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(71) Applicant *(for all designated States except US)*: **VIRGINIA TECH INTELLECTUAL PROPERTIES, INC.** [US/US]; 2200 Kraft Drive, Suite 1050, Blacksburg, VA 24060 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **HEMP, Sean, T.** [US/US]; 12600 Foxridge Lane, Apt. J, Blacksburg, VA 24060 (US). **ALLEN, Michael, H.** [US/US]; 1813 Sussex Road, Blacksburg, VA 24060 (US). **SMITH, Adam, E.** [US/US]; 105 Amethyst Drive, Christiansburg, VA 24073 (US). **LONG, Timothy, E.** [US/US]; 607 Gigi Drive, Blacksburg, VA 24060 (US).

(74) Agent: **MAYBERRY, Michele, L.**; NEW RIVER VALLEY IP LAW, P.o. Box 10944, Blacksburg, VA 24062 (US).

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(54) Title: PHOSPHONIUM-CONTAINING POLYELECTROLYTES FOR NONVIRAL GENE DELIVERY

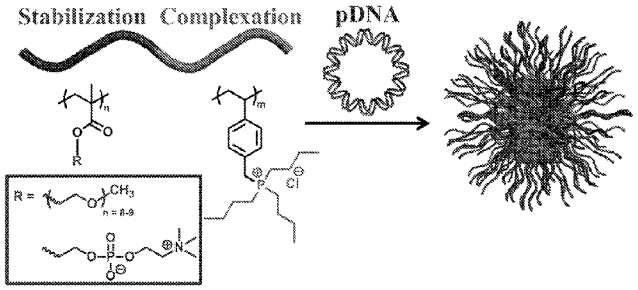
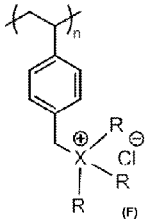


FIG. 1C



(57) Abstract: The present invention provides compositions comprising polymers and biologically active compounds, especially polymeric drug delivery systems. More specifically, the present invention provides ammonium and/or phosphonium-containing polymers and/or block copolymers as a viable and improved option for gene delivery. In an embodiment, the present invention provides a method of using a composition comprising a block copolymer that comprises: a stabilization block and a complexation block, and optionally an endosomolytic block for nonviral gene delivery. Preferred compositions include such co-polymers, wherein the complexation block is chosen from polymers of styrenic-based phosphonium-containing monomers chosen from: (F) wherein X is phosphorus; R is Cl-24 alkyl; and n is a number ranging from 2 to 1,000.

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PHOSPHONIUM-CONTAINING POLYELECTROLYTES FOR NONVIRAL GENE DELIVERY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of the filing date of U.S. Provisional Application No. 61/497,648, filed on June 16, 2011, the disclosure of which is hereby incorporated by reference herein in its entirety.

STATEMENT OF GOVERNMENT INTEREST

[0002] This material is based upon work supported in part by the U.S. Army Research Office under grant number W911NF-07-1-0452 Ionic Liquids in Electro-Active Devices (ILEAD) MURI. This material is based upon work also supported in part by the U.S. Army Research Laboratory and the U.S. Army Research Office under the Army Materials Center of Excellence Program, contract W911NF-06-2-0014. This material is additionally based upon work supported by the Army Research Office (ARO) under Award No. W911NF-10-1-0307 (DURIP). Funding is also acknowledged from NSF (CHE-0722638). The United States government has certain rights in and to the invention.

BACKGROUND OF THE INVENTION

Field of the Invention

[0003] The present invention provides compositions comprising polymers and biologically active compounds, especially polymeric drug delivery systems. More specifically, the present invention provides ammonium and/or phosphonium-containing polymers and/or block copolymers as a viable and improved option for gene delivery.

Description of Related Art

[0004] DNA delivery has been a flagship in nonviral gene delivery. The promise of therapeutic DNA delivery as a potential cure for many genetic diseases has stimulated much interest over the past decade. *See* Mintzer, M. A.; Simanek, E. E., *Chemical Reviews* 2009, *109* (2), 259-302; Li, S. D.; Huang, L., *Gene Therapy* 2006, *13* (18), 1313-1319; and Heath, W. H.; Senyurt, A. F.; Layman, J.; Long, T. E., *Macromolecular Chemistry and Physics* 2007, *208* (12), 1243-1249. With unacceptable immune responses and other adverse events recently reported for viral delivery, nonviral gene delivery becomes even more attractive. Viral vectors exhibit a number of significant drawbacks (deleterious immuno-response, lack of cell specificity, and high

manufacturing costs) that limit their widespread clinical impact despite their efficient delivery of therapeutics. These shortcomings of viral vectors and the advent of controlled, modular synthetic strategies have stimulated the development of nonviral vectors for the successful delivery of nucleic therapeutics to a cell. *See* Yue, X.; Qiao, Y.; Qiao, N.; Guo, S.; Xing, J.; Deng, L.; Xu, J.; Dong, A. *Biomacromolecules* 2010, *11*, 2306.

[0005] However, the main limitation with nonviral delivery is the inefficient transfection, caused mainly by the poor transport of DNA across cell membranes.

[0006] Various cationic polymers have shown promising effects in facilitating gene delivery as these polymers readily conjugate with DNA to neutralize the net negative charges from DNA molecules. However, recent research has indicated that successful polymeric candidates must satisfy a set of requirements: (i) the polymer must not destabilize the helical structure of the DNA to an extent that its bioactivity is lost, (ii) the polymer must not impose cytotoxicity to cells, and (iii) the conjugated particles must be readily dispersable in aqueous solution. Unstable aggregates are difficult to administer and are rapidly cleared from systemic administration. These conditions together with the level of the high cost in the synthesis of some of the cationic polymers mean that few existing polymers can meet this set of requirements.

[0007] Nonviral gene delivery is a rapidly growing field of biomedical research for polymer chemistry focused on the therapeutic delivery of DNA to treat and potentially cure various genetic diseases. Cationic macromolecular delivery vehicles bind and compact nucleic acids into nanoparticles termed "polyplexes." *See* Lai, E.; van Zanten, J. H., *Journal of Controlled Release* 2002, *82* (1), 149-158; and Allen, M. H.; Green, M. D.; Getaneh, H. K.; Miller, K. M.; Long, T. E., *Biomacromolecules* 2011, *12* (6), 2243-2250 ("Allen 2011"). Cationic polymers reversibly bind nucleic acids to offer protection from enzymatic degradation and facilitate cellular uptake through various endocytic mechanisms. *See* Reineke, T. M. *J. Polym. Sci., Part A: Polym. Chem.* 2006, *44*, 6895. Cationic polymers or lipids electrostatically complex and compact DNA to form a polyplex or lipoplex, respectively, to effectively deliver DNA to cells. *See* Lai, E.; van Zanten, J. H., *Biophysical Journal* 2001, *80* (2), 864-873; and Lai, E.; van Zanten, J. H., *Journal of Controlled Release* 2002, *82* (1), 149-158. These nanoparticles inhibit cellular

enzymatic degradation of DNA during delivery to the nucleus and also provide an avenue for cellular uptake, endosomal escape, and trafficking to the nucleus with subsequent release of the DNA. *See* Richardson, S. C. W.; Kolbe, H. V. J.; Duncan, R., *International Journal of Pharmaceutics* 1999, *178* (2), 231-243; Ruponen, M.; Rönkkö, S.; Honkakoski, P.; Pelkonen, J.; Tammi, M.; Urtti, A., *Journal of Biological Chemistry* 2001, *276* (36), 33875-33880; Funhoff, A. M.; van Nostrum, C. F.; Koning, G. A.; Schuurmans-Nieuwenbroek, N. M. E.; Crommelin, D. J. A.; Hennink, W. E., *Biomacromolecules* 2003, *5* (1), 32-39; and Forrest, M. L.; Pack, D. W., *Molecular Therapy* 2002, *6* (1), 57-66.

[0008] Common cationic polymers and lipids of major focus commercially and academically for gene delivery include poly(ethylene imine) (PEI), Lipofectamine™, Superfect® poly(2-dimethylaminoethyl methacrylate) (PDMAEMA), and chitosan. *See* Hardy, J. G.; Love, C. S.; Gabrielson, N. P.; Pack, D. W.; Smith, D. K., *Organic & Biomolecular Chemistry* 2009, *7* (4), 789-793; Dalby, B.; Cates, S.; Harris, A.; Ohki, E. C.; Tilkins, M. L.; Price, P. J.; Ciccarone, V. C., *Methods* 2004, *33* (2), 95-103; Ahn, H. H.; Lee, J. H.; Kim, K. S.; Lee, J. Y.; Kim, M. S.; Khang, G.; Lee, I. W.; Lee, H. B., *Biomaterials* 2008, *29* (15), 2415-2422; Godbey, W. T.; Wu, K. K.; Mikos, A. G., *Proceedings of the National Academy of Sciences* 1999, *96* (9), 5177-5181; Dufès, C.; Uchegbu, I. F.; Schätzlein, A. G., *Advanced Drug Delivery Reviews* 2005, *57* (15), 2177-2202; Luo, D.; Haverstick, K.; Belcheva, N.; Han, E.; Saltzman, W. M., *Macromolecules* 2002, *35* (9), 3456-3462; Koping-Hoggard, M.; Tubulekas, I.; Guan, H.; Edwards, K.; Nilsson, M.; Varum, K. M.; Artursson, P., *Gene Therapy* 2001, *8*, 1108-1121; and Strand, S. P.; Lelu, S.; Reitan, N. K.; de Lange Davies, C.; Artursson, P.; Vårum, K. M., *Biomaterials* 2010, *31* (5), 975-987. These systems and others concentrate primarily on modifying the chemical composition and architecture of nitrogen-containing polymers including ammonium and imidazolium cations for gene delivery to investigate various structure-property relationships including acetylation, PEGylation, attachment of targeting ligands, controlling charge density, incorporating hydrogen bonding, and topology. *See* Nimesh, S.; Aggarwal, A.; Kumar, P.; Singh, Y.; Gupta, K. C.; Chandra, R., *International Journal of Pharmaceutics* 2007, *337* (1-2), 265-274; Gabrielson, N. P.; Pack, D. W., *Biomacromolecules* 2006, *7* (8), 2427-2435; Tang, G. P.; Zeng, J. M.; Gao, S. J.; Ma, Y. X.; Shi, L.; Li, Y.; Too, H. P.; Wang, S., *Biomaterials* 2003, *24* (13), 2351-2362 ("Tang 2003");

Brumbach, J. H.; Lin, C.; Yockman, J.; Kim, W. J.; Blevins, K. S.; Engbersen, J. F. J.; Feijen, J.; Kim, S. W., *Bioconjugate Chemistry* 2010, *21* (10), 1753-1761; Ramirez, S. M.; Layman, J. M.; Bissel, P.; Long, T. E., *Macromolecules* 2009, *42* (21), 8010-8012; Chan, P.; Kurisawa, M.; Chung, J. E.; Yang, Y.-Y., *Biomaterials* 2007, *28* (3), 540-549; Kursa, M.; Walker, G. F.; Roessler, V.; Ogris, M.; Roedl, W.; Kircheis, R.; Wagner, E., *Bioconjugate Chemistry* 2002, *14* (1), 222-231; Rungsardthong, U.; Ehtezazi, T.; Bailey, L.; Armes, S. P.; Garnett, M. C.; Stolnik, S., *Biomacromolecules* 2003, *4* (3), 683-690; Allen 2011; Prevette, L. E.; Kodger, T. E.; Reineke, T. M.; Lynch, M. L., *Langmuir* 2007, *23* (19), 9773-9784; Georgiou, T. K.; Vamvakaki, M.; Patrickios, C. S.; Yamasaki, E. N.; Phylactou, L. A., *Biomacromolecules* 2004, *5* (6), 2221-2229; and Deshpande, M. C.; Garnett, M. C.; Vamvakaki, M.; Bailey, L.; Armes, S. P.; Stolnik, S., *Journal of Controlled Release* 2002, *81* (1-2), 185-199.

[0009] Multiple barriers exist for *in vivo* delivery of nucleic acids, including colloidal instability, rapid renal clearance, and insufficient biodistribution. *See* Davis, M. E.; Chen, Z. G.; Shin, D. M. *Nat. Rev. Drug Discovery* 2008, *7*, 771. In addition, many nonviral gene delivery vehicles, such as PEI, exhibit poor colloidal stability in serum-containing media. Salt induces polyplex aggregation through charge neutralization, and negatively-charged serum proteins associate to the positively-charged polyplexes, which induces aggregation that causes inefficient cellular uptake and delivery of the nucleic acid payload. *See* Prevette, L. E.; Lynch, M. L.; Kizjakina, K.; Reineke, T. M. *Langmuir* 2008, *24*, 8090 ("Prevette 2008"); and de Wolf, H. K.; Luten, J.; Snel, C. J.; Oussoren, C.; Hennink, W. E.; Storm, G. *Journal of Controlled Release* 2005, *109*, 275 ("de Wolf 2005"). The inclusion of a neutral, hydrophilic segment that provides steric shielding prevents salt- and serum-induced aggregation for many cationic homopolymers. *See* Neu, M.; Fischer, D.; Kissel, T. *The Journal of Gene Medicine* 2005, *7*, 992. Additionally, the incorporation of a hydrophilic stabilizing block reduces opsonization and reticuloendothelial system clearance leading to increased blood circulation time. *See* Hwang, S. J.; Davis, M. E. *Curr. Opin. Mol. Ther.* 2001, *3*, 183; Kakizawa, Y.; Kataoka, K. *Adv. Drug Delivery Rev.* 2002, *54*, 203; and Knop, K.; Hoogenboom, R.; Fischer, D.; Schubert, U. S. *Angew. Chem. Int. Ed.* 2010, *49*, 6288.

[0010] Conventional nonviral gene therapy almost exclusively focuses on nitrogen-containing macromolecules and lipids to condense and deliver nucleic acids. Phosphorus-containing

macromolecules are widely studied in a variety of fields and applications due to their improved thermal stability, flame retardancy, biocompatibility, ionic aggregation, and molecular recognition in block and random copolymers. *See* Monge, S.; Canniccioni, B.; Graillot, A.; Robin, J.-J., *Biomacromolecules* 2011, *12* (6), 1973-1982; Ren, H.; Sun, J.; Wu, B.; Zhou, Q., *Polymer Degradation and Stability* 2007, *92* (6), 956-961; Xie, W.; Xie, R.; Pan, W.-P.; Hunter, D.; Koene, B.; Tan, L.-S.; Vaia, R., *Chemistry of Materials* 2002, *14* (11), 4837-4845; Cheng, S.; Zhang, M.; Wu, T.; Hemp, S. T.; Mather, B. D.; Moore, R. B.; Long, T. E. *J. Polym. Sci., Part A: Polym. Chem.* 2012, *50*, 166; and Mather, B. D.; Baker, M. B.; Beyer, F. L.; Green, M. D.; Berg, M. A. G.; Long, T. E. *Macromolecules* 2007, *40*, 4396. Substitution of cationic phospholipid head groups from ammonium to phosphonium or arsenium in antitumor lipids decreased cytotoxicity while maintaining efficacy. *See* Stekar, J.; Nössner, G.; Kutscher, B.; Engel, J.; Hilgard, P., *Angewandte Chemie International Edition in English* 1995, *34* (2), 238-240. Few references concentrate on the structure-property relationship between different cationic centers for nonviral gene delivery. Clément *et al.* first successfully synthesized and examined ammonium-, phosphonium-, and arsenium-containing lipids for nonviral gene delivery. *See* Picquet, E.; Le Ny, K.; Delépine, P.; Montier, T.; Yaouanc, J.-J.; Cartier, D.; des Abbayes, H.; Férec, C.; Clément, J.-C., *Bioconjugate Chemistry* 2005, *16* (5), 1051-1053; Floch, V.; Loisel, S.; Guenin, E.; Hervé, A. C.; Clément, J. C.; Yaouanc, J. J.; des Abbayes, H.; Férec, C., *Journal of Medicinal Chemistry* 2000, *43* (24), 4617-4628; and Guénin, E.; Hervé, A.-C.; Floch, V.; Loisel, S.; Yaouanc, J.-J.; Clément, J.-C.; Férec, C.; des Abbayes, H., *Angewandte Chemie International Edition* 2000, *39* (3), 629-631. In these lipid-based gene delivery vectors, they found modifying the cationic head group from ammonium to phosphonium or arsenium improved gene delivery *in vivo* and *in vitro* and decreased cytotoxicity. The phosphonium and arsenium lipid-based vectors also displayed improved solution stability. Chitosan has been functionalized through amidation to generate a chitosan with roughly 3-4 mol% incorporation of a phosphonium substituent. The water-soluble chitosan displayed negligible cytotoxicity but gene transfection with these polymers was not investigated. *See* Wang, L.; Xu, X.; Guo, S.; Peng, Z.; Tang, T., *International Journal of Biological Macromolecules* 2011, *48* (2), 375-380.

[0011] Cellular uptake of polyplexes predominately occurs through either clathrin- or caveolae-

mediated endocytosis. *See* van der Aa, M.; Huth, U.; Häfele, S.; Schubert, R.; Oosting, R.; Mastrobattista, E.; Hennink, W.; Peschka-Süss, R.; Koning, G.; Crommelin, D., *Pharmaceutical Research* 2007, *24* (8), 1590-1598 ("van der Aa 2007"). Clathrin and dynamin cause invagination and pinching-off of vesicles respectively in the clathrin pathway. *See* Khalil, I. A.; Kogure, K.; Akita, H.; Harashima, H., *Pharmacological Reviews* 2006, *58* (1), 32-45. Vesicles originating from clathrin-mediated endocytosis undergo acidification to form an endosome (pH ~ 5-6) and eventually fuse with lysosomes (pH ~5) where enzymatic degradation occurs. The proton sponge hypothesis is often invoked throughout the literature as an avenue for polymers with large buffering capacity to display increased transfection efficiency. *See* Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P., *Proceedings of the National Academy of Sciences* 1995, *92* (16), 7297-7301; Ramirez, S. M.; Layman, J. M.; Long, T. E., *Macromolecular Bioscience* 2009, *9* (11), 1127-1134; Dai, J.; Zou, S.; Pei, Y.; Cheng, D.; Ai, H.; Shuai, X., *Biomaterials* 2011, *32* (6), 1694-1705; and Behr, J.-P., *CHIMIA International Journal for Chemistry* 1997, *51*, 34-36. The proton sponge hypothesis relies on protonatable sites on cationic vectors that buffer the endosome during acidification causing the influx of Cl^- ions with the increased osmotic pressure rupturing the endosome. Caveolae-mediated endocytosis requires a high concentration of caveolin (a protein) and cholesterol on the cell membrane surface to generate a caveosome. *See* Pelkmans, L.; Helenius, A., *Traffic* 2002, *3* (5), 311-320. The resulting caveosome undergoes an indirect pathway to the lysosome enabling more efficient transfection. *See* van der Aa 2007; Rejman, J.; Bragonzi, A.; Conese, M., *Molecular Therapy* 2005, *12* (3), 468-474; Gabrielson, N. P.; Pack, D. W., *Journal of Controlled Release* 2009, *136* (1), 54-61; and McLendon, P. M.; Fichter, K. M.; Reineke, T. M., *Molecular Pharmaceutics* 2010, *7* (3), 738-750.

[0012] The present inventors disclosed for the first time the utility of phosphonium-containing macromolecules for nonviral gene delivery. *See* Hemp, S. T.; Allen, M. H.; Green, M. D.; Long, T. E. *Biomacromolecules* 2012, *13*, 231. Specifically, the inventors directly compared ammonium- and phosphonium-containing polystyrene homopolymers with variable alkyl substituent lengths attached to the cationic center. Phosphonium vectors mediated higher gene transfection than ammonium analogs; the longer tributyl alkyl substituent lengths attached to the cationic center

also imparted enhanced pDNA delivery relative to triethyl-based analogs. Fréchet et al. recently demonstrated the improved siRNA delivery of phosphonium-containing polyacrylate homopolymers compared to ammonium-containing polyacrylates. *See* Ornelas-Megiatto, C.; Wich, P. R.; Fréchet, J. M. J. *Journal of the American Chemical Society* 2012, *134*, 1902.

[0013] Despite advancements in the field, there are still unmet, critical and immediate needs such as the need for better designed macromolecules for nonviral gene therapy. Therefore, what is needed is the synthesis of more diverse cationic structures that can provide more efficient vehicles for gene delivery.

SUMMARY OF THE INVENTION

[0014] The numerous limitations inherent in nonviral gene therapy described above provide great incentive for a new and better synthetic cationic macromolecules and methods of using such macromolecules to provide better delivery efficacy and therapeutic results. The present invention provides compositions comprising polymers and biologically active compounds, especially polymeric drug delivery systems, including ammonium and/or phosphonium-containing polymers and/or block copolymers as viable and improved options for gene delivery.

[0015] Herein, the present invention provides the synthesis and characterization of phosphonium-containing macromolecules for nonviral gene delivery. A direct comparison of phosphonium-containing macromolecules to ammonium-containing analogs elucidates the influence of the cationic site on transfection efficiency. The effect of varying alkyl substituent length on DNA delivery is also demonstrated in the present invention. The vectors of the present invention do not contain protonatable sites pointing to a different endosomal escape mechanism other than the proton sponge effect. Improved ability of the polyelectrolytes to bind and deliver DNA to HeLa cells is demonstrated using DNA binding assays, dynamic light scattering (DLS), cytotoxicity assays, luciferase expression assays, and wide-field fluorescence optical microscopy. Both substitutions (cation and alkyl substituent lengths) greatly influence transfection efficiency of the vectors. The present invention also provides the preferred endocytic pathway for the described polyelectrolytes.

[0016] In some embodiments, the present invention provides a composition comprising a block copolymer that comprises: a stabilization block, a complexation block, and an endosomolytic

block for use in nonviral gene delivery. In such embodiments, the complexation blocks include ammonium and/or phosphonium-containing polymers and demonstrate effective nucleic acid complexation to encapsulate and protect the nucleic acid during gene delivery. The endosomolytic blocks may exhibit amphiphilic behavior at physiological pH and upon uptake into the endosome become cationic in nature resulting in endosomal escape. The stabilizing blocks are selected from the group consisting of poly(2-methacryloyloxyethyl phosphoryl-choline) (poly(MPC)), poly[(ethylene glycol)$_9$ methyl ether methacrylate] (poly(EG$_9$MEMA)), poly(ethylene glycol), and any combination thereof to provide salt and serum stability preventing polyplex aggregation, subsequently improving transfection.

[0017] In another embodiment, the present invention provides a composition comprising polymers of styrenic-based ammonium and phosphonium monomers for use in nonviral gene delivery. In such embodiments, conventional free radical polymerization of the ammonium and phosphonium monomers results in ammonium and phosphonium containing polymers.

[0018] Other embodiments of the present invention disclose a method of using the compositions above for improved nonviral gene delivery of nucleic acid.

[0019] The features and advantages of the present invention will be apparent to those skilled in the art. While numerous changes may be made by those skilled in the art, such changes are within the spirit of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] These drawings illustrate certain aspects of some of the embodiments of the present invention, and should not be used to limit or define the invention.

[0021] FIGS. 1A, 1B, and 1C are schematic representations of block copolymer architectures of embodiments of the invention that can be used to achieve efficient nucleic acid delivery, such as siRNA or pDNA delivery, while providing salt and serum stability, and strong complexation, optionally in combination with providing endosomolytic activity, with FIG. 1B showing in particular core-shell polyplexes between siRNA and AB diblock copolymers with a hydrophilic, stabilizing A block and a cationic phosphonium B block, and FIG. 1C showing phosphonium-containing diblock copolymers of the invention.

[0022] FIG. 2 is a graphical representation of Aqueous SEC curves for the ammonium- and phosphonium-containing polyelectrolytes. MALLS detector traces are shown highlighting the similarity in absolute molecular weights of all the samples.

[0023] FIGS. 3A-D are gel pictures showing the selection of DNA binding assays for the ammonium- and phosphonium-containing polyelectrolytes: a) PTEA b) PTEP c) PTBA d) PTBP.

[0024] FIG. 4 is a gel picture showing siRNA electrophoretic gel shift assays of TBP_{61} and $MPC_{87}TBP_{81}$ as a representative agarose gel for the diblock copolymers demonstrating complete siRNA binding at a +/- ratio of 2.0.

[0025] FIGS. 5A-D are graphical representations of Polyplex diameter (squares) and zeta potential (circles) of various ammonium- and phosphonium-containing polyplexes: a) PTEA b) PTEP c) PTBA d) PTBP.

[0026] FIG. 6 is a graphical representation of dynamic light scattering analysis showing the salt stability of polyplexes derived from the complexation of poly(EG_9MEMA-*b*-TBP) with siRNA.

[0027] FIG. 7 is a graphical representation showing the hydrodynamic diameter of the siRNA polyplexes formed using phosphonium-based diblock copolymers, TBP_{61}, and Jet-PEI and challenged under salt conditions for 24 h to probe colloidal stability. Error bars represent the standard deviation of three measurements.

[0028] FIG. 8 is a graphical representation showing the hydrodynamic diameter of the siRNA polyplexes (+/- ratios of 2.0) prepared with phosphonium-based diblock copolymers of various embodiments of the invention at varying concentrations of siRNA. Error bars represent the standard deviation of three measurements.

[0029] FIG. 9 is a graphical representation showing the cytotoxicities of ammonium- and phosphonium-containing gene delivery vectors (n = 8) of embodiments of the invention. All polymers exhibited similar toxicities due to their 100% charge density.

[0030] FIG. 10 is a graphical representation of the polyplex cytotoxicities of ammonium- and phosphonium-containing gene delivery vectors (n = 8) in comparison with other systems.

[0031] FIG. 11 is a graph of cytotoxicities of diblock copolymer/siRNA polyplexes (+/- ratios of 2.0) according to embodiments of the invention, at siRNA doses varying between 25 and 1000 nM in HepaRG cells. Error bars represent the standard deviation of three replicates.

[0032] FIG. 12 is a graphical representation of serum-free luciferase expression of ammonium- and phosphonium-containing polyelectrolytes (n = 4) of embodiments of the invention compared with other systems.

[0033] FIGS. 13A-B provide pictures showing the cellular uptake of Cy5-labeled DNA for phosphonium polyelectrolytes (+/- ratio of 4) of embodiments of the invention, showing successful cellular uptake of polyplexes into the HeLa cells: a) PTEP b) PTBP. The channels illustrated are as follows: 1) DAPI stained nuclei, 2) Cy5-labeled DNA showing polyplexes, 3) Alexa Fluor® 488 Phalloidin stained F-actin, and 4) Overlay of all three channels highlighting cellular uptake of polyplexes. Scale bar = 50 μm.

[0034] FIG. 14 is a graphical representation showing the serum-containing luciferase expression of ammonium- and phosphonium-containing polyelectrolytes (n = 4) of embodiments of the invention in comparison with other systems.

[0035] FIG. 15 is a graphical representation showing the relative luciferase expression for PTBA and PTBP with various endocytic inhibitors (n = 4). Genistein and methyl β-cyclodextrin inhibited caveolae-mediated endocytosis while amantadine inhibited clathrin-mediated endocytosis.

[0036] FIG. 16 is a graphical representation of RNase degradation assays for the polyplexes (+/-ratio of 2.0) formed with $OEG_{52}TBP_{78}$ and $MPC_{87}TBP_{81}$ as representative samples demonstrating the protection of siRNA from nuclease degradation.

[0037] FIGS. 17A-B are graphs showing aqueous SEC LS curves for: a) the initial OEG_{52} macroCTA and the final $OEG_{52}TBP_y$ diblock copolymers and b) the initial MPC_{87} macroCTA and the resulting $MPC_{87}TBP_y$ diblock copolymers.

[0038] FIG. 18 is a gel picture showing DNA gel shift assays of TBP_{61} and $OEG_{52}TBP_{78}$ as a representative DNA gel shift assay for the diblock copolymers demonstrating complete DNA binding at a +/- ratio of 1.0.

[0039] FIG. 19 is a graph showing hydrodynamic diameter of the polyplexes formed using the phosphonium-based diblock copolymers, TBP_{61}, and Jet-PEI challenged under serum-free media conditions for 24 h to probe their colloidal stability. Polyplexes prepared in water at +/- ratios of 2.0 for the phosphonium-based vehicles and N/P ratio of 5.0 for Jet-PEI with subsequent

dilution into serum-free DMEM. Error bars represent the standard deviation of three measurements.

[0040] FIG. 20 is a graph showing hydrodynamic diameter of the polyplexes prepared with the phosphonium-based diblock copolymers, TBP_{61}, and Jet-PEI challenged under serum-containing media conditions for 24 h to probe their colloidal stability. Polyplexes prepared in water at +/- ratios of 2.0 for the phosphonium-based vehicles and N/P ratio of 5.0 for Jet-PEI and then diluted into serum-containing DMEM. Error bars represent the standard deviation of three measurements.

[0041] FIGS. 21A-E are pictures showing GFP expression of successfully transfected HepaRG cells using the phosphonium-containing diblock copolymers at a +/- ratio of 2.0 and dosages of 1.4 μg DNA/well.

[0042] FIG. 22 is a graph showing luciferase expression and cell viability of transfected HepaRG cells using the phosphonium-containing diblock copolymers (+/- ratios of 2.0) and Jet-PEI (N/P = 5.0). The histogram bars correlate to the luciferase expression and the individual points correspond to the cell viability. Error bars represent the standard deviation of three measurements. All delivery vehicles transfected statistically higher than the negative controls, cells and DNA only ($p < 0.02$).

DETAILED DESCRIPTION OF VARIOUS EMBODIMENTS OF THE INVENTION

[0043] The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and examples included herein. However, before the present compounds, compositions, and methods are disclosed and described, it is to be understood that this invention is not limited to specific nucleic acids, specific polypeptides, specific cell types, specific host cells, specific conditions, or specific methods, etc., as such may, of course, vary, and the numerous modifications and variations therein will be apparent to those skilled in the art. It is also to be understood that the terminology used herein is for the purpose of describing specific embodiments only and is not intended to be limiting.

[0044] The present invention provides compositions comprising polymers and biologically active compounds, especially polymeric drug delivery systems, including ammonium and/or

phosphonium-containing polymers and/or block copolymers, as a viable and improved option for gene delivery. Compositions of the invention can comprise a stabilization block and a complexation block, optionally in combination with an endosomolytic block.

[0045] The invention is of most value where the biologically active compound is anionic, preferably polyanionic, in nature. The invention is of most value where the active compound is a nucleic acid, for instance an oligonucleotide, having 5 to 50 base residues usually of DNA. For instance the oligonucleotide may be an active anti-sense molecule. The nucleic acid may alternatively be a single strand RNA molecule or a single or double strand DNA molecule and can include pDNA and siRNA. Double stranded DNA may, for instance, comprise genes encoding useful products, especially a plasmid, including control sequences enabling it to be transcribed and translated when transfected into a cell. Embodiments of the invention are useful as gene delivery systems. Other anionic actives may be saccharide-containing compounds, proteins or peptides and amphiphilic anionic compounds such as retinoic acid and derivatives to name a few.

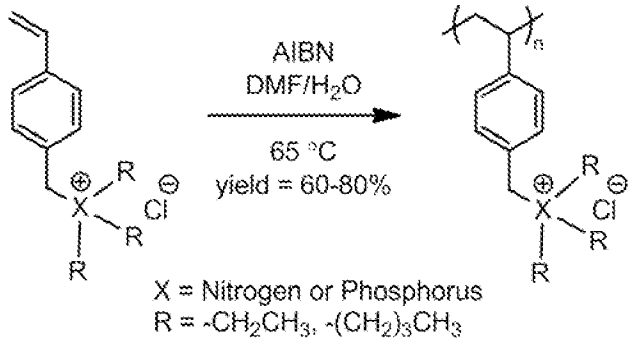
[0046] The invention may also be useful where the biologically active compound is a cationic drug, especially a polycationic drug or an amphiphilic cationic drug. Examples include, but are not limited to, cetyl and other long chain alkyl-pyridinium compounds, anaesthetics, such as procaine-HCl, rhodamine probes, and low molecular weight drugs such as mexilitine, amiloride HCl, diminazene aceturate and amikicin sulphate.

[0047] The composition of the invention is preferably in the form of an aqueous composition or a non-aqueous composition which may be made up to form an aqueous composition by addition of water. In the context of this specification, the term “associated with” in relation to the interaction between the polymer and the biologically active compound means that the polymer and the active compound are electrostatically bound to one another. In embodiments, the polymer and active compound are not covalently bound to one another.

[0048] More preferably the compositions can comprise a polymer and biologically active compound associated with one another in the form of particles having an average diameter of less than about 500 nm, such as about 300 nm, or about 200 nm, preferably less than about 150 nm, such as 100 nm. Preferably, the particles are in suspension and compositions

according to embodiments of the invention are provided as suspensions in aqueous form. Particles of size less than the indicated maximum, are capable of being taken up by cells, so that the biologically active compounds may be delivered intracellularly. Such particles may also be stabilized against settlement in an aqueous composition. Such a composition thus retains useful rheology, enabling it to be handled by usual liquid handling techniques, without having to be thickened or gelled to stabilize the particles against settlement.

[0049] A new composition according to the invention comprises polymers of styrenic-based ammonium and phosphonium monomers for use in nonviral gene delivery. In an embodiment, these polymers are made through conventional free radical polymerization as shown in Scheme 1 below.



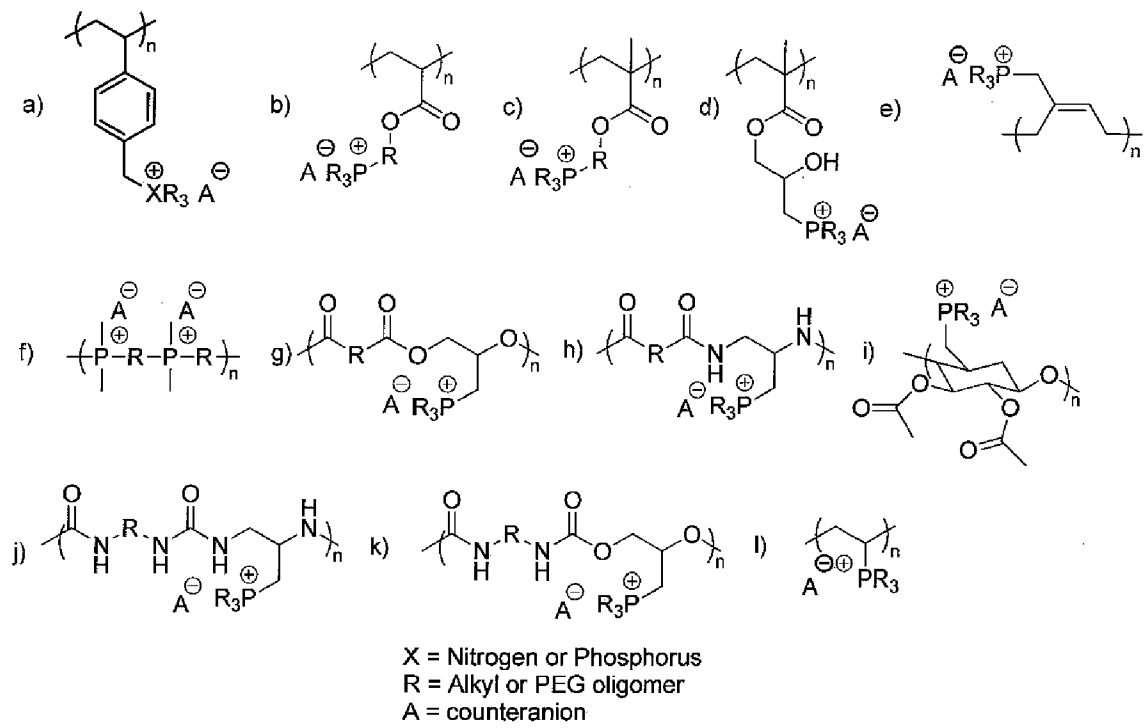
Scheme 1. Conventional free-radical polymerization of ammonium- and phosphonium-containing styrenic homopolymers to afford gene delivery vectors with different alkyl substituent lengths.

[0050] Polymerization of functional monomers enabled the synthesis of a fully quaternized polyelectrolyte in contrast to post-polymerization quaternization of poly(4-vinylbenzyl chloride) that does not ensure quantitative functionalization. The ammonium- and phosphonium-containing polyelectrolytes may include, but are not limited to, poly(triethyl-(4-vinylbenzyl)ammonium chloride) (PTEA), poly(tributyl-(4-vinylbenzyl)ammonium chloride) (PTBA), poly(triethyl-(4-vinylbenzyl)phosphonium chloride) (PTEP), and poly(tributyl-(4-vinylbenzyl)phosphonium chloride) (PTBP), and any combination thereof. In addition, one or more of the R-groups can be methyl or propyl, or any length alkyl group with 1-24 carbon atoms, such as from 1-5, or from 2-8, or from 1-10 carbons and so on, including branched or unbranched, substituted or unsubstituted R-groups with additional alkyl groups. For example,

one or more of the R-groups can be an ethyl or methyl group, while the remaining R-group(s) are butyl or propyl groups. Similarly, one or more of the R-groups can be butyl or propyl groups with the remaining R-group(s) being ethyl or methyl groups. In embodiments, one or more of the R groups can be hydrogen.

[0051] Even further, in addition to styrenics, phosphonium polymers according to embodiments of the invention may include ionenes, acrylates, methacrylates, isoprenes, polyesters, polyamides, polyurethanes, and polyureas, some of which are shown below in Scheme 2. Indeed, any olefinic monomer can be used as the building blocks of the polymers. Even further, step-growth polymers can be used as the complexation polymer.

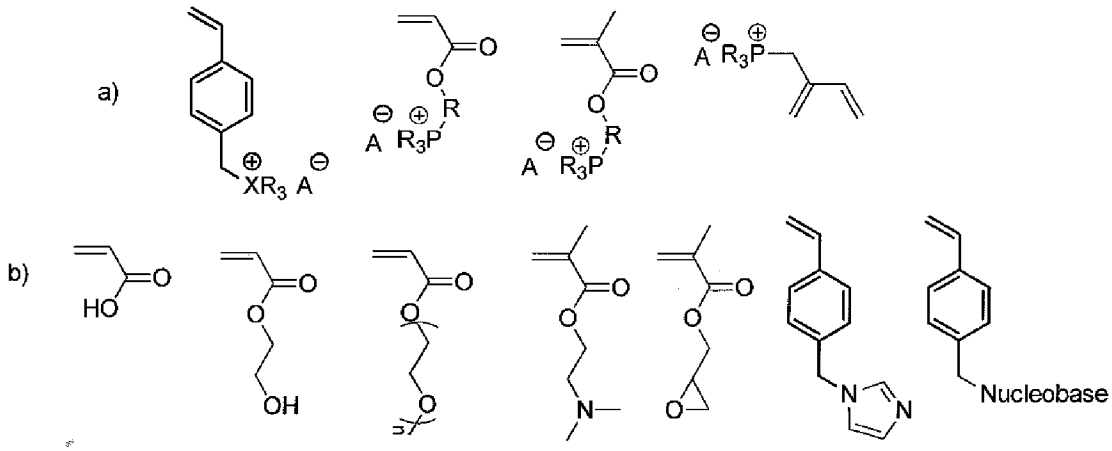
[0052]



Scheme 2. Phosphonium-comprising polymers for non-viral gene delivery: (a) styrenics, (b) acrylics, (c) methacrylics, (d) glycidyl methacrylate phosphoniums, (e) dienes, (f) ionenes, (g) polyesters, (h) polyamides, (i) cellulose derivatives (j) polyureas, (k) polyurethanes, (l) vinyl phosphoniums.

[0053] A wide variety of phosphonium-comprising copolymers can be synthesized according to embodiments of the invention. The different copolymers with have different characteristics,

such as charge density, hydrogen bonding, PEG incorporation, and buffering amines, thus differing effects on cytotoxicity and transfection. Monomers and co-monomers that can be used to make phosphonium-containing copolymers of the invention include those in Scheme 3.



[0054]

Scheme 3. Phosphonium-comprising monomers (a) and co-monomers (b) for synthesis of a wide variety of phosphonium-containing co-polymers according to the invention.

[0055] In an embodiment, phosphonium-containing macromolecules were compared to the respective ammonium analogs and both were examined for their ability to condense DNA and transfect cells. Phosphonium-containing polymers bound DNA at an earlier +/- ratio and exhibited improved DNA delivery with higher luciferase expression than the ammonium-containing polymers. A person of ordinary skill in the art, with the benefit of this disclosure, would know which type of ion to use for the intended purposes.

[0056] In an embodiment of the present invention, phosphonium-containing and/or ammonium-containing monomers also allowed the synthesis of a wide variety of copolymers capable of controlling one or more of charge density, DNA binding affinity, cytotoxicity, and transfection in a desired range.

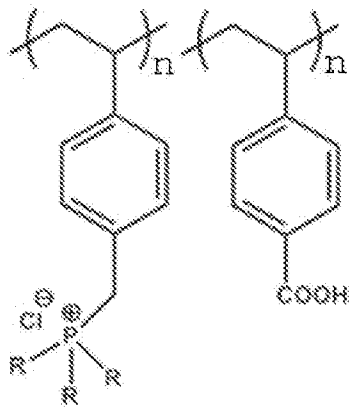
[0057] The block copolymer may be a simple A-B block copolymer, or may be an A-B-A or B-A-B block copolymer. In some embodiments, it may be a star-type polymer with more than two arms of blocks A extending from a core block B or vice versa. It may be a comb type polymer in which the back bone is considered as block A and each tine is a B block or vice versa. It may

also be an A-B-C, A-C-B or B-A-C block copolymer, where C is a different type of block. C blocks may for instance comprise a function, e.g. cross-linking or ionic groups, to allow for reactions of the copolymer in composition. Crosslinking reactions especially of A-C-B type copolymers, may confer useful stability on drug-containing micelles. Cross-linking may be covalent, or sometimes, electrostatic in nature. Crosslinking may involve addition of a separate reagent to link functional groups, such as using a difunctional alkylating agent to link two amino groups.

[0058] The block copolymers according to embodiments of the invention preferably have controlled molecular weights within a desired range. It is preferable for each of the blocks to have molecular weight controlled within a narrow band, that is to have a narrow polydispersity. The polydispersity of molecular weight should, for instance, be less than about 2.0, more preferably less than about 1.5.

[0059] The present invention includes preferred block copolymer architectures that exhibit significant promise for nonviral gene delivery as shown in FIGS. 1A and 1B. These triblock and diblock copolymer structures incorporate a multi-component platform allowing for significant modification and tailoring to achieve efficient nucleic acid delivery. The desired block copolymers may contain a salt and serum stabilizing block and a nucleic acid complexation block as shown in FIGS. 1A-C. In the context of this specification, the complexation block portion of the molecule can also be referred to as a spacer. In embodiments, triblock copolymers are provided, which can comprise another block added to the diblock copolymers, for example to generate a pH-responsive endosomolytic block for improved nucleic acid delivery, such as is shown in FIG. 1A.

[0060] Representative endosomolytic blocks may include polymers chosen from:

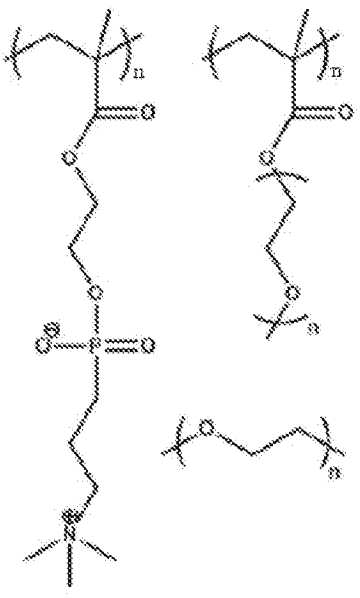


[0061]

[0062] wherein R is an alkyl with 1-24 carbon atoms, such as from 2-15 carbons, or from 5-12, or from 1-10 carbon atoms and n is a number ranging from 2-1,000. In preferred embodiments, the degree of polymerization for each block of the co-polymer can range from 250-400, or from 500-750, or from 600-1,000. Likewise, in preferred embodiments, the degree of polymerization for the block co-polymer as a whole can range from 100-1,000, such as from 200-700, or from 350-900, or up to 1,000. In further embodiments, polymers of any of the co-polymer blocks can have any number of repeating units, for example, wheren n is 10, 20, 25, 50, 75, 100, 125, 150, or 200. In the context of this specification, the degree of polymerization for a polymer of the composition is a value equal to n number of monomer repeating units for that polymer. The degree of polymerization for the block co-polymer can be equal to the sum of the n number of polymer repeating units for each polymer block.

[0063] The specific stabilizing blocks include, but are not limited to, poly(2-methacryloyloxyethyl phosphoryl-choline) (poly(MPC)), poly[(ethylene glycol)$_9$ methyl ether methacrylate] (poly(EG$_9$MEMA)), poly(ethylene glycol), and any combination thereof. In preferred embodiments, the chosen blocks provide salt and serum stability preventing polyplex aggregation and subsequently improving transfection.

[0064] More particularly, in embodiments, the block co-polymers of the invention can comprise a stabilization block comprising polymers chosen from:



[0065] wherein n is a number ranging from 2 to 100, or up to 1,000.

[0066] In some embodiments the blocks may comprise a stabilization block, a complexation block and an endosomolytic block. The complexation blocks include ammonium and/or phosphonium-containing styrenics and methacrylics (FIG. 1) which demonstrate effective nucleic acid complexation to encapsulate and protect the nucleic acid during delivery. Finally, the endosomolytic blocks shown in FIG. 1A exhibit amphiphilic behavior at physiological pH. Upon uptake into the endosome, the endosomolytic block becomes cationic in nature due to the lower pH resulting in endosomal escape. A person of ordinary skill in the art, with the benefit of this disclosure would know the type, number, and combination of blocks to use for the intended purpose.

[0067] Any suitable method may be used to synthesize the polymers and copolymers of the present invention. In some embodiments, controlled radical polymerization may be used to enable the synthesis of a diblock copolymer with a PEG-containing block for serum stability and a cationic ammonium- or phosphonium-containing block for DNA condensation. One of ordinary skill in the art, with the benefit of this disclosure, would know how to synthesize the compounds disclosed.

[0068] The present invention discloses ammonium and/or phosphonium-containing macromolecules as novel gene delivery compounds. Further the present invention discloses the use such of ammonium and/or phosphonium cations for nonviral gene delivery through the synthesis of phosphonium-containing and/or ammonium-containing random and block copolymers to minimize cytotoxicity, charge density, and serum aggregation while improving gene transfection. This invention broadens the potential avenues for synthetic gene delivery vehicles to include phosphonium-containing and/or ammonium-containing polymers and copolymers as viable and improved options for the delivery of nucleic acids (DNA and siRNA).

[0069] To facilitate a better understanding of the present invention, the following examples of certain aspects of some embodiments are given. In no way should the following examples be read to limit, or define, the scope of the invention.

[0070] **Example I.**

[0071] **Materials.** Triethylamine (99.5%), tributylamine (≥98.5%), triethylphosphine (99%),

tributylphosphine (≥93.5%), and 4-vinylbenzyl chloride (≥90%) were purchased from Sigma Aldrich and used as received. α,α'-Azoisobutyronitrile (AIBN) was purchased from Sigma Aldrich and recrystallized from methanol. Triethyl-(4-vinylbenzyl)ammonium chloride, tributyl-(4-vinylbenzyl)ammonium chloride, and tributyl-(4-vinylbenzyl)phosphonium chloride were synthesized as previously reported in the literature. *See* Hatakeyama , E. S.; Ju, H.; Gabriel, C. J.; Lohr, J. L.; Bara, J. E.; Noble, R. D.; Freeman, B. D.; Gin, D. L., *Journal of Membrane Science* 2009, *330* (1-2), 104-116 ("Hatakeyama 2009"). All solvents were obtained from Sigma Aldrich and used as received.

[0072] **Analytical Methods.** ^{1}H NMR spectroscopy was performed on a Varian Unity 400 at 400 MHz in $CDCl_3$ or D_2O. Mass spectrometry was performed with an Agilent 6220 LC-TOF-MS system. Aqueous size-exclusion chromatography (SEC) was performed using a Waters 1515 Isocratic HPLC Pump and Waters 717plus Autosampler with Waters 2414 Refractive Index and Wyatt MiniDAWN MALLS detectors at a flow rate of 0.8 mL/min. Two Waters Ultrahydrogel Linear and one Waters Ultrahydrogel 250 columns were utilized. The aqueous solvent was composed of 54/23/23 (v/v/v %) water/methanol/acetic acid with 0.1 M sodium acetate. DLS confirmed the absence of polymer aggregates in the aqueous SEC solvent. Absolute molecular weights were obtained from the MALLS detector after determining the d*n*/d*c* offline using a Wyatt Optilab T-rEX Differential Refractometer at 658 nm and 35 °C. Statistical analysis of the transfection experiments was performed using the Student's t-test.

[0073] **Monomer Synthesis.** All monomers were synthesized following similar procedures in existing literature. *See* Hatakeyama 2009. The synthesis of triethyl-(4-vinylbenzyl)-phosphonium chloride follows as an example. Dry acetonitrile (30 mL) was added to a flame-dried 100-mL round-bottomed flask, containing a magnetic stir bar, using a cannula. 6.0 mL 4-vinylbenzyl chloride (42.6 mmol) and 5.4 mL triethylphosphine (36.7 mmol) were added to the flask. The yellow solution was heated to 40 °C for 24 h and the monomer was precipitated into 1 L of a 75:25 hexanes:ethyl acetate mixture. The solid was vacuum filtered and washed with hexanes. The resulting white crystals were dried at reduced pressure (0.5 mmHg) to obtain a final yield of 9.4 g (95% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) (δ, ppm): 1.21 (m, CH_3-, 9H), 2.46 (m, -CH_2-, 6H), 4.24 (d, -Ar-CH_2-P-, 2H), 5.26 (d, CH_2=, 1H), 5.73 (d, CH_2=, 1H), 6.63 (dd,

=CH, 1H), 7.34 (d, ArH, 2H), 7.41 (dd, ArH, 2H). ^{13}C NMR: 6.11 (d, CH_3-), 12.27 (d, -PCH_2-), 26.14 (d, ArCH_2P), 115.05 (d, CH_2=), 127.21 (d, Ar), 127.89 (d, Ar), 130.49 (d, Ar), 135.87 (d, Ar), 137.74 (d, Ar). ^{31}P NMR: 36.79. Mass Spectrometry: Theoretical m/z 270.1304 Experimental m/z 270.1296.

[0074] **Polymer Synthesis.** In a typical polymerization, 1.0032 g of triethyl-(4-vinylbenzyl)-ammonium chloride monomer was added to a 25-mL, round-bottomed flask with 50/50 (v/v %) DMF/dH_2O, 3.2 mg of AIBN (0.02 mmol, 0.5 mol%), and stir bar at a concentration of 10 wt% solids. The flask was sealed and purged with argon for 30 min to remove oxygen. The polymerization was then conducted at 65 °C for 24 h. The resulting polymer solution was dialyzed against dH_2O for 2 days to remove monomer and DMF and then lyophilized to obtain a white powder in 60-80% yield.

[0075] Phosphonium-containing monomers also allowed the synthesis of a wide variety of copolymers to control charge density, DNA binding affinity, cytotoxicity, and transfection. Ones with improved potential for gene delivery are discussed below.

[0076] Aqueous SEC-MALLS determined the absolute molecular weights of all the ammonium- and phosphonium-containing styrenic-based polymers. The aqueous SEC solvent, i.e. 54/23/23 (v/v/v/ %) water/methanol/acetic acid with 0.1 M sodium acetate, dissolved the polyelectrolytes without aggregation, and the polyelectrolytes successfully eluted from the SEC columns shown in **FIG 2**. *See* Layman, J. M.; Borgerding, E. M.; Williams, S. R.; Heath, W. H.; Long, T. E., *Macromolecules* 2008, *41* (13), 4635-4641. **Table 1** below summarizes the absolute molecular weights for the ammonium and phosphonium polyelectrolytes. **Table 2** below summarizes the absolute molecular weights for the resulting diblock copolymers. Long et al. showed previously that molecular weight variance influences transfection as higher molecular weight PDMAEMA-HCl samples improved gene delivery compared to lower molecular weight PDMAEMA-HCl. *See* Layman, J. M.; Ramirez, S. M.; Green, M. D.; Long, T. E., *Biomacromolecules* 2009, *10* (5), 1244-1252.

[0077] All polymers displayed similar absolute number-average molecular weights ($\overline{M}_n$) with typical polydispersities for conventional free-radical polymerization, therefore, minimizing the influence of molecular weight on transfection efficiency.

[0078] **Table 1**

Sample	$\overline{M}_n$ (kg/mol)	$\overline{M}_w$ (kg/mol)	$\overline{M}_w / \overline{M}_n$
PTEA	230	384	1.67
PTBA	224	389	1.74
PTEP	304	484	1.59
PTBP	254	462	1.82

[0079] **Table 2.** Molecular weight analysis of the macroCTAs and resulting diblock copolymers.

Polymer	M_n (g/mol)	M_w/M_n	DP of A block	DP of B Block
TBP_{61}	21,700	1.24	0	61
OEG_{52}	25,400	1.02	52	0
$OEG_{52}TBP_{27}$	34,900	1.05	52	27
$OEG_{52}TBP_{56}$	45,100	1.10	52	56
$OEG_{52}TBP_{78}$	53,100	1.13	52	78
MPC_{87}	25,600	1.02	87	0
$MPC_{87}TBP_{23}$	33,800	1.04	87	23
$MPC_{87}TBP_{59}$	46,500	1.05	87	59
$MPC_{87}TBP_{81}$	54,400	1.09	87	81

Molecular weight analysis of the polymers and the resulting diblock copolymers.

[0080] **DNA Binding Assay.** Agarose gels were prepared with 0.6 g of agarose in 60 mL of 1X Tris-acetate-EDTA (TAE, Sigma Aldrich) buffer and 6 µL of SYBR Green I (Sigma Aldrich) as a fluorescent stain for DNA. Polyplexes were prepared using 0.2 µL of gWiz-Luc plasmid DNA (1 µg/µL in H_2O, Aldevron) and the required amount of polymer to obtain a desired +/- ratio (positively charged cation in the polymeric vector to negatively charged phosphate in DNA) in a 1X TAE buffer solution (28 µL total volume). The polyplexes were incubated for 30 min at 23 °C and then 7 µL of gel loading buffer (Sigma Aldrich) was added. The polyplexes were loaded onto the gel and metered at 70 V for 90 min. The gels were imaged using a MultiDoc-it™ Digital Imaging System (UVP).

[0081] DNA gel shift assays examined the affinity of all four polymers for DNA: PTEA, PTBA,

PTEP, and PTBP (**FIG. 3**) and for the diblock copolymers **(FIG. 4)**. Typically, researchers utilize N/P ratios to create different polyplexes where N corresponds to protonated/protonatable nitrogens and P corresponds to negatively charged phosphates in the DNA backbone. *See* Chan, P.; Kurisawa, M.; Chung, J. E.; Yang, Y.-Y., *Biomaterials* 2007, *28* (3), 540-549. Here, a similar ratio called a +/- ratio (charge ratio) is defined where + corresponds to quaternized cationic charges and - corresponds to negatively charged phosphates in the DNA backbone. The ammonium polyelectrolytes bound DNA at a +/- ratio of 4 while the phosphonium polyelectrolytes bound DNA at a +/- ratio of 2, which suggested improved DNA binding of phosphonium cations over ammonium cations. Phosphorus, structurally a larger and less electronegative atom than nitrogen, forms larger cations with different electron density distributions compared to ammonium cations. Colby et. al. reported *ab initio* calculations of the charge distribution on the cationic atom and the surrounding carbons for tetrabutylphosphonium and tetrabutylammonium cations. *See* Wang, S.-W.; Liu, W.; Colby, R. H., *Chemistry of Materials* 2011, *23* (7), 1862-1873. Since nitrogen has a higher electronegativity than carbon, the positive charge was distributed on the adjacent carbons (+0.375e for each carbon) while the nitrogen atom had a negative charge (-0.5e). For the tetrabutylphosphonium cation, the charge distribution was reversed with a positive charge on the phosphorus (+1.1e) and a negative charge on the adjacent carbons (-0.025e for each carbon). It is believed that a combination of different charge densities and cation sizes influenced the DNA binding affinity of the polyelectrolytes causing the phosphonium polyelectrolytes with a larger cation and less diffuse positive charge to bind DNA more effectively.

[0082] The DNA gel shift assay also showed differences between the triethyl- and tributyl-based polyelectrolytes. Upon complete DNA binding, the triethyl-containing polyelectrolytes quenched SYBR Green I fluorescence while the tributyl-containing polyelectrolytes required higher +/- ratios to fully quench fluorescence. SYBR Green I must bind to dsDNA to fluoresce green; therefore, the absence of fluorescence indicated tight polyplex formation blocking access to the DNA strands for binding. *See* Simpson, D. A.; Feeney, S.; Boyle, C.; Stitt, A. W., *Molecular Vision* 2000, *6*, 178-83. While not quantitative, the DNA binding gel highlighted an

improved DNA binding ability for shorter alkyl substituent lengths due to either lower steric hindrance or hydrophobicity than the longer alkyl substituent length polyelectrolytes. *See* Yang, Y.; Lee, J.; Cho, M.; Sheares, V. V., *Macromolecules* 2006, *39* (25), 8625-8631.

[0083] **Dynamic Light Scattering.** Dynamic light scattering (DLS) was performed on a Malvern Zetasizer Nano ZS utilizing disposable zeta potential cells to obtain both polyplex diameter and zeta potential. 2.0 μg of gWiz-Luc DNA was added to 0.5 mL of Opti-MEM (Invitrogen) while the appropriate amount of polymer required to reach a desired +/- ratio was added to another vial of 0.5 mL Opti-MEM. The polymer Opti-MEM solution was added to the DNA Opti-MEM solution and incubated for 30 min prior to measurement. All size and zeta potential measurements were repeated in triplicate at 25 °C.

[0084] DLS determined the polyplex diameter and zeta potential for the ammonium and phosphonium polyelectrolytes (**FIG. 5**). All polyelectrolytes except for PTBP condensed DNA into polyplexes near 200 nm or less at +/- ratios of 4 or higher. These polyelectrolytes also exhibited a plateau in their zeta potential without significant change from a +/- ratio of 2 to 10. PTBP polyplexes generated at a +/- ratio of 2 had zeta potentials near neutral, and the polyplexes were greater than 300 nm until a +/- ratio of 6, which was significantly different from the other polyelectrolytes. The zeta potentials of the triethyl-based polyplexes were more positive than the tributyl-based polyplexes due to hydrophobic screening of the cationic charge with longer alkyl chains. *See* Santos, J. L.; Oliveira, H.; Pandita, D.; Rodrigues, J.; Pêgo, A. P.; Granja, P. L.; Tomás, H., *Journal of Controlled Release* 2010, *144* (1), 55-64. Zeta potentials of the free polymers in Opti-MEM (1 mg/mL) showed a similar trend of higher zeta potentials for the triethyl-containing polyelectrolytes. The polyplex diameter and zeta potential for all polyelectrolytes plateaued at higher +/- ratios suggesting that additional polymer remained as free polymer in solution uncomplexed to DNA.

[0085] The effectiveness of the stabilizing block on salt stability was demonstrated using dynamic light scattering (DLS) shown in **FIG. 6**. As an example, poly(EG_9MEMA-*b*-TBP) bound siRNA effectively at a charge ratio of 2 with a hydrodynamic diameter of 86 nm. In the presence of salt, the resulting polyplexes resisted aggregation and only grew slightly in size after 24 h as shown in **FIG. 6** to a hydrodynamic diameter of 138 nm. The initial polyplex size

and the demonstrated salt stability highlight the significant potential for these diblock and triblock copolymers for effective nucleic acid delivery in the presence of salt and serum. The hydrodynamic diameter of the siRNA polyplexes formed using the phosphonium-based diblock copolymers, TBP_{61}, and Jet-PEI challenged under salt conditions for 24 hours to probe colloidal stability is shown in **FIG. 7**. Also the hydrodynamic diameter of the siRNA polyplexes (+/- ratios of 2.0) prepared with the phosphonium-based diblock copolymers at varying concentrations of siRNA is shown in **FIG. 8**.

[0086] **Cell Culture.** Human cervical cancer (HeLa) cells were obtained from ATCC (Manassas, VA) and incubated in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were incubated at 37 °C in 95% humidity with 5% CO_2. All components were obtained from Mediatech.

[0087] **Cytotoxicity Assay.** The 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich) colorimetric assay was utilized to determine polymer cytotoxicity. 100 μL of a 50,000 HeLa cells/mL solution was added to each well of a 96-well plate. The cells were incubated for 24 h at 37 °C with 5% CO_2. Each well was aspirated and rinsed with DMEM prior to application of polymer solutions. Polymer solutions were prepared containing varying amounts of polymer and Opti-MEM to obtain a range of polymer concentrations. Polymer solutions were applied and the cells were incubated for 24 h. After incubation, the polymer solutions were removed and the cells rinsed with 100 μL of DMEM. 100 μL of a 0.5 mg/mL MTT solution in DMEM was added to each well and the cells were incubated for 4 h. The MTT solution was removed using suction and then 100 μL of DMSO was added to dissolve the formazan product. A Molecular Devices SpectraMax M2 was utilized to measure the resulting solutions absorbance at 570 nm. Cell viabilities were compared to control wells containing no polymer to determine the cytotoxicity of the polymers. For polyplex cytotoxicity, 100 μL of a 50,000 HeLa cells/mL solution was added to each well of a 96-well plate and allowed to incubate for 24 h. After aspirating and rinsing with 100 μL of Hank's buffered salt solution (HBSS), 100 μL of the polyplex solution (2 μg DNA/mL and the required polymer amount to obtain the desired +/- ratio in Opti-MEM) were applied and the cells were incubated for 4 h.

The polyplex solutions were removed and 100 µL of complete media was added to each well. After 48 h of incubation, the complete media was aspirated and the cells were rinsed with 100 µL of DMEM. The above procedure involving the addition and incubation of the MTT solution was performed, and the cell viability was analyzed in the same manner as the free polymer MTT cytotoxicity assay.

[0088] MTT colorimetric assays determined the cytotoxicity of both free polymer and polyplexes in HeLa cells (**FIG. 9**). These polyelectrolytes demonstrated high toxicity to HeLa cells primarily due to their high charge density. *See* Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T., *Biomaterials* 2003, *24* (7), 1121-1131. All polyelectrolytes exhibited similar cytotoxicities and were non-toxic to 3 µg/mL with significant toxicity occurring at 5 µg/mL. The polyplexes with these polyelectrolytes were also toxic at the +/- ratio of 2 as shown in **FIG. 10**. Their polyplex cytotoxicity approximately equaled Jet-PEI's cytotoxicity at a +/- ratio of 2. Ammonium- and phosphonium-containing polyplexes exhibited similar cytotoxicities. Cytotoxicities of the diblock copolymer/siRNA polyplexes at a +/- ratio of 2 what shown for siRNA doses varying between 25 and 1000nM in HepaRG cells. All cytotoxicities were similar as shown in **FIG. 11**. DLS analysis suggests that cytotoxicity at higher +/- ratios may result from free polymer in solution.

[0089] **Luciferase Expression Assay.** Polyplexes were formed in Opti-MEM with final gWiz-Luc concentrations of 2.0 µg/mL and the appropriate amount of polymer required to reach the desired +/- ratio. Superfect® and Jet-PEI polyplexes were prepared and applied to cells according to manufacturer specifications. Upon addition of the polymer, the polyplexes were incubated for 30 min prior to application to the cells. Wells in a 24-well plate were seeded with 100,000 HeLa cells 24 h prior to transfection and the cells were rinsed with 300 µL HBSS before polyplex application. 500 µL of each polyplex solution corresponding to 1 µg DNA/well was applied to each well. After 4 h of incubation, the polyplex solutions were aspirated and 500 µL of complete media was added. The cells were incubated for a total of 48 h after transfection. The media was aspirated at 48 h, the cells were rinsed with 300 µL of PBS, and then 120 µL of a 1 X lysis buffer (Promega) was added. The cells were incubated for 30 min at 37 °C then subjected to multiple freeze-thaw cycles to fully lyse the cells. A Promega luciferase assay kit

was utilized according to the manufacturer's protocol to determine the luciferase activity. Protein concentration was determined using a Pierce BCA Protein Assay kit according to the enclosed directions. Gene expression was reported as relative light units per mg of cell protein lysate (RLU/mg). Experiments were repeated twice in quadruplicate. Serum transfections were performed similarly to the above except 400 μL of complete media was added to each well and then 100 μL of the polyplex solutions (10 μg DNA/mL) in Opti-MEM were added for a total of 1 μg DNA/well.

[0090] The endocytic inhibition luciferase assay followed the same procedure as the above serum-free luciferase transfection except for the initial pre-incubation of the cells with the inhibitory drugs. Prior to polyplex addition, 500 μL of genistein (100 μg/mL in Opti-MEM), methyl β-cyclodextrin (20 mg/mL in Opti-MEM), and amantadine (2 mM in Opti-MEM) solutions were applied to individual wells. After 1 h incubation, 500 μL of the polyplex solution (+/- ratio of 4, 2μg DNA/mL in Opti-MEM) was applied to each well for a total transfection volume of 1 mL and half the initial inhibitory drug concentrations. The serum-free luciferase transfection protocol was then followed from the point of the polyplex incubation. Luciferase expressions from each drug inhibition were compared to control transfection wells with no drug inhibition.

[0091] Serum-free luciferase expression for each styrenic polyelectrolyte compared to negative and positive controls determined their efficacy for gene transfection and protein expression (**FIG. 12**). Both PTEA and PTEP displayed poor transfection efficiency compared to Superfect® and Jet-PEI while PTBA and PTBP exhibited excellent transfection efficiency similar to Superfect®. In fact, PTBP showed higher transfection efficiency than PTBA and Superfect® ($p < 0.05$). Stayton et al. focused on the synthesis of diblock copolymers containing a cationic block for siRNA condensation and a terpolymer amphiphilic block for endosomal release. *See* Convertine, A. J.; Diab, C.; Prieve, M.; Paschal, A.; Hoffman, A. S.; Johnson, P. H.; Stayton, P. S., *Biomacromolecules* 2010, *11* (11), 2904-2911; and Convertine, A. J.; Benoit, D. S. W.; Duvall, C. L.; Hoffman, A. S.; Stayton, P. S., *Journal of Controlled Release* 2009, *133* (3), 221-229. When Stayton incorporated higher mol% of n-butyl methacrylate into the endosomolytic block, siRNA delivery improved and the hemolytic activity of the diblock copolymers increased. Upon endocytosis and endosomal acidification, the terpolymer block became cationic enabling the

block to presumably electrostatically associate with the endosome membrane. Upon association, the hydrophobic n-butyl methacrylate in the endosomolytic block inserted into the hydrophobic membrane ultimately disrupting and lysing the endosome membrane achieving polyplex escape. Both PTEP and PTBP polyplexes (+/- ratio of 4) successfully entered the cell as determined using Cy5-labeled DNA and wide-field fluorescence optical microscopy (**FIG. 13**). The larger and higher intensity polyplexes presumably correlated to aggregated intracellular polyplexes. Since the triethyl-based polyplexes were successfully taken up into HeLa cells, their poor transfection resulted from other intracellular mechanisms preventing transfection such as endosomal escape or DNA release. In polyelectrolytes of the invention, it is expected that the tributyl-containing polyelectrolytes with longer, more hydrophobic, alkyl chains aided in membrane destabilization and endosomal release similar to Stayton's endosomolytic block. Additionally, the DNA gel shift assays demonstrated tighter DNA binding potentially leading to reduced DNA release and transfection for the triethyl-based polyplexes. *See* Song, Y.; Wang, H.; Zeng, X.; Sun, Y.; Zhang, X.; Zhou, J.; Zhang, L., *Bioconjugate Chemistry* 2010, *21* (7), 1271-1279.

[0092] When comparing PTBA and PTBP, PTBP showed enhanced DNA delivery over PTBA at all +/- ratios ($p < 0.05$). PTBP also exhibited significantly improved gene transfection over Superfect® at +/- ratios of 4 and 6 ($p < 0.05$). GFP transfection microscopy results (+/- ratio of 4) qualitatively correlated with the quantitative luciferase expression results. Results demonstrated a marked improvement in nonviral gene delivery upon modification of the cationic center from an ammonium to a phosphonium. Endo et al. focused on the investigation of the antibacterial properties of PTBA and PTBP and the influence of the cation on antibiotic activity. *See* Kanazawa, A.; Ikeda, T.; Endo, T., *Journal of Polymer Science Part A: Polymer Chemistry* 1993, *31* (2), 335-343; and Kanazawa, A.; Ikeda, T.; Endo, T., *Journal of Applied Polymer Science* 1994, *53* (9), 1245-1249. Cationic polymeric biocides primarily function through cellular membrane destabilization resulting in cellular death. PTBP exhibited improved antibiotic activity over PTBA presumably due to improved cellular membrane destabilization. Results comparing the triethyl-based and tributyl-based polyelectrolytes also point to the importance of endosomolytic activity and the improved transfection of PTBP over PTBA may result from improved endosomolytic activity.

[0093] All polyelectrolytes exhibited poor transfection in serum-containing media as shown in **FIG. 14**. Since the vectors are fully charged, they generated polyplexes with large, positive zeta potentials causing significant protein aggregation rendering the vectors ineffective as transfection agents in serum. *See* Dash, P. R.; Read, M. L.; Barrett, L. B.; Wolfert, M. A.; Seymour, L. W., *Gene Therapy* 1999, *6*, 643-650.

[0094] Endocytic inhibition of either clathrin-mediated or caveolae-mediated endocytosis elucidated the preferred method of cellular uptake for both PTBA and PTBP. Pack et al. previously utilized the same endocytic inhibitors (genistein, methyl β-cyclodextrin, and amantadine) to elucidate the preferred endocytic pathway for PEI. *See* Gabrielson, N. P.; Pack, D. W., *Journal of Controlled Release* 2009, *136* (1), 54-61. They showed that PEI's primary avenue of effective delivery was caveolae-mediated endocytosis. A similar procedure as Pack et al. was followed with some modifications to inhibit caveolae-mediated endocytosis using genistein or methyl β-cyclodextrin and clathrin-mediated endocytosis using amantadine. Relative luciferase expressions were compared to a positive control with the polyelectrolyte vector in the absence of endocytic inhibition (**FIG. 15**). Genistein and methyl β-cyclodextrin (caveolae-mediated endocytosis inhibitors) knocked down luciferase expression showing PTBA and PTBP efficiently delivered through caveolae-mediated endocytosis ($p < 0.05$) while amantadine (clathrin-mediated endocytosis inhibitor) improved gene transfection compared to the control ($p < 0.05$). Improved transfection during inhibition of clathrin-mediated endocytosis resulted from increased cellular uptake of polyplexes through more efficient endocytic pathways such as caveolae-mediated endocytosis. PTBA and PTBP more efficiently transfected HeLa cells when cellular uptake occurred through caveolae-mediated endocytosis versus clathrin-mediated endocytosis.

[0095] **Wide-Field Optical Microscopy. Polyplex Uptake.** Cy5-labeled gWiz-Luc plasmid (0.1 μg/μL in H_2O) was diluted in Opti-MEM to a concentration of 4.0 μg/mL. Simultaneously, the polyelectrolytes were diluted in Opti-MEM to a final concentration corresponding to a +/- ratio of 4. These solutions were incubated for 10 min before adding the polymer to the pDNA and then incubated at 23 ^{o}C for 30 min. HeLa cells were plated into 24 well plates at a cell density of 100,000 cells/well 24 h prior to polyplex exposure. The cells were rinsed with HBSS and 0.5

mL of transfection solution was added to each well. The cells were incubated at 37 °C and 5% CO_2 for 2 h. Cellular nuclei were stained through the addition of 1 μL of 4',6-diamidino-2-phenylindole (DAPI, 1 μg/μL in PBS) to the transfection solution and incubated for 10 min at 37 °C. The cells were then rinsed twice with PBS, fixed with 0.5 mL paraformaldehyde (2 wt% in PBS) for 10 min at 37 °C, and cellular membranes were permeabilized with 0.5 mL TritonX-100 (0.1 vol% in PBS) for 10 min at 37 °C. The cells were rinsed with PBS, and the cellular F-Actin were stained with 0.5 mL of Alexa Fluor 488 phalloidin (5 U/mL in PBS) for 10 min at 37 °C. The cells were rinsed with PBS, and then stored in PBS. Images were acquired using Cy5, UV-2EC, and F/EGFP fluorescence filters using a Nikon Eclipse TE2000-U inverted microscope equipped with a Nikon C-HGFI Intensilight light source and Nikon DS-Qi,Mc B&W CCD camera.

[0096] **GFP Expression.** gWiz-GFP plasmid (1 μg/μL in H_2O) was diluted in Opti-MEM to a concentration of 4.0 μg/mL. Simultaneously, the vectors were diluted in Opti-MEM to a final concentration corresponding to a +/- ratio of 4. These solutions were incubated for 10 min before adding the polymer to the pDNA and then incubated at 23 °C for 30 min. Superfect® and Jet-PEI polyplexes were prepared according to the manufacturer's suggestion. HeLa cells were plated into 24 well plates at a cell density of 100,000 cells/well 24 h prior to polyplex exposure. The cells were rinsed with HBSS and 0.5 mL of transfection solution was added to each well. The cells were incubated at 37 °C and 5% CO_2 for 4 h. The transfection media was then removed and replaced with complete DMEM, and the cells were incubated at 37 °C, 5% CO_2 for 48 h. After 48 h, cellular nuclei were stained through the addition of 1 μL DAPI (1 μg/μL in PBS) to the transfection solution and incubated for 10 min at 37 °C. The cells were then rinsed twice with PBS, fixed with 0.5 mL paraformaldehyde (2 wt% in PBS) for 10 min at 37 °C, and the cellular membrane was permeabilized with 0.5 mL TritonX-100 (0.1 vol% in PBS) for 10 min at 37 °C. The cells were rinsed with PBS, and the cellular F-Actin was stained with 12.5 μL Alexa Fluor 647 phalloidin (200 U/mL in methanol) for 10 min at 37 °C. The cells were rinsed with PBS, and then stored in PBS. Images were acquired using Cy5, UV-2EC, and F/EGFP fluorescence filters using a Nikon Eclipse TE2000-U inverted microscope equipped with a Nikon C-HGFI Intensilight light source and Nikon DS-Qi,Mc B&W CCD camera.

[0097] **Degradation Assays.** RNase degradation assays were performed on the polyplexes at a

ratio of +/- 2. $OEG_{52}TBP_{78}$ and $MPC_{87}TBP_{81}$ were used as representative samples demonstrating the protection of siRNA from nuclease degradation as shown in **FIG. 16**.

[0098] **Example II.**

[0099] In this embodiment, RAFT polymerization successfully controlled the synthesis of phosphonium-based AB diblock copolymers for nonviral gene delivery. A stabilizing block of either oligo(ethylene $glycol_9$) methyl ether methacrylate or 2-(methacryloxy)ethyl phosphorylcholine provided colloidal stability, and the phosphonium-containing cationic block of 4-vinylbenzyltributylphosphonium chloride induced electrostatic nucleic acid complexation. RAFT polymerization generated well-defined stabilizing blocks (M_n = 25,000 g/mol) and subsequent chain extension synthesized diblock copolymers with DPs of 25, 50, and 75 for the phosphonium-containing block. All diblock copolymers bound DNA efficiently at +/- ratios of 1.0 in H_2O, and polyplexes generated at +/- ratios of 2.0 displayed hydrodynamic diameters between 100 to 200 nm. The resulting polyplexes exhibited excellent colloidal stability under physiological salt or serum conditions, and maintained constant hydrodynamic diameters over 24 h. Cellular uptake studies using Cy5-labeled DNA confirmed reduced cellular uptake in COS-7 and HeLa cells, and consequently, resulted in low transfection in these cell lines. Serum transfection in HepaRG cells, which are a predictive cell line for *in vivo* transfection studies, showed successful transfection using all diblock copolymers with luciferase expression on the same order of magnitude as Jet-PEI. All diblock copolymers exhibited low cytotoxicity (>80% cell viability). Promising *in vitro* transfection and cytotoxicity results suggest *in vivo* applicability of these phosphonium-based diblock copolymer delivery vehicles.

[00100] As shown in greater detail below, embodiments of the invention provide phosphonium-containing AB diblock copolymers for enhanced nucleic acid delivery where the A block provides colloidal stability and the phosphonium-based B block efficiently complexes pDNA to generate core-shell nanoparticles. The A block consisted of either oligo(ethylene $glycol_9$) methyl ether methacrylate (OEG) or 2-methacryloyloxyethyl phosphorylcholine (MPC) since both polymeric units exhibit protein resistance and extended circulation times due to steric shielding of the nanoparticles. *See* Monge, S.; Canniccioni, B.; Graillot, A.; Robin, J.-J. *Biomacromolecules* 2011, *12*, 1973; and Knop, K.; Hoogenboom, R.; Fischer, D.; Schubert, U. S.

Angew. Chem. Int. Ed. 2010, *49*, 6288. RAFT polymerization controlled OEG and MPC to yield macroCTAs with similar number-average molecular weights, and subsequent chain extension with 4-vinylbenzyltributylphosphonium chloride (TBP) provided AB diblock structures with variable block lengths. DNA binding studies probed the effective nucleic acid binding of the TBP block, and dynamic light scattering (DLS) examined the colloidal stability of the polyplexes under physiological salt and serum conditions. Transfection studies assessed the ability of the diblock copolymer polyplexes to deliver DNA to three cell lines (COS-7, HeLa, and HepaRG cells) and elucidated the cytotoxicities of the diblock copolymer polyplexes.

[00101] **Materials.** OEG (485 g/mol) was purchased from Sigma Aldrich and purified using a basic alumina column prior to use. MPC was purchased from Polysciences, dissolved in water, and washed with diethyl ether to remove the inhibitor. 4,4'-Azobis(4-cyanopentanoic acid) (V-501) was purchased from Sigma Aldrich and recrystallized twice from methanol prior to use. TBP and 4-cyano-4-(propylsulfanylthiocarbonyl)sulfanylpentanoic acid (CPP) were synthesized as previously reported in literature. *See* Hatakeyama 2009. All solvents were obtained from Sigma Aldrich and used as received.

[00102] **Analytical Methods.** ^{1}H NMR spectroscopy was performed on a Varian Inova 400 in D_2O. Aqueous size exclusion chromatography (SEC) was utilized to determine the number-average molecular weight (M_n) and polydispersity indices (PDIs) for the macroCTAs and the diblock copolymers using an aqueous eluent of 54/23/23 (v/v/v %) water/methanol/acetic acid with 0.1 M sodium acetate at a flow rate of 0.8 mL/min, a Waters 1515 isocratic HPLC pump, a Waters 717plus autosampler, two Waters ultrahydrogel linear columns, one Waters ultrahydrogel 250 column, a Wyatt MiniDAWN, and a Waters 2414 refractive index detector. An Optilab T-rEX refractometer (λ = 658 nm) was used to measure d*n*/dc values offline for determination of absolute molecular weights.

[00103] **Polymer Synthesis.** A solution of CPP (82.3 mg, 0.297 mmol), OEG (10.8 g, 22.3 mmol), and V-501 (16.8 mg, 0.060 mmol) in 90 mL of DMSO was added to a 250-mL, round-bottomed flask equipped with a magnetic stir bar to synthesize the OEG_{52} macroCTA. The solution was sparged with nitrogen for 30 min and then placed in a preheated oil bath at 70 °C

for 3 h. The OEG_{52} macroCTA was recovered as viscous yellow oil after dialysis against water (pH 4 - 5) and lyophilization.

[00104] The MPC_{87} macroCTA was synthesized according to a similar procedure as above. Briefly, MPC (5.52 g, 18.7 mmol), CPP (51.8 mg, 0.187 mmol), and V-501 (5.2 mg, 0.019 mmol) were dissolved in 70 mL of 4:1 acetate buffer (pH 5.2)/DMSO in a 250-mL, round-bottomed flask equipped with a magnetic stir bar. The solution was sparged with nitrogen for 30 min and then placed in a preheated oil bath at 70 °C for 3.5 h. After dialysis against water (pH 4 - 5) and lyophilization, the MPC_{87} macroCTA was recovered as a white powder.

[00105] The two macroCTAs were subsequently chain extended with TBP to yield diblock copolymers following a similar procedure. As an example, TBP (0.350 g, 0.986 mmol), OEG_{52} (0.250 g), and V-501 (2.8 mg, 9.86 x 10^{-3} mmol) were dissolved in 4 mL of 1:1 acetate buffer (pH 5.2)/DMSO and was added to a 10-mL, round-bottomed flask equipped with a magnetic stir bar. After sparging with nitrogen for 30 min, the reaction was allowed to proceed at 70 °C for 2.5 h. The product was dialyzed against DI water (pH 4 - 5), lyophilized, and recovered as a white powder.

[00106] **DNA Binding Assay.** gWiz-Luc plasmid DNA (0.2 μL of 1 μg/μL in H2O, Aldevron) was mixed with the required amount of polymer to obtain the desired +/- ratio (ratio of positively charged phosphonium cation on the polymeric vector to negatively charged phosphate on DNA) in H_2O (28 μL total volume). After an incubation time of 30 min at room temperature, 7 μL of gel loading buffer (30% glycerol in H_2O) was added. The polyplex solution (20 μL) was loaded onto an agarose gel (0.6 g of agarose and 6 μL of SYBR Green I (Sigma Aldrich) in 60 mL of 1× Tris-acetate-EDTA) and electrophoresed at 70 V for 30 min. A MultiDoc-it Digital Imaging System (UVP) was utilized to image the agarose gels.

[00107] **Dynamic Light Scattering.** A Malvern Instruments Zetasizer Nano ZS (633 nm) was used to measure the polyplex sizes. gWiz-Luc DNA (2 μg in 100 μL of H_2O) was incubated for 30 min at room temperature with the required amount of each diblock copolymer in 200 μL of H_2O to give a +/- ratio of 2.0. After the incubation period, 800 μL of H_2O, Dulbecco's modified Eagle's media (DMEM), or DMEM supplemented with 10% fetal bovine serum (FBS) was added to assess the colloidal stability of the polyplexes in salt- and serum-containing

media. The particle sizes were then measured at time intervals of 0, 1, 2, 4, and 24 h after dilution. The zeta potential measurements were also performed on the samples diluted with H_2O using a Malvern Instruments Zetasizer Nano ZS. All measurements were performed in triplicate and the data are represented as the mean ± the standard deviation.

[00108] **Cell culture.** HepaRG cells (Life Technologies, Carlsbad, CA) were maintained in supplemented Williams Medium E (65 mL HepaRG maintenance supplement, 5 mL GlutaMAX-I™, and 500 mL Williams Medium E) (Invitrogen). Cells were incubated in 95% humidity with 5% CO_2 at 37 °C.

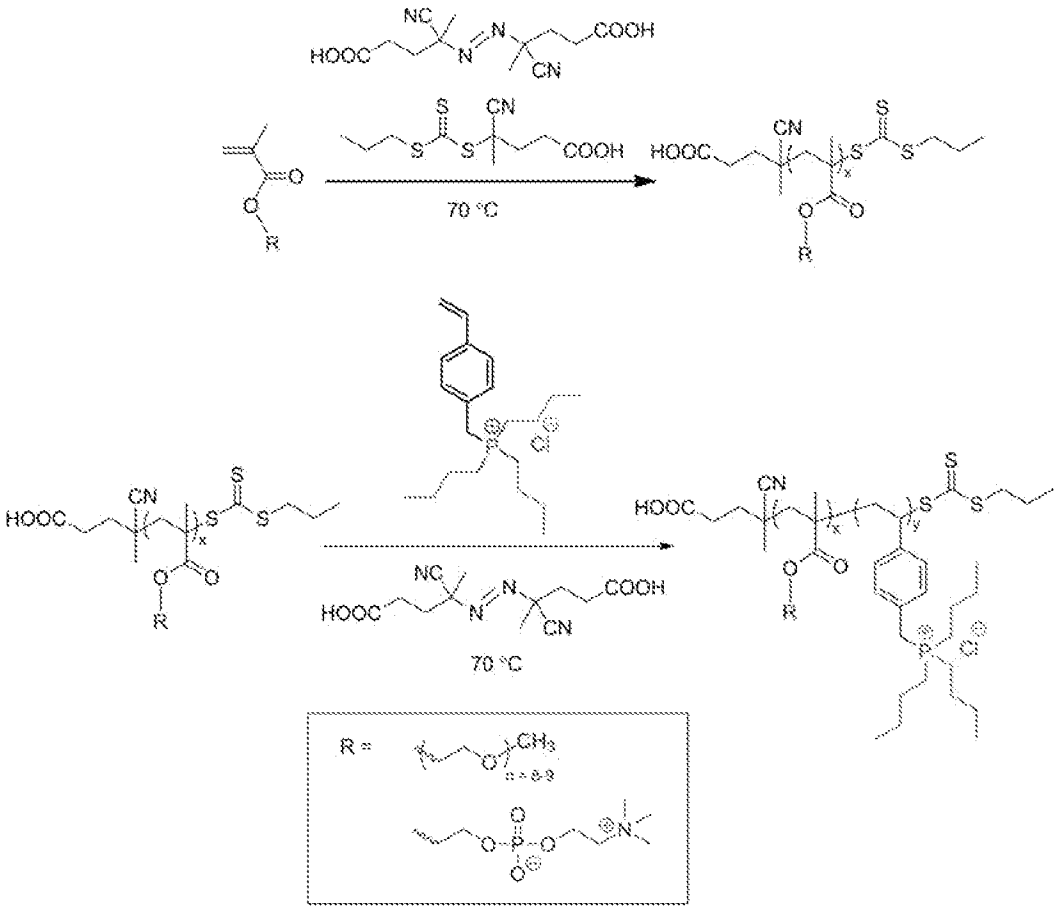
[00109] **Luciferase Expression and Cytotoxicity Assay.** Prior to transfection, HepaRG cells were plated on 24-well plates at a density of 100,000 cells per well, approximately 95% confluency. Cells were incubated in 400 µL of supplemented Williams Medium E for 24 h at 37 °C in a 5% CO_2 environment. Control reagents were formulated with pDNA based upon their recommended protocols. Polymers were formulated with pDNA at a +/- ratio of 2.0 in 100 µL of Williams Medium E (no supplements). Solutions of pDNA (gWiz-Luciferase, Aldevron, Fargo, ND) complexes for each polymer were added in triplicate to corresponding wells (1.5 µg pDNA per well, V_t=500 µL). After 48 h of incubation, the media was evacuated from wells and cells were lysed in 100 µL Cell Lysis Buffer (Promega, Madison, WI). Cell lysates were deposited on 96-well plates and analyzed for luciferase activity on a luminometer plate reader (Promega GloMax® 96 Microplate Luminometer). Protein lysates were stained using a Pierce BCA Protein Assay kit according to manufacturer protocols. Cell viability was determined from sample absorbance relative to the cells only control. Student's t test analysis of the luciferase transfection results was performed to determine statistical significance ($p < 0.02$).

[00110] **Wide-field Fluorescence Optical Microscopy.** HepaRG cells were plated at a density of 500,000 cells per well on 6-well plates 24 h prior to transfection and polyplexes were formed using the same methods reported above in the luciferase assay instead using a plasmid encoding enhanced green florescent protein (EGFP-C1, 7.5 µg total per well). Transfection conditions were consistent with those reported above. After 48 h, cells were directly imaged on an AMG Evos-Fl microscope for both differential interference contrast images and for GFP florescence (ex. 488 nm, em. 509 nm).

[00111] **Polymer Synthesis and Characterization.** Controlled radical polymerization enables the synthesis of well-defined block copolymers where each block serves a specific function in nucleic acid delivery. *See* Chu, D. S. H.; Schellinger, J. G.; Shi, J.; Convertine, A. J.; Stayton, P. S.; Pun, S. H. *Accounts of Chemical Research* 2012; and Xu, F. J.; Yang, W. T. *Progress in Polymer Science* 2011, *36*, 1099. Chain transfer agents, such as dithioesters and trithiocarbonates, utilized in RAFT polymerization enable controlled free radical polymerization through degenerative chain transfer. *See* Moad, G.; Rizzardo, E.; Thang, S. H. *Australian Journal of Chemistry* 2009, *62*, 1402. RAFT polymerization allows a modular design in block chemical composition, block molecular weight, and end group functionality to optimize delivery vehicles. *See* York, A. W.; Kirkland, S. E.; McCormick, C. L. *Advanced Drug Delivery Reviews* 2008, *60*, 1018; Scales, C. W.; Huang, F.; Li, N.; Vasilieva, Y. A.; Ray, J.; Convertine, A. J.; McCormick, C. L. *Macromolecules* 2006, *39*, 6871; and York, A. W.; Zhang, Y.; Holley, A. C.; Guo, Y.; Huang, F.; McCormick, C. L. *Biomacromolecules* 2009, *10*, 936.

[00112] Initially, it was desired to examine the influence of two A block structures on colloidal stability and ultimately gene transfection for a series of diblock copolymers, which included a PEG brush block (OEG) and a zwitterionic block (MPC). Similar A block molecular weights were synthesized which resulted in different degrees of polymerization (DP) due to the disparity in repeat unit molecular weight for each monomer. RAFT polymerization successfully controlled the molecular weights using a trithiocarbonate chain transfer agent (CTA) and V-501 (an azo initiator) to achieve M_n = 25,000 g/mol and PDIs < 1.10 for each macroCTA as shown in **Table 2** above in Example I.

[00113] Chain extension of the macroCTAs with TBP, as shown in **Scheme 4**, achieved the desired AB diblock copolymers.



[00114]

Scheme 4. RAFT polymerization of OEG and MPC with subsequent chain extension using TBP to synthesize phosphonium-containing diblock copolymers OEG_xTBP_y and MPC_xTBP_y

[00115] The TBP block DP was varied to investigate the influence of the A/B block ratio on gene transfection. **Figure 17** highlights the monotonic shift in elution time for each peak as the molecular weight of the TBP block increased for the $OEG_{52}TBP_y$ and $MPC_{87}TBP_y$ diblock copolymers. **Table 2** (as provided above in Example I) summarizes the *absolute* molecular weights from aqueous SEC for the final diblock copolymer compositions. All diblock copolymers exhibited PDIs < 1.20 demonstrating polymerization control using the RAFT process. Three DPs (25, 50, and 75) of the TBP block were targeted and the resulting OEG- and MPC-based diblock copolymers closely matched the target DPs. In addition, RAFT polymerization allowed the controlled synthesis of a TBP_{61} homopolymer to achieve a DP similar to the $OEG_{52}TBP_{56}$ and $MPC_{87}TBP_{59}$ diblock copolymers as a control for DNA binding and colloidal stability assays.

[00116] **DNA Binding and Colloidal Stability.** Initial studies focused on the DNA complexation and compaction of these novel diblock copolymers using DNA gel shift assays and

DLS. TBP_{61} and all the diblock copolymers bound DNA completely at a +/- ratio of 1.0 (**Figure 18**). There was not a detectable effect on the onset of DNA binding as a function of the A block composition or TBP block length. DLS confirmed DNA binding and compaction, forming polyplexes (sizes of 100 – 200 nm) in water at +/- ratios of 2.0 for all diblock copolymers. Zeta potentials for all diblock copolymers except $MPC_{87}TBP_{23}$ exhibited positive values between 20 – 35 mV; $MPC_{87}TBP_{23}$ demonstrated a slightly negative zeta potential of -3 mV presumably due to the shorter TBP block and zwitterionic nature of the MPC block.

[00117] *In vivo* nucleic acid delivery requires a sufficiently small polyplex (< 200 nm) to enable long circulation times and cellular uptake. Scholz, C.; Wagner, E. *Journal of Controlled Release*, *In Press*. In addition, polyplexes must resist salt and serum induced aggregation. In sharp contrast, nonviral gene delivery vehicles for *in vitro* applications typically rely on poor colloidal stability to induce polyplex aggregation and sedimentation onto the cell monolayer, which enables efficient cellular uptake and transfection. Nguyen, J.; Szoka, F. C. *Accounts of Chemical Research* 2012. DLS enabled kinetic analysis of the polyplex aggregation in serum-free and serum-containing media conditions to investigate the colloidal stability of the diblock copolymers, TBP_{61}, and Jet-PEI. Smith, A. E.; Sizovs, A.; Grandinetti, G.; Xue, L.; Reineke, T. M. *Biomacromolecules* 2011, *12*, 3015. Serum-free DMEM effectively mimics the physiological salt and nutrient conditions required for cell growth, and serum-containing media contains anionic proteins similar to those found in blood, which may electrostatically associate with polyplexes and induce aggregation. Jeong, J.; Park, T.; Kim, S. *Pharmaceutical Research* 2011, *28*, 2072.

[00118] Physiological salt conditions induce polyplex aggregation through neutralization of the polyplex surface charge resulting in neutral polyplexes that aggregate. Prevette 2008. **Figure 19** depicts the hydrodynamic diameter of each polyplex prepared using the diblock copolymers (+/- ratio of 2.0), TBP_{61} (+/- ratio of 2.0), and Jet-PEI (N/P = 5.0). TBP_{61} initially produced polyplexes with sizes of 77 nm in water; upon dilution into serum-free DMEM, the TBP_{61} polyplexes rapidly increased 1300% in size to 1051 nm after 2 h under physiological salt conditions. TBP_{61} polyplexes precipitated from serum-free DMEM conditions after 2 h. In contrast, the phosphonium-based diblock copolymers generated polyplexes (~110 nm in size), which remained colloidally stable without aggregation under physiological salt conditions for

24 h suggesting the efficiency of the A block to generate stable polyplex colloids. Neither A block composition nor TBP block length significantly influenced the polyplex colloidal stability under physiological salt conditions. Jet-PEI, a popular positive control for nonviral gene delivery, demonstrated reduced colloidal stability, increasing in size from 63 nm to 606 nm over 2 h in serum-free DMEM with subsequent sedimentation after 2 h.

[00119] Colloidal stability assays also probed the diblock copolymer polyplexes' resistance to serum-induced polyplex aggregation. Anionic serum proteins stimulate polyplex aggregation through electrostatic association to the polyplex surface causing charge neutralization and consequently polyplex aggregation. *See* de Wolf 2005. **Figure 20** highlights the change in the hydrodynamic diameters of the diblock copolymer (+/- ratio of 2.0), TBP_{61} (+/- ratio of 2.0), and Jet-PEI (N/P = 5.0) polyplexes after dilution into serum-containing DMEM. TBP_{61} polyplexes rapidly increased 250% in hydrodynamic diameter to 190 nm and remained ~190 nm in size over 24 h. Substantial increase in polyplex size did not occur after the initial growth, presumably due to anionic protein association to the TBP_{61} polyplex surface providing an overall negative surface charge and therefore providing colloidal stability. Ogris, M.; Steinlein, P.; Kursa, M.; Mechtler, K.; Kircheis, R.; Wagner, E. *Gene Ther.* 1998, *5*, 1425. All diblock copolymer polyplexes resisted serum-induced polyplex aggregation and remained a similar size over a 24 h period without an obvious difference in the efficacy of the A block composition or TBP block length on serum colloidal stability. Preferably, polyplexes of embodiments of the invention exhibit no change or relatively little change in hydrodynamic diameter over a 24 h period. For example, after dilution in DMEM (serum-containing or not) polyplexes of the invention may exhibit an increase in hydrodynamic diameter of no more than about 100%, or about 150%, or about 200%, or about 250%. Polyplexes of the invention that exhibit greater increases in hydrodynamic diameter are still viable options for drug delivery, however, it is expected such polyplexes will have a reduction in one or more beneficial characteristics as compared with polyplexes having a smaller change in hydrodynamic diameter. As such, it is expected that some embodiments of the present invention may have a change in hydrodynamic diameter ranging from about 100-2000% and still may be viable for some applications. Both OEG and MPC stabilizing blocks prevented polyplex aggregation

presumably through steric repulsion of the brush PEG block or zwitterionic block, respectively. Jet-PEI exhibited diminished colloidal stability under serum-containing DMEM conditions with a rapid 1200% growth in polyplex size over 2 h to 744 nm.

[00120] **Cytotoxicity and Transfection.** Three different cell lines (COS-7, HeLa, and HepaRG) were focused on to investigate the transfection efficiency and cytotoxicity of the phosphonium-based diblock copolymers. COS-7 (African green monkey kidney fibroblasts) and HeLa (Human cervical cancer epithelia) cells are two typical cell lines utilized in the nonviral gene delivery field to elucidate the *in vitro* transfection capabilities of novel delivery vehicles. Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *Proceedings of the National Academy of Sciences* 1995, *92*, 7297. HepaRG cells are terminally differentiated human hepatocytes, which grow with similar hepatic morphologies and enzymatic activity. Kanebratt, K. P.; Andersson, T. B. *Drug Metabolism and Disposition* 2008, *36*, 137. *In vitro* drug metabolism studies typically utilize HepaRG cells as a predictive metric for drug metabolism and hepatic toxicity concerns preceding *in vivo* testing. Kanebratt, K. P.; Andersson, T. B. *Drug Metabolism and Disposition* 2008, *36*, 1444. Also investigated were the cytotoxicity and transfection efficacy of the diblock copolymers in HepaRG cells to provide justification for *in vivo* administration. McGill, M. R.; Yan, H.-M.; Ramachandran, A.; Murray, G. J.; Rollins, D. E.; Jaeschke, H. *Hepatology* 2011, *53*, 974.

[00121] Initial transfection experiments focused on the delivery of luciferase-encoded pDNA (1 μg/well) to HeLa and COS-7 cells at low +/- ratios of 2.0 and 4.0. Ideally, *in vivo* nucleic acid delivery vehicles must bind and deliver DNA efficiently under serum conditions at low charge ratios to minimize cytotoxicity and noncomplexed polymer in solution. All diblock copolymers failed to efficiently deliver pDNA to HeLa and COS-7 cells at +/- ratios of 2.0 and 4.0 compared to the positive control Jet-PEI. Wide-field optical fluorescence microscopy studies focused on the cellular uptake of Cy5-labeled DNA under serum-free conditions did not show cellular uptake of diblock copolymer polyplexes after 4 h; however, TBP_{61} and Jet-PEI polyplexes demonstrated cellular uptake within 4 h. While steric stabilizing blocks such as OEG and MPC provide superior colloidal stability, these functionalities also hinder cellular uptake through screening of the cationic charge and steric repulsion against the cellular membrane. Zhang, X.;

Pan, S.-R.; Hu, H.-M.; Wu, G.-F.; Feng, M.; Zhang, W.; Luo, X. *Journal of Biomedical Materials Research Part A* 2008, *84A*, 795. The poor colloidal stability of TBP_{61} and Jet-PEI compared to the diblock copolymers also presumably aided in cellular uptake through aggregation and sedimentation of the polyplexes onto the cell monolayer inducing cellular uptake. Ogris, M.; Steinlein, P.; Kursa, M.; Mechtler, K.; Kircheis, R.; Wagner, E. *Gene Ther.* 1998, *5*, 1425; De Smedt, S. C.; Demeester, J.; Hennink, W. E. *Pharmaceutical Research* 2000, *17*, 113; and Luo, D.; Saltzman, W. M. *Nat Biotech* 2000, *18*, 893.

[00122] Dose-dependent GFP expression studies performed in HepaRG cells elucidated the optimal DNA/well concentration required for successful transfection in serum-containing HepaRG media. HepaRG cells were dosed at various GFP-encoded pDNA concentrations (0.35 μg DNA/mL to 14 μg DNA/mL) using the diblock copolymers (+/- ratios of 2.0). **Figure 21** shows representative wide-field fluorescence optical microscopy for all the diblock copolymers at a dosage of 1.4 μg/well, which revealed the largest proportion of GFP expressing HepaRG cells compared to other DNA dosages. Therefore, we performed luciferase transfections at 1.5 μg DNA/mL to quantify nucleic acid delivery compared to the GFP expression.

[00123] HepaRG luciferase transfections established a significant influence of cell type on the transfection results for the diblock copolymers. Although the diblock copolymers failed to transfect COS-7 and HeLa cells, the diblock copolymers (+/- ratio of 2.0) delivered DNA effectively on the same order of magnitude as Jet-PEI (N/P = 5.0) in HepaRG cells (**Figure 22**). The diblock copolymers all performed statistically higher than cells and DNA only ($p < 0.02$), however, the diblock copolymers did not demonstrate a statistically significant trend in transfection capability for different A block compositions or TBP block lengths.

[00124] Overall, the diblock copolymers demonstrated selective *in vitro* transfection in HepaRG cells with minimal cytotoxicity. In summary, in this embodiment, Example II, the first synthesis and characterization of phosphonium-based diblock copolymers for nonviral nucleic acid delivery are provided. RAFT polymerization successfully controlled the synthesis of $OEG_{52}TBP_y$ and $MPC_{87}TBP_y$ diblock copolymers with M_n's of 25,000 g/mol for the stabilizing A block and variable DPs of the TBP block (25, 50, and 75). All diblock copolymers and the TBP_{61} homopolymer initially bound DNA at a +/- ratio of 1.0. The $OEG_{52}TBP_y$ and $MPC_{87}TBP_y$ diblock

copolymers at +/- ratios of 2.0 exhibited enhanced colloidal stability compared to TBP_{61} (+/- ratio of 2.0) and Jet-PEI (N/P = 5.0) under physiological salt and serum conditions due to the stabilizing A block. Serum transfections in COS-7, HeLa, and HepaRG cells demonstrated cell specificity for cellular uptake and transfection. Cellular uptake studies demonstrated poor cellular uptake for the diblock copolymers, which led to inadequate transfection in HeLa and COS-7 cells. GFP microscopy studies in HepaRG cells showed successful transfection in a dose-dependent manner and all diblock copolymers delivered pDNA on the same order of magnitude as Jet-PEI with minimal cytotoxicity. Equipped with the teachings in this specification, one of skill in the art will know how to and the type of blocks to prepare with different A block molecular weights in order to optimize colloidal stability and improve cellular uptake to improve transfection across multiple cell lines.

[00125] The present invention has been described with reference to particular embodiments having various features. It will be apparent to those skilled in the art that various modifications and variations can be made in the practice of the present invention without departing from the scope or spirit of the invention. One skilled in the art will recognize that these features may be used singularly or in any combination based on the requirements and specifications of a given application or design. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention. It is intended that the specification and examples be considered as exemplary in nature and that variations that do not depart from the essence of the invention are intended to be within the scope of the invention.

[00126] Therefore, the present invention is well adapted to attain the ends and advantages mentioned as well as those that are inherent therein. The particular embodiments disclosed above are illustrative only, as the present invention may be modified and practiced in different but equivalent manners apparent to those skilled in the art having the benefit of the teachings herein. Furthermore, no limitations are intended to the details of construction or design herein shown, other than as described in the claims below. It is therefore evident that the particular illustrative embodiments disclosed above may be altered or modified and all such variations are considered within the scope and spirit of the present invention. While

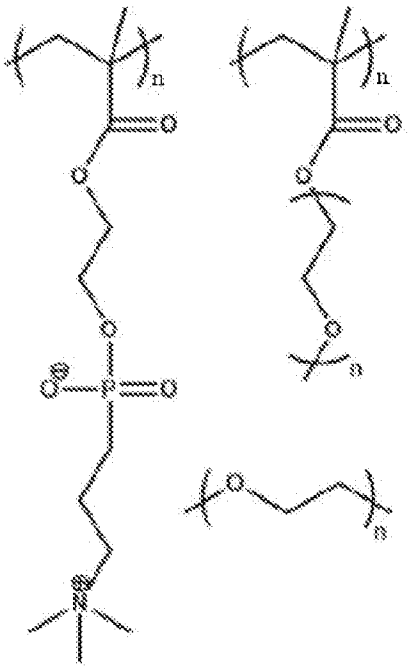
compositions and methods are described in terms of "comprising," "containing," or "including" various components or steps, the compositions and methods can also "consist essentially of" or "consist of" the various components and steps. All numbers and ranges disclosed above may vary by some amount. Whenever a numerical range with a lower limit and an upper limit is disclosed, any number and any included range falling within the range is specifically disclosed. In particular, every range of values (of the form, "from about a to about b," or, equivalently, "from approximately a to b," or, equivalently, "from approximately a-b") disclosed herein is to be understood to set forth every number and range encompassed within the broader range of values. Also, the terms in the claims have their plain, ordinary meaning unless otherwise explicitly and clearly defined by the patentee. Moreover, the indefinite articles "a" or "an," as used in the claims, are defined herein to mean one or more than one of the element that it introduces. If there is any conflict in the usages of a word or term in this specification and one or more patent or other documents that may be incorporated herein by reference, the definitions that are consistent with this specification should be adopted.

[00127] Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this pertains and to provide basic information about how to make and use embodiments of the invention. For example, it is well known in the art how monomers and polymers may be synthesized according to the requirements of the particular compounds and compositions being made, and synthesis details of such is provided at length in the cited literature and need not be reiterated here. Further, the references disclosed are also individually and specifically incorporated by reference herein for the material contained in them that is discussed in the sentence in which the reference is relied upon.

CLAIMS

1. A composition comprising a block copolymer that comprises:

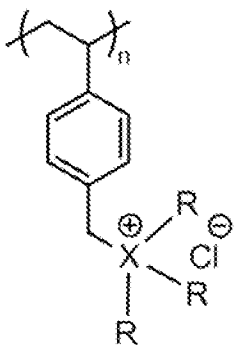
(A) a stabilization block chosen from polymers chosen from:



wherein n is a number ranging from 2 to 1,000; and

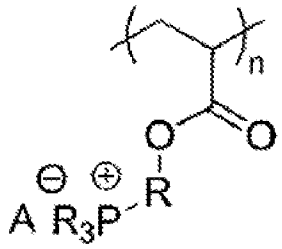
(B) a complexation block chosen from phosphonium-containing polymers chosen from:

(a)



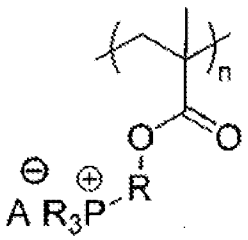
;

(b)



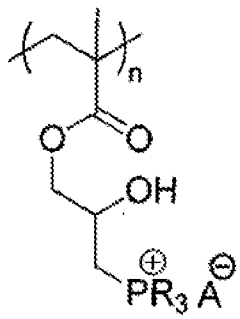
;

(c)



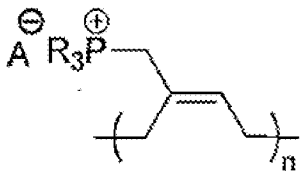
;

(d)



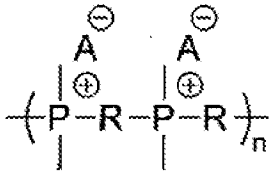
;

(e)



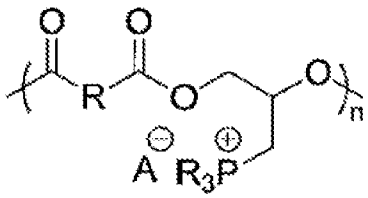
;

(f)



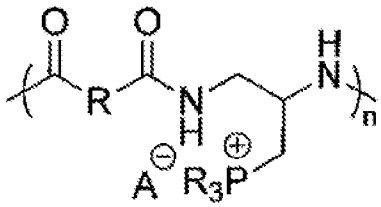
;

(g)



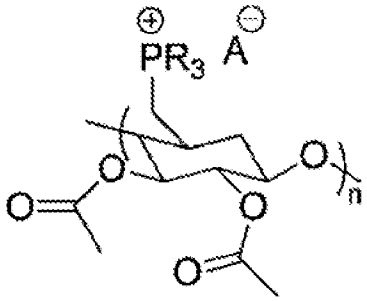
;

(h)



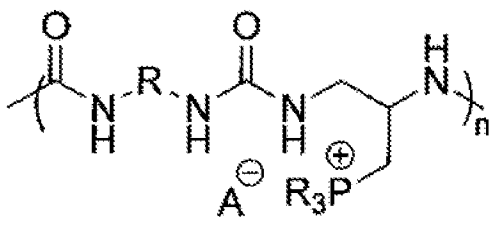
;

(i)



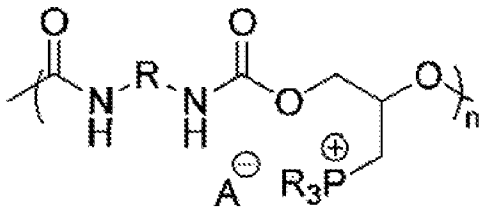
;

(j)



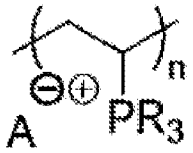
;

(k)



; and

(l)



wherein X is Phosphorus;

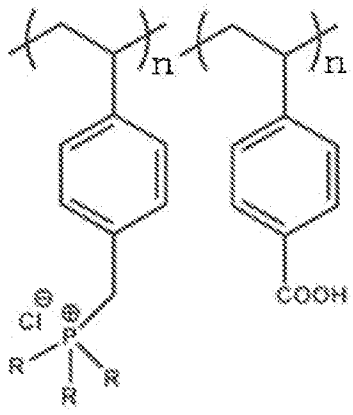
R is chosen from C_{1-24} alkyl;

A is a counterion; and

n is a number ranging from 2 to 1,000.

2. The composition of claim 1 further comprising:

an endosomolytic block chosen from polymers chosen from:



wherein R is C_{1-24} alkyl; and

n is a number ranging from 2-1,000.

3. The composition of claim 1, wherein the complexation block encapsulates the nucleic acid during gene delivery.

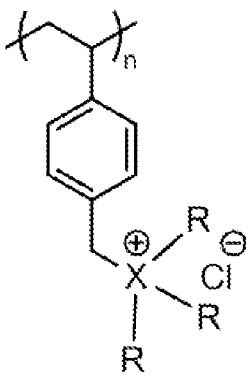
4. The composition of claim 2, wherein the endosomolytic block exhibits amphiphilic behavior at physiological pH and upon uptake into cell endosome is cationic resulting in endosomal escape.

5. The composition of claim 1, wherein the stabilizing block is chosen from poly(2-methacryloyloxyethyl phosphoryl-choline) (poly(MPC)), poly[(ethylene glycol)$_9$ methyl ether methacrylate] (poly(EG$_9$MEMA)), poly(ethylene glycol), and any combination thereof.

6. The composition of claim 1, wherein the stabilization block provides salt and serum stability to reduce polyplex aggregation and improve transfection.

7. The composition of claim 1, wherein the complexation block comprises phosphonium-containing polymers chosen from styrenics, acrylics, methacrylics, glycidyl methacrylate phosphoniums, dienes, ionenes, isoprenes, polyesters, polyamides, cellulose derivatives, polyureas, polyurethanes, and vinyl phosphoniums.

8. The composition of claim 7, wherein the complexation block is chosen from polymers of styrenic-based phosphonium-containing monomers chosen from:



wherein X is phosphorus;

R is C_{1-24} alkyl; and

n is a number ranging from 2-1,000.

9. The composition of claim 8, wherein R is methyl, ethyl, propyl, or butyl.

10. The composition of claim 9, wherein phosphonium-containing polymers are chosen from poly(triethyl-(4-vinylbenzyl)phosphonium chloride) (PTEP), and poly(tributyl-(4-vinylbenzyl)phosphonium chloride) (PTBP), and any combination thereof.

11. A method of using any of the compositions of claims 1-10 for nonviral gene delivery into a cell.

12. The method of claim 11, comprising the delivery of nucleic acid.

13. The method of claim 12, wherein the nucleic acid is chosen from oligo nucleotides, having 5 to 80 bases, single stranded RNA, single stranded DNA and double stranded DNA, pDNA, and siRNA.

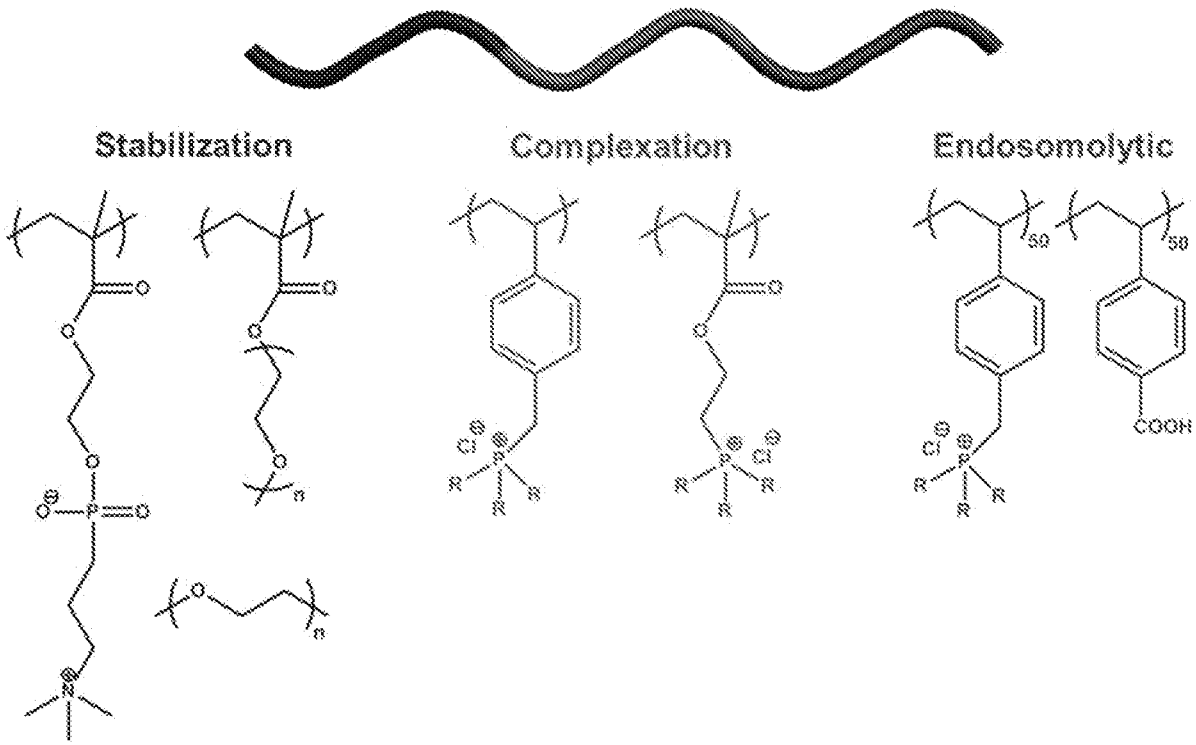


FIG. 1A

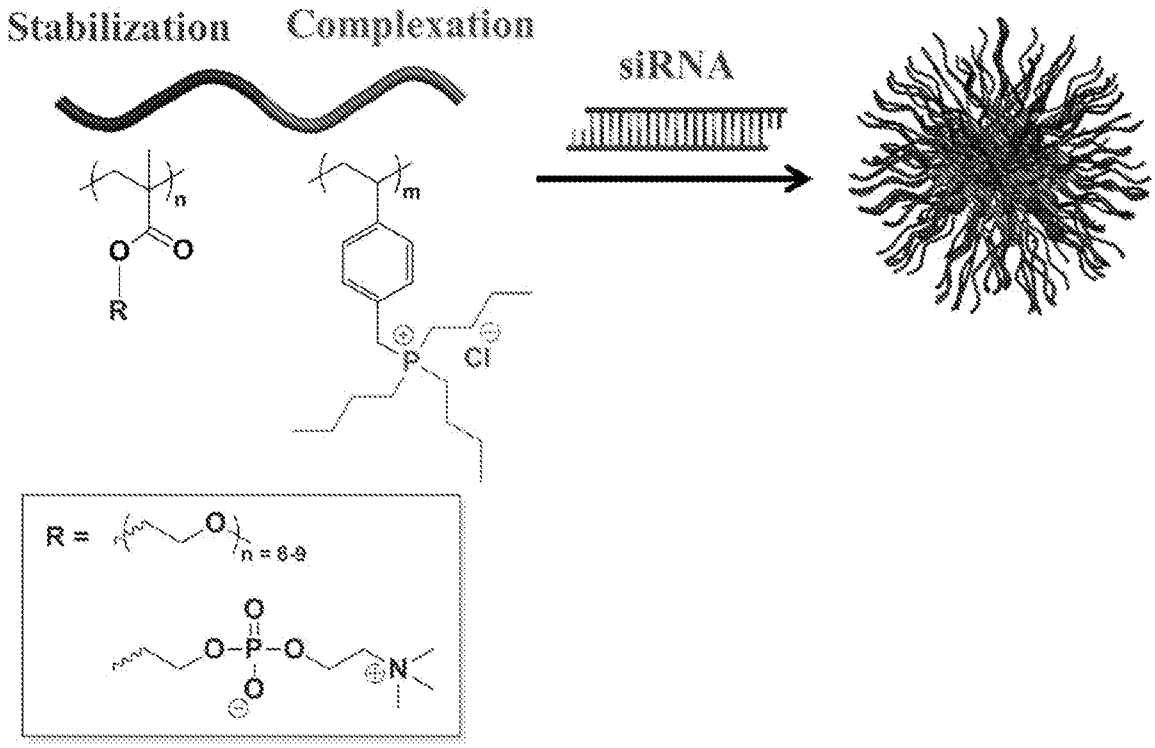


FIG. 1B

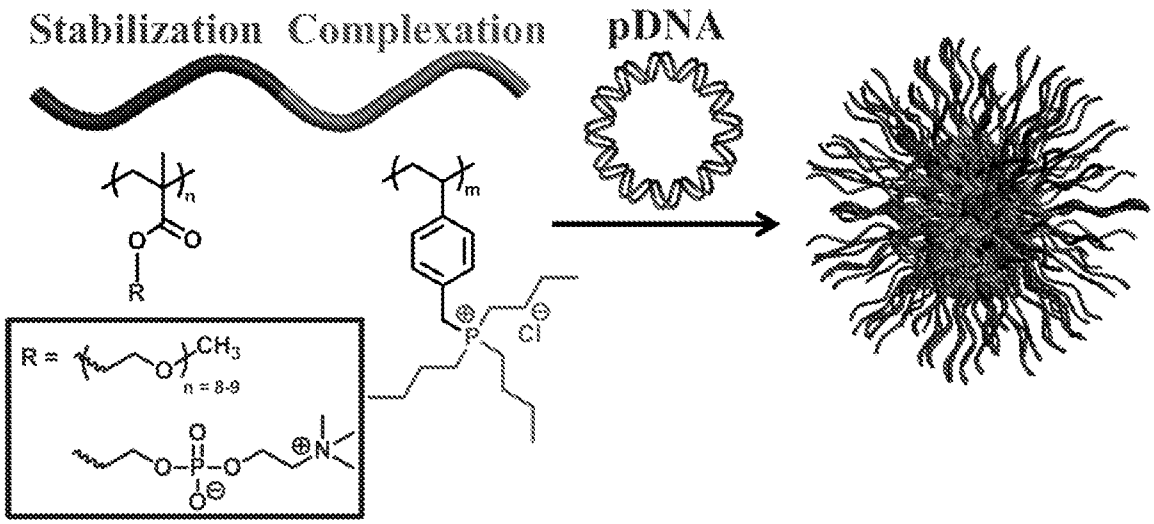


FIG. 1C

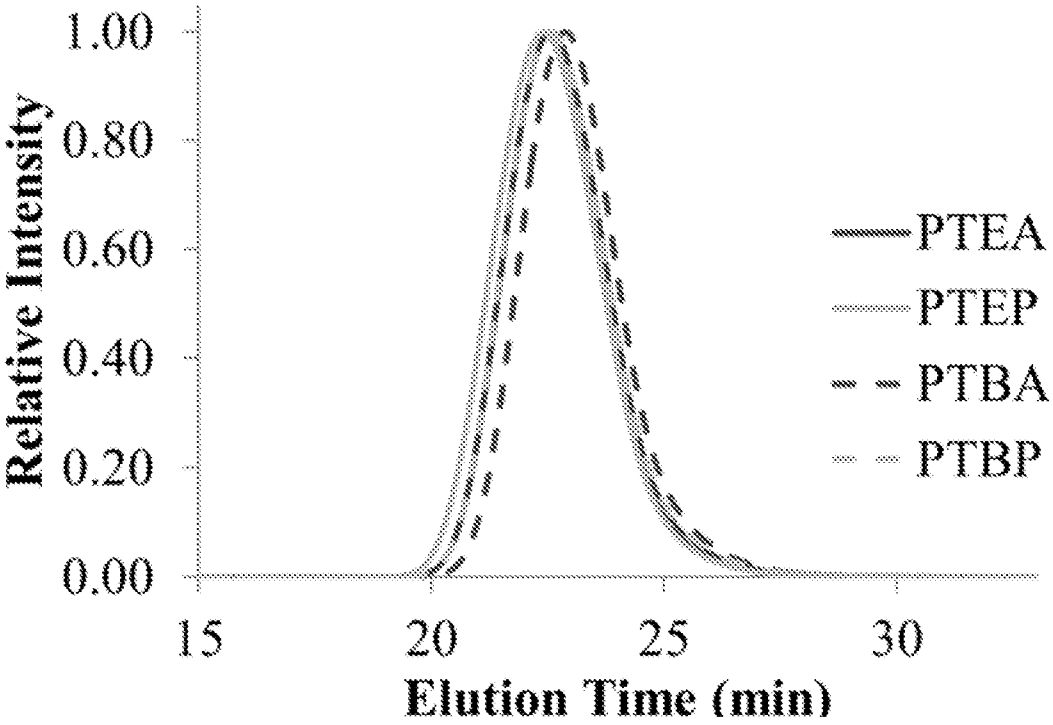
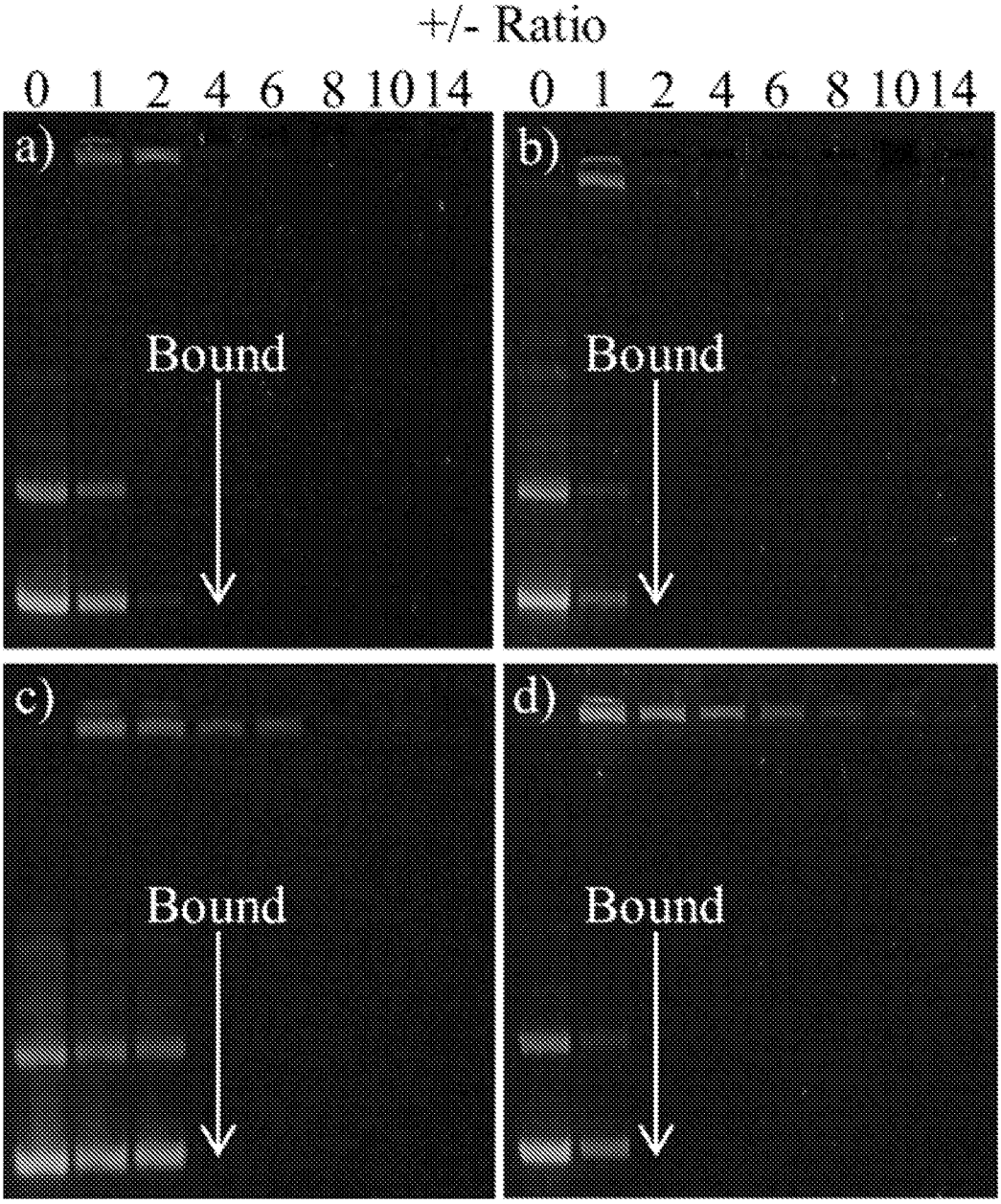

1.00
0.80
0.60
0.40
0.20
0.00
Relative Intensity
15
20
25
30
Elution Time (min)
PTEA
PTEP
PTBA
PTBP

FIG. 2



FIGS. 3A-D

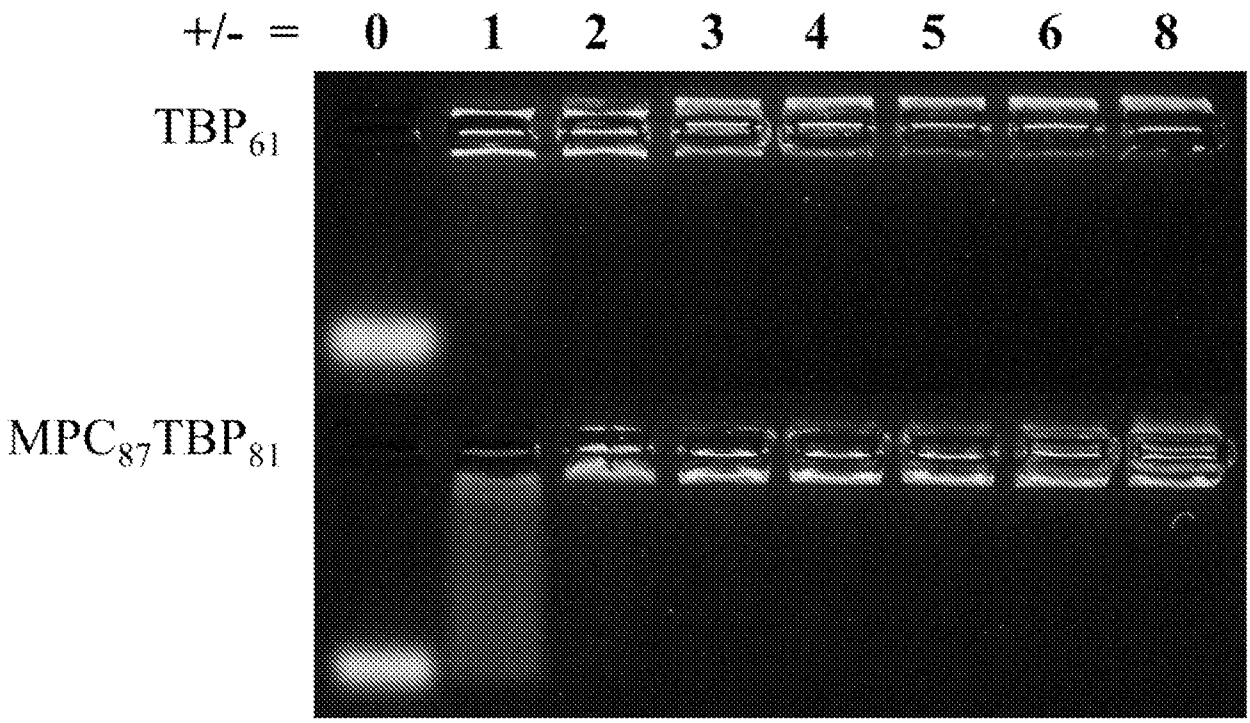
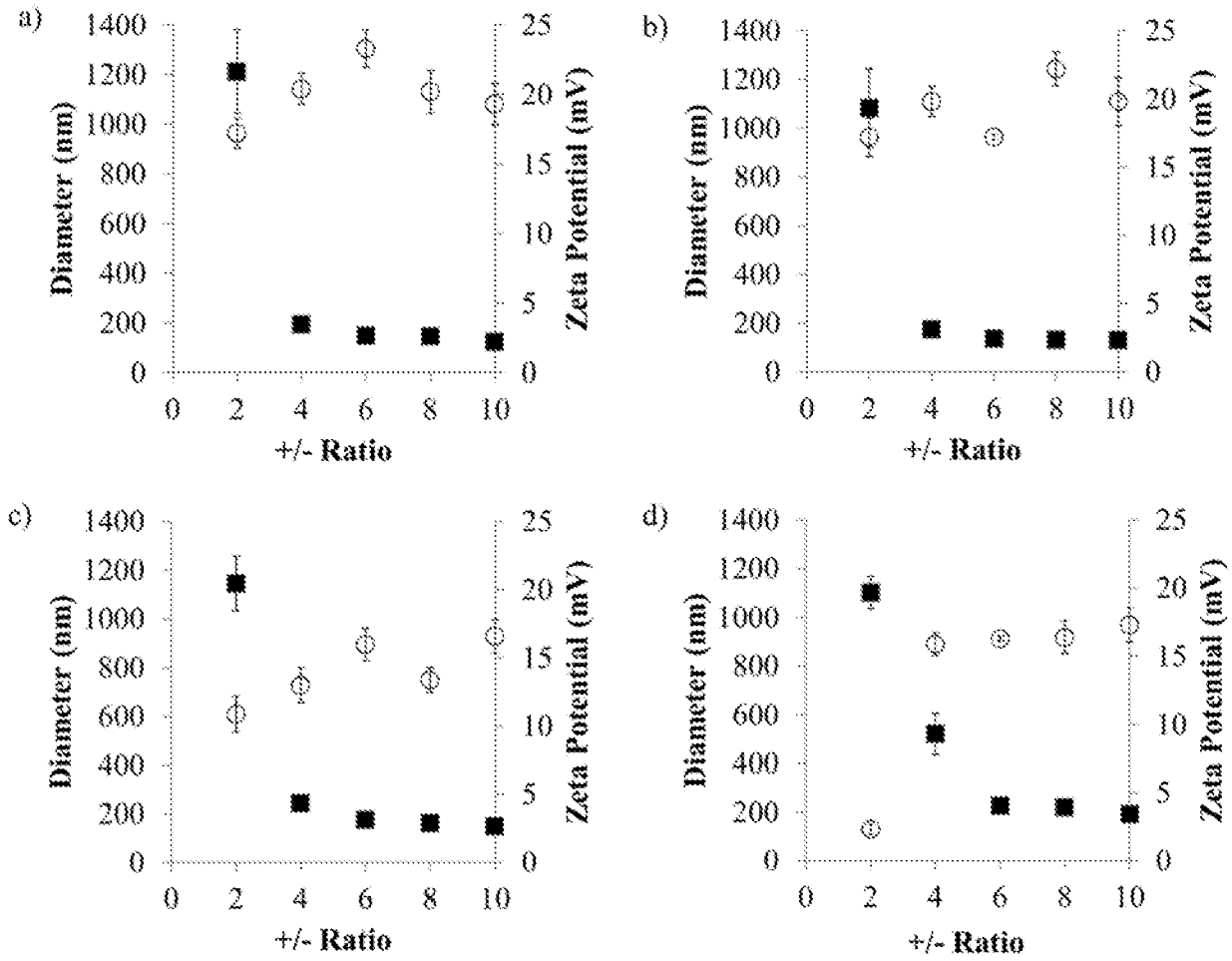


FIG. 4


a)
b)
c)
d)
Diameter (nm)
Zeta Potential (mV)
+/- Ratio
0
200
400
600
800
1000
1200
1400
0
5
10
15
20
25
0
2
4
6
8
10

FIGS. 5A-D

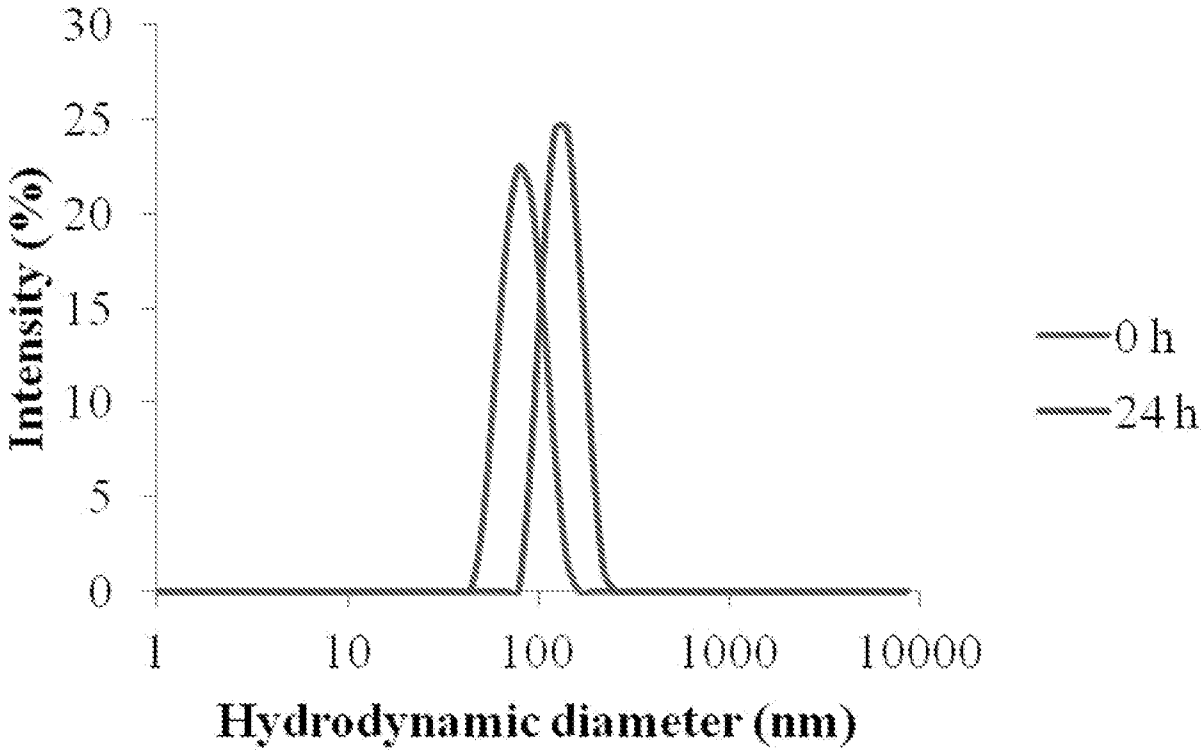

30
25
20
15
10
5
0
Intensity (%)
1
10
100
1000
10000
Hydrodynamic diameter (nm)
0 h
24 h

FIG. 6

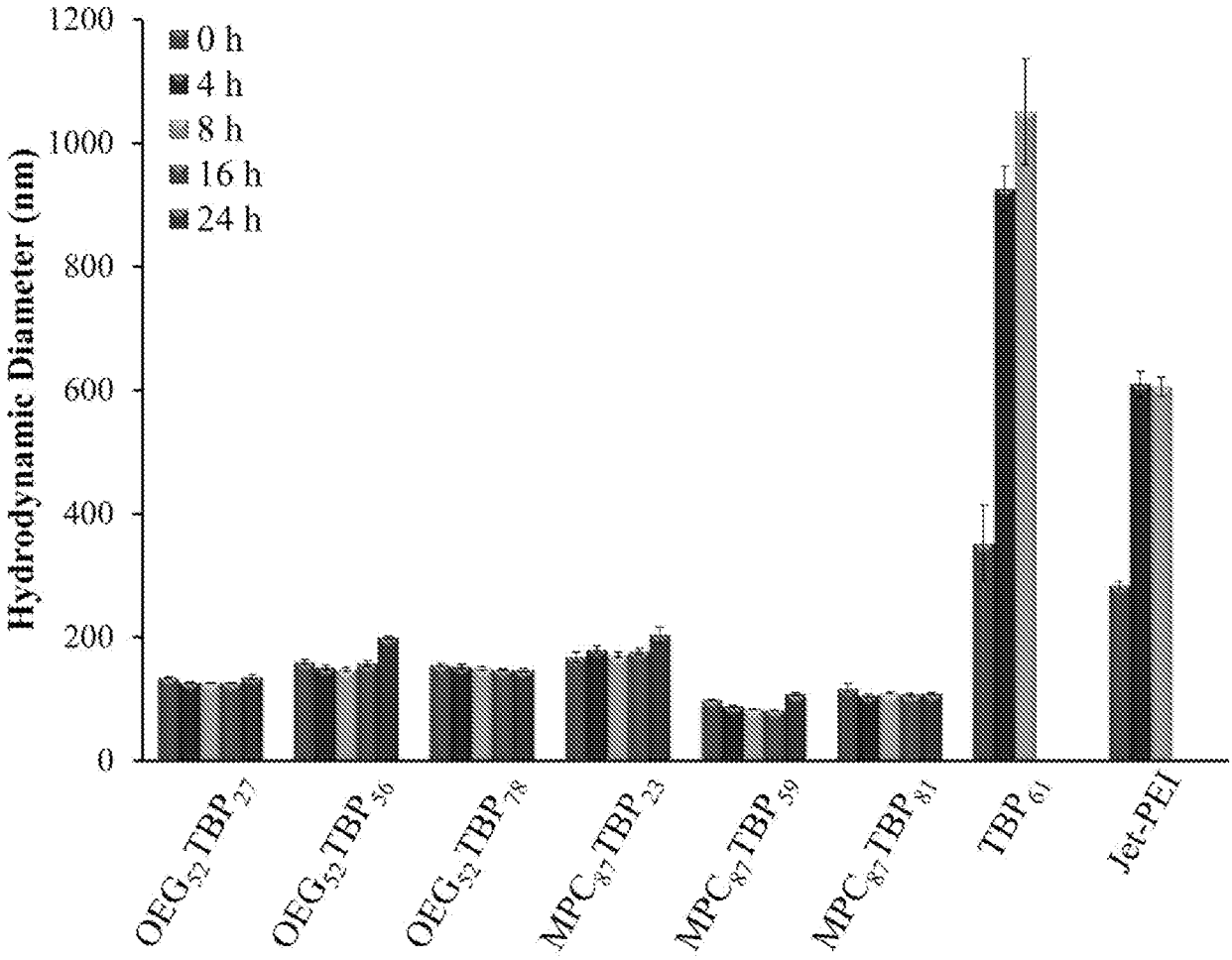

Hydrodynamic Diameter (nm)
1200
1000
800
600
400
200
0
0 h
4 h
8 h
16 h
24 h
OEG52TBP27
OEG52TBP56
OEG52TBP78
MPC87TBP23
MPC87TBP59
MPC87TBP81
TBP61
Jet-PEI

FIG. 7

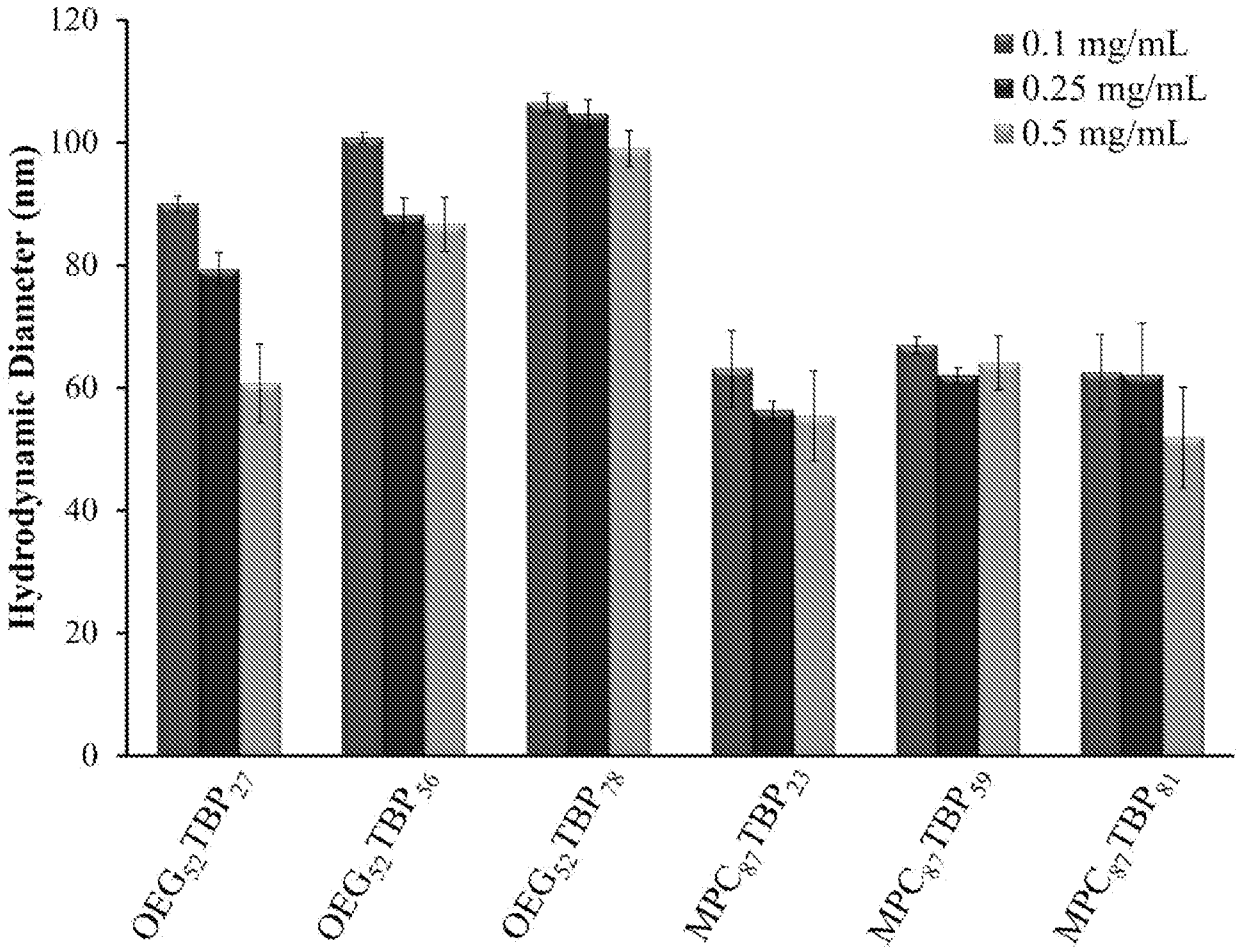

Hydrodynamic Diameter (nm)
120
100
80
60
40
20
0
0.1 mg/mL
0.25 mg/mL
0.5 mg/mL
OEG52TBP27
OEG52TBP56
OEG52TBP78
MPC87TBP23
MPC87TBP59
MPC87TBP81

FIG. 8

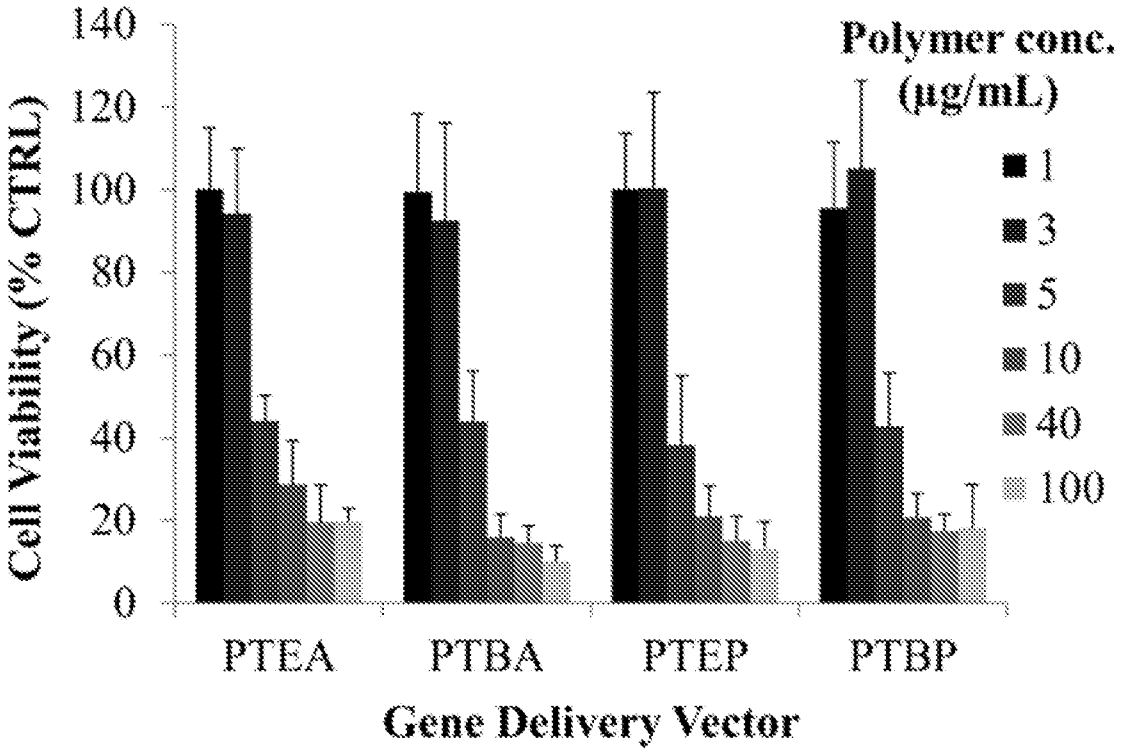

Cell Viability (% CTRL)
140
120
100
80
60
40
20
0
PTEA
PTBA
PTEP
PTBP
Gene Delivery Vector
Polymer conc. (µg/mL)
1
3
5
10
40
100

FIG. 9

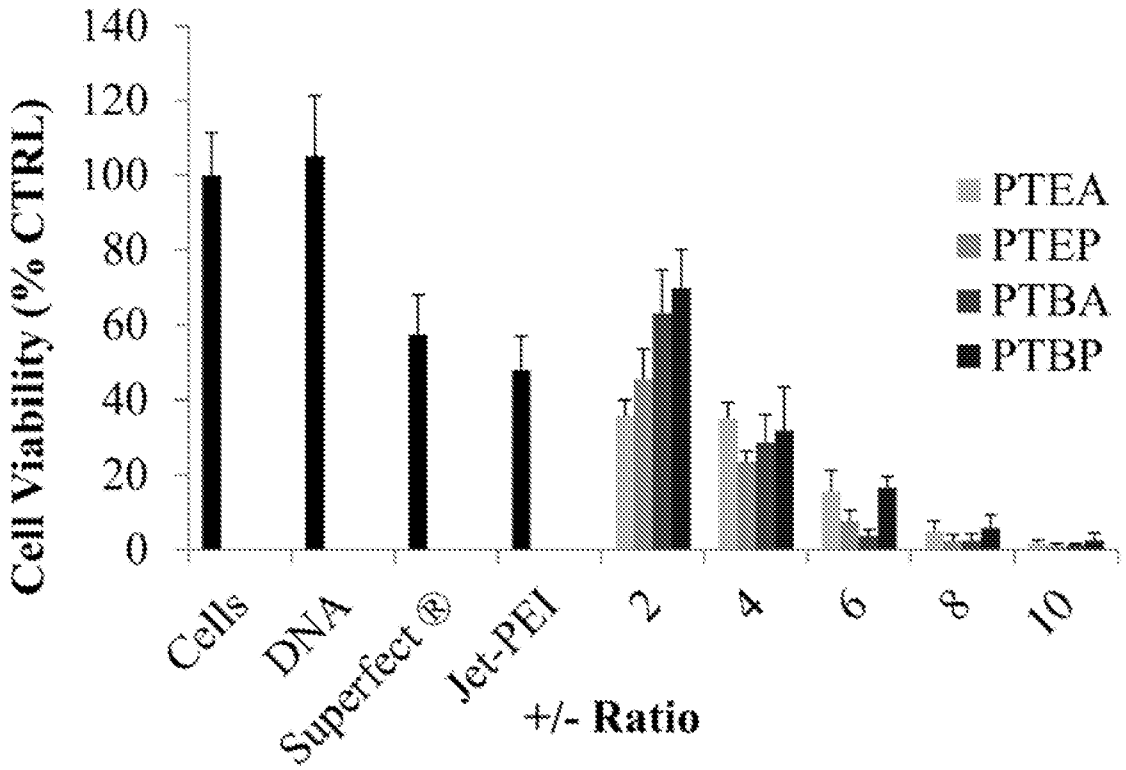

Cell Viability (% CTRL)
140
120
100
80
60
40
20
0
PTEA
PTEP
PTBA
PTBP
Cells
DNA
Superfect ®
Jet-PEI
2
4
6
8
10
+/- Ratio

FIG. 10

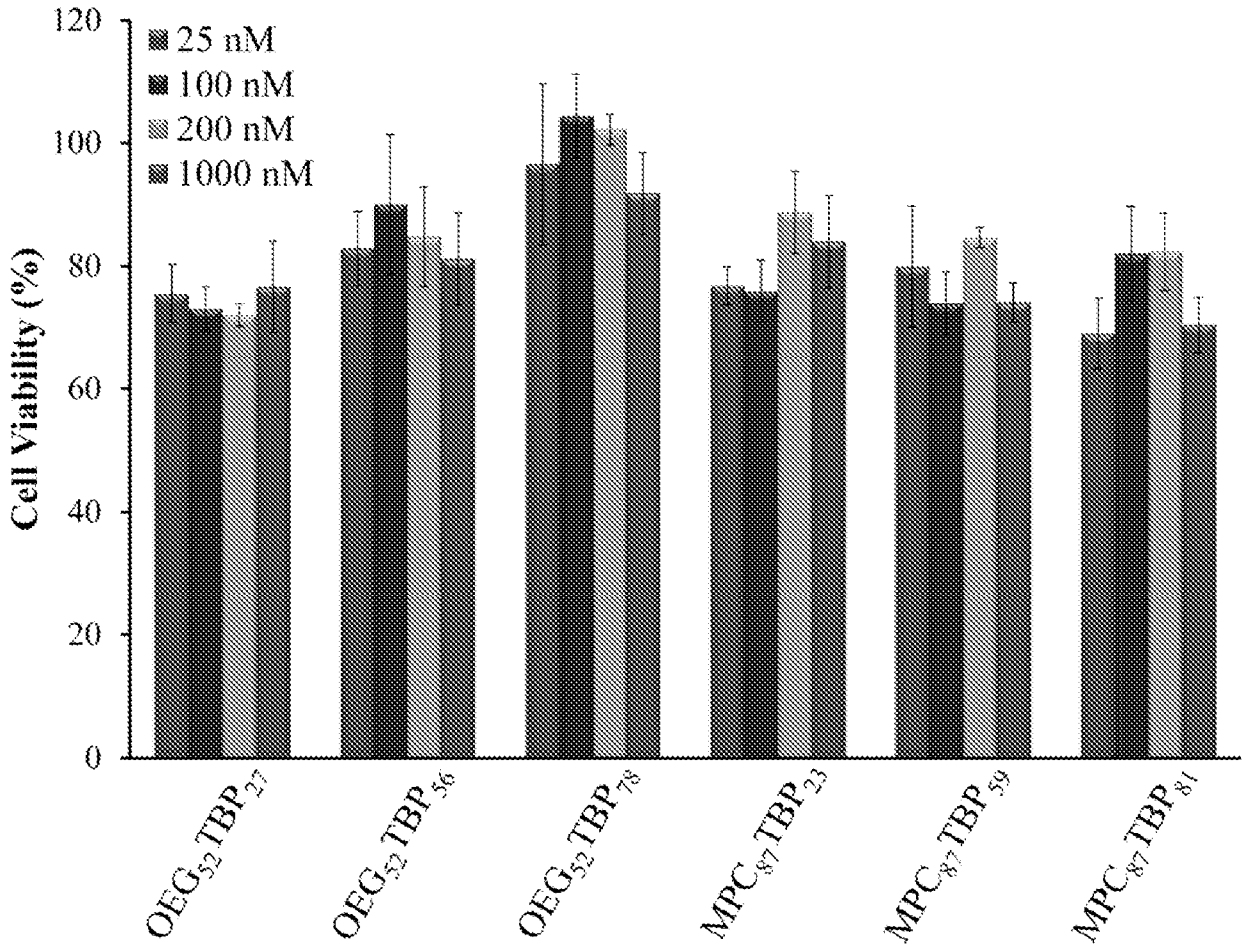

120
100
80
60
40
20
0
Cell Viability (%)
25 nM
100 nM
200 nM
1000 nM
OEG52 TBP27
OEG52 TBP56
OEG52 TBP78
MPC87 TBP23
MPC87 TBP59
MPC87 TBP81

FIG. 11

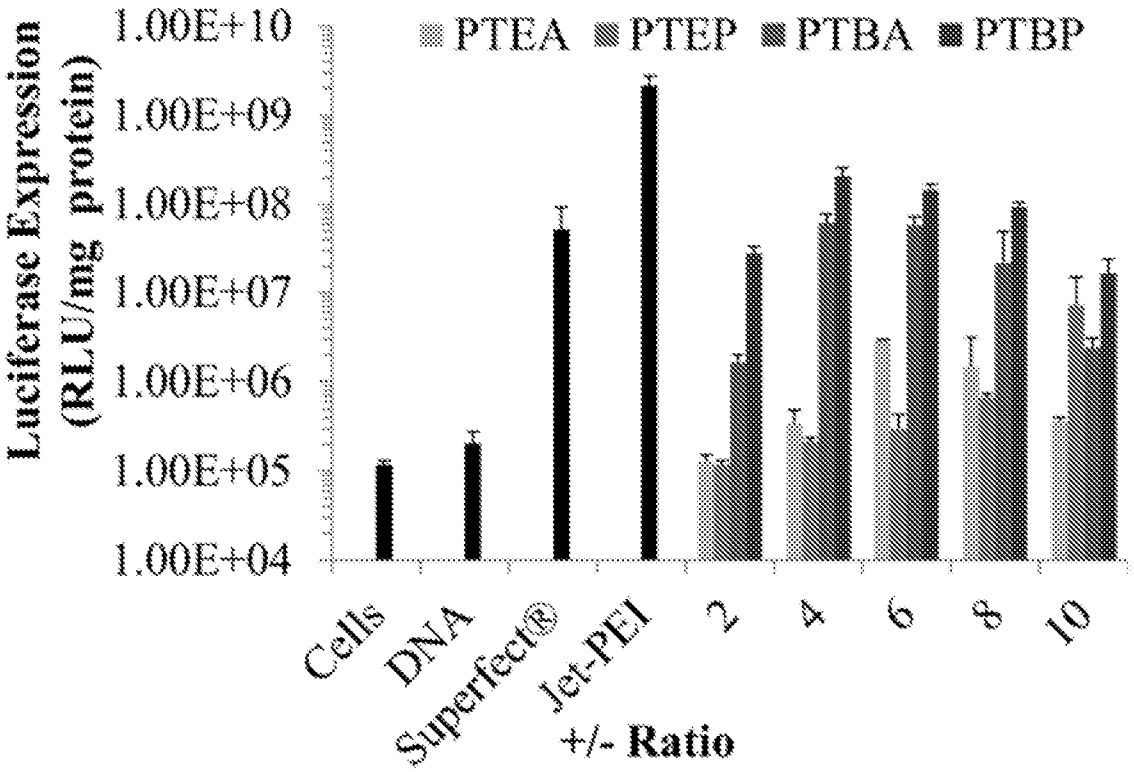
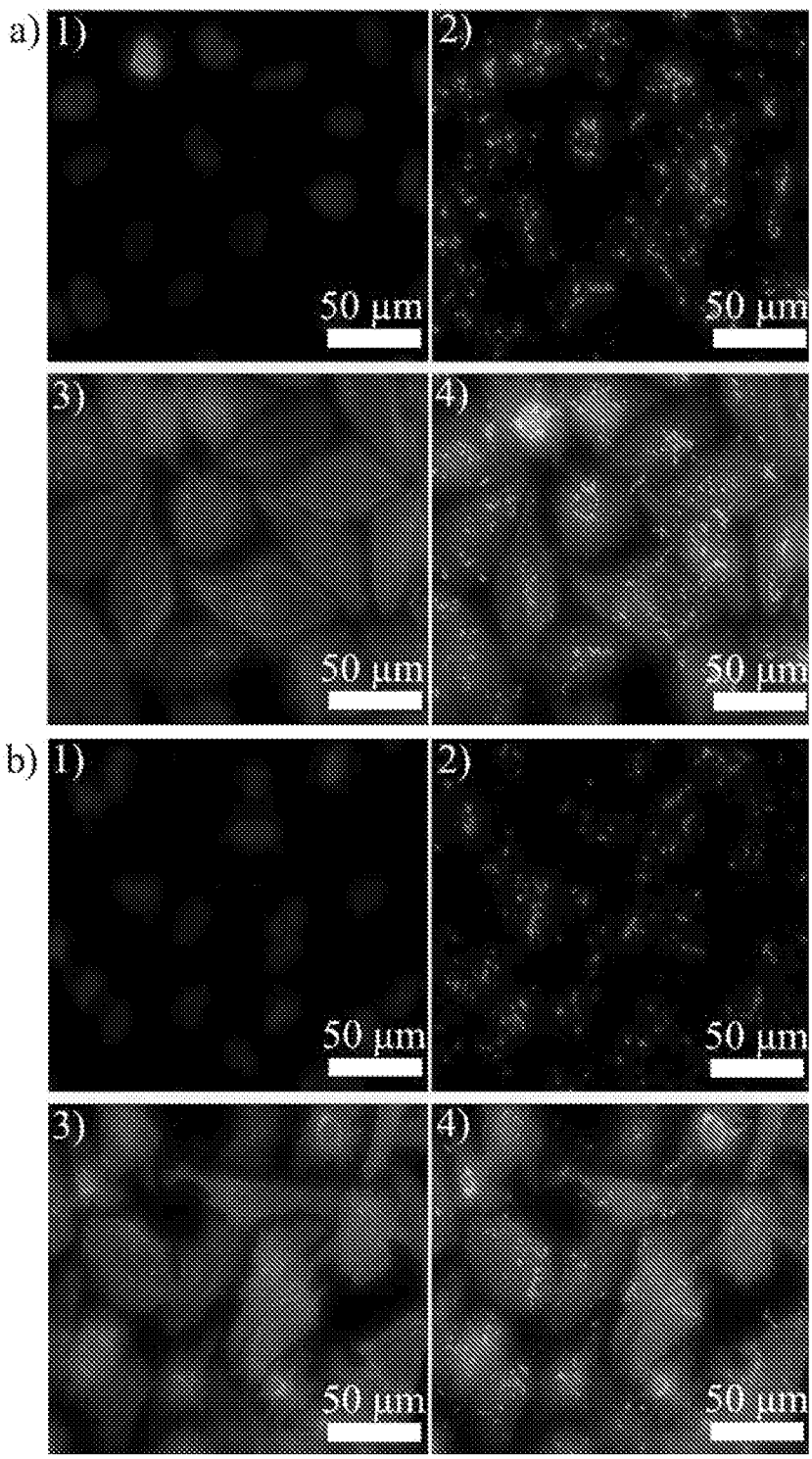

Luciferase Expression
(RLU/mg protein)
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
PTEA
PTEP
PTBA
PTBP
Cells
DNA
Superfect®
Jet-PEI
2
4
6
8
10
+/- Ratio

FIG. 12



FIGS. 13A-B

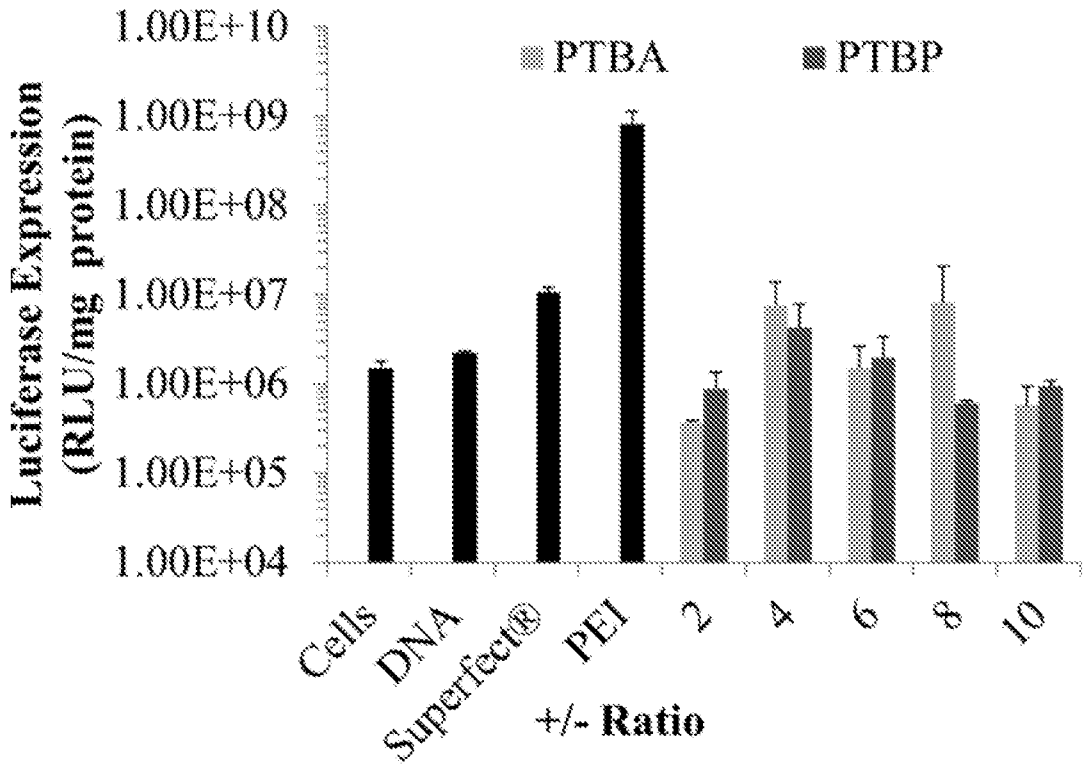

Luciferase Expression (RLU/mg protein)
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
PTBA
PTBP
Cells
DNA
Superfect®
PEI
2
4
6
8
10
+/- Ratio

FIG. 14

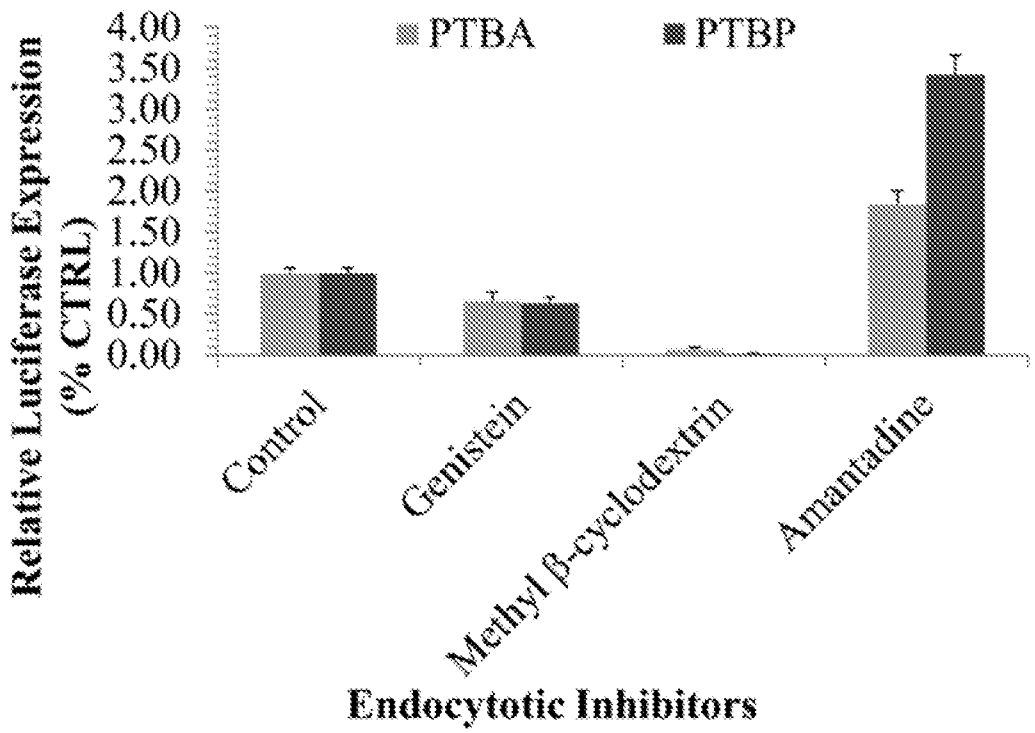

Relative Luciferase Expression (% CTRL)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
PTBA
PTBP
Control
Genistein
Methyl β-cyclodextrin
Amantadine
Endocytotic Inhibitors

FIG. 15

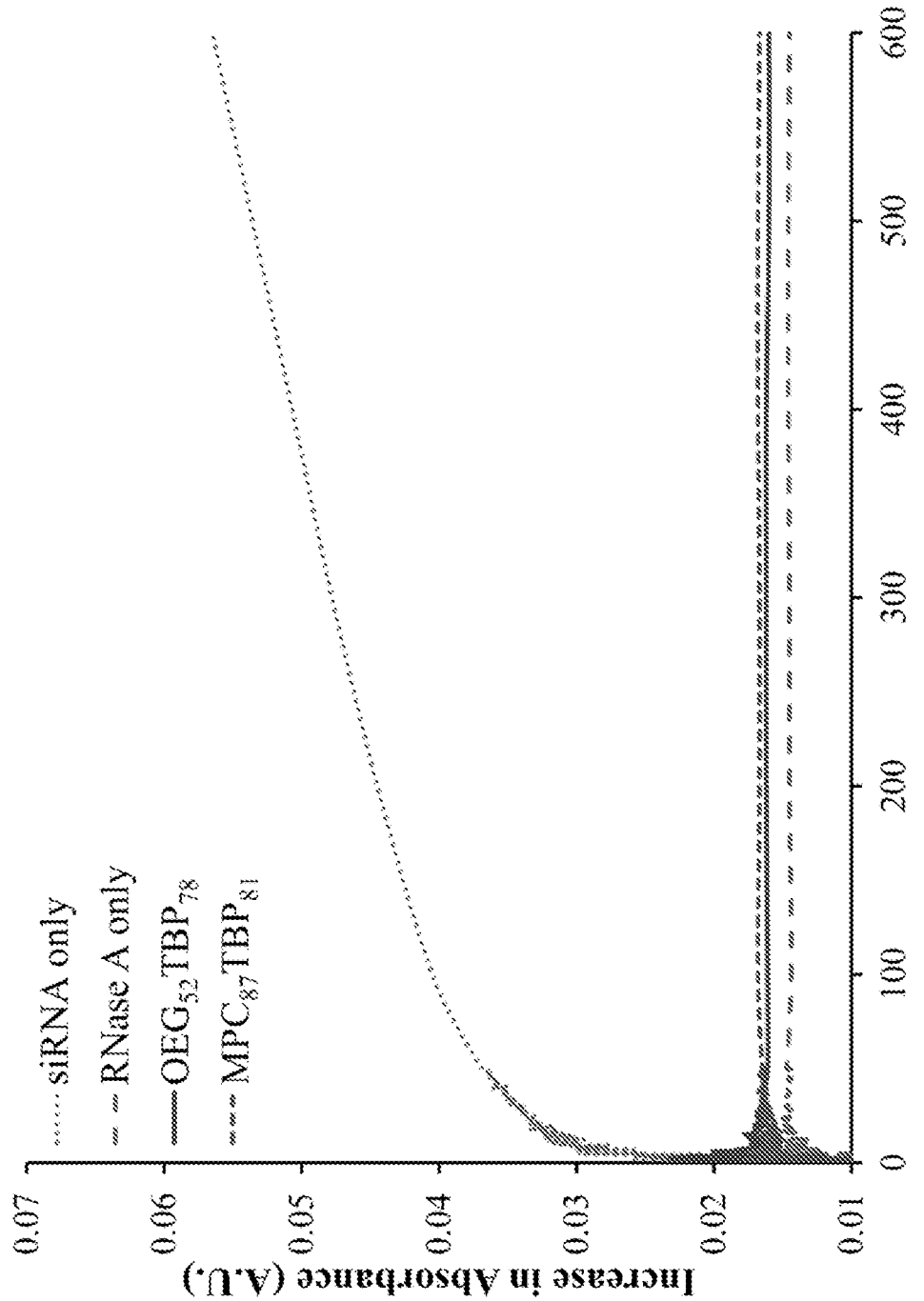

siRNA only
RNase A only
$OEG_{52}TBP_{78}$
$MPC_{87}TBP_{81}$
Increase in Absorbance (A.U.)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
100
200
300
400
500
600
Time (min)

FIG. 16

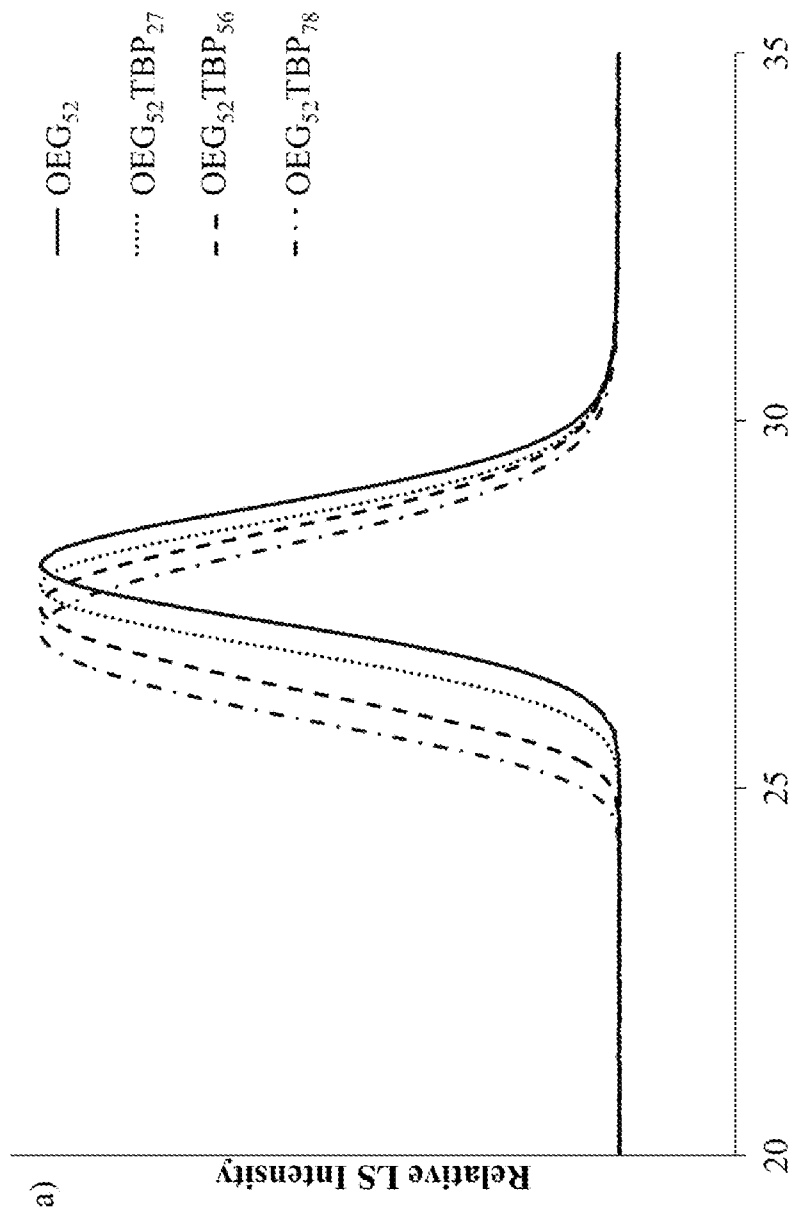

a)
Relative LS Intensity
20
25
30
35
Elution Time (min)
OEG52
OEG52TBP27
OEG52TBP56
OEG52TBP78

FIG. 17A

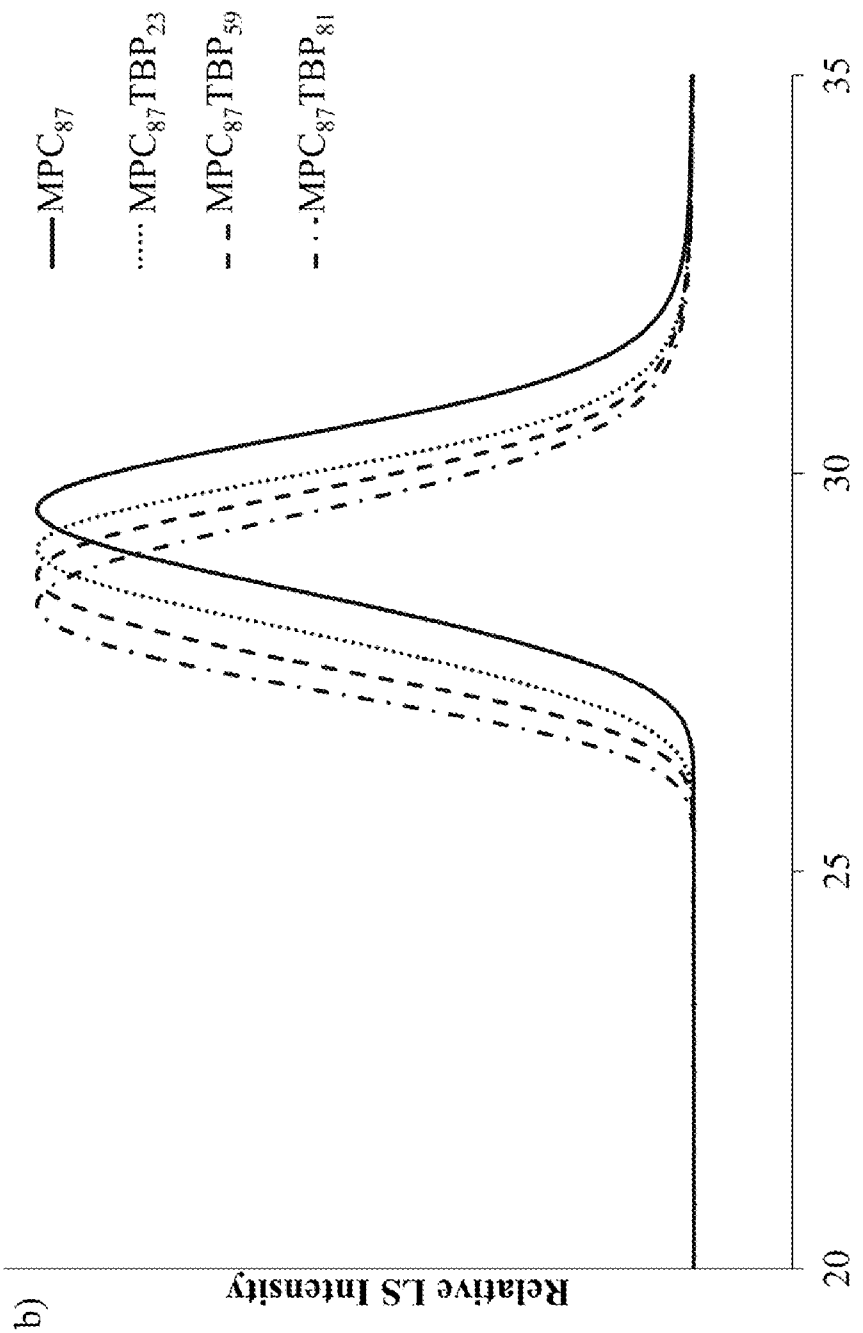

b)
Relative LS Intensity
20
25
30
35
Elution Time (min)
MPC87
MPC87TBP23
MPC87TBP59
MPC87TBP81

FIG. 17B

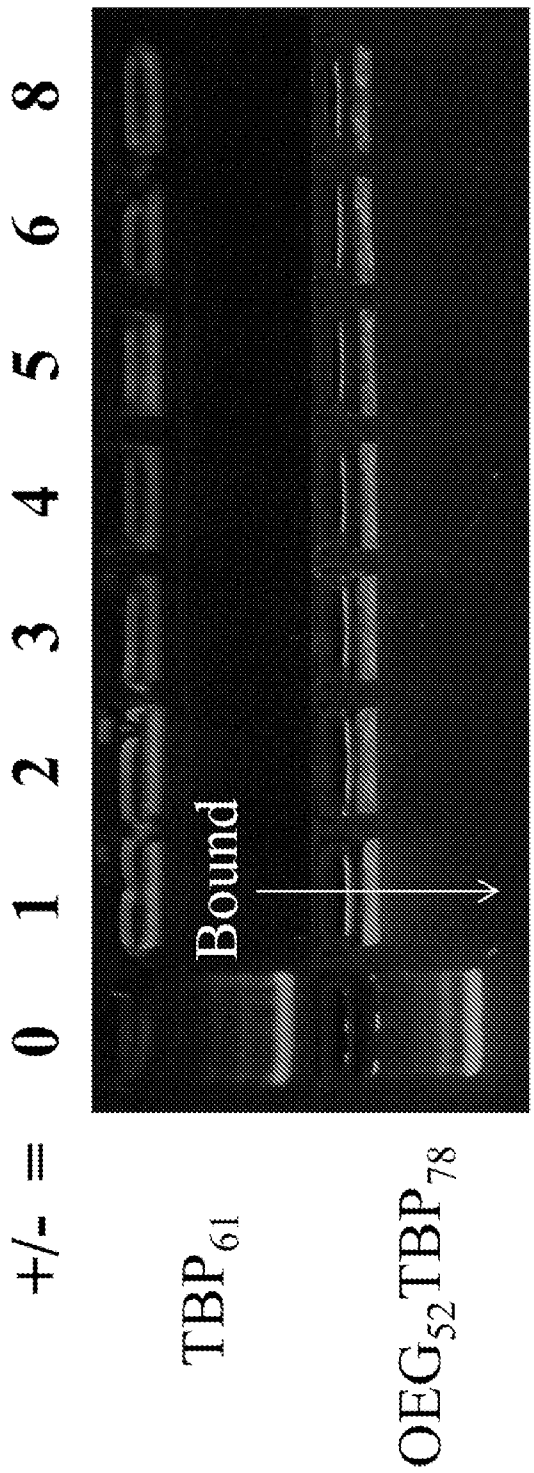


FIG. 18

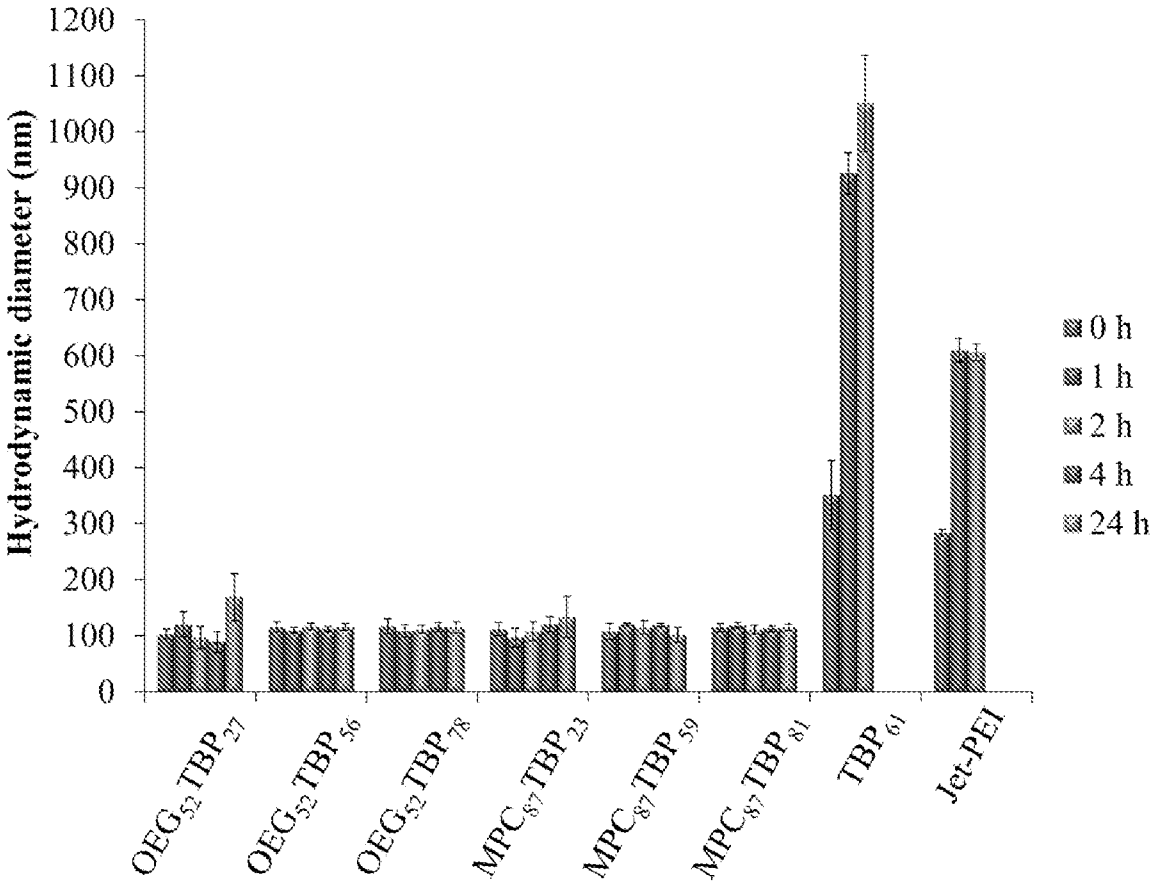

Hydrodynamic diameter (nm)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0 h
1 h
2 h
4 h
24 h
OEG52TBP27
OEG52TBP56
OEG52TBP78
MPC87TBP23
MPC87TBP59
MPC87TBP81
TBP61
Jet-PEI

FIG. 19

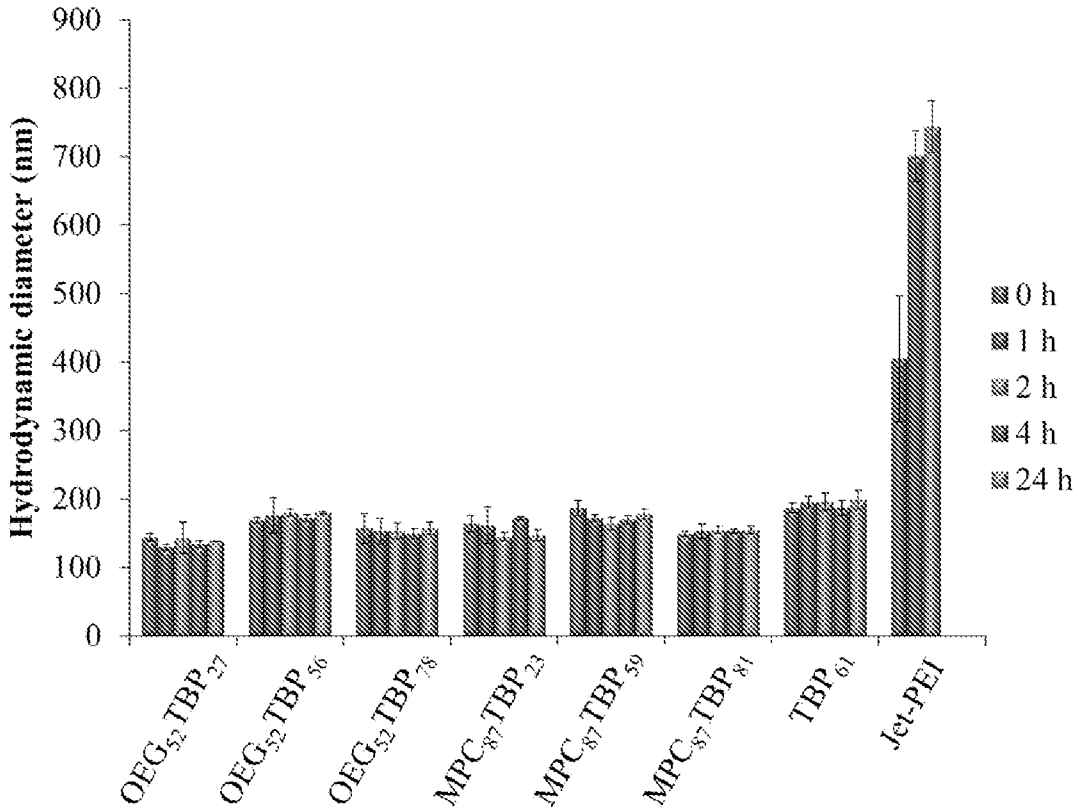
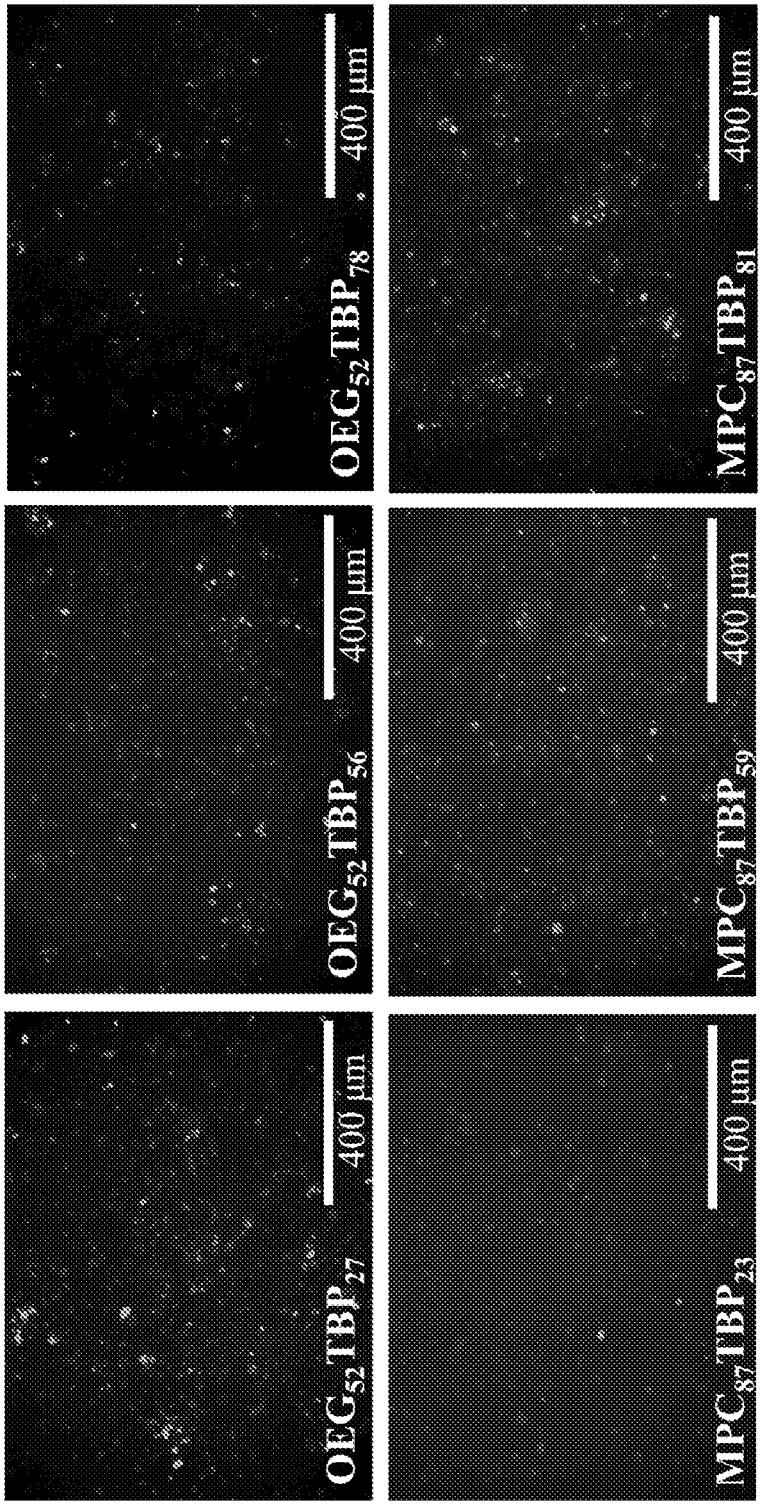

Hydrodynamic diameter (nm)
900
800
700
600
500
400
300
200
100
0
0 h
1 h
2 h
4 h
24 h
OEG52TBP27
OEG52TBP56
OEG52TBP78
MPC87TBP23
MPC87TBP59
MPC87TBP81
TBP61
Jet-PEI

FIG. 20


OEG52TBP27
400 μm
OEG52TBP56
400 μm
OEG52TBP78
400 μm
MPC87TBP23
400 μm
MPC87TBP59
400 μm
MPC87TBP81
400 μm

FIGS. 21A-E

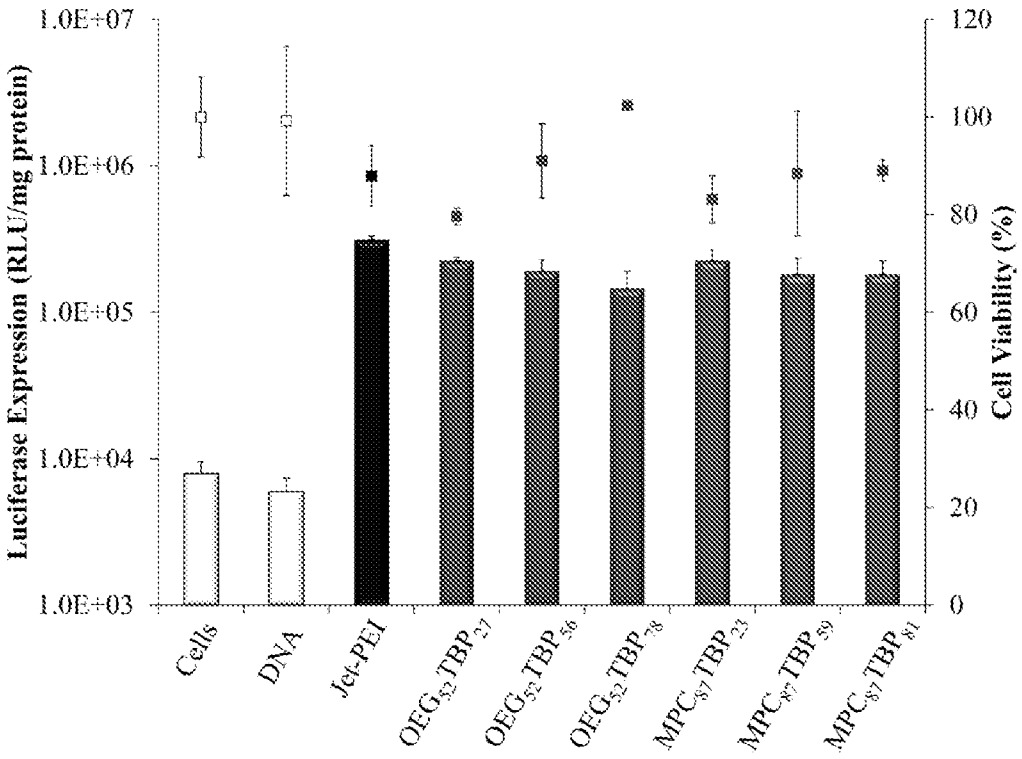

Luciferase Expression (RLU/mg protein)
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
Cell Viability (%)
120
100
80
60
40
20
0
Cells
DNA
Jet-PEI
OEG52TBP27
OEG52TBP56
OEG52TBP78
MPC87TBP23
MPC87TBP59
MPC87TBP81

FIG. 22