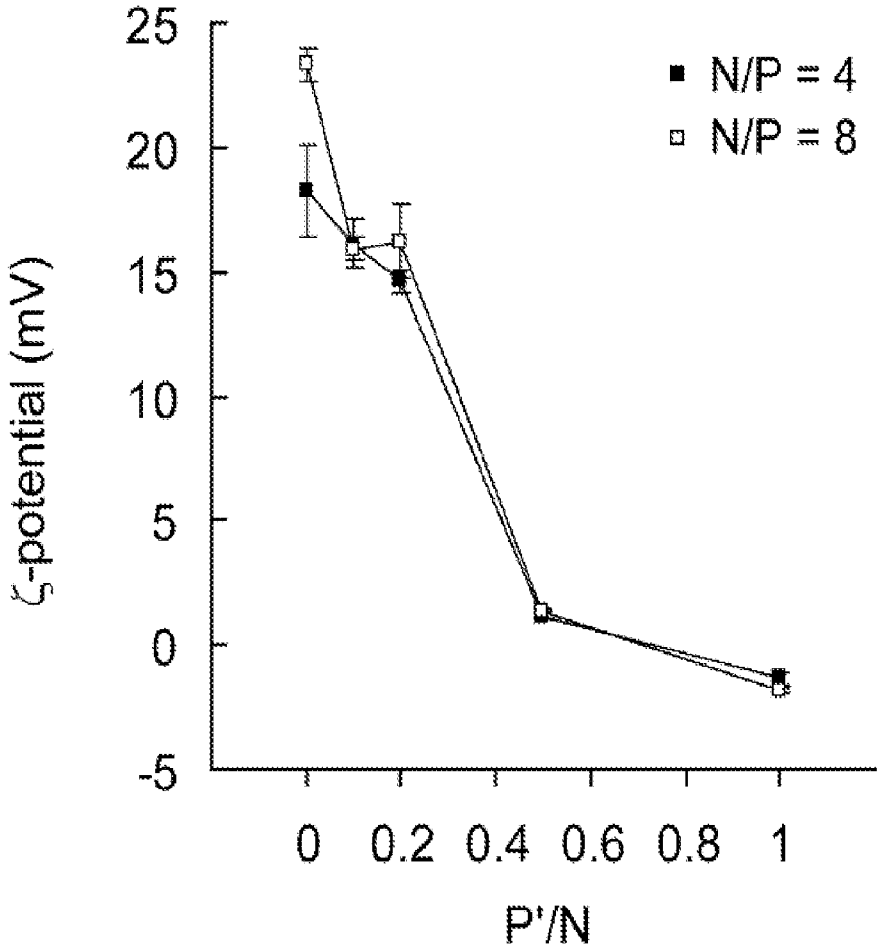
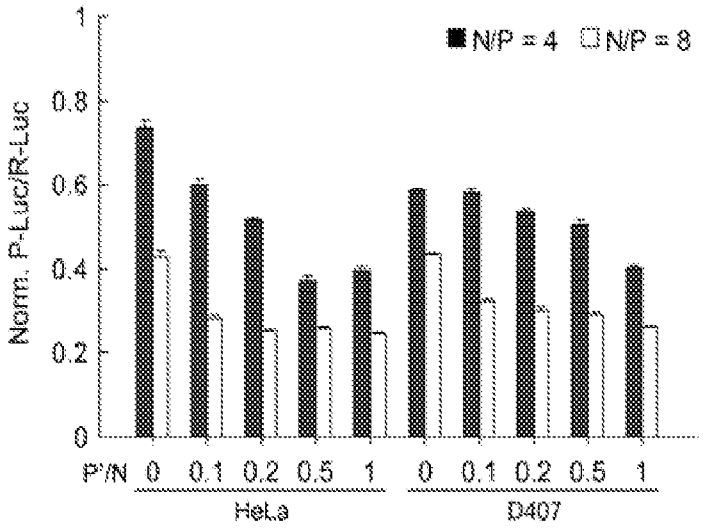


FIGURE 6 6/10



7A


N/P = 4
N/P = 8
Norm. P-Luc/R-Luc
1
0.8
0.6
0.4
0.2
0
P'/N 0 0.1 0.2 0.5 1 0 0.1 0.2 0.5 1
HeLa
D407

7B

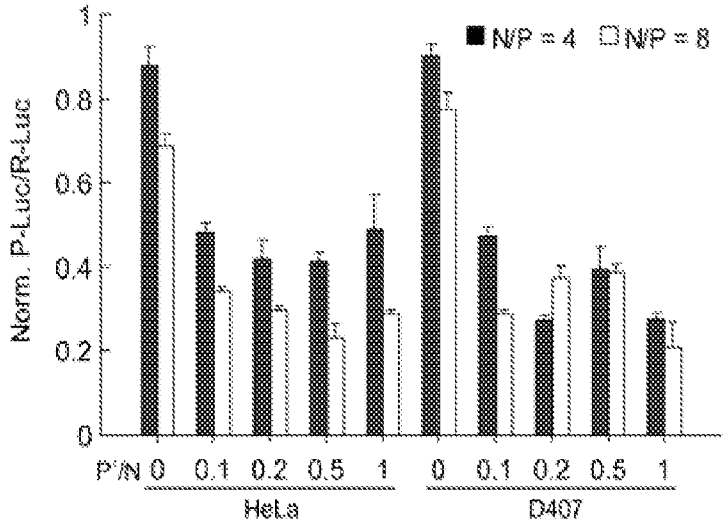

N/P = 4
N/P = 8
Norm. P-Luc/R-Luc
1
0.8
0.6
0.4
0.2
0
P'/N 0 0.1 0.2 0.5 1 0 0.1 0.2 0.5 1
HeLa
D407

FIGURE 8 8/10

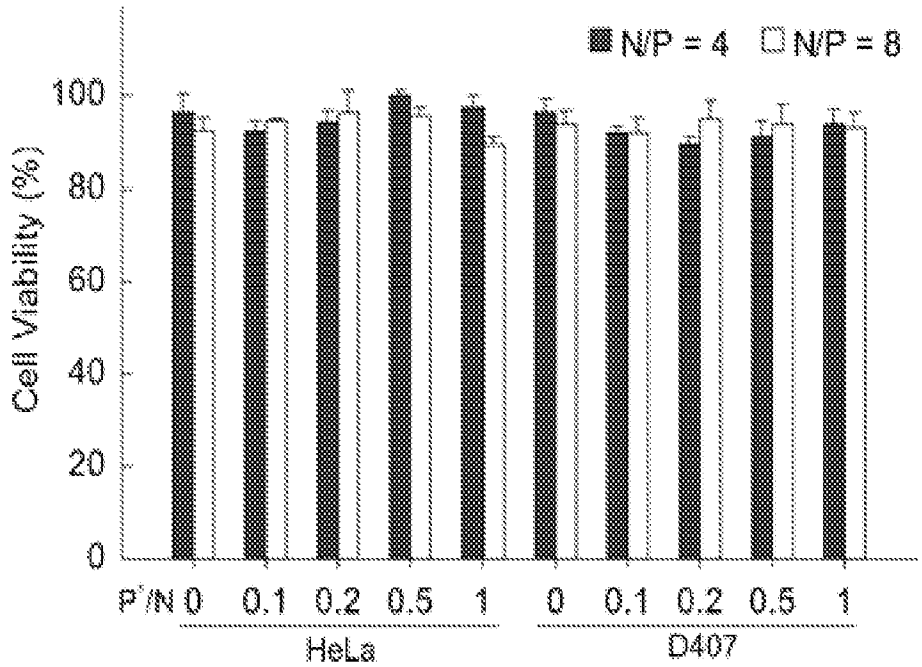

N/P = 4
N/P = 8
Cell Viability (%)
100
80
60
40
20
0
P'/N 0 0.1 0.2 0.5 1 0 0.1 0.2 0.5 1
HeLa
D407

FIGURE 9 9/10

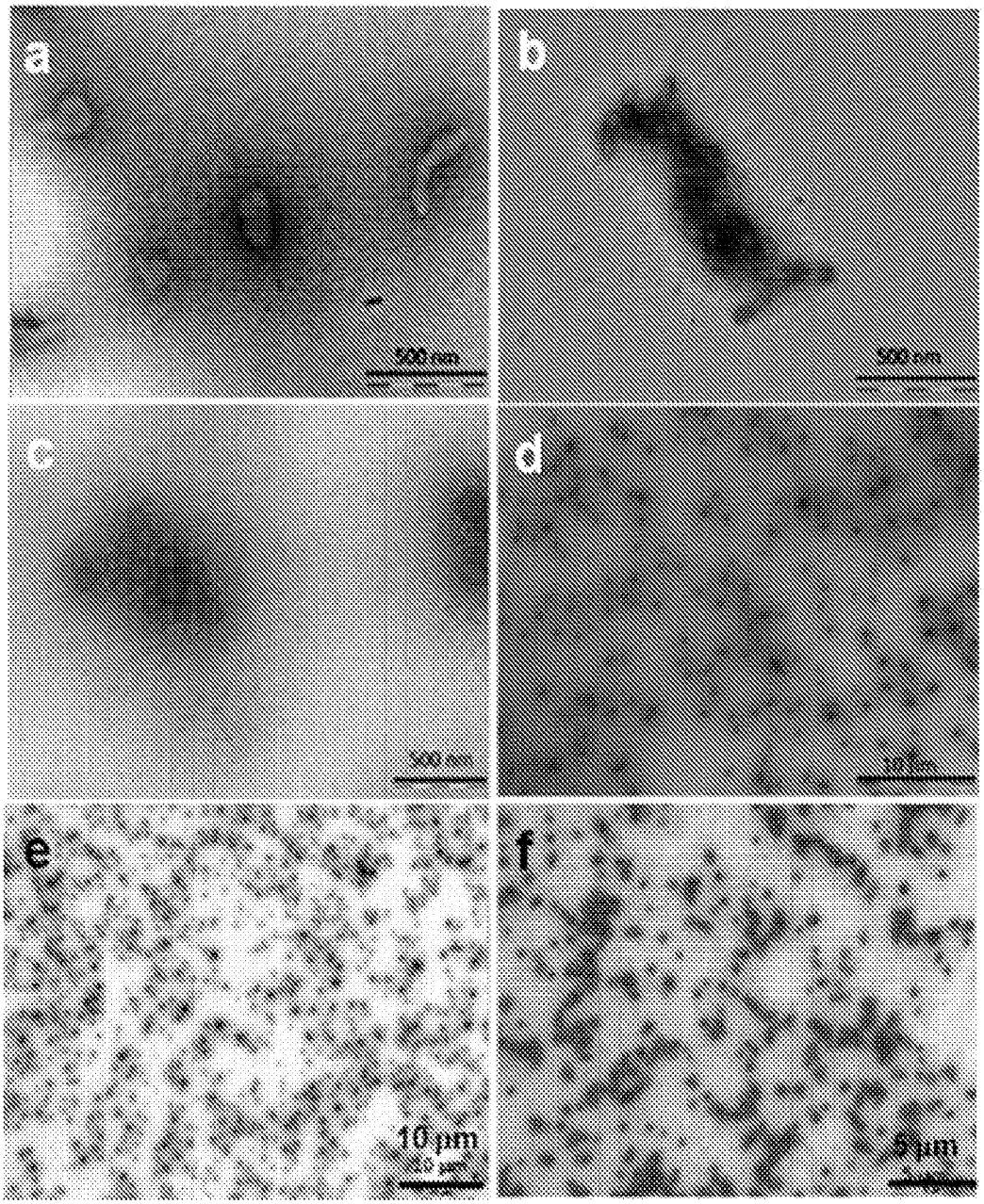
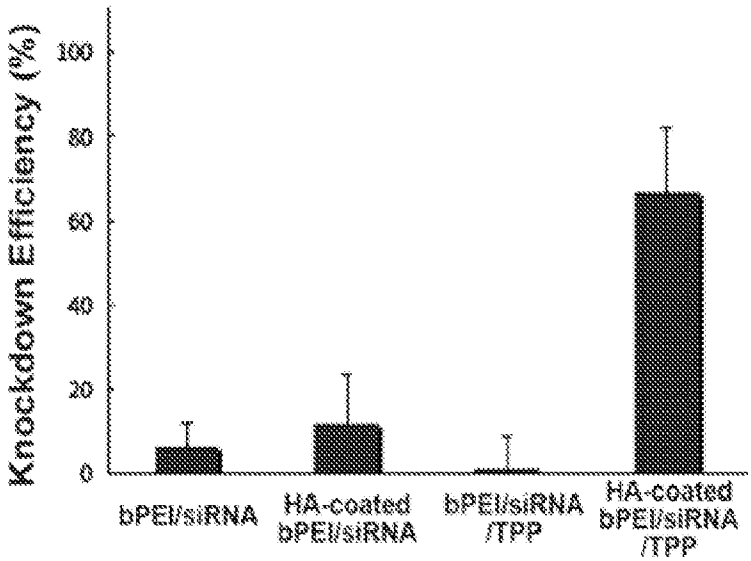


FIGURE 10 10/10

10A



10B