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(54) Title: BLOCK COPOLYMERS AND USES THEREOF

(57) Abstract: An encoding/decoding apparatus and method using a low-density parity-check code (LDPC code) is disclosed. Basic column group information, serving as a set of information regarding positions of rows with weight 1, is extracted from a reference column in each column group of a predetermined parity-check matrix. Column group information transforms the positions of rows with weight 1 into positions whose lengths are within a required parity length. A parity-check matrix is generated according to the generated column group information. Data is encoded or decoded based on the generated parity-check matrix.

BLOCK COPOLYMERS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional Application No. 61/117,892, filed November 25, 2008, which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

The invention relates to the field of polymer chemistry.

In the field of pharmaceutical agent delivery there exists an extensive interest in amphiphilic block copolymers that can self-assemble in aqueous environments into stable supramolecular structures. A variety of supramolecular structures can be generated such as micellar and vesicular assemblies, both of which can be important for pharmacological applications. Extensive investigations have been conducted in poly(ethylene glycol) (PEG)-containing block copolymers, such as copolymers with poly(propylene glycol) and poly(ethylethylene). Such block copolymers are generally prepared via ionic polymerization under strictly anhydrous conditions, making it difficult to obtain asymmetric block copolymers or to introduce biological molecules.

Accordingly, there is a need for new block copolymers.

SUMMARY OF THE INVENTION

Block copolymers containing charged blocks or chemical moieties sensitive to oxidation or hydrolysis have been developed. We describe the use of such block copolymers in supramolecular structures, e.g., micelles or vesicles, and pharmaceutical compositions and in methods of preparing the supramolecular structures and pharmaceutical compositions. The invention is particularly useful for the delivery of pharmaceutical agents, e.g., nucleic acids, to cells.

Accordingly, in one aspect, the invention features a block copolymer including a hydrophilic block and a hydrophobic block wherein at least one of the blocks is interrupted with a hydrolysable or oxidation-sensitive chemical moiety. Desirably, the block copolymer is capable of self-assembling into a supramolecular structure, such as a micelle or vesicle. In certain embodiments, the hydrolysable chemical moiety is an ester, amide, thioester, anhydride, or ketal. In another embodiment, the hydrophilic block is poly(ethylene glycol) (PEG), and the hydrophobic block is poly(propylene sulfide) (PPS).

In another aspect, the invention features a supramolecular structure, e.g., a micelle or a vesicle, containing (i) a block copolymer of a hydrophilic block and a hydrophobic block and (ii) an excipient. Desirably, the excipient is an amphipathic molecule. In preferred embodiments, the excipient is 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or PEG of molecular weight between 400 and 800. The supramolecular structure may further contain a hydrophobic or hydrophilic pharmaceutical agent. The pharmaceutical agent is, for example, a peptide, a nucleic acid, an antibiotic, or a chemotherapeutic drug. In certain embodiments, the pharmaceutical agent is selected from dexamethasone, paclitaxel, cyclosporine A, sirolimus, everolimus, tacrolimus, amphotericin B, or adriamycin. In other embodiments, the pharmaceutical agent is a polypeptide, such as a protein or an antibody or an antigen-binding fragment of an antibody. In preferred embodiments, the pharmaceutical agent is encapsulated in the supramolecular structure at an efficiency greater than 5%, 25%, 50%, 75%, 90%, or even 95%. The supramolecular structure may be included in a pharmaceutical composition with a pharmaceutically acceptable diluent.

In a related aspect, the invention features a method of encapsulating a pharmaceutical agent in a supramolecular structure, e.g., a micelle or a vesicle. The method includes contacting the pharmaceutical agent with an excipient and a block copolymer containing a hydrophilic block and a hydrophobic block, applying heat to homogenize the mixture of the pharmaceutical agent, excipient, and block copolymer, and diluting the homogenized mixture in an

aqueous solution. In certain embodiments, the excipient is DBU or PEG of molecular weight between 400 and 800.

The invention further features a method of making a vesicle including forming micelles from a block copolymer containing a hydrophilic block and a hydrophobic block, wherein a vesicle formed by the block copolymer is thermodynamically more stable than a micelle formed by the block copolymer, and heating the micelles to form the vesicle. In certain embodiments, the vesicles formed by the method are 70 to 800 nm in diameter. In other embodiments, the micelles are suspended in a solution containing a pharmaceutical agent, and the pharmaceutical agent is encapsulated in the vesicles upon heating the solution. In another embodiment, the hydrophilic block of the block copolymer contains PEG, and the hydrophobic block of the block copolymer contains PPS. The invention also provides a vesicle prepared by this method.

In another aspect, the invention features a dry formulation containing micelles of a block copolymer having a hydrophilic block and a hydrophobic block, wherein the water content of the formulation is less than 5%, e.g., less than 2%. The dry formulation may further contain a pharmaceutical agent. In certain embodiments, the hydrophilic block of the block copolymer contains PEG, and the hydrophobic block of the block copolymer contains PPS.

The invention further features a supramolecular structure containing a block copolymer containing a positively charged block and a nucleic acid, wherein the supramolecular structure has a maximal diameter of less than 60 nm. The block copolymer may further include a hydrophilic block, e.g., PEG, and a hydrophobic block, e.g., PPS. In one embodiment, the block copolymer contains PPS, PEG, and polyethylene imine (PEI). In another embodiment, the nucleic acid is a single-stranded oligonucleotide, a short interfering RNA, an aptamer, or plasmid DNA. Desirably, the maximal diameter of the supramolecular structure is less than 40 nm. In a related aspect, the invention features a method of transfecting a cell with a nucleic acid including contacting

the cell with a supramolecular structure containing a block copolymer containing a positively charged block and the nucleic acid.

In another aspect, the invention features a block copolymer containing PPS, PEG, and PEI. In certain embodiments, the PPS block and the PEI block are attached via a bond that is labile in an endosome, e.g., a disulfide bond, vinyl ether, orthoester, acyl hydrazone, or a –N–PO_3– group. In another embodiment, the block copolymer includes a nucleic acid that is bound to the PEI block. The block copolymer may be included in a pharmaceutical composition containing a pharmaceutical agent and a pharmaceutically acceptable diluent.

The invention further features a micelle between 10 and 60 nm in diameter containing two block copolymers, the first of which contains a hydrophilic block and a hydrophobic block, the second of which contains a hydrophilic block, a hydrophobic block, and a positively charged block. In one particular embodiment, the first block copolymer contains PEG and PPS, and the second block copolymer contains PEG, PPS, and PEI. In other embodiments, the micelle has a maximal diameter between 20 and 50 nm. The micelle may be included in a pharmaceutical composition containing a pharmaceutical agent and a pharmaceutically acceptable diluent.

In another aspect, the invention features a supramolecular structure containing block copolymers containing a hydrophilic block, e.g., PEG, and a hydrophobic block, e.g., PPS, wherein 5-25% of the repeating units in the block copolymer have a charged chemical moiety disposed at the outer surface of the supramolecular structure. In certain embodiments, the charged chemical moiety is carboxylic acid, sulfate, or sulfone. The supramolecular structure may be included in a pharmaceutical composition containing a pharmaceutical agent and a pharmaceutically acceptable diluent.

In any of the embodiments of the invention, the hydrophilic block may contain poly(ethylene glycol), poly(ethylene oxide)-co-poly(propylene oxide) di- or multiblock copolymers, poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(N-vinyl pyrrolidone), poly(acrylic acid),

poly(ethyloxazoline), poly(alkylacrylates), poly(acrylamide), poly(N-alkylacrylamides), polypeptide, polysaccharide, poly(N,N-dialkylacrylamides), hyaluronic acid, or poly (N-acryloylmorpholine). The hydrophobic block may contain poly(propylene sulfide), poly(propylene glycol), esterified poly(acrylic acid), esterified poly(glutamic acid), esterified poly(aspartic acid), or a polypeptide. In certain embodiments, the charged block is PEI, a polypeptide, poly(amidoamine), poly(sodium 1-(*N*-acryloylpiperazin-1-yl)diazen-1-ium-1,2-diolate), poly(sodium 1-(*N*-acryloylhomopiperazin-1-yl)diazen-1-ium-1,2-diolate) or poly(sodium 1-(*N*-acryloyl-2,5-dimethylpiperazin-1-yl)diazen-1-ium-1,2-diolate).

By a "block copolymer" is meant a compound containing at least two blocks that each contain two or more repeating units of a chemical moiety. The chemical moiety of one block is distinct from a chemical moiety present in another block of the block copolymer. For example, a block copolymer may contain a poly(ethyleneglycol) (PEG) block and a poly(propylene sulfide) (PPS) block. Typically, the number of repeating units in a block is between 4 and 250. An exemplary hydrophilic block contains up to 250 repeating units, and an exemplary hydrophobic block contains up to 100 repeating units. Repeating units of a block may be interrupted or modified by a group that confers a desirable functionality, e.g., the ability to be hydrolyzed.

By a block that is "interrupted with" a hydrolysable chemical moiety is meant a block of the same repeating unit that includes within it the hydrolysable chemical moiety so that, when the chemical moiety is hydrolyzed, the number of repeating units in the block decreases. Upon hydrolysis, the block may decrease in size by at least, e.g., 2, 4, 10, 15, 20, 30, 50, 75, 100, or 115 repeating units. Hydrolysable moieties include, e.g., esters, amides, thioesters, anhydrides, and ketals. An exemplary block that is interrupted with a hydrolysable chemical moiety is PEG_{46} esterified to PEG_4.

By a "hydrolysable chemical moiety" is meant a chemical moiety that is cleaved in aqueous solution with a half life of 1 year or less at pH 7.4 and 37

°C. Preferably, the half life of the moiety at pH 7.4 and 37 °C is one month or less.

By "nucleic acid" is meant any nucleobase oligomer. For the purposes of this specification, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone are also considered to be nucleobase oligomers. Non-limiting examples of nucleic acids are antisense oligonucleotides, small interfering RNAs (siRNAs), aptamers, and plasmid DNA.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A. Conversion of poly(ethylene glycol) monomethyl ether (PEG MME) to PEG thioacetate (PEG TAc). PEG MME was dried using a Dean Stark trap, and 2.5 eq of thionyl bromide was added. The reaction was then refluxed for 4 h at 140 °C. The toluene was evaporated, and the polymer was dissolved in dichloromethane (DCM) and precipitated in cold diethyl ether. The final conversion was accomplished by dissolving the PEG-Br in dimethylformamide (DMF) with 5 eq K_2CO_3 and 5 eq thiolacetic acid, and stirring overnight. The product was filtered, and the DMF was evaporated and dissolved in DCM. The PEG-TAc was purified over activated charcoal and precipitated in cold diethyl ether.

Fig. 1B. PEG-TAc was converted to PEG-ester-EG_4-acrylate by reacting PEG-TAc with PEG(200) diacrylate in excess. PEG-TAc was dissolved in tetrahydrofuran (THF) and degassed thoroughly. Next, the solution was added to 50 eq of PEG diacrylate in THF with 1 eq of triethylamine as base. The reaction was stirred overnight and purified by precipitation twice in cold diethyl ether.

Fig. 1C. The final polymer was synthesized by forming the PPS block initiated from benzenethiol, and using the PEG-acrylate monomer as an end-capping reagent. This was accomplished by dissolving DBU base into THF and degassing. The benzenethiol was added under argon flow, and propylene sulfide monomer was added through a gastight septum. After 1 hr, the PEG-

ester-EG_4-ester-acrylate was added, dissolved in THF, and degassed (0.5 eq). The reaction was allowed to stir overnight. The final product was purified by dissolving the dried mixture in toluene and filtering. After evaporating the toluene, the polymer was dissolved in DCM and precipitated in cold diethyl ether.

Fig. 2A. Hydrolysis of PEG-PPS at 25°C over time at various pH values. The polymer was prepared by solvent dispersion from THF. The THF was removed under vacuum prior to the start of the study. Degradation was quantified via gel permeation chromatography, and the peaks for the free PPS and PEG blocks were quantified. At elevated pH (8.4) degradation occurred more rapidly, whereas at pH 5.4 no degredation was observed.

Fig. 2B. Degradation of the same polymer preparation as that shown in Fig. 2A was quantified at 37°C. Degradation was greatest at high pH owing to rapid hydrolysis of the ester bonds under these conditions. Unexpectedly, after 20 days degradation was also observed at pH 5.4.

Fig. 3. Formulation excipients DBU and PEG600 were blended with the block copolymer PEG-PPS at 95°C with stirring.

Fig. 4. Dynamic scanning calorimetry of PEG-PPS blended with DBU-HCl. The polymer and salt melted together creating a homogeneous blend of the two materials.

Fig. 5. Characterization of PEG-PPS processing methods using optical density. Samples were analyzed directly after preparation at the same concentration (5 mg/mL). Legend represents solvent dispersion (SD), thin film extrusion (TFE), and direct hydration (DH).

Fig. 6. Gel permeation chromatograph of a formulation incubated at 95°C for three hours versus control. The data were acquired using a refractive index detector. For clarity, the control is shown on the bottom, and the 180 min sample is offset by 5%. Both chromatograms were normalized.

Fig. 7. Degradation study of PEG-PPS at 95°C over time. The bar graph represents the average number average molecular weight (Mn) of the injected samples. From left to right are unheated polymer control (Control); 15

min control heated without salt (15C); 15 and 30 min heated with salt (15, 30); 60 min heated without salt (60C); 60, 120, and 180 min heated with salt (60, 120, 180). Student's t-test comparing the unheated control with the 180 min at 95°C revealed the difference was not statistically significant. Mp represents peak molecular weight, and Mw represents weight average molecular weight. The PDI using gel permeation chromatography (GPC) is calculated by Mw/Mn. The Mn = Sum (NiMi) / Sum (Ni), and Mw = Sum ($NiMi^2$) / Sum (NiMi).

Fig. 8. Degradation study analyzed using proton NMR. Unheated, heated, and heated with salt preparations using the polymer EG_{46}-PS_{12} were compared. The polymer and salt with polymer samples were heated at 95°C for three hours prior to extraction in THF and precipitation in diethyl ether. The purified fractions were measured via proton-NMR in chloroform-D containing 0.1% TMS as an internal standard.

Fig. 9. Dynamic scanning calorimetry of the constituents separately and mixed together. The mixture was prepared by mixing EG_{46}-PS_{12} 50/50 wt/wt with DBU-HCl and heating at 95°C for 60 min. Samples were mixed thoroughly and measured on the DSC. For comparison, the polymer and DBU-HCl salt were also measured separately. The salt and polymer dissolved into a molten state upon heating.

Fig. 10. Imaging vesicles formed via direct hydration using cryogenic TEM. The formed lamellar phases were vitrified on a holey carbon grid prior to imaging. Aggregates were extruded prior to analysis using cryo-TEM.

Fig. 11A. Encapsulation efficiency of DBU-HCl formulation compared to thin film hydration with extrusion. Ovalbumin was reduced using TCEP and purified using Sephadex G50 and freeze-dried. 26 mg of the reduced ovalbumin was dissolved in 500 µl of distilled water and added to a preparation of PEG-PPS blended with DBU-HCl at 95°C for 15 min. Above, 10 µl of this solution was added, mixed, and slowly diluted with distilled water. Results were calculated from a standard curve made with the same

reduced ovalbumin sample. Thin film hydration with extrusion results were taken from Jousma *et al* Int. J. Pharm. 35 (1987) 263-274.

Fig. 11B. Encapsulation efficiency of dexamethasone and paclitaxel in PEG formulations. Dexamethasone or paclitaxel were incubated with the indicated block copolymers and the indicated PEG or control formulations according to the methods of the invention.

Fig. 11C. Bovine serum albumin and ovalbumin were prepared at 50 mg/mL in distilled water. The formulations were prepared as follows. Ten milligrams of PEG-PPS was heated with 10mg of PEG500 dimethyl ether and heated at 95°C for 15 minutes. The melt was mixed and allowed to cool to room temperature. After, a volume of protein solution (5μL or 10μL) was added and slowly diluted with distilled water up to 1 mL volume with mixing. To calculate the encapsulation efficiencies, standard curves were generated for both BSA (using fluorescamine) or ovalbumin (using FITC-ovalbumin). The dispersed vesicle solutions were centrifuged for 10 minutes at 10,000g to sediment the vesicles, and we measured the free protein in the supernatant.

Fig. 12. Optical density change over time with heating of the polymer at 95°C. PEG-PPS (EG_{46}-PS_{64}) micelles were prepared from solvent dispersion in water using THF. After removing the THF under vacuum for 1 h, the suspension at 10 mg/mL was placed 1 mL each into 1.5 mL eppendorf tubes. To this aliquot, 200μL of THF was added. Initial samples for time=0 optical density (OD) and dynamic light scattering (DLS) were removed, and the tubes were placed into a pre-heated incubator at 95°C. Samples were drawn at specific time points, 100 μl per sample and aliquoted into a 96 well plate on ice. After the final sample was removed, 50 μl of each sample was added to a new 96 well plate, and the optical density was measured at 400 nm using a plate reader in absorbance mode.

Fig. 13. The samples for optical density (50 μl) were dispersed into 550 μl of double distilled water and measured using DLS. Both the OD and DLS

data clearly display a trend towards larger particle size, and changing morphology.

Fig. 14. Cryogenic Transmission Electron Microscopy of the thermal transition of PEG-PPS micelles into vesicles. The micelle sample (left) displays small aggregates of PEG-PPS in good agreement with the DLS results from Fig 2. The 30 min sample (right) shows the vesicles (polymersomes) created during heating.

Fig. 15. The aggregation of PEG-PPS micelles during heating captured using negative staining TEM. Here the 3 min sample from the heating experiment was added to a 400 mesh carbon coated copper grid which had been prepared by glow discharge. The sample was then blotted off after 60 sec, and stained 30 sec with 2% uranyl acetate. The image above was taken at 75,000x. The 2-D surface vesicles form slowly into short worm like micelles.

Fig. 16A. Particle size distribution by dynamic light scattering of PEG44-PPS20 cyclosporine A-loaded polymer micelles.

Fig. 16B. Particle size distribution of the micelles of Fig. 16A after drying and rehydration.

Fig. 17A. Stability of PEG_{44}-PPS_{20} after exposure to gastric pH, as measured by gel permeation chromatography.

Fig. 17B. Stability of PEG_{44}-PPS_{20} after exposure to gastric pH, as measured by dynamic light scattering.

Fig. 18. A synthetic route to PEG-PPS-PEI. A disulfide link between the PPS block and the PEI block allows destabilization of the polymer after endocytosis.

Fig. 19. PEG-PPS-PEI was demonstrated to condense plasmid DNA into nanoparticles of size distribution for transfection.

Fig. 20. PEG-PPS-PEI was demonstrated to transfect cells very efficiently, even difficult-to-transfect cells such as 3T3 fibroblasts, shown here. Other cells were transfected at even higher transfection efficiency, including 239T cells at 96% with PEG2kDa-PPS_{27}-PEI_{96}.

Fig. 21. Cytotoxicity with PEG-PPS-PEI was much lower than that with linear PEI of the same molecular weight at the same PEI concentration.

Fig. 22. The size of the resulting gene pharmaceutical agent complexes could be controlled by a number of means, including the ratio of the polymers used to construct mixed micelles.

Fig. 23. PEG-PPS-based polymers were able to condense oligonucleotide pharmaceutical agents here with an siRNA sequence, for example to prevent gel migration. This demonstrates the versatility of these polymers with oligonucleotides sequences as well as plasmid DNA.

Fig. 24. PEG-PPS-based polymers were able to induce high transfection efficiency with oligonucleotides pharmaceutical agents here with siRNA knocking down expression of lamin A/C in HeLa cells.

Fig. 25. Gene complexes formed with a 10:1 ratio of PEG-PPS-PEI:PEG-PPS efficiently transfect cells. PEG-PPS was used to reduce the size of PEG-PPS-PEI micelles resulting in smaller complexes with gene-based pharmaceutical agents. Here, cells were incubated with 30 nm complexes containing the green fluorescent protein (GFP) gene sequence.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides various block copolymers having at least two blocks, one hydrophilic and one hydrophobic. Block copolymers may further include additional blocks, be interrupted with hydrolysable chemical moieties, or be otherwise modified. Each block of the copolymer is important for self-assembly and biological function. The size of each block may be determined independently of the other blocks, e.g., to tailor the function of each block. Each block may be synthesized and bound to the other blocks using methods known in the art, e.g., as described in US 2003/0059906 and WO 2007/008300, which are hereby incorporated by reference.

Block Copolymers

Hydrophilic Blocks.

The hydrophilic block, e.g., PEG, may be utilized to (i) prevent non-specific nucleic acid/positively charged polymer complex interactions with serum proteins, cells, and tissues in the body, which allows for specific interactions to be designed via incorporated ligands, and (ii) increase the solubility of the complexes in aqueous milieu.

Polymers or molecules that are soluble or swell in an aqueous environment will prevent protein absorption while still enhancing the solubility of the particles. For example, carbohydrate polymers such as hyaluronic acid (HA) may swell to about 1000 times their volume and are used in nature to prevent protein absorption. Other carbohydrate polymer or molecule candidates are found in nature. Exemplary hydrophilic blocks include poly(ethylene glycol), poly(ethylene oxide)-co-poly(propylene oxide) di- or multiblock copolymers, poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(N-vinyl pyrrolidone), poly(acrylic acid), poly(ethyloxazoline), poly(alkylacrylates), poly(acrylamide), poly(N-alkylacrylamides), polypeptides, polysaccharides, poly(N-acryloylmorpholine), or poly(N,N-dialkylacrylamides), potentially bearing polar, ionic, or ionizable groups in the aliphatic chains.

Hydrophilic blocks having molecular weights between 500 and 10,000 Da are practical and convenient, although higher molecular weight hydrophilic blocks may be employed. For hydrophilic blocks, a number of repeating units between about 10 and about 250 is preferable because of the ease with which these materials may be eliminated from the body by renal filtration. A PEG hydrophilic block is preferably between 750 and 5500 Da, e.g., between 2 and 5 kDa (e.g., a block containing 115 units). Hydrophilic blocks with a larger number of repeating units may also be cleared by the kidney but at slower rates than hydrophilic polymers of lower number of repeating units, which may place limits on doses that can be applied.

Hydrophobic blocks.

The hydrophobic block may include any polymer that is hydrophobic in context. A preferred hydrophobic block is PPS. Poly(propylene glycol) (PPG), a structural homolog of PPS with an oxygen atom instead of a sulfur atom in the backbone, may also be employed. Larger PPG chains may be required relative to the useful length of PPS chains. In general, polymers that have low melting or glass transition temperatures are most desirable because this characteristic is most conducive to effective micellization.

Other polymers that are otherwise hydrophilic but are derivatized with hydrophobic functionalities on their side chains may be used in the hydrophobic block. Examples include esterified poly(acrylic acid), esterified poly(glutamic acid) or poly(aspartic acid), and hydrophobic peptides or peptoids (e.g., *N*-substituted glycines). Hydrophobic blocks having molecular weights between 300 and 5000 Da are practical and convenient, although higher molecular weight hydrophobic blocks may also be employed. For hydrophobic blocks, the number of repeating units is, for example, between about 4 and about 240, preferably between 4 and 70. For example, a polypropylene sulfide hydrophobic block can vary from 150 to 16,000 Da, e.g., from 200 to 15,000 Da, depending on the initial hydrophilic block (e.g., PEG) used and the desired application. A higher number of repeating units may also be utilized; however, self-assembled structures from such polymers may be far from their equilibrium morphology.

Additional Blocks.

In certain embodiments, the copolymers of the invention have only two polymeric blocks, although other chemical moieties, e.g., hydrolysable, charged, or biologically active moieties, may be present. In other embodiments, the copolymers may include three or more polymeric blocks. Such additional blocks may be employed, for example, (i) to bind nucleic acids or other charged molecules via electrostatic interactions, (ii) to help a self-assembled structure enter the cell, and/or (iii) to execute another biological

function. When employed to bind charged molecules, e.g., a nucleic acid, the additional block is preferably sized to produce reversible binding with the charged molecule. For example, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or even 95% of the charged molecule may dissociate from the block under appropriate cellular conditions, e.g., in the cytosol or within the nucleus. In addition, short blocks may be employed to reduce or eliminate any toxicity.

The use of a positively charged block in a block copolymer is particularly advantageous for the formation of small complexes with, e.g., nucleic acids. Exemplary positively charged blocks are a polypeptide (e.g., polylysine), poly(ethyleneimine) (PEI), poly(amidoamine), poly(sodium 1-(*N*-acryloylpiperazin-1-yl)diazen-1-ium-1,2-diolate), poly(sodium 1-(*N*-acryloylhomopiperazin-1-yl)diazen-1-ium-1,2-diolate) or poly(sodium 1-(*N*-acryloyl-2,5-dimethylpiperazin-1-yl)diazen-1-ium-1,2-diolate). Blocks having a positive charge between 5 and 20 at physiological pH are practical and convenient, although blocks with a larger number of positive charges may also be employed. Molecular weights of a positively charged block between 500 and 10000 Da are preferred. For polymers such as PEI, a number of repeating units between about 10 and about 250 is preferred. The longer a charged block becomes, in general the higher the corresponding cytotoxicity.

Additional blocks may also be peptides. Peptides have been extensively utilized in the field of pharmaceutical agent delivery and other medical applications owing to the multitude of chemical and biological functionalities they can embody. Examples of charged peptides include the biologically active TAT peptide and oligo(lysine) (e.g., Lys_9) peptides. Both peptides are charged and can bind to nucleic acids and other negatively charged molecules. Additional examples of charged blocks include oligo(histidine), oligo(arginine) (e.g., Arg_9), and copolymers of Lys, Arg, and His.

Furthermore, poly(amidoamine) (PAMAM) dendrimers have been used to complex nucleic acids and may be included in a charged block. PAMAM

has been shown to efficiently escape the endosome, allowing release of the complexed contents in the cytosol.

Polymer Modifications.

Polymeric blocks in the copolymers of the invention may also be modified. Such modifications include adding charged groups (e.g., carboxylic acids groups, sulfates, sulfones, and amines), hydrophilic groups (e.g., hydroxyl), hydrophobic groups (e.g., phenyl or methyl), hydrolysable groups (e.g., ester, amide, thioester, anhydride, or ketal moieties), or groups sensitive to oxidation. In particular, portions of a polymeric block that are exposed to aqueous solution in supramolecular structures may be modified to include one or more charged groups to allow for more efficient uptake in vivo, as described herein. Blocks copolymers may also be end capped with various groups as described herein and known in the art.

One particular example of a block copolymer modified by a hydrolysable chemical moiety is illustrated in Example 1. For such a hydrolysable block copolymer, the PEG diacrylate preferably has a molecular weight from 200 to 600 Da. Other hydrolysable blocks that may be used include lactide or caprolactone groups.

Degradation in vivo.

In order to avoid irreversible accumulation in the targeted organs, the self-assembled carriers may demonstrate some form of degradation in vivo. Polysulfides are known to readily undergo oxidation to polysulfoxides and even to polysulfones, e.g., by the action of mild oxidizing agents, such as hydrogen peroxide. Under biological conditions, this oxidation can be performed extracellularly, e.g., by macrophages, or intracellularly after cellular uptake into an endosomal or lysosomal compartment. A similar kind of reaction is used for oxidizing thioether-terminated PEGs (used as emulsifiers in pulp and paper processing) in order to break wastewater foams (see, e.g., U.S. Patent No. 4,618,400).

The conversion of the polysulfides to polysulfoxides can solubilize the block copolymers in water, allowing elimination through excretion (Napoli A et al., Nature Materials, 2004. 3(3):p. 183-189.). The conversion can trigger the instability of self-assembled aggregates, e.g., the conversion of gels to micelles or soluble polymers, the conversion of vesicles to micelles or soluble polymers, or the conversion of micelles into micelles of different size and shape or to soluble polymers. Destabilizing the aggregate can also trigger the release of any encapsulated pharmaceutical agents, e.g., a nucleic acid. The mechanisms of clearance of soluble polymers are relatively well understood. The most important such mechanism is clearance via renal filtration, the effective molecular weight cutoff of which is approximately 30,000. Particles of size less than approximately 100 nm can be cleared from the bloodstream in the liver. Lymphatic uptake also may play a role in clearance.

Copolymers of the invention may also be synthesized such that they respond to the changing environment of the endosome. For example, a disulfide bond may be introduced into the copolymer so that, as the environment of the endosome becomes reducing, the bond is cleaved, thereby destabilizing the complex within the endosome. An N-PO_3 bond, which responds to low pH, e.g., as in an endosome, may also be introduced into the structure. Additional bonds that are sensitive to intracellular degradation, such as vinyl ether, orthoester, and acyl hydrazone, may also be employed.

Copolymers of the invention may also be synthesized so that a hydrophobic or hydrophilic block is interrupted with a hydrolysable chemical moiety, e.g., an ester or amide. Hydrolysis of the moiety leads to a change in the relative amount of hydrophobic or hydrophilic block, which in turn can lead to destabilization of a supramolecular complex by changing its favorable self-assembled morphology. In one embodiment, the half-life of the hydrolysable bond is between 1 hour and 1 year in an aqueous solution at pH 7.4 and 37 °C. Desirably, the half-life is between 1 day and 9 months, more preferably between 2 days and 6 months, and most preferably between 4 days

and 3 weeks. In certain embodiments, a thioether or secondary amine is present at the alpha or beta position relative to the hydrolysable bond.

Self Assembly.

Amphiphilic block copolymers have long been used as surfactants and dispersants in a wide variety of applications; the formation of organized structures in a solvent that is selective for one of the blocks is the basis of this behavior.

Well-defined self-assembled structures, such as spherical or cylindrical micelles, lamellae, or vesicles (Booth et al., Macromol. Chem., Rapid Commun. 2000, 21, 501-527; Won, Science 1999, 283, 960-963; Discher et al., Science 1999, 284, 1143-1146; and Eisenberg et al., Macromolecules 1998, 31, 3509) have been observed in poly(oxyalkylene) block copolymers. The concentration of the polymer solution and the temperature greatly influence the kind of aggregates that can be formed: changing, e.g., from liquid spherical micellar phases to cubic phases of spherical micelles and finally to hexagonal phases of cylindrical micelles upon an increase in temperature (Mortensen, Progr. Coll. Polym. Sci. 1993, 93, 72-75). The phase diagram and accessible structures of the amphiphilic block copolymers exhibit a dependence on the block length and number, i.e., basically, on the hydrophilic/lipophilic balance.

Block copolymers of PEG with poly(ethylethylene) have shown a propensity to form worm-like micelles at a ratio 55/45 between hydrophilic and hydrophobic repeating units (total MW=4900), and to form lamellar structures at a ratio 40:37 (total MW=3900).

This invention provides materials capable of generating a wide variety of structures; for example, a material containing long sequences of hydrophilic groups is able to form micelles, while a high hydrophobic content facilitates the formation of lamellar gels, and, under suitable conditions, vesicles.

The formation of vesicles can also be achieved by adding to water a solution or colloidal suspension of the copolymer in an organic solvent and subsequently removing the organic solvent.

Combinations of two or more block copolymers of the invention may also be employed to form supramolecular structures. Typically, PEG-PPS with Mw fractions (*f*PEG) of approximately 0.99-0.7 form micelles, whereas Mw fractions (*f*PEG) of approximately 0.30 to 0.25 form vesicles.

Thermal Transitions of Block Copolymer Assemblies.

The invention also features a method of making micelles from vesicles. In these embodiments, micelles are formed from block copolymers that are thermodynamically disposed to form vesicles. Upon heating the micelles, they spontaneously form vesicles. When the micelles are in aqueous suspension with a dissolved compound, e.g., pharmaceutical agent, the process of forming vesicles results in encapsulation of the pharmaceutical agent in the interior of the vesicles.

For example, polymer micelles are formed using a polymer composition with an fPEG that thermodynamically favors vesicle formation. In suspension, the micelles are metastable and can be highly concentrated. Application of heat to the metastable micelles induces spontaneous formation of vesicles, which are very small and homogeneous in size distribution. Pharmaceutical agent incorporated in the micelle suspension is loaded within the vesicles during their formation. Micelles can be made from copolymers that would otherwise form vesicles, i.e., under nonequilibrium conditions, by means that include rapidly dissolving/dispersing the polymer without heating, for example from powdered or lyophilizied polymer. This rapid dissolution kinetically traps the polymer in a micellar form. Addition of mobility provides the opportunity for the nonequilibium form to approach equilibrium, here by forming the more favored vesicle morphology.

We have shown that when heat is applied to a mixture containing block copolymers and a salt, the mixture melts into a homogeneous composition. Small molecule pharmaceutical agents also melt in the formulation during heating. This process leads to encapsulation of the pharmaceutical agents in

the supramolecular structures, e.g., micelles or vesicles, formed by the block copolymers.

Drying and Rehydrating Block Copolymer Assemblies.

Supramolecular structures of the invention, e.g., vesicles, may be dehydrated and made into dry formulations. Such dry formulations may be rehydrated in vivo upon administration or in vitro prior to administration or other use. Preferably, a dry formulation includes less than 5% water by weight. The limit of water content that is acceptable in a dry formulation may be determined by measurement of storage lifetime by standard methods. Dry formulations may be stable for greater than two weeks, one month, six months, or one year. A pharmaceutical agent encapsulated within the supramolecular structures, e.g., by a method described herein, may be reconstituted to an active form upon rehydration. The dried compositions may be rehydrated in any aqueous solution, e.g., in a pharmaceutically acceptable diluent. In conventional pharmaceutical processing by a number of drying methods, water contents substantially less than 5% may be easily reached.

Pharmaceutical Compositions.

In suitable conditions for the generation of micelles, e.g., by treatment with heat, the block copolymers of the invention can be used for the encapsulation of pharmaceutical agents such as peptides, nucleic acids, antibiotics (e.g., ampicillin or tetracycline), chemotherapeutics (e.g., doxorubicin), or other small molecule pharmaceutical agents. When lamellar phases are to be formed, vesicles can be generated from the lamellar structure bending; in this way, water-dissolved pharmaceutical agents can be entrapped in the internal cavity of the vesicle. Pharmaceutical compositions may also employ excipients that increase the encapsulation efficiency of one or more block copolymers for pharmaceutical agents that are hydrophilic, hydrophobic, or amphiphilic. The excipients may increase the compatibility of the pharmaceutical agent with one or more blocks in the block copolymer, e.g., by

reducing repulsive forces or increasing attractive forces between the pharmaceutical agent and one or more blocks of the block copolymer.

Suitable exemplary excipients are 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and polyethylene glycol (e.g., PEG 600). PEG having a molecular weight between 400 and 800 Da is effective as an excipient. Polyethylene glycols outside of this range have not been effective. Other excipients that may be used are PPS-PEG copolymers and hydrobromide or hydrochloride salts of common organic bases such as triethanolamine, triethylamine, or pyridine. The addition of an excipient to a mixture containing a block copolymer and a pharmaceutical agent may increase the efficiency of encapsulation of the pharmaceutical agent by greater than 1.5-fold, 3-fold, 5-fold, 10-fold, or 50-fold. Examples of improved pharmaceutical agent encapsulation in the presence of excipients are provided herein.

The copolymers of the invention may be dispersed in a pharmaceutically acceptable diluent. In addition, self-assembled structures of the invention may include pharmaceutical agents or biologically active compounds. In various embodiments, the pharmaceutical composition includes about 1 ng to about 20 mg of pharmaceutical agent, e.g., a nucleic acid or a hydrophobic compound (e.g., paclitaxel or dexamethasone). In some embodiments, the composition contains about 10 ng to about 10 mg, about 0.1 mg to about 500 mg, about 1 mg to about 350 mg, about 25 mg to about 250 mg, or about 100 mg of pharmaceutical agents. Those of skill in the art of clinical pharmacology can readily arrive at dosing amounts using routine experimentation.

Suitable diluents include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The composition can be adapted for the mode of administration and can be in the form of, for example, a pill, tablet, capsule, spray, powder, or liquid. In some embodiments, the pharmaceutical composition contains one or more pharmaceutically acceptable additives suitable for the selected route and mode of administration. These compositions may be administered by, without

limitation, any parenteral route including intravenous, intra-arterial, intramuscular, subcutaneous, intradermal, intraperitoneal, intrathecal, as well as topically, orally, and by mucosal routes of delivery such as intranasal, inhalation, rectal, vaginal, buccal, and sublingual. In some embodiments, the pharmaceutical compositions of the invention are prepared for administration to vertebrate (e.g., mammalian) subjects in the form of liquids, including sterile, non-pyrogenic liquids for injection, emulsions, powders, aerosols, tablets, capsules, enteric coated tablets, or suppositories. Conventional procedures and ingredients for the selection and preparation of suitable formulations are described, for example, in Remington's Pharmaceutical Sciences (2003 - 20th edition) and in The United States Pharmacopeia: The National Formulary (USP 24 NF19), published in 1999.

Pharmaceutical agents may be hydrophilic, hydrophobic, or amphoteric. The type of supramolecular structure employed to encapsulate an agent will depend on the solubility characteristics of the agent and the copolymer. Typically, hydrophilic agents will be encapsulated in the interior of vesicles, and hydrophobic agents will be encapsulated in the interior of micelles. Agents that may be employed with copolymers of the invention include but are not limited to natural and synthetic compounds, e.g., a nucleic acid, having the following therapeutic activities: anti-arthritic, anti-arrhythmic, anti-bacterial, anticholinergic, anticancer, anticoagulant, antidiuretic, antidote, antiepileptic, antifungal, anti-inflammatory, antimetabolic, antimigraine, antineoplastic, antiparasitic, antipyretic, antiseizure, antisera, antispasmodic, analgesic, anesthetic, beta-blocking, biological response modifying, bone metabolism regulating, cardiovascular, diuretic, enzymatic, fertility enhancing, growth-promoting, hemostatic, hormonal, hormonal suppressing, hypercalcemic alleviating, hypocalcemic alleviating, hypoglycemic alleviating, hyperglycemic alleviating, immunosuppressive, immunoenhancing, muscle relaxing, neurotransmitting, parasympathomimetic, sympathominetric plasma extending, plasma expanding, psychotropic, thrombolytic, chemotherapeutic, and vasodilating.

Nucleic Acids.

In certain embodiments of the invention, the block copolymer composition contains a nucleic acid. The nucleic acid may associate with one or more blocks of a copolymer and may be incorporated into supramolecular structures containing block copolymers, e.g., micelles or vesicles. In preferred embodiments, the nucleic acid is present in a therapeutically effective amount in a pharmaceutical composition containing one or more block copolymers. Antisense oligonucleotides, small interfering RNAs (siRNAs), aptamers, and plasmid DNA are examples.

Oligonucleotides containing modified backbones or non-natural internucleoside linkages may be employed. Nucleobase oligomers that have modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity, wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included. Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Patent Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, each of which is herein incorporated by reference.

Nucleobase oligomers having modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by

short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 components. Representative United States patents that teach the preparation of the above oligonucleotides include, but are not limited to, U.S. Patent Nos.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference.

In other nucleobase oligomers, both the sugar and the internucleoside linkage, i.e., the backbone, are replaced with novel groups. One such nucleobase oligomer, is referred to as a Peptide Nucleic Acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Methods for making and using these nucleobase oligomers are described, for example, in "Peptide Nucleic Acids: Protocols and Applications" Ed. P.E. Nielsen, Horizon Press, Norfolk, United Kingdom, 1999. Representative United States patents that teach the preparation of PNAs include, but are not limited to, U.S. Patent Nos.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., Science, 1991, 254, 1497-1500.

In particular embodiments of the invention, the nucleobase oligomers have phosphorothioate backbones and nucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ (known as a methylene (methylimino) or MMI backbone), $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$, and $-O-N(CH_3)-CH_2-CH_2-$. In other embodiments, the oligonucleotides have morpholino backbone structures described in U.S. Patent No. 5,034,506.

Nucleobase oligomers may also contain one or more substituted sugar moieties. Nucleobase oligomers comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl, and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred nucleobase oligomers include one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl, or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of a nucleobase oligomer, a group for improving the pharmacodynamic properties of a nucleobase oligomer, or other substituents having similar properties. Preferred modifications are 2'-O-methyl and 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE). Another desirable modification is 2'-dimethylaminooxyethoxy (i.e., $O(CH_2)_2ON(CH_3)_2$), also known as 2'-DMAOE. Other modifications include, 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on an oligonucleotide or other nucleobase oligomer, particularly the 3' position of the sugar of the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of the 5' terminal nucleotide. Nucleobase oligomers may also have sugar

mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Patent Nos. 4,981,957, 5,118,800, 5,319,080, 5,359,044, 5,393,878, 5,446,137, 5,466,786, 5,514,785, 5,519,134, 5,567,811, 5,576,427, 5,591,722, 5,597,909, 5,610,300, 5,627,053, 5,639,873, 5,646,265, 5,658,873, 5,670,633, and 5,700,920, each of which is herein incorporated by reference in its entirety.

Nucleobase oligomers may also include nucleobase modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases, such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine, 2-thiocytosine, 5-halouracil, cytosine, 5-propynyl uracil, 6-azo uracil, thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo (e.g., 5-bromo), 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 7-methyladenine, 8-azaguanine, 8-azaadenine, 7-deazaguanine, 7-deazaadenine, and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in The Concise Encyclopedia of Polymer Science and Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y. S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of an antisense or (partially) complementary oligonucleotide of the invention to a target nucleic acid. These include 5-substituted pyrimidines, 6-azapyrimidines, and N-2, N-6, and O-6 substituted purines, including 2-aminopropyladenine, 5-

propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C. (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., eds., Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are desirable base substitutions, even more particularly when combined with 2'-O-methoxyethyl or 2'-O-methyl sugar modifications. Representative United States patents that teach the preparation of certain of the above-noted modified nucleobases as well as other modified nucleobases include U.S. Patent Nos. 4,845,205, 5,130,302, 5,134,066, 5,175,273, 5,367,066, 5,432,272, 5,457,187, 5,459,255, 5,484,908, 5,502,177, 5,525,711, 5,552,540, 5,587,469, 5,594,121, 5,596,091, 5,614,617, 5,681,941, and 5,750,692, each of which is herein incorporated by reference.

Another modification of a nucleobase oligomer of the invention involves chemically linking to the nucleobase oligomer one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 86:6553-6556, 1989), cholic acid (Manoharan et al., Bioorg. Med. Chem. Let, 4:1053-1060, 1994), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 660:306-309, 1992Manoharan et al., Bioorg. Med. Chem. Let., 3:2765-2770, 1993), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 20:533-538, 1992), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 10:1111-1118, 1991; Kabanov et al., FEBS Lett., 259:327-330, 1990; Svinarchuk et al., Biochimie, 75:49-54, 1993), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett., 36:3651-3654, 1995; Shea et al., Nucl. Acids Res., 18:3777-3783, 1990), a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 14:969-973, 1995), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 36:3651-3654, 1995), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1264:229-

237, 1995), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 277:923-937, 1996). Representative United States patents that teach the preparation of such nucleobase oligomer conjugates include U.S. Patent Nos. 4,587,044, 4,605,735, 4,667,025, 4,762,779, 4,789,737, 4,824,941, 4,828,979, 4,835,263, 4,876,335, 4,904,582, 4,948,882, 4,958,013, 5,082,830, 5,109,124, 5,112,963, 5,118,802, 5,138,045, 5,214,136, 5,218,105, 5,245,022, 5,254,469, 5,258,506, 5,262,536, 5,272,250, 5,292,873, 5,317,098, 5,371,241, 5,391,723, 5,414,077, 5,416,203, 5,451,463, 5,486,603, 5,510,475, 5,512,439, 5,512,667, 5,514,785, 5,525,465, 5,541,313, 5,545,730, 5,552,538, 5,565,552, 5,567,810, 5,574,142, 5,578,717, 5,578,718, 5,580,731, 5,585,481, 5,587,371, 5,591,584, 5,595,726, 5,597,696, 5,599,923, 5,599,928, 5,608,046, and 5,688,941, each of which is herein incorporated by reference.

The present invention also includes nucleobase oligomers that are chimeric compounds. "Chimeric" nucleobase oligomers are nucleobase oligomers, particularly oligonucleotides, that contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide. A chimeric nucleobase oligomer may contain one or more regions to confer increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid.

Chimeric nucleobase oligomers may be formed as composite structures of two or more nucleobase oligomers as described above. Such nucleobase oligomers, when oligonucleotides, have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include U.S. Patent Nos. 5,013,830, 5,149,797, 5,220,007, 5,256,775, 5,366,878, 5,403,711, 5,491,133, 5,565,350, 5,623,065, 5,652,355, 5,652,356, and 5,700,922, each of which is herein incorporated by reference in its entirety.

Locked nucleic acids (LNAs) are nucleobase oligomers that can be employed in the present invention. LNAs contain a 2'O, 4'-C methylene

bridge that restrict the flexibility of the ribofuranose ring of the nucleotide analog and locks it into the rigid bicyclic N-type conformation. LNAs show improved resistance to certain exo- and endonucleases and activate RNAse H, and can be incorporated into almost any nucleobase oligomer. Moreover, LNA-containing nucleobase oligomers can be prepared using standard phosphoramidite synthesis protocols. Additional details regarding LNAs can be found in PCT publication No. WO 99/14226 and U.S. Patent Application Publication No. US 2002/0094555 A1, each of which is hereby incorporated by reference.

Arabinonucleic acids (ANAs) can also be employed in the present invention. ANAs are nucleobase oligomers based on D-arabinose sugars instead of the natural D-2'-deoxyribose sugars. Underivatized ANA analogs have similar binding affinity for RNA as do phosphorothioates. When the arabinose sugar is derivatized with fluorine (2' F-ANA), an enhancement in binding affinity results, and selective hydrolysis of bound RNA occurs efficiently in the resulting ANA/RNA and F-ANA/RNA duplexes. These analogs can be made stable in cellular media by a derivatization at their termini with simple L sugars. The use of ANAs in therapy is discussed, for example, in Damha et al., Nucleosides Nucleotides & Nucleic Acids 20: 429-440, 2001.

Polypeptides

Polypeptides may be employed in embodiments of the invention. The amino acids of the polypeptide may be natural, non-natural, or a mixture thereof. The polypeptide may be produced by recombinant genetic technology or chemical synthesis using methods known in the art. Examples of polypeptides are a single-chain peptide fragment (e.g., a polypeptide of 5-20 amino acids joined by conventional peptide bonds), a naturally occurring protein, and an antibody or antigen-binding fragment thereof.

Methods for Internalization and Therapeutic Methods

The copolymers may utilize biological pathways for both delivery and therapeutic action. In one embodiment, a block copolymer that self-assembles in aqueous environments into nanoscale micelles or vesicles may be employed for the delivery of pharmaceutical agents, such as siRNA or other nucleic acids. Moreover, a block copolymer of the invention can exploit changing intracellular environments, e.g., the reductive environment of the endosome, for efficient delivery of the pharmaceutical agent and a biological pathway for therapeutic action, e.g., the activation of the RNAi pathway for gene silencing. The development of biologically responsive materials to induce release of a therapeutic agent within the early endosome and destabilize it holds promise for the development of delivery systems that can overcome limitations of current delivery systems.

Colloidal particles such as nanospheres, liposomes, and micelles have been studied extensively for site-specific pharmaceutical agent delivery. Unless the reticuloendothelial system (RES) is a target, the particles must escape capture by the RES of the liver and the filtration activity of the lungs. Prolonged survival of colloidal systems in the blood has been obtained by the use of PEG-containing amphiphiles (Lasic et al., Ed. *Stealth Liposomes*; CRC Press: Boca Raton, FL, 1995). By virtue of marked reduction of opsonization by plasma proteins, the macrophages clearance of PEG-based liposomes has been drastically reduced (Torchilin et al., Biochim Biophys Acta 1994, 1195, 11-20).

A variety of internalization agents, i.e., compounds or species that enhance the internalization of the copolymers of the invention, such as antibodies, growth factors, cytokines, adhesion factors, oligonucleotide sequences and nuclear localization sequences has served to enhance the delivery capabilities of PEG-coated liposomes, and it has been demonstrated that the maximal activity is shown by ligands tethered to the distal end of PEG chains (Blume et al., Biochim. Biophys. Acta 1993, 1149, 180-184; Zalipsky et al., Bioconjugate Chem. 1995, 6, 705-708; Zalipsky, J. Controlled Release

1996, 39, 153-161; Gabizon, Bioconjugate Chem. 1999, 10, 289-298). This approach can be employed with the polymers of the invention. Some internalization agents can lead to very efficient cellular uptake, such as the use of growth factors, for example, fibroblast growth factor to effect cellular uptake of DNA formulations. Other internalization agents can lead to very efficient intracellular trafficking, such as nuclear localization sequences, and these may be used in the present invention. Additional internalization agents include transferrin, folate, a lectin, growth factor, an RGD peptide, and a mannose-containing glycopeptide.

The copolymers of the present invention are useful for any application in the controlled release, e.g., in the cytosol or nucleus, of a pharmaceutical agent, e.g., nucleic acid. The release of the contents, e.g., the nucleic acid, of the self-assembled aggregate, such as a micelle or vesicle, may be achieved through sensitivity of the aggregate to the environment, such as triggering a release based on the lowering of pH, increase in the extent of oxidation, and increase in the concentration of proteases during the process of intracellular trafficking from the endosome to the lysosome. Excipients may also be incorporated along with the pharmaceutical agent to help it in reaching its final biological target, such as incorporation of agents that assist in destabilizing or permeabilizing biological membranes, such as the endosomal or lysosomal membranes, to enhance transport of the nucleic acid into the cytoplasm or ultimately into the nucleus.

The polymers may also be employed to deliver mixtures of pharmaceutical agents, e.g., two or more different nucleic acids or a nucleic acid and a pharmaceutical agent, such as an antibiotic.

Gene-based Pharmaceutical Agents.

The block copolymers of the invention may be used to deliver nucleic acids for the up- or down-regulation of genes. Examples of nucleic acids include siRNA, ODN (antisense), and pDNA, including pDNA encoding therapeutic proteins.

The internalization of DNA/positively charged polymer complexes can be enhanced by the covalent attachment of ligands, such as transferrin, folate, lectins, epidermal growth factor (EGF), RGD peptides, and mannose-containing species such as mannose-containing glycopeptides to bind to the mannose receptor (Kircheis, R., et al., Gene Ther, 1997. 4(5): p. 409-18; Gottschalk, S., et al., Gene Ther, 1994. 1(3): p. 185-91; Erbacher, P., et al., Hum Gene Ther, 1996. 7(6): p. 721-9; Blessing, T., et al., Bioconjug Chem, 2001. 12(4): p. 529-37; Harbottle, R.P., et al., Hum Gene Ther, 1998. 9(7): p. 1037-47; East L, Isacke CM. Biochimica et Biophysica Acta, 2002 1572:p. 364-386.). The ligand functions to direct the DNA complex to the cell surface by specifically binding to a receptor, and mediating endocytosis. Fusogenic peptides and other functional groups have been attached to enhance endosomal escape of the DNA complex (Carlisle, R.C., Curr Opin Mol Ther, 2002. 4(4): p. 306-12; Bell, P.C., et al., J Am Chem Soc, 2003. 125(6): p. 1551-8).

There exists a parallel need for delivery of other gene-based pharmaceutical agents, including ODN and pDNA. Here, the agent must also be delivered to the cell and its cytoplasm, and eventually to the nucleus. With ODNs, the need is even more acute, since they function by stoichiometric competition. With plasmids, the challenge is even higher, since the large size of the plasmid greatly inhibits its passage through the membranes of the cell, e.g., the plasma membrane and the endosomal membranes.

Methods for Delivering Pharmaceutical Agents.

The invention provides methods for delivering a pharmaceutical agent, e.g., a nucleic acid, to a cell or an animal, e.g., a mammal, or plant by contacting the cell or administering to the animal a pharmaceutical composition of the invention. The delivery may reduce or inhibit the expression of a target gene in a cell (e.g., a eukaryotic cell, a plant cell, an animal cell, an invertebrate cell, a vertebrate cell, such as a mammalian or human cell, or a pathogen cell) or may treat the animal or cell by any mechanism specific to the pharmaceutical agent contained in the

pharmaceutical composition. The method may be used to treat infection by a pathogen or to treat a nonpathogenic disease, e.g., cancer, postsurgical adhesions, scar formation, or restenosis after removal of arterial block (e.g., via balloon angioplasty or stenting). Typically, a nucleic acid internalized in the cell specifically reduces or inhibits the expression of a target gene, e.g., one associated with the disease (e.g., all or a region of a gene, a gene promoter, or a portion of a gene and its promoter). Exemplary pathogens include bacteria, protozoan, yeast, and fungi. In some embodiments, the nucleic acid or other molecule inhibits the expression of an endogenous gene in a vertebrate cell or a pathogen cell (e.g., a bacterial, a yeast cell, or a fungal cell), or inhibits the expression of a pathogen gene in a cell infected with the pathogen (e.g., a plant or animal cell). The nucleic acid or other molecule may also reduce or inhibit the expression of an endogenous gene, e.g., in a cancer cell or in cells that produce undesirable effects, e.g., restenosis, scar formation, and postsurgical adhesions. In some embodiments, the target gene is a gene associated with cancer, such as an oncogene, or a gene encoding a protein associated with a disease, such as a mutant protein, a dominant negative protein, or an overexpressed protein.

Alternatively, the nucleic acid or other pharmaceutical agent delivered may increase the expression of a gene. For example, the copolymer of the invention may be used to deliver a plasmid or other gene vector to the nucleus where one or more genes contained on the plasmid may be expressed. Such a system may be employed to enable expression of gene products that are not expressed endogenously, to increase expression of endogenous gene products, and to replace gene products that are mutated or otherwise non-functional. In some cases, local expression of these genes is mostly desired, as with, without limitation, vascular endothelial growth factor, transforming growth factor beta, platelet derived growth factor, fibroblast growth factor, insulin-like growth factor, bone morphogenetic protein, growth and differentiation factor, nerve growth factor, neurotrophin, cytokines, and transcription factors, such as hif-1alpha, runx2, and sox-9.

The nucleic acid or other pharmaceutical agent may reduce, inhibit, or increase expression of a target gene by at least 20, 40, 60, 80, 90, 95, or 100%. The methods of the invention may also be used to simultaneously reduce or inhibit the expression of one or more target genes while increasing the expression of one or more other target genes.

Treatment of Disease.

The compositions of the inventions may be used to treat a disease, .e.g., cancer, in an animal, e.g., a human. Exemplary cancers that can be treated using the methods described herein include prostate cancers, breast cancers, ovarian cancers, pancreatic cancers, gastric cancers, bladder cancers, salivary gland carcinomas, gastrointestinal cancers, lung cancers, colon cancers, melanomas, brain tumors, leukemias, lymphomas, and carcinomas. Benign tumors may also be treated or prevented using the methods of the present invention. Other cancers and cancer related genes that may be targeted are known in the art.

Exemplary endogenous proteins that may be associated with disease include ANA (anti-nuclear antibody) found in SLE (systemic lupus erythematosis), abnormal immunoglobulins including IgG and IgA, Bence Jones protein associated with various multiple myelomas, and abnormal amyloid proteins in various amyloidoses including hereditary amyloidosis and Alzheimer's disease. In Huntington's Disease, a genetic abnormality in the HD (huntingtin) gene results in an expanded tract of repeated glutamine residues. In addition to this mutant gene, HD patients have a copy of chromosome 4 which has a normal sized CAG repeat. Thus, methods of the invention can be used to silence the abnormal gene, but not the normal gene.

Exemplary diseases that may be treated with the methods include infection by pathogens, such as a virus, a bacterium, a yeast, a fungus, a protozoan, or a parasite. The nucleic acid may be delivered to the pathogen or to a cell infected with the pathogen. The pathogen may be an intracellular or extracellular pathogen. The target nucleic acid sequence is, for example, a

gene of the pathogen that is necessary for replication and/or pathogenesis, or a gene encoding a cellular receptor necessary for a cell to be infected with the pathogen. Such methods may be employed prior to, concurrent with, or following the administration of the in-dwelling device to a patient to prevent infections. In-dwelling devices include, but are not limited to, surgical implants, prosthetic devices, and catheters, i.e., devices that are introduced to the body of an individual and remain in position for an extended time. Such devices include, for example, artificial joints, heart valves, pacemakers, vascular grafts, vascular catheters, cerebrospinal fluid shunts, urinary catheters, and continuous ambulatory peritoneal dialysis (CAPD) catheters.

A bacterial infection may be due to one or more of the following bacteria: *Chlamydophila pneumoniae, C. psittaci, C. abortus, Chlamydia trachomatis, Simkania negevensis, Parachlamydia acanthamoebae, Pseudomonas aeruginosa, P. alcaligenes, P. chlororaphis, P. fluorescens, P. luteola, P. mendocina, P. monteilii, P. oryzihabitans, P. pertocinogena, P. pseudalcaligenes, P. putida, P. stutzeri, Burkholderia cepacia, Aeromonas hydrophilia, Escherichia coli, Citrobacter freundii, Salmonella typhimurium, S. typhi, S. paratyphi, S. enteritidis, Shigella dysenteriae, S. flexneri, S. sonnei, Enterobacter cloacae, E. aerogenes, Klebsiella pneumoniae, K. oxytoca, Serratia marcescens, Francisella tularensis, Morganella morganii, Proteus mirabilis, Proteus vulgaris, Providencia alcalifaciens, P. rettgeri, P. stuartii, Acinetobacter calcoaceticus, A. haemolyticus, Yersinia enterocolitica, Y. pestis, Y. pseudotuberculosis, Y. intermedia, Bordetella pertussis, B. parapertussis, B. bronchiseptica, Haemophilus influenzae, H. parainfluenzae, H. haemolyticus, H. parahaemolyticus, H. ducreyi, Pasteurella multocida, P. haemolytica, Branhamella catarrhalis, Helicobacter pylori, Campylobacter fetus, C. jejuni, C. coli, Borrelia burgdorferi, V. cholerae, V. parahaemolyticus, Legionella pneumophila, Listeria monocytogenes, Neisseria gonorrhea, N. meningitidis, Kingella dentrificans, K. kingae, K. oralis, Moraxella catarrhalis, M. atlantae, M. lacunata, M. nonliquefaciens, M. osloensis, M. phenylpyruvica, Gardnerella vaginalis, Bacteroides fragilis,*

Bacteroides distasonis, Bacteroides 3452A homology group, Bacteroides vulgatus, B. ovalus, B. thetaiotaomicron, B. uniformis, B. eggerthii, B. splanchnicus, Clostridium difficile, Mycobacterium tuberculosis, M. avium, M. intracellulare, M. leprae, C. diphtheriae, C. ulcerans, C. accolens, C. afermentans, C. amycolatum, C. argentorense, C. auris, C. bovis, C. confusum, C. coyleae, C. durum, C. falsenii, C. glucuronolyticum, C. imitans, C. jeikeium, C. kutscheri, C. kroppenstedtii, C. lipophilum, C. macginleyi, C. matruchoti, C. mucifaciens, C. pilosum, C. propinquum, C. renale, C. riegelii, C. sanguinis, C. singulare, C. striatum, C. sundsvallense, C. thomssenii, C. urealyticum, C. xerosis , Streptococcus pneumoniae, S. agalactiae, S. pyogenes, Enterococcus avium, E. casseliflavus, E. cecorum, E. dispar, E. durans, E. faecalis, E. faecium, E. flavescens, E. gallinarum, E. hirae, E. malodoratus, E. mundtii, E. pseudoavium, E. raffinosus, E. solitarius, Staphylococcus aureus, S. epidermidis, S. saprophyticus, S. intermedius, S. hyicus, S. haemolyticus, S. hominis, and/or *S. saccharolyticus*.

A viral infection may be due to one or more of the following viruses: Hepatitis B, Hepatitis C, picornarirus, polio, HIV, coxsacchie, herpes simplex virus Type I and 2, St. Louis encephalitis, Epstein-Barr, myxoviruses, JC, coxsakieviruses B, togaviruses, measles, paramyxoviruses, echoviruses, bunyaviruses, cytomegaloviruses, varicella-zoster, mumps, equine encephalitis, lymphocytic choriomeningitis, rhabodoviruses including rabies, simian virus 40, human polyoma virus, parvoviruses, papilloma viruses, primate adenoviruses, coronaviruses, retroviruses, Dengue, yellow fever, Japanese encephalitis virus, BK, Retrovirus, Herpesvirus, Hepadenovirus, Poxvirus, Parvovirus, Papillornavirus, and Papovavirus. The target viral nucleic acid sequence is, for example, necessary for replication and/or pathogenesis of the virus in an infected cell. Such viral target genes are necessary for the propagation of the virus and include, e.g., the HIV gag, env, and pol genes, the HPV6 LI and E2 genes, the HPV I I LI and E2 genes, the HPV 16 E6 and E7 genes, the BPV 18 E6 and E7 genes, the HBV surface antigens, the HBV core antigen, HBV reverse transcriptase, the HSV gD gene, the HSVvp 16 gene, the

HSV gC, gH, gL and gB genes, the HSV ICPO, ICP4 and ICP6 genes, Varicella zoster gB, gC and gH genes, and the BCR-abl chromosomal sequences, and non-coding viral polynucleotide sequences which provide regulatory functions necessary for transfer of the infection from cell to cell, e.g., the HIV LTR, and other viral promoter sequences, such as HSV vp 16 promoter, HSV-ICPO promoter, HSV- ICP4, ICP6 and gD promoters, the HBV surface antigen promoter, the HBV pre-genomic promoter, among others.

The copolymers of the invention can be used to treat subjects already infected with a virus, such as HIV, in order to shut down or inhibit a viral gene function essential to virus replication and/or pathogenesis, such as HIV gag. Alternatively, this method can be employed to inhibit the functions of viruses, which exist in mammals as latent viruses, e.g., Varicella zoster virus, the causative agent of shingles. Similarly, diseases such as atherosclerosis, ulcers, chronic fatigue syndrome, and autoimmune disorders, recurrences of HSV- I and HSV-2, HPV persistent infection, e.g., genital warts, and chronic BBV infection among others, which have been shown to be caused, at least in part, by viruses, bacteria, or another pathogen, can be treated according to this method by targeting certain viral polynucleotide sequences essential to viral replication and/or pathogenesis in the mammalian subject.

Preferably, the nucleic acid or other molecule is administered in an amount sufficient to treat the disease or condition, e.g., to prevent, stabilize, or inhibit the growth of the pathogen or to kill the pathogen or to increase or decrease the expression of an endogenous gene whose under- or overexpression results in a disease.

Examples

Example 1. *Block copolymer functionality can be designed to enable hydrolytic and oxidative sensitivity.*

PPS serves as a useful hydrophobic block in micelle and vesicle formation, as it can be oxidized to form the hydrophilic sulfone and sulfoxide products, after which it can presumably be excreted from the body by renal

filtration. It is possible to build into the block copolymeric amphiphile other degradable segments, for example hydrolyzable chemical moieties. Such moieties may be placed within the hydrophilic domain of the block copolymer, so that moieties' accessibility to water does not become a limiting factor in hydrolysis rate and thus release rate. One route by which to accomplish this is shown in Figure 1.

The effect of the synthesis shown in Fig. 1 is to insert a hydrolytic link within the PEG chain, from which a PEG-PPS block copolymer is made, i.e., the PEG block is interrupted with a hydrolysable chemical moiety. As hydrolysis ensues, the fraction of the block copolymer comprised by the hydrophile (f_{PEG}) changes dramatically, thus changing the nature of the assembly that forms, releasing the contents of a vesicle, for example, formed from the block copolymer. This instability is illustrated in Figure 2.

Example 2. *Excipients can be used to enhance encapsulation efficiency of hydrophobic pharmaceutical agents in micelles.*

Encapsulation of hydrophobic molecules within polymer micelles during micellization can be a difficult challenge. We have demonstrated a novel method for formation of polymer micelles that involves the use of an excipient in the encapsulation process that is both soluble in the polymer and soluble in water, considering the organic base DBU and the polymer PEG as examples, as shown in Figure 3. Figure 4, with Tables 1 and 2, demonstrate that the excipients can be used to obtain very high encapsulation efficiency of agents for which encapsulation is difficult, using paclitaxel and dexamethasone as examples.

Excipient	Pharmaceutical agent	Z-Avg(nm)	Volume(nm)	PDI
PEG600	Paclitaxel	66.36	24.54	0.292
DBU-	Paclitaxel	52.02	22.55	0.610

HCl				
PEG600	Dexamethasone	48.59	19.26	0.440
DBU-HCl	Dexamethasone	29.79	20.67	0.295

Table 1. Dynamic Light Scattering of pharmaceutical agent loaded PEG-PPS micelles. 100 μl of sample was dispersed into 900 μl of distilled water before performing the DLS measurement. PDI represents the polydispersity index. PDI for DLS measurements is defined in ISO 13321:1996. The PDI is a dimensionless parameter defined as the broadness of the size distribution, which is defined as:

$$PDI = \frac{\mu_2}{(\Gamma)^2}$$

Where μ_2 is the second cumulant and Γ is the decay rate. In this case, the decay rate is representative of the Gaussian distribution of decay rates observed in the sample.

Excipient	Pharmaceutical agent	EE
None	Dexamethasone	5.5%
PEG600	Paclitaxel	75.9%
DBU-HCl	Paclitaxel	40.6%
PEG600	Dexamethasone	37.7%
DBU-HCl	Dexamethasone	46.1%

Table 2. Encapsulation efficiency (EE) of small molecule pharmaceutical agents using excipient formulations.

Samples used in Table 2 were prepared as follows. 10 mg of PEG-PPS was added to a 1.5 mL centrifuge tube with either 90 mg of PEG600 or DBU-HCl, and 2 mg of either paclitaxel or dexamethasone. This was heated at 95°C for 15 min and mixed thoroughly. After cooling to RT, the blend was slowly diluted to 1 mL with distilled water. The free pharmaceutical agent was pelleted via centrifugation for 10 minutes at 10,000 g, and the pellet and supernatant were separately freeze dried, and analyzed in THF via gel permeation chromatography. Dexamethasone and paclitaxel results were quantified using a standard curve.

Dexamethasone and amphotericin B were more efficiently encapsulated via solvent dispersion than via the method of the invention. In contrast, paclitaxel was more efficiently encapsulated via the method of the invention. Other pharmaceutical agents such as sirolimus and everolimus were also efficiently encapsulated using the methods of the invention. The encapsulation efficiency depends on the structure of the pharmaceutical agent. The flexibility of the PEG-PPS system to encapsulate pharmaceutical agents is very large because the system accommodates a variety of techniques to encapsulate most pharmaceutical agents at high efficiencies.

Example 3. *Excipients can be used to enhance encapsulation efficiency of hydrophilic pharmaceutical agents in vesicles.*

Polymeric vesicles represent very powerful tools for protection and delivery of hydrophilic pharmaceutical agents, such as peptides, proteins, nucleic acids, and genes; however, they are difficult to load. We have developed novel mechanisms to load vesicles at very high loading efficiency. One method is to dissolve in the polymer an excipient that is soluble both in the polymer and in water, such as DBU or PEGs, as illustrated above in the formation of polymer micelles. An aqueous solution of the pharmaceutical agent to be encapsulated is added to the polymer mixture with the excipient (the so-called direct hydration method). Typical results are illustrated in Table 3 and Figures 5-11.

*f*PEG	SD	TFE	DH	DHE
0.16	83.3	240.8	416.8	281.0
0.18	74.9	224.3	531.8	227.8
0.23	121.9	230.3	1417.0	125.5
0.33	102.9	196.4	458.3	139.4
0.39	43.4	315.7	695.4	117.7
0.49	20.0	159.4	278.6	97.8
0.60	15.9	46.5	113.6	46.4

*f*PEG	SD	TFE	DH	DHE
0.68	18.4	55.5	244.3	77.5
0.72	25.2	42.7	225.1	83.6
0.87	14.6	56.4	558.4	114.0

Table 3. Particle size determination using dynamic light scattering. Values are reported as zeta-size. The processing methods are solvent dispersion using tetrahydrofuran (SD), thin film extrusion (TFE), direct hydration (DH), and direct hydration with extrusion (DHE).

Example 4. *Thermal transitions can induce vesicle formation from micelles.*

As mentioned above, vesicles are powerful tools with which to encapsulate hydrophilic pharmaceutical agents, to modulate their release, to target their release, and to protect them from biological clearance and degradation mechanisms. We have developed a method to form polymer micelles involving application of heat. Polymer micelles are formed, using a polymer composition with an fPEG that would thermodynamically form vesicles instead. In suspension, the micelles can be metastable and can be concentrated to a high degree. Application of heat to the metastable micelles induces spontaneous formation of vesicles, which can be very small and homogeneous in size distribution. Pharmaceutical agent incorporated in the micelle suspension will be loaded within the vesicles during their formation. The approach is illustrated in Figures 12-15.

The ultrasmall size of the polymer vesicles formed by this method may be particularly useful in some applications. For example, in targeting tumors from the bloodstream via the enhanced permeation and retention effect, smaller particles are more effective than larger particles in penetration of the fenestrated endothelium in the tumor microcirculation. Smaller particles are more effective than larger ones in penetration of the arterial wall under physiological pressure or mild overpressure, in penetration of mucosal surfaces and targeting cells beneath, such as dendritic cells, in permeation of the interstitium to target lymph nodes draining the tissue site, and in targeting the lymphatics in the gut.

Example 5. *PEG-PPS vesicle formulations can be stable upon drying and rehydration.*

Formulations that can solubilize hydrophobic pharmaceutical agents and can be administered in dry form are useful in a number of pharmaceutical applications. We have demonstrated that PEG-PPS micelles can be dried into a tablet and subsequently resuspended rapidly, to the same size distribution, without loss of encapsulated pharmaceutical agent. For example, PEG_{44}-PPS_{20} micelles were formed with size mean of 21 nm (Figure 16A), loaded with cyclosporine A. The suspension was dried, and then the dried sample was placed in water to allow brief rehydration. The measured size distribution showed a mean of 20.3 nm (Figure 16B). Throughout the process, high encapsulation efficiency was maintained (Table 7). The particles, being primarily sensitive to oxidation, are stable at gastric pH (Figure 17).

Polymer	CsA added (mg)	CsA loading (mg/mg)	Encapsulation efficiency (%)	Mean aggregate sixe (nm)
PEG_{44}-PPS_{20}	4	0.130	64	21
PEG_{44}-PPS_{20} after rehydration	4	0.123	62	20

Table 7. High loading of cyclosporine A (CsA) was obtained in PEG_{44}-PPS_{20} micelles, and this loading was maintained after the micelle suspension was dried at 80°C and rehydrated in water.

Example 6. *PEG-PPS-PEI copolymers can efficiently deliver gene-based pharmaceutical agents.*

Previous work described, among other embodiments, PEG-PPS-polycation triblock copolymers, including the case where the polycation blocks were based on peptides. We have developed easier synthetic routes to analogous polymers, including PEG-PPS-PEI block copolymers (Figure 18). These copolymers achieve high transfection efficiency, both with plasmid

DNA and also with siRNA. The polymers also demonstrated much lower cytotoxicity than did linear PEI of the same PEI molecular weight at the same PEI concentration. These results are shown in Figures 19-21.

Example 7. Blends of PEG-PPS and PPS-PEI can form very small complexes with gene-based pharmaceutical agents.

It is sometimes particularly desirable to obtain nanoparticles with gene-based pharmaceutical agents that are very small. This was possible by using mixed micelles of PEG-PPS and PPS-PEI to obtain very small complexes. Results with this approach are shown in Table 8 and Figures 22-24. It is also possible to incorporate hydrophobic agents within the PPS domains of these complexes to induce an additional effect, such as present a bioactive agent or deliver an agent that enhances transfection efficiency.

As mentioned above, the ultrasmall size of the polymer micelles and other particles may be important; the sizes formed by this method may be particularly useful in some applications. For example, in targeting tumors from the bloodstream via the enhanced permeation and retention effect, in penetration of the arterial wall under physiological pressure or mild overpressure, in penetration of the fenestrated endothelium in the tumor microcirculation, in penetration of mucosal surfaces and targeting cells beneath, such as dendritic cells, and in targeting the lymphatics in the gut, smaller particles are more effective than larger particles.

Polymer	Size of the particles alone	Size of the particles complexed with siRNA	Size of the particles complexed with DNA
PEG_{44}-PPS_{27}-PEI_{96}	~160 nm	~150 nm	~180 nm
PEG_{44}-PPS_{20}-PEI_{70}	~170 nm	~160 nm	-
PEG_{44}-PPS_{20} / PPS_{10}-PEI_{50}	~60 nm	~40 nm	~45 nm

Table 8. PEG-PPS / PPS-PEI mixed micelles condense gene-based pharmaceutical agents into very small nanoparticles.

Example 8. *Blends of PEG-PPS and PEG-PPS-PEI can form very small complexes with gene-based pharmaceutical agents.*

In many situations of gene and gene-based pharmaceutical agent delivery (plasmid DNA, siRNA, antisense oligonucleotides, aptamers, or microRNA, for example), the size of the particle is critical for effective delivery. Small particles penetrate tissues better than larger particles and may also lead to higher cytoplasmic delivery and transfection efficiency. For example, complex delivery to tumor beds is more effective with very small complexes. The gene complexes formed from PEG-PPS-PEI can be substantially reduced in size by incorporation of PEG-PPS in the micelle into which the gene is incorporated. As such, the gene-non-binding PEG-PPS drives the formation of smaller size micelles from PEG-PPS-PEI. Figure 25 demonstrates that ca. 30 nm plasmid DNA complexes can be formed from a 9:1 ratio of PEG-PPS and PEG-PPS-PEI.

Example 9. *Surface modified nanoparticles.*

Given that PEG-PPS nanoparticles can survive harsh conditions such as gastric pH, we contemplated their transport in the gut lymphatics, determining their potential to be processed by mechanisms related to fat uptake in the gut. This was measured using a coculture model of caco-2 cells (enterocytes) and lymphatic endothelial cells (LECs), allowing full characterization of uptake, packaging, and transit (collectively referred to as transport). It was determined that the surface characteristics of PPS-based nanoparticles can be engineered to provide for effective transport: for example, particles on which about 10% of the terminal chain groups were substituted with a carboxyl functionality were transported at a level 5-fold higher than that of equivalent particles lacking a surface charge. Other surface charging moieties, such as sulfates and sulfones, are similarly effective after optimization.

The uptake of PEG-PPS nanoparticles is biospecific, as the control macromolecule dextran was not well transported, and the transport was blocked at cold temperatures.

Other surface characteristics also lead to enhanced transport. Particles formed with a terminal hydroxyl group were about 10-fold better transported than analogous particles with terminal methoxy groups. Thus, PPS nanoparticles with 90% -OCH_3 and 10% COOH are actively transported across LECs (5x better than others). Moreover, PPS nanoparticles with 90% -OH and 10% COOH are actively transported across Caco-2 cells (10x better than others).

OTHER EMBODIMENTS

The description of the specific embodiments of the invention is presented for the purposes of illustration. It is not intended to be exhaustive nor to limit the scope of the invention to the specific forms described herein. Although the invention has been described with reference to several embodiments, it will be understood by one of ordinary skill in the art that various modifications can be made without departing from the spirit and the scope of the invention, as set forth in the claims. All patents, patent applications, and publications referenced herein are hereby incorporated by reference.

Other embodiments are within the claims.

What is claimed is:

CLAIMS

1. A block copolymer comprising a hydrophilic block and a hydrophobic block, wherein said hydrophilic or hydrophobic block is interrupted with a hydrolysable chemical moiety.

2. The block copolymer of claim 1, wherein said hydrophilic block has a molecular weight between 500 and 10,000 Da.

3. The block copolymer of claim 1, wherein said hydrophobic block has a molecular weight between 300 and 5,000 Da.

4. The block copolymer of any preceding claim, wherein said block copolymer is capable of self-assembling into a supramolecular structure.

5. The block copolymer of claim 4, wherein said supramolecular structure is a micelle or vesicle.

6. The block copolymer of any preceding claim, wherein said hydrophilic block comprises poly(ethylene glycol), poly(ethylene oxide)-co-poly(propylene oxide) di- or multiblock copolymers, poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(N-vinyl pyrrolidone), poly(acrylic acid), poly(ethyloxazoline), poly(alkylacrylates), poly(acrylamide), poly(N-alkylacrylamides), polypeptide, polysaccharide, poly(N,N-dialkylacrylamides), hyaluronic acid, or poly (N-acryloylmorpholine).

7. The block copolymer of any preceding claim, wherein said hydrophobic block comprises poly(propylene sulfide), poly(propylene glycol), esterified poly(acrylic acid), esterified poly(glutamic acid), esterified poly(aspartic acid), or a polypeptide.

8. The block copolymer of any preceding claim, wherein said hydrophilic block comprises PEG, and said hydrophobic block comprises PPS.

9. The block copolymer of any preceding claim, wherein said hydrolysable chemical moiety is an ester, amide, thioester, anhydride, or ketal.

10. A pharmaceutical composition comprising the block copolymer of any preceding claim and a pharmaceutically acceptable diluent.

11. A supramolecular structure comprising a block copolymer comprising a hydrophilic block and a hydrophobic block and an excipient.

12. The supramolecular structure of claim 11, wherein said structure is a micelle or vesicle.

13. The supramolecular structure of claim 11 or 12, further comprising a pharmaceutical agent.

14. The supramolecular structure of claim 13, wherein said pharmaceutical agent is hydrophobic.

15. The supramolecular structure of claim 14, wherein said hydrophobic pharmaceutical agent is selected from dexamethasone, paclitaxel, cyclosporine A, sirolimus, everolimus, tacrolimus, amphotericin B, or adriamycin.

16. The supramolecular structure of claim 13, wherein said pharmaceutical agent is hydrophilic.

17. The supramolecular structure of claim 16, wherein said hydrophilic pharmaceutical agent is a polypeptide or nucleic acid.

18. The supramolecular structure of claim 17, wherein said polypeptide is an antibody or an antigen-binding fragment thereof.

19. The supramolecular structure of claim 16, wherein said hydrophilic pharmaceutical agent is an antibiotic or a chemotherapeutic drug.

20. The supramolecular structure of claim 13, wherein said pharmaceutical agent is encapsulated at an efficiency of greater than 5%.

21. The supramolecular structure of claim 13, wherein said pharmaceutical agent is encapsulated at an efficiency of greater than 50%.

22. The supramolecular structure of claim 13, wherein said pharmaceutical agent is encapsulated at an efficiency of greater than 90%.

23. The supramolecular structure of any of claims 11-22, wherein said excipient is selected from 1,8-diazabicyclo[5.4.0]undec-7-ene and poly(ethylene glycol).

24. The supramolecular structure of any of claims 11-23, wherein said hydrophilic block comprises poly(ethylene glycol), poly(ethylene oxide)-co-poly(propylene oxide) di- or multiblock copolymers, poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(N-vinyl pyrrolidone), poly(acrylic acid), poly(ethyloxazoline), poly(alkylacrylates), poly(acrylamide), poly(N-alkylacrylamides), polypeptide, polysaccharide, poly(N,N-dialkylacrylamides), hyaluronic acid, or poly (N-acryloylmorpholine).

25. The supramolecular structure of any of claims 11-24, wherein said hydrophobic block comprises poly(propylene sulfide), poly(propylene

glycol), esterified poly(acrylic acid), esterified poly(glutamic acid), esterified poly(aspartic acid), or a polypeptide.

26. The supramolecular structure of any of claims 11-25, wherein said hydrophilic block is PEG, and said hydrophobic block is PPS.

27. A pharmaceutical composition comprising the supramolecular structure of any of claims 13-23 and a pharmaceutically acceptable diluent.

28. The pharmaceutical composition of claim 27, wherein the supramolecular structure is a vesicle.

29. The pharmaceutical composition of claim 27, wherein the supramolecular structure is a micelle.

30. A method of encapsulating a pharmaceutical agent in a supramolecular structure comprising

a. applying heat to a mixture of (i) a block copolymer comprising a hydrophilic block and a hydrophobic block, (ii) an excipient, and (iii) a pharmaceutical agent in an amount sufficient to homogenize said mixture, and

b. diluting said mixture in an aqueous solution.

31. The method of claim 30, wherein said supramolecular structure is a micelle.

32. The method of claim 30, wherein said supramolecular structure is a vesicle.

33. The method of any of claims 30-32, wherein said excipient is DBU or PEG.

34. A method of making a vesicle comprising:

a. forming micelles from a block copolymer comprising a hydrophilic block and a hydrophobic block, wherein vesicles of said block copolymer are thermodynamically more stable than micelles; and

b. heating said micelles to form said vesicle.

35. The method of claim 34, wherein said vesicle has a size between 70-800 nm.

36. The method of claim 34 or 35, wherein said micelles are suspended in a solution comprising a pharmaceutical agent that is encapsulated into said vesicles in step (b).

37. The method of any of claims 34-36, wherein said hydrophilic block comprises poly(ethylene glycol), poly(ethylene oxide)-co-poly(propylene oxide) di- or multiblock copolymers, poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(N-vinyl pyrrolidone), poly(acrylic acid), poly(ethyloxazoline), poly(alkylacrylates), poly(acrylamide), poly(N-alkylacrylamides), polypeptide, polysaccharide, poly(N,N-dialkylacrylamides), hyaluronic acid, or poly (N-acryloylmorpholine).

38. The method of any of claims 34-37, wherein said hydrophobic block comprises poly(propylene sulfide), poly(propylene glycol), esterified poly(acrylic acid), esterified poly(glutamic acid), esterified poly(aspartic acid), or a polypeptide.

39. The method of any of claims 34-38, wherein said hydrophilic block is PEG, and said hydrophobic block is PPS.

40. A vesicle made by the method of any of claims 34-39.

41. A dry formulation comprising micelles comprising a block copolymer comprising a hydrophilic block and a hydrophobic block, wherein said formulation comprises less than 5% water by weight.

42. The formulation of claim 41, wherein said formulation comprises less than 2% water by weight.

43. The formulation of claim 41 or 42, further comprising a pharmaceutical agent encapsulated in said micelles.

44. The formulation of any of claims 41-43, wherein said hydrophilic block comprises poly(ethylene glycol), poly(ethylene oxide)-co-poly(propylene oxide) di- or multiblock copolymers, poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(N-vinyl pyrrolidone), poly(acrylic acid), poly(ethyloxazoline), poly(alkylacrylates), poly(acrylamide), poly(N-alkylacrylamides), polypeptide, polysaccharide, poly(N,N-dialkylacrylamides), hyaluronic acid, or poly (N-acryloylmorpholine).

45. The formulation of any of claims 41-44, wherein said hydrophobic block comprises poly(propylene sulfide), poly(propylene glycol), esterified poly(acrylic acid), esterified poly(glutamic acid), esterified poly(aspartic acid), or a polypeptide.

46. The formulation of any of claims 41-45, wherein said hydrophilic block is PEG, and said hydrophobic block is PPS.

47. A supramolecular structure comprising a (i) a block copolymer comprising a positively charged block, and (ii) a nucleic acid, wherein said supramolecular structure has a maximal diameter of 60 nm or less.

48. The supramolecular structure of claim 47, wherein said supramolecular structure is a molecule or a vesicle.

49. The supramolecular structure of claim 47 or 48, wherein said block copolymer further comprises a hydrophilic block or a hydrophobic block.

50. The supramolecular structure of claim 49, wherein said block copolymer further comprises a hydrophilic block and a hydrophobic block.

51. The supramolecular structure of any of claims 47-50, wherein said maximal diameter is 40 nm or less.

52. The supramolecular structure of any of claims 47-51, wherein said block copolymer comprises PEG-PPS-PEI.

53. The supramolecular structure of any of claims 47-52, wherein said nucleic acid comprises a single-stranded oligonucleotide, a short interfering RNA, an aptamer, or plasmid DNA.

54. A method of transfecting a cell with a nucleic acid comprising contacting said cell with the supramolecular structure of any of claims 47-53.

55. A block copolymer comprising PEG-PPS-PEI.

56. The block copolymer of claim 55, wherein the PPS block and the PEI block are attached via a bond that is labile in an endosome.

57. The block copolymer of claim 56, wherein said bond comprises a disulfide bond, vinyl ether, orthoester, acyl hydrazone, or a $-N-PO_3-$ group.

58. The block copolymer of any of claims 55-57, further comprising a nucleic acid complexed to said PEI.

59. A pharmaceutical composition comprising the block copolymer of any of claims 55-58 and a pharmaceutically acceptable diluent.

60. A micelle comprising first and second block copolymers and having a size between 10 and 60 nm, wherein said first and second block copolymers each comprise a hydrophilic block and a hydrophobic block.

61. The micelle of claim 60, wherein said micelle has a size between 10 and 40 nm.

62. The micelle of claim 60 or 61, wherein said hydrophilic block comprises poly(ethylene glycol), poly(ethylene oxide)-co-poly(propylene oxide) di- or multiblock copolymers, poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(N-vinyl pyrrolidone), poly(acrylic acid), poly(ethyloxazoline), poly(alkylacrylates), poly(acrylamide), poly(N-alkylacrylamides), polypeptide, polysaccharide, poly(N,N-dialkylacrylamides), hyaluronic acid, or poly (N-acryloylmorpholine).

63. The micelle of any of claims 60-62, wherein said hydrophobic block comprises poly(propylene sulfide), poly(propylene glycol), esterified poly(acrylic acid), esterified poly(glutamic acid), esterified poly(aspartic acid), or a polypeptide.

64. The micelle of any of claims 60-63, wherein said first block copolymer comprises PEG and PPS, and said second block copolymer comprises PEI and PPS.

65. A micelle comprising a first block copolymer comprising a hydrophilic block and a hydrophobic block and a second block copolymer comprising a hydrophilic block, a hydrophobic block, and a positively charged block.

66. The micelle of claim 65, wherein said hydrophilic blocks comprise moieties selected independently from poly(ethylene glycol), poly(ethylene oxide)-co-poly(propylene oxide) di- or multiblock copolymers, poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(N-vinyl pyrrolidone), poly(acrylic acid), poly(ethyloxazoline), poly(alkylacrylates), poly(acrylamide), poly(N-alkylacrylamides), polypeptide, polysaccharide, poly(N,N-dialkylacrylamides), hyaluronic acid, and poly (N-acryloylmorpholine).

67. The micelle of claim 65 or 66, wherein said hydrophobic blocks comprise moieties selected independently from poly(propylene sulfide), poly(propylene glycol), esterified poly(acrylic acid), esterified poly(glutamic acid), esterified poly(aspartic acid), and a polypeptide.

68. The micelle of any of claims 65-67, wherein said charged block comprises a polypeptide, poly(ethyleneimine), poly(amidoamine), poly(sodium 1-(*N*-acryloylpiperazin-1-yl)diazen-1-ium-1,2-diolate), poly(sodium 1-(*N*-acryloylhomopiperazin-1-yl)diazen-1-ium-1,2-diolate), or poly(sodium 1-(*N*-acryloyl-2,5-dimethylpiperazin-1-yl)diazen-1-ium-1,2-diolate).

69. The micelle of any of claims 65-68, wherein said first block copolymer is PEG-PPS, and said second block copolymer is PEG-PPS-PEI.

70. The micelle of any of claims 65-69, wherein said micelle is between 20 and 50nm in diameter.

71. A pharmaceutical composition comprising the micelle of any of claims 60-70 and a pharmaceutically acceptable diluent.

72. A supramolecular structure comprising block copolymers comprising a hydrophilic block and a hydrophobic block, wherein 5-25% of said block copolymers have a charged chemical moiety disposed at the outer surface of said structure.

73. The supramolecular structure of claim 72, wherein said hydrophilic block comprises poly(ethylene glycol), poly(ethylene oxide)-co-poly(propylene oxide) di- or multiblock copolymers, poly(ethylene oxide), poly(vinyl alcohol), poly(ethylene-co-vinyl alcohol), poly(N-vinyl pyrrolidone), poly(acrylic acid), poly(ethyloxazoline), poly(alkylacrylates), poly(acrylamide), poly(N-alkylacrylamides), polypeptide, polysaccharide, poly(N,N-dialkylacrylamides), hyaluronic acid, or poly (N-acryloylmorpholine).

74. The supramolecular structure of claim 72 or 73, wherein said hydrophobic block comprises poly(propylene sulfide), poly(propylene glycol), esterified poly(acrylic acid), esterified poly(glutamic acid), esterified poly(aspartic acid), or a polypeptide.

75. The supramolecular structure of any of claims 72-74, wherein said hydrophilic block is PEG, and said hydrophobic block is PPS.

76. The supramolecular structure of any of claims 72-75, wherein said charged chemical moiety is carboxylic acid, sulfate, or sulfone.

77. A pharmaceutical composition comprising the supramolecular structure of any of claims 72-76 and a pharmaceutically acceptable diluent.

78. The pharmaceutical composition of claim 10, 27, 59, or 77, further comprising a pharmaceutical agent.

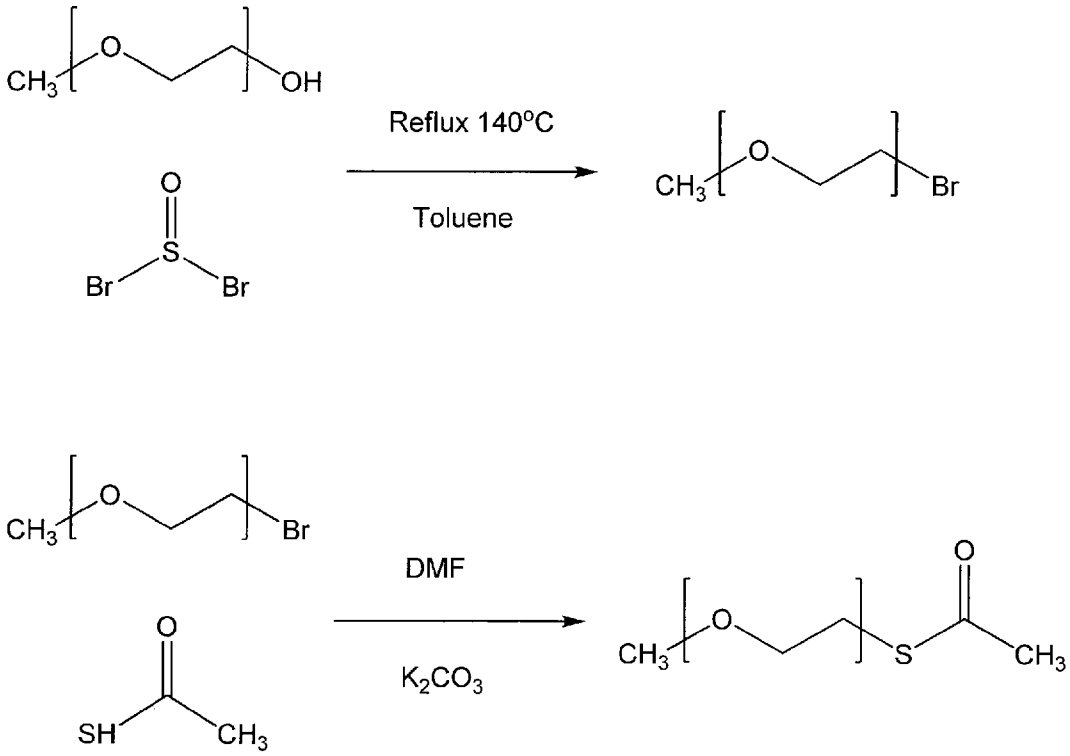

CH3
O
OH
Reflux 140°C
Toluene
O
S
Br
Br
CH3
O
Br
DMF
K2CO3
O
SH
CH3
CH3
O
O
S
CH3

FIGURE 1A

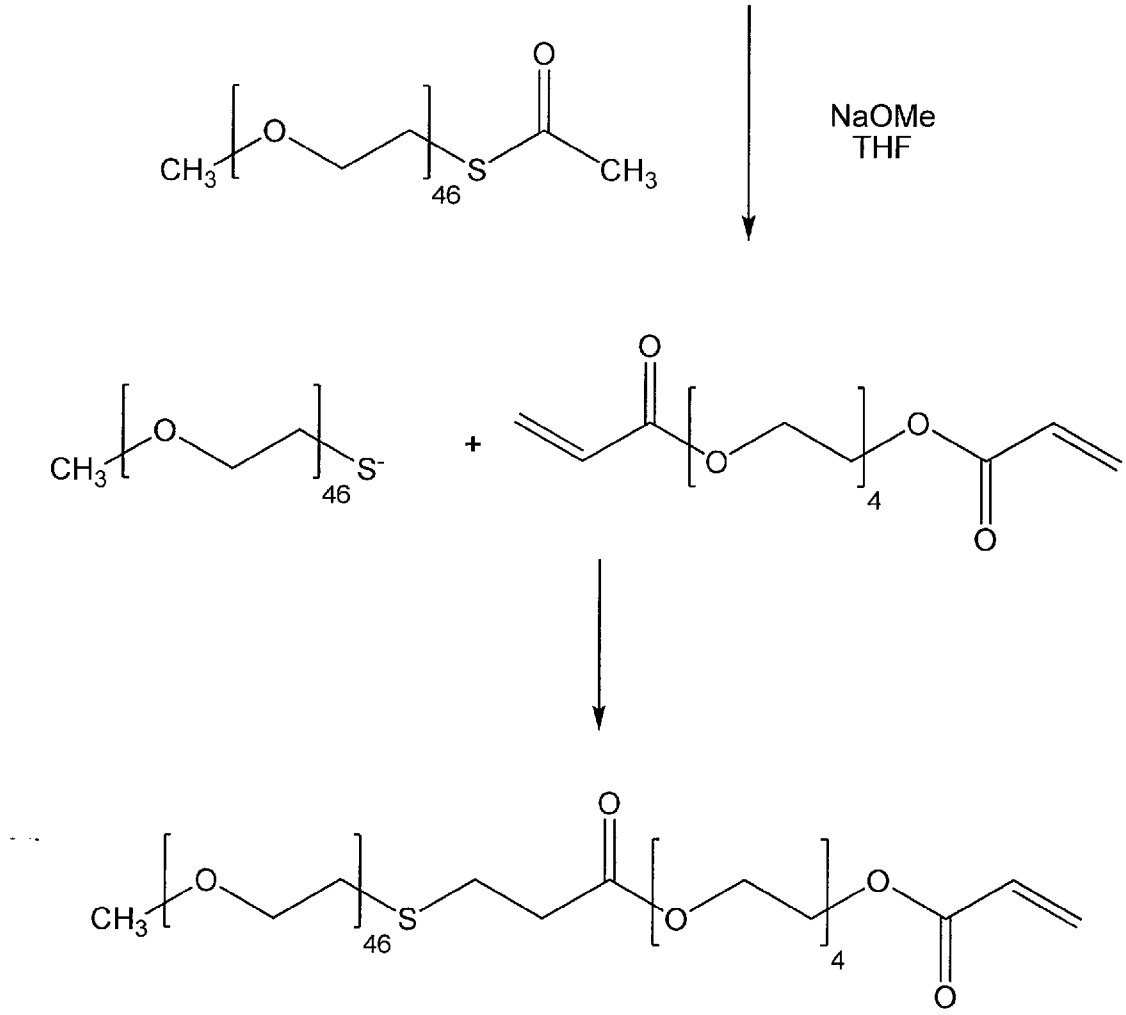
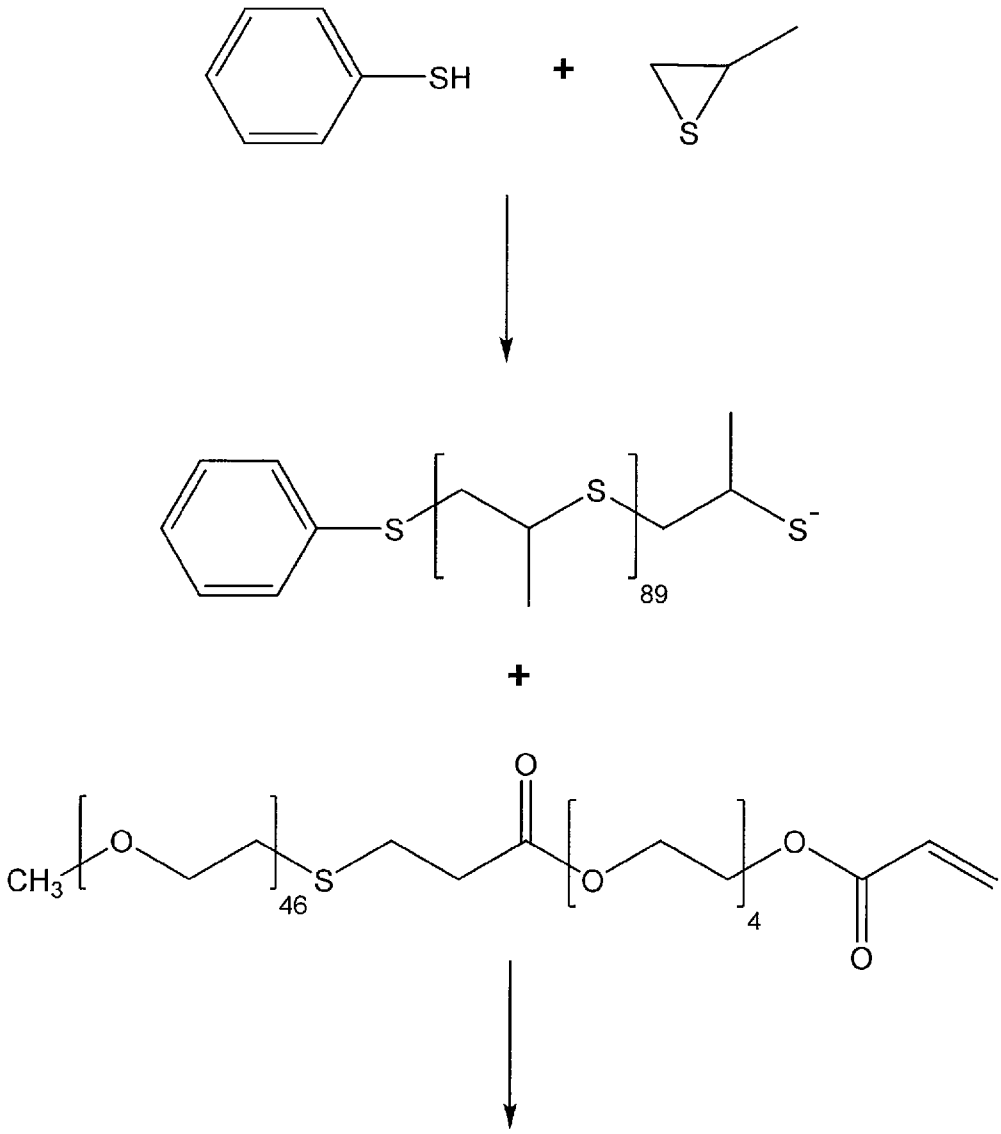


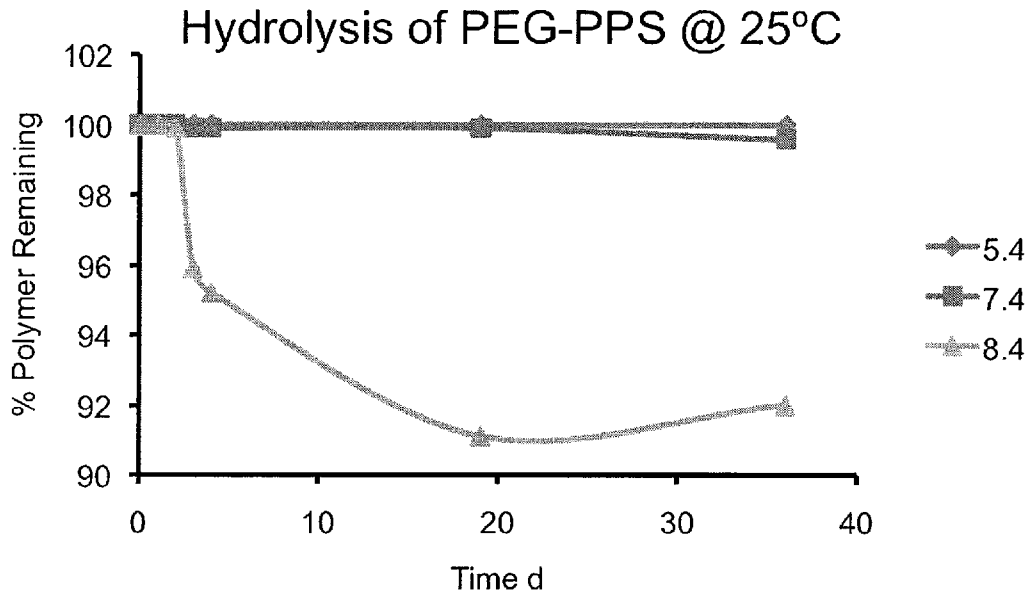
FIGURE 1B



$_3$HCO-EG$_{46}$-ester-EG$_4$-ester-PS$_{89}$-Benzene

FIGURE 1C

A


Hydrolysis of PEG-PPS @ 25°C
% Polymer Remaining
102
100
98
96
94
92
90
0
10
20
30
40
Time d
5.4
7.4
8.4

B

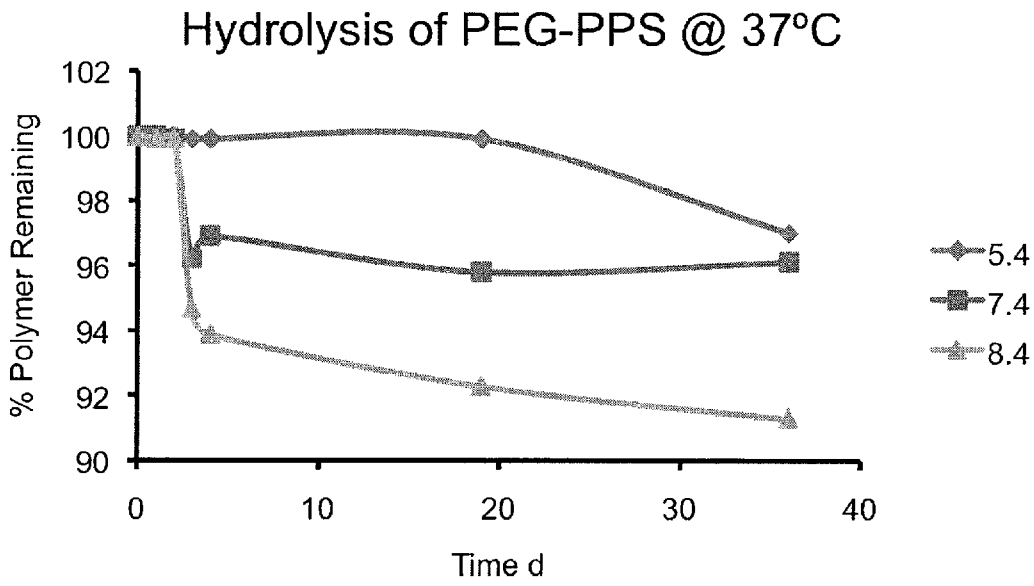
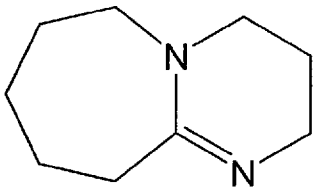
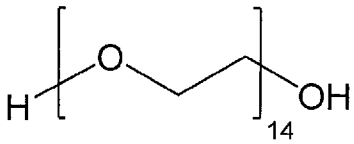

Hydrolysis of PEG-PPS @ 37°C
% Polymer Remaining
102
100
98
96
94
92
90
0
10
20
30
40
Time d
5.4
7.4
8.4

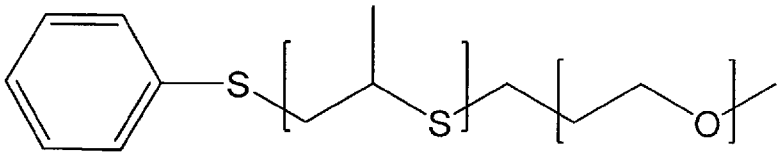
FIGURE 2



1,8-Diazabicyclo[5.4.0]undec-7-ene



Polyethylene glycol Mw 400



Poly(ethylene glycol)-*bl*-Poly(propylene sulfide) (PEG-PPS)

FIGURE 3

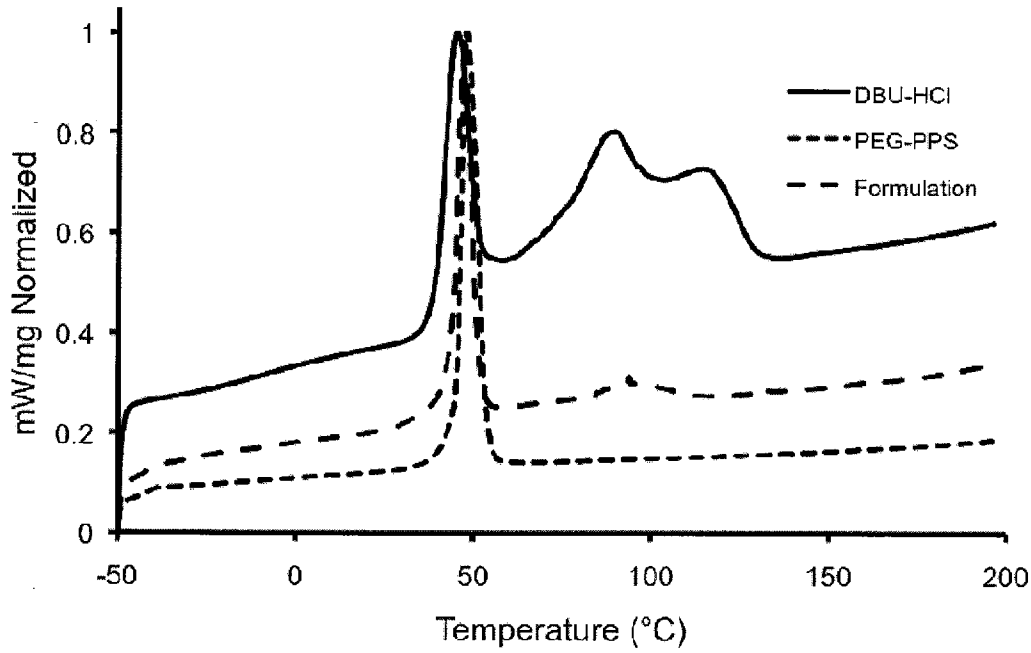

1
0.8
0.6
0.4
0.2
0
mW/mg Normalized
-50
0
50
100
150
200
Temperature (°C)
DBU-HCl
PEG-PPS
Formulation

FIGURE 4

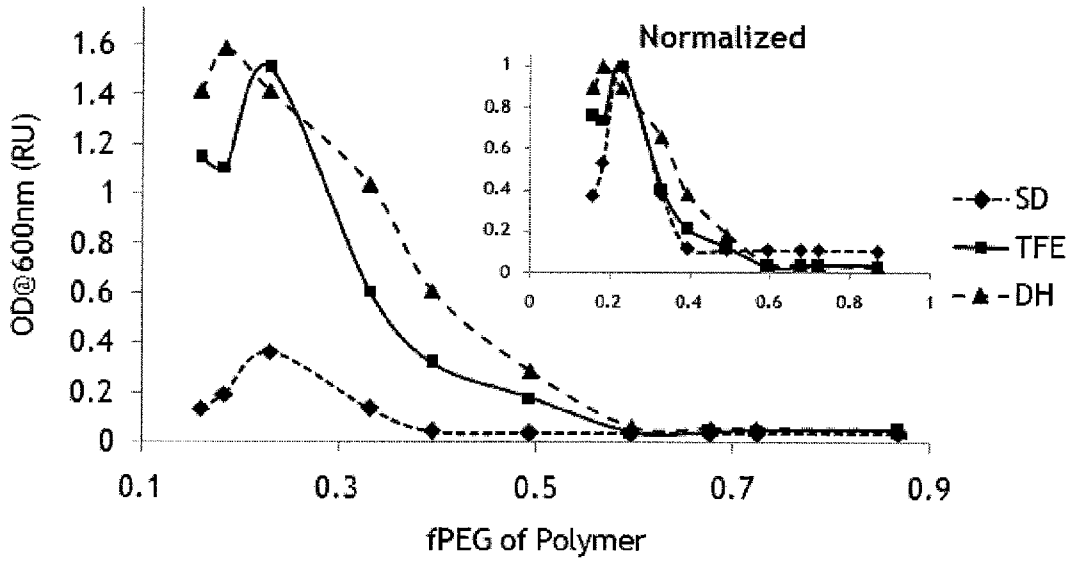

OD@600nm (RU)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.1
0.3
0.5
0.7
0.9
fPEG of Polymer
Normalized
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
SD
TFE
DH

FIGURE 5

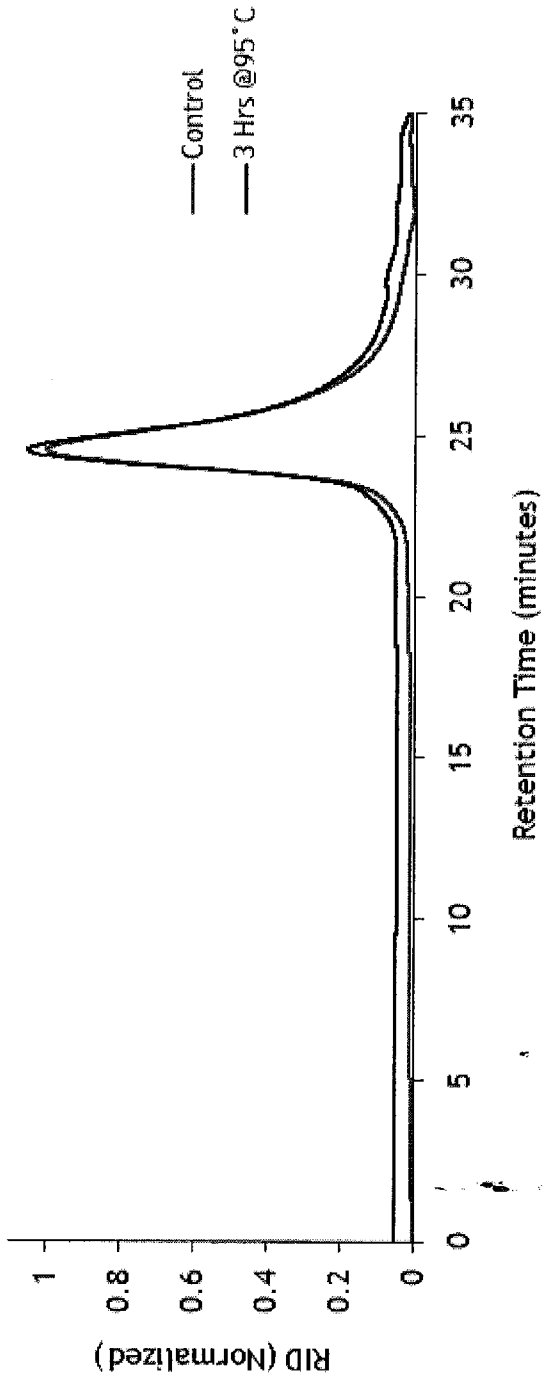
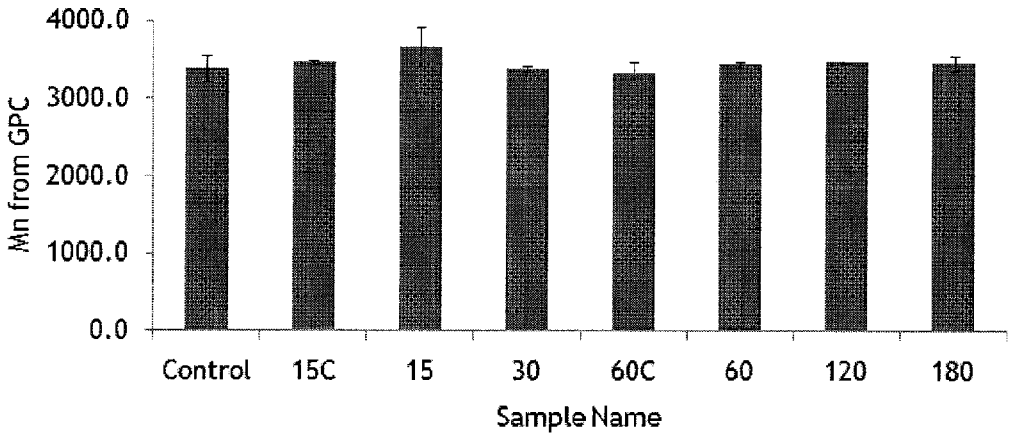

Control
3 Hrs @95°C
RID (Normalized)
0
0.2
0.4
0.6
0.8
1
0
5
10
15
20
25
30
35
Retention Time (minutes)

FIGURE 6

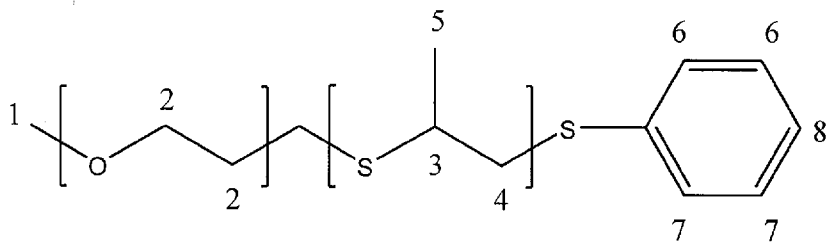
A



B

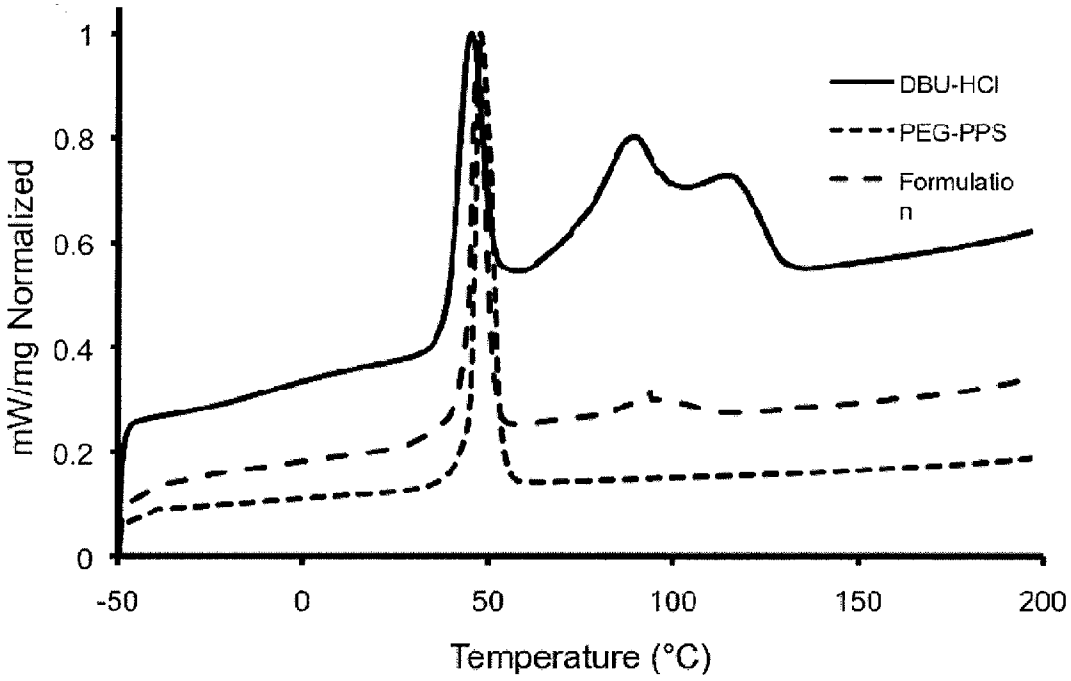
	Mp	Mn	Mw	PDI
AVG	3677.96	3451.46	4098.65	1.19
STDEV	87.69	143.02	137.55	0.01
%CV	2.4%	4.1%	3.4%	1.2%

FIGURE 7



	PEG		*PPS*			*Benzene*		
	CH3 (1)	CH2CH2O (2)	CH (3)	CH2 (4)	CH3 (5)	CH (6)	CH (7)	CH (8)
Control	3.006	183.782	12.396	24.222	37.684	1.996	1.998	1.012
Heated	2.950	183.782	12.109	24.697	37.584	1.991	2.039	1.075
Heat+Salt	2.978	183.782	12.129	24.732	37.396	1.918	2.008	1.061
Average	2.98	183.78	12.21	24.55	37.55	1.97	2.02	1.05
Std. Dev.	0.03	0.00	0.16	0.28	0.15	0.04	0.02	0.03
%CV	0.9%	0.0%	1.3%	1.2%	0.4%	2.2%	1.1%	3.2%

FIGURE 8



Sample	PEG-PPS	Mix		Salt		
Peak	1	1	2	1	2	3
Average	48.15	47.65	104.86	44.92	89.95	118.40
STDEV	0.78	1.28	8.70	0.20	0.76	4.58
%CV	1.6%	2.7%	8.3%	0.4%	0.8%	3.9%

FIGURE 9

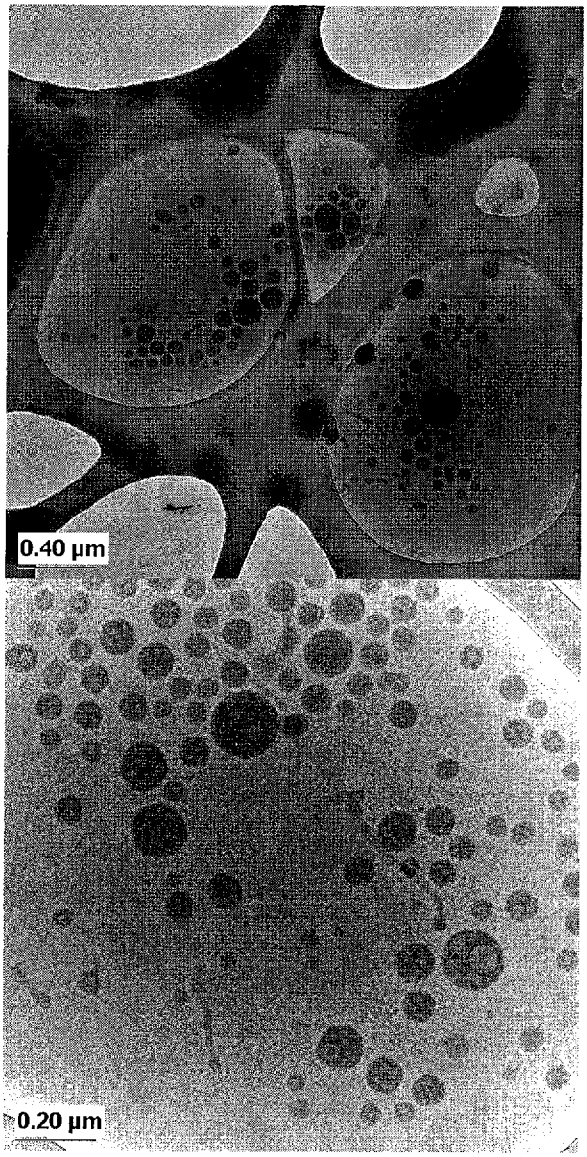
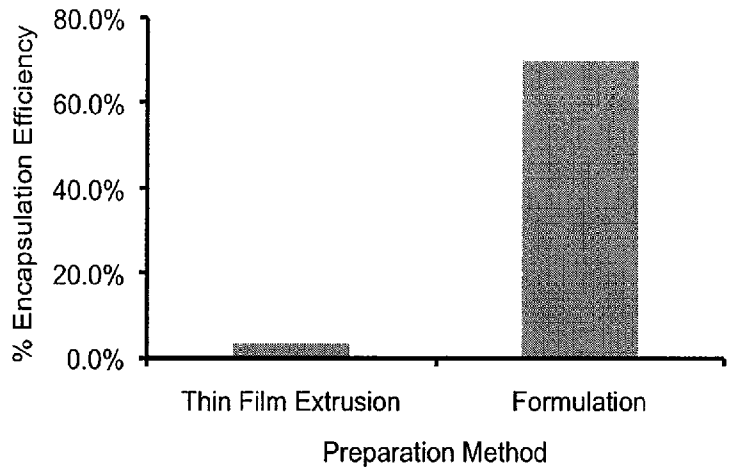


FIGURE 10

A



B

Formulation	*Drug*	*PEG-PPS*	*E. Efficiency*	*Loading*
Control	Dexamethasone	EG46-PS21	88.7%	8.3%
PEG600	Dexamethasone	EG46-PS21	64.5%	65.2%
PEG600	Dexamethasone	EG46-PS10	36.1%	4.5%
Control	Paclitaxel	EG46-PS21	30.4%	4.6%
PEG600	Paclitaxel	EG46-PS21	8.1%	10.9%
PEG600	Paclitaxel	EG46-PS10	96.2%	14.6%

C

Encapsulation efficiency using the direct hydration method with PEG 500 DME

Polymer	*Protein*	*Protein added (μg)* [a]	*Protein encapsulated (μg)* [a]	*Protein loading, mean ± SD (μg/mg)*	*Encapsulation efficiency, mean ± SD (%)*
EG_{46}-PS_{66}	bSA	281.9	15.4 ± 15.1	1.5 ± 1.5	5.5 ± 5.4
EG_{46}-PS_{66}	bSA	659.2	128.0 ± 32.6	12.8 ± 3.3	19.4 ± 4.9
EG_{46}-PS_{66}	bSA	659.2	61.9 ± 6.9	6.2 ± 0.7	8.4 ± 10.5 [b]
EG_{46}-PS_{66}	Ova	43.6	10.4 ± 7.4	1.0 ± 0.7	23.9 ± 17.1
EG_{46}-PS_{66}	Ova	83.0	25.2 ± 4.7	2.5 ± 0.5	30.3 ± 5.7
EG_{46}-PS_{66}	ß-Gal	0.01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a] Per 10 mg copolymer

[b] bSA freeze dried and formulated with polymer and PEG 500 DME

FIGURE 11

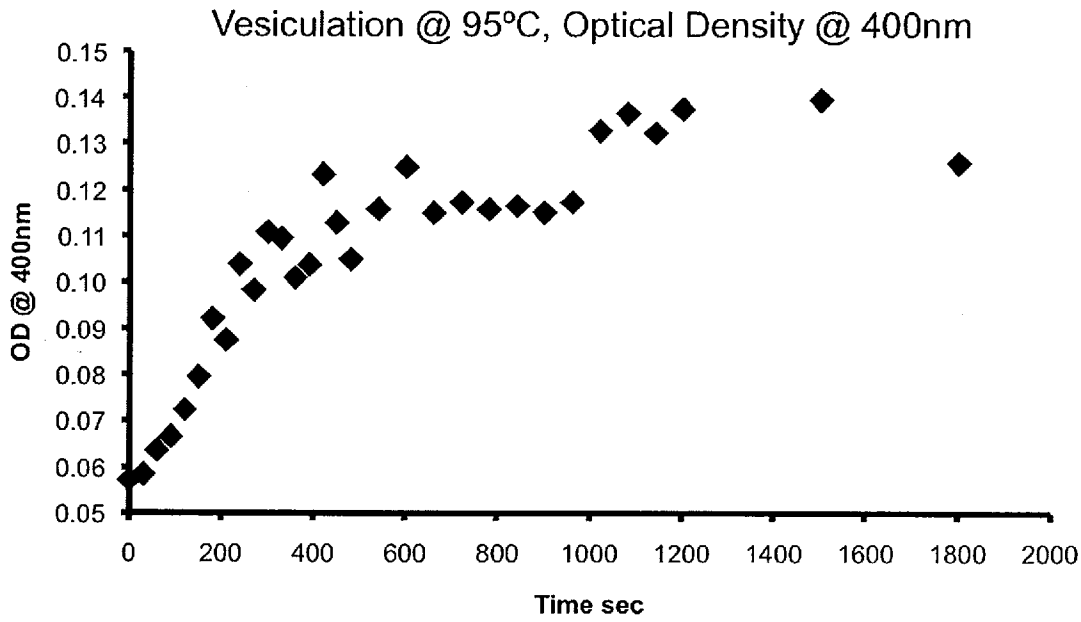

Vesiculation @ 95°C, Optical Density @ 400nm
OD @ 400nm
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Time sec

FIGURE 12

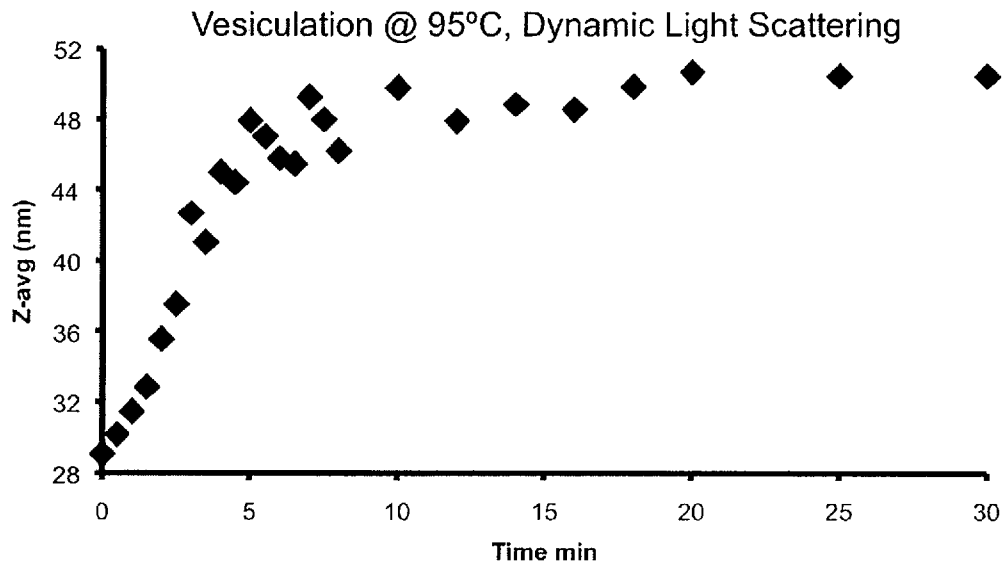

Vesiculation @ 95°C, Dynamic Light Scattering
Z-avg (nm)
52
48
44
40
36
32
28
0
5
10
15
20
25
30
Time min

FIGURE 13

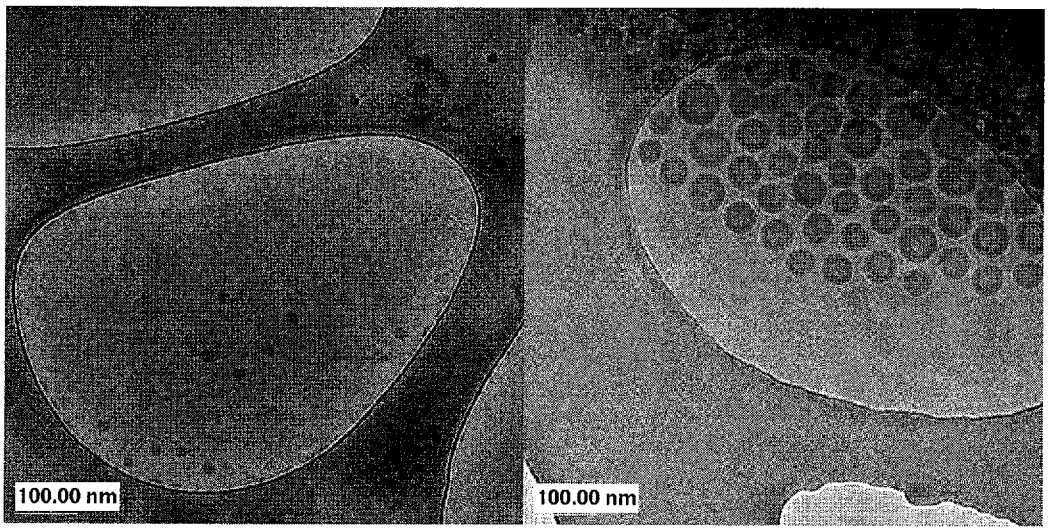


FIGURE 14

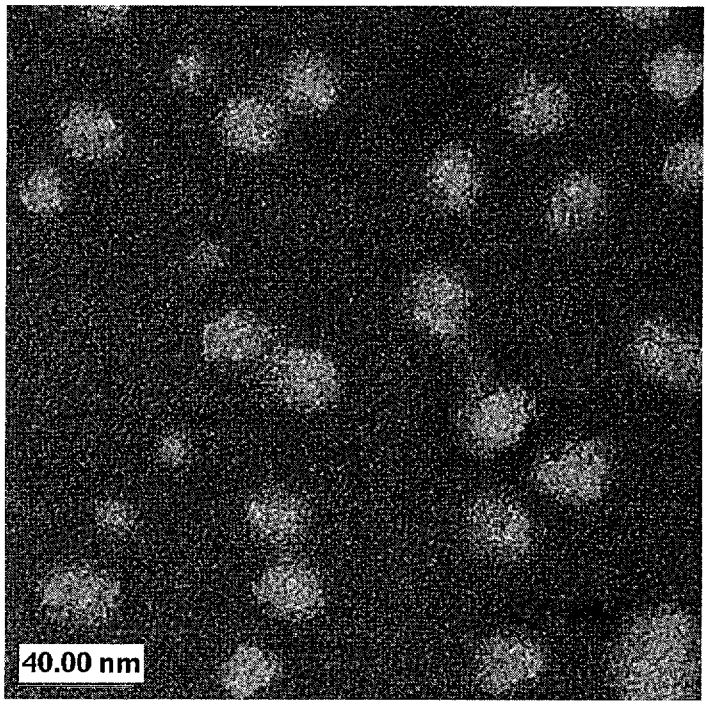


FIGURE 15

A

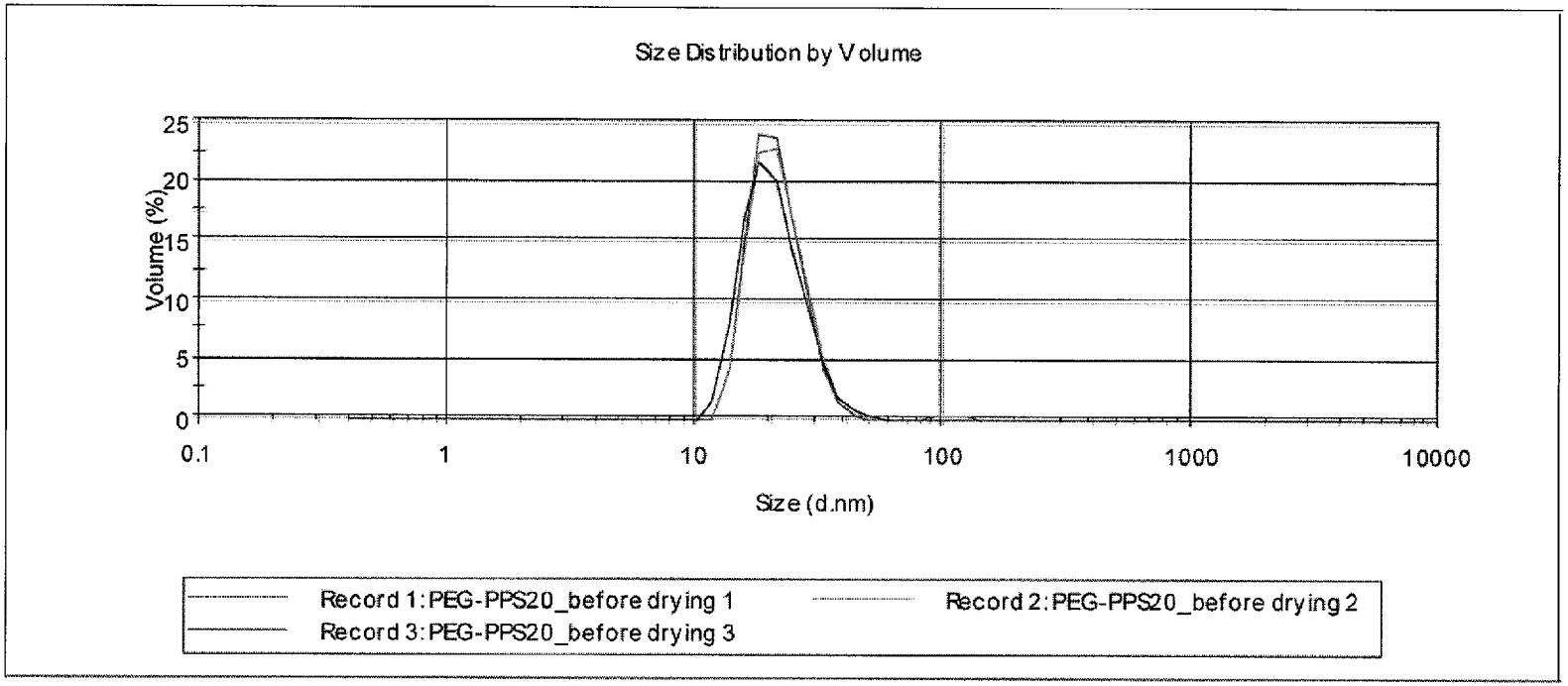

Size Distribution by Volume
Volume (%)
25
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 1:PEG-PPS20_before drying 1
Record 2:PEG-PPS20_before drying 2
Record 3:PEG-PPS20_before drying 3

FIGURE 16

B

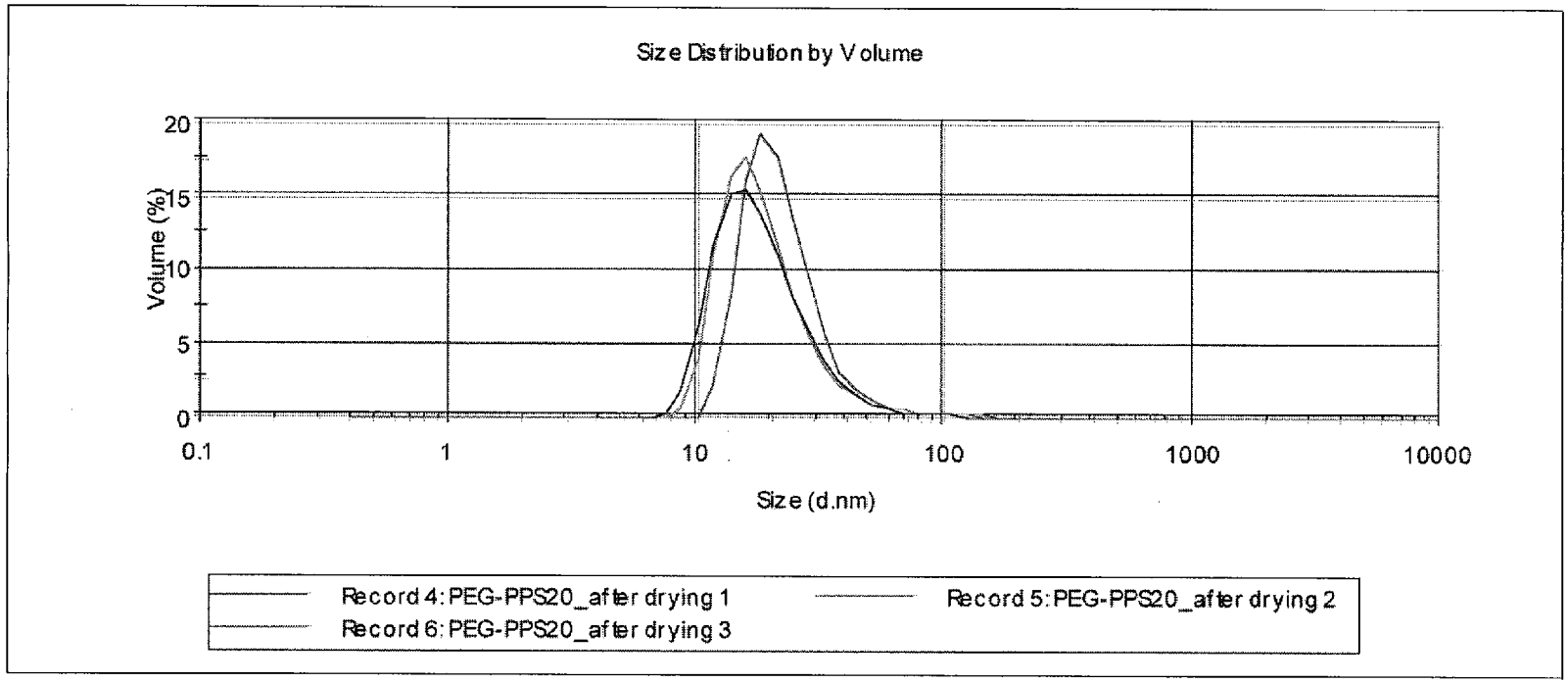

Size Distribution by Volume
Volume (%)
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 4: PEG-PPS20_after drying 1
Record 5: PEG-PPS20_after drying 2
Record 6: PEG-PPS20_after drying 3

FIGURE 16

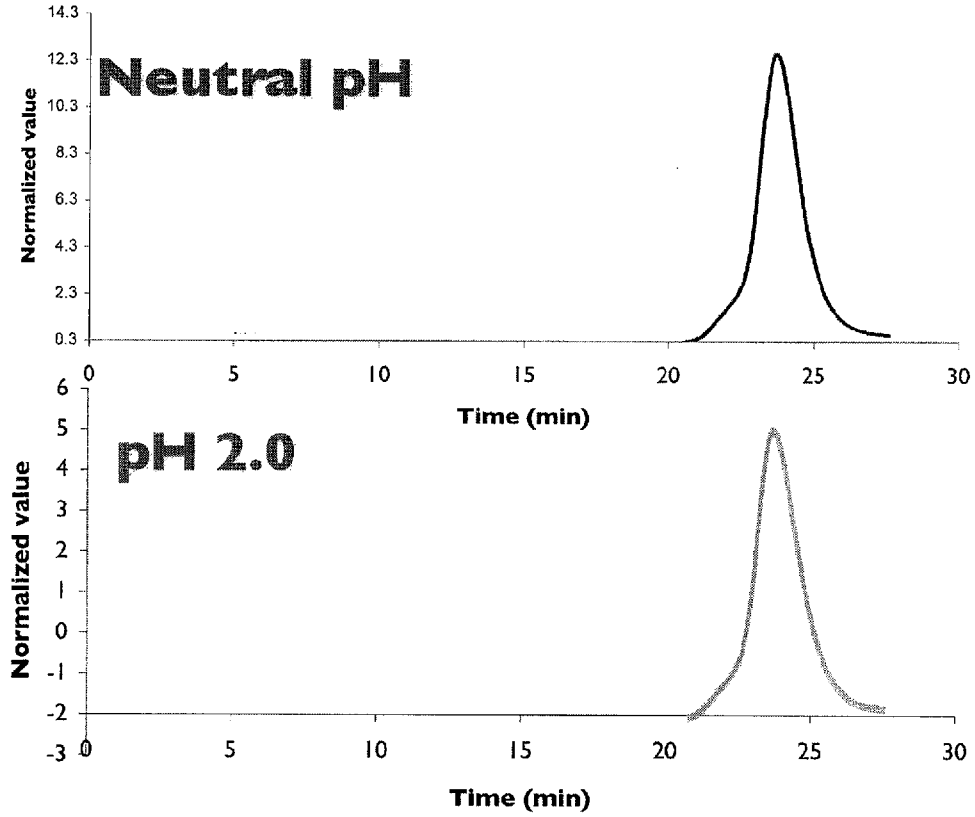

Neutral pH
Normalized value
14.3
12.3
10.3
8.3
6.3
4.3
2.3
0.3
0
5
10
15
20
25
30
Time (min)
pH 2.0
Normalized value
6
5
4
3
2
1
0
-1
-2
-3
0
5
10
15
20
25
30
Time (min)

FIGURE 17A

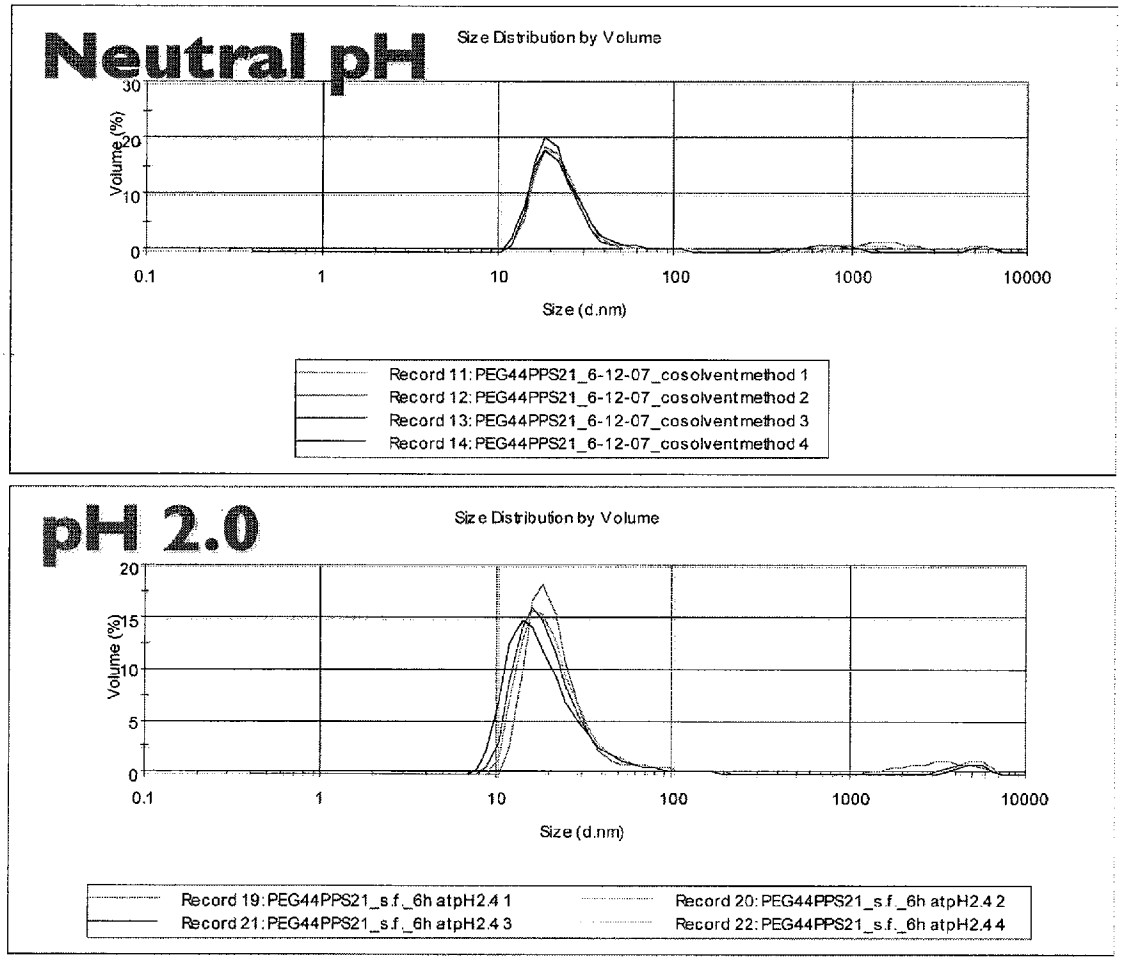

Neutral pH
Size Distribution by Volume
Volume (%)
30
20
10
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 11: PEG44PPS21_6-12-07_cosolventmethod 1
Record 12: PEG44PPS21_6-12-07_cosolventmethod 2
Record 13: PEG44PPS21_6-12-07_cosolventmethod 3
Record 14: PEG44PPS21_6-12-07_cosolventmethod 4
pH 2.0
Size Distribution by Volume
Volume (%)
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 19: PEG44PPS21_s.f._6h atpH2.4 1
Record 20: PEG44PPS21_s.f._6h atpH2.4 2
Record 21: PEG44PPS21_s.f._6h atpH2.4 3
Record 22: PEG44PPS21_s.f._6h atpH2.4 4

FIGURE 17B

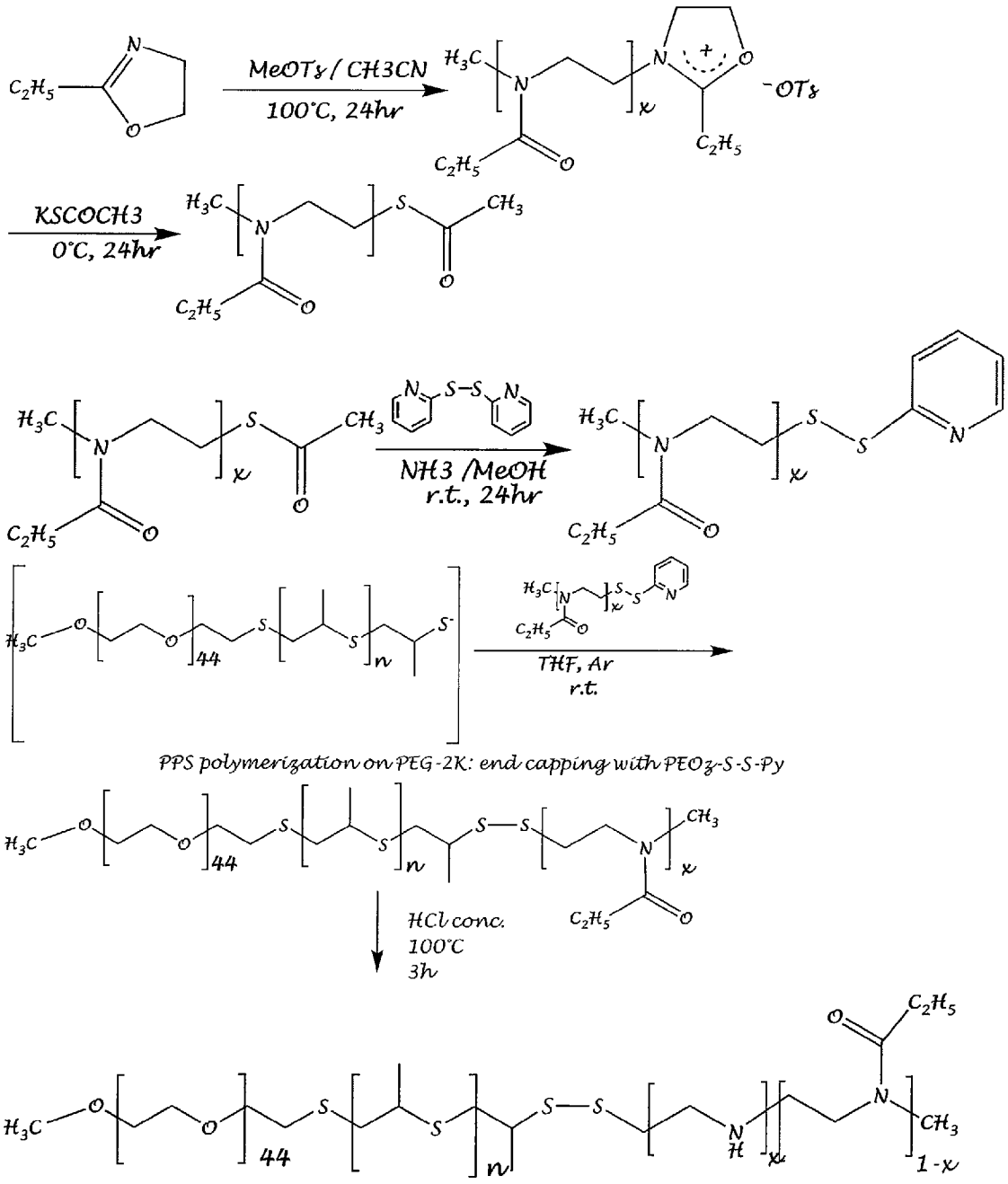
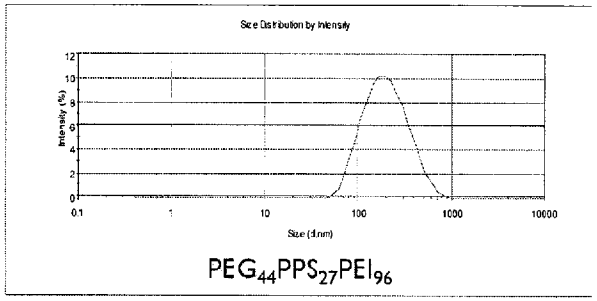
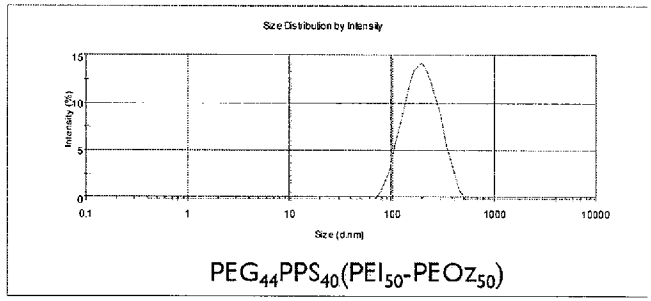

MeOTs / CH3CN
100°C, 24hr
-OTs
KSCOCH3
0°C, 24hr
NH3 /MeOH
r.t., 24hr
THF, Ar
r.t.
PPS polymerization on PEG-2K: end capping with PEOz-S-S-Py
HCl conc.
100°C
3h
44
n
1-x

FIGURE 18

Dynamic Light Scattering (DLS): determines the size distribution of small particles in diluted solution



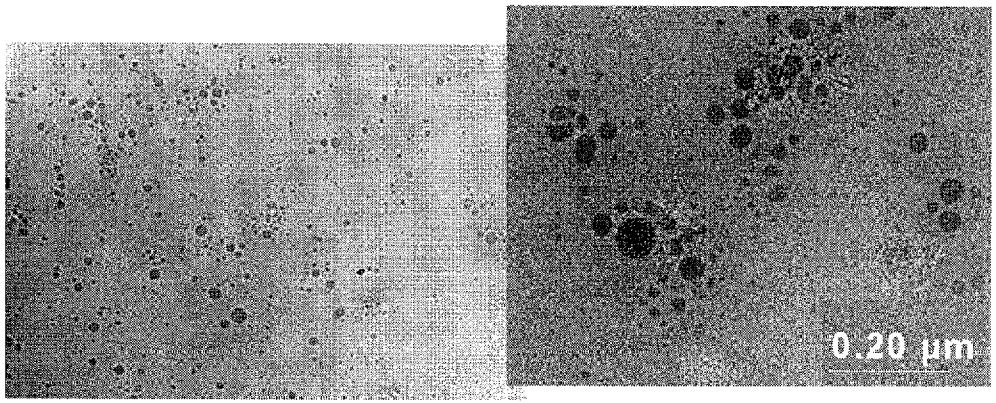
$PEG_{44}PPS_{27}PEI_{96}$



$PEG_{44}PPS_{40}(PEI_{50}\text{-}PEOz_{50})$

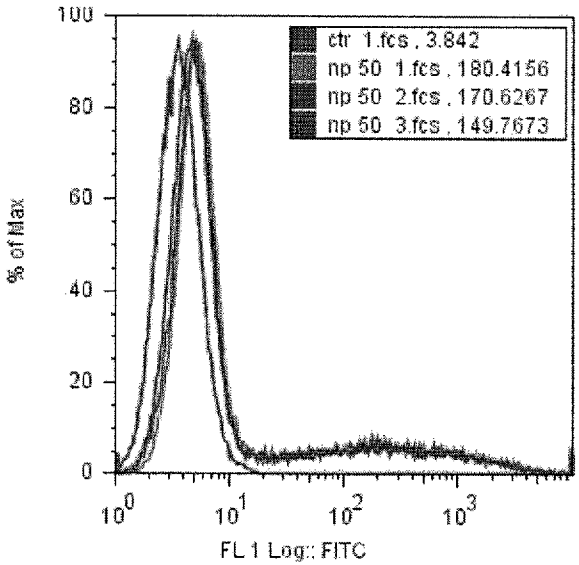
d = 170 nm

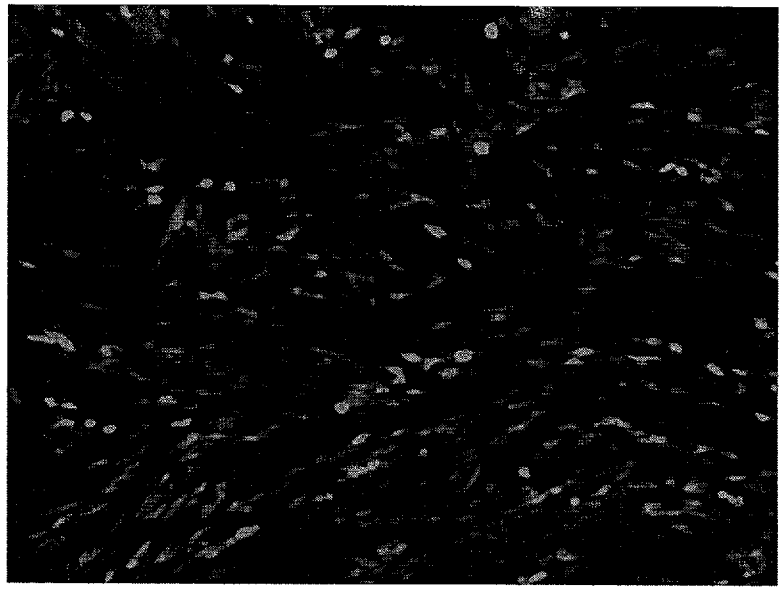
Cryo-Trasmission Electron Mycroscopy (Cryo-TEM): yields insight into aggregate morphology. Samples need to be higlhy concentrated (8mg/mL)



More packed aggregates:
PEI chains are not completely extended

FIGURE 19





TB-copolymer-DNA	serum	Incubation time	Time between transfection and counting
PEG2k-PPS27-PEI96	Yes	24h	48h

47% cells transfected (FACS analysis)

FIGURE 20

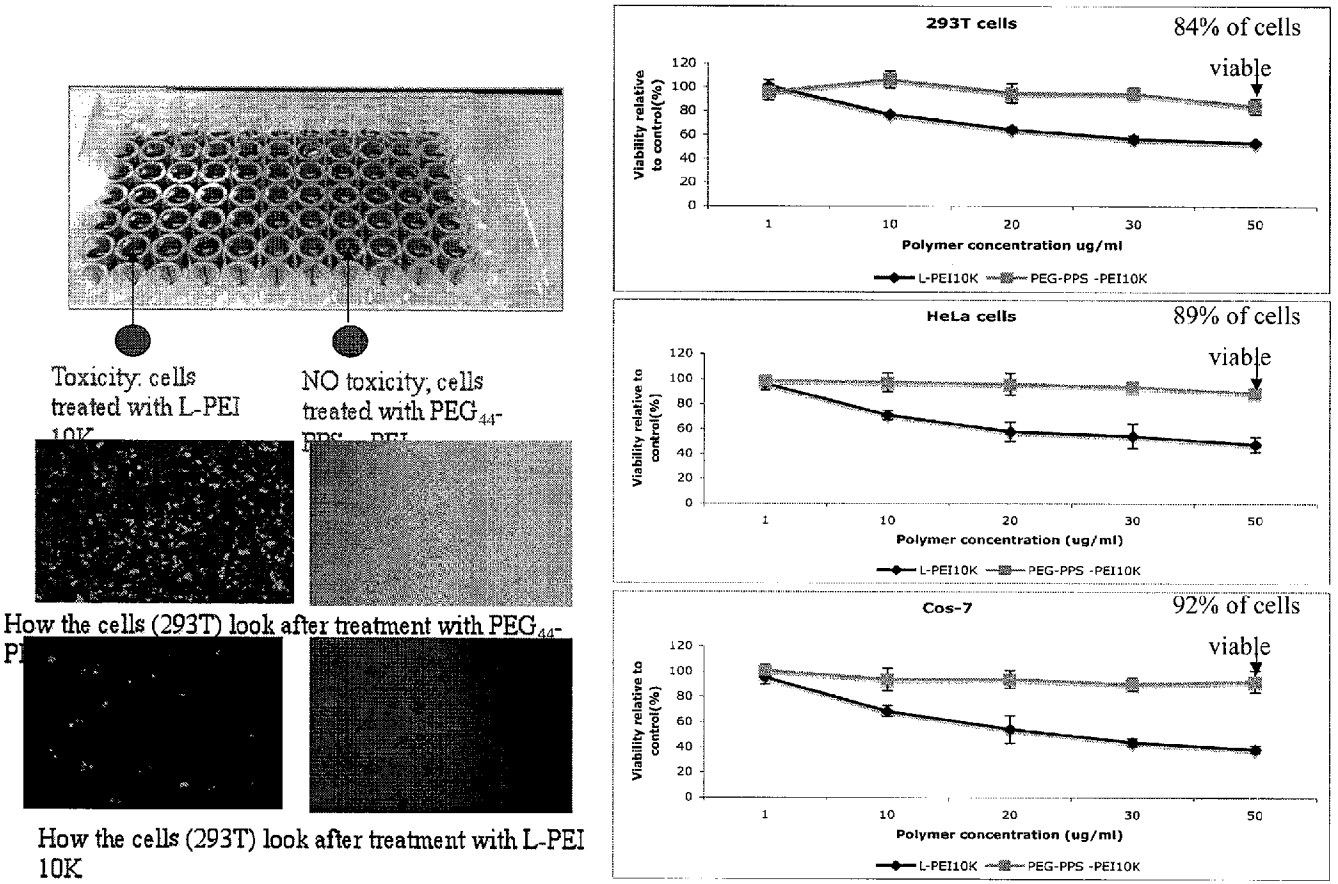

Toxicity: cells treated with L-PEI 10K
NO toxicity, cells treated with PEG44-
How the cells (293T) look after treatment with PEG44-
How the cells (293T) look after treatment with L-PEI 10K
293T cells
84% of cells viable
Viability relative to control(%)
Polymer concentration ug/ml
L-PEI10K
PEG-PPS -PEI10K
HeLa cells
89% of cells viable
Viability relative to control(%)
Polymer concentration (ug/ml)
L-PEI10K
PEG-PPS -PEI10K
Cos-7
92% of cells viable
Viability relative to control(%)
Polymer concentration (ug/ml)
L-PEI10K
PEG-PPS -PEI10K

FIGURE 21

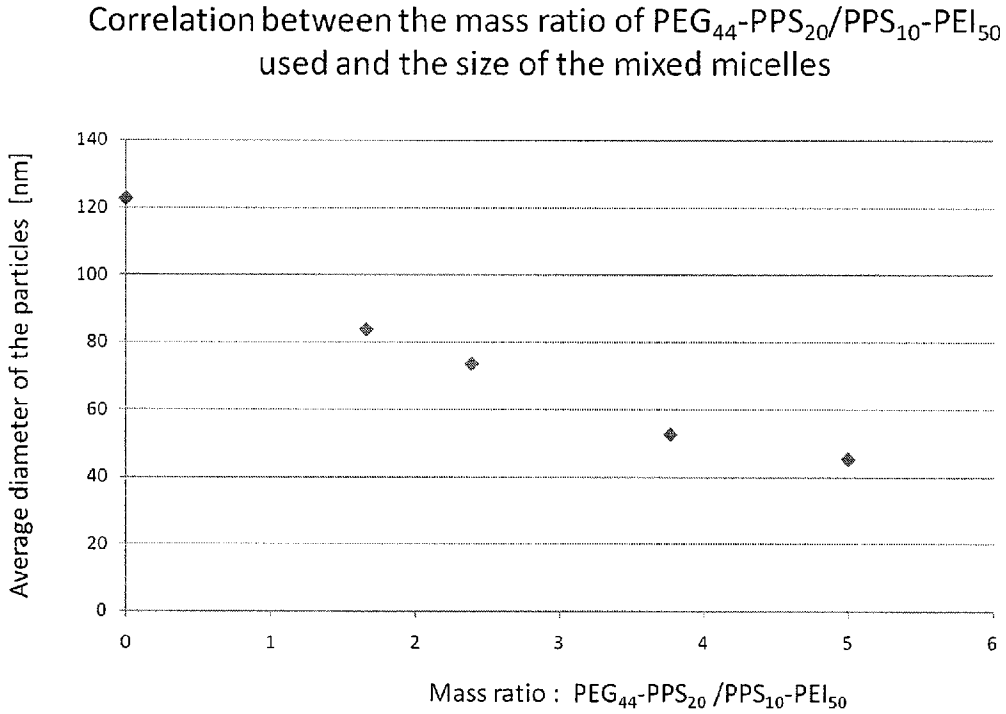
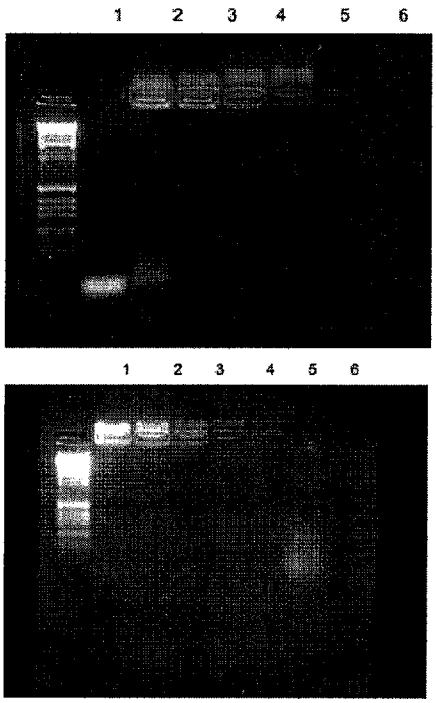

Correlation between the mass ratio of PEG44-PPS20/PPS10-PEI50 used and the size of the mixed micelles
Average diameter of the particles [nm]
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Mass ratio : PEG44-PPS20 /PPS10-PEI50

FIGURE 22



PEG_{44} - PPS_{27} - PEI_{96}

Sample	Polymer	siRNA
1	0	100 pmole
2	10μg	100 pmole
3	20μg	100 pmole
4	30μg	100 pmole
5	50μg	100 pmole
6	95μg	100 pmole

PEG_{44}-PPS_{20} & PPS_{10}-PEI_{50}

Sample	Polymer	siRNA
1	120μg	100 pmole
2	240μg	100 pmole
3	360μg	100 pmole
4	600μg	100 pmole
5	1140μg	100 pmole
6	0	100 pmole

FIGURE 23

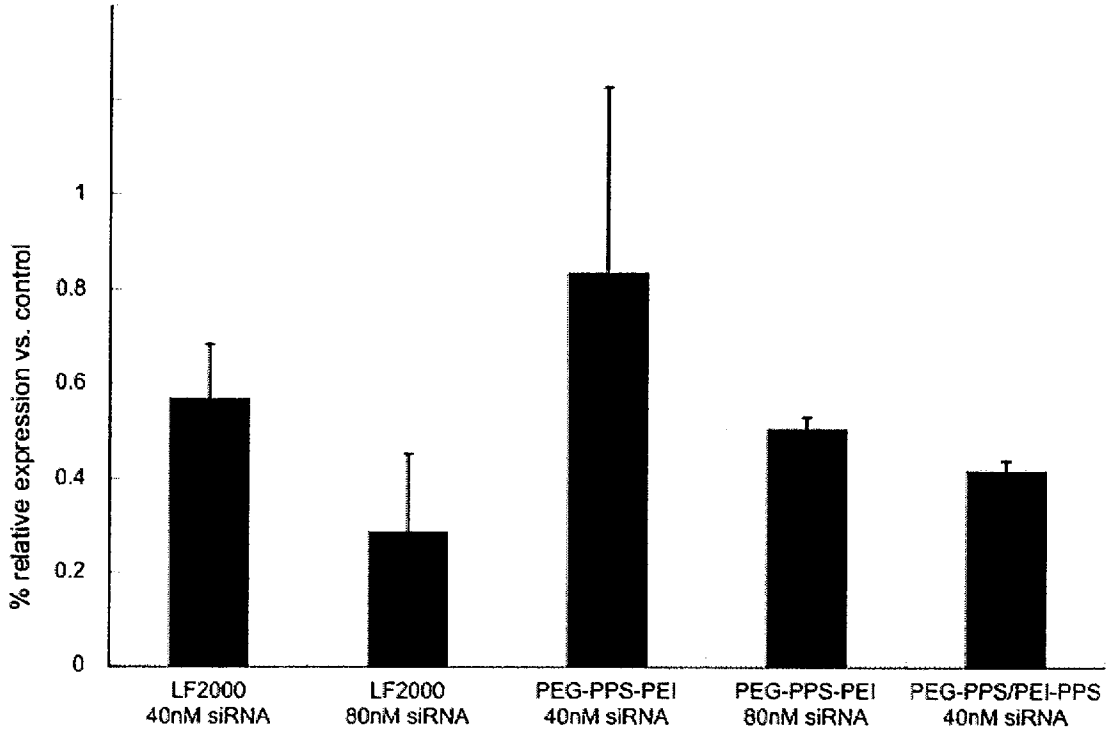
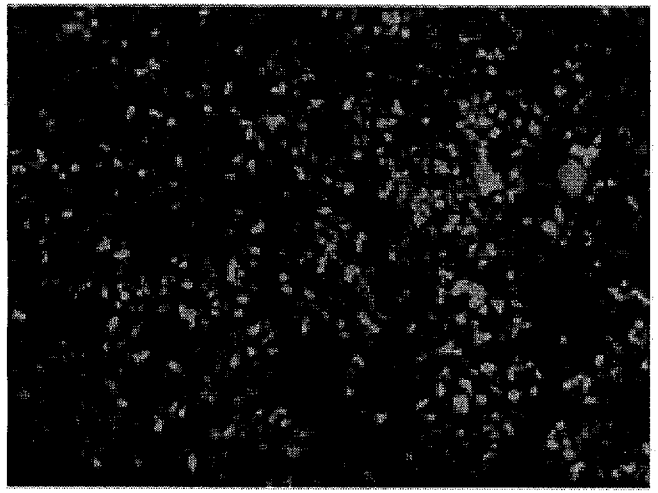
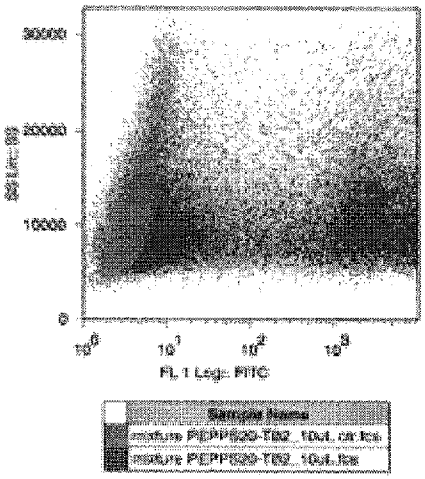

% relative expression vs. control
0
0.2
0.4
0.6
0.8
1
LF2000 40nM siRNA
LF2000 80nM siRNA
PEG-PPS-PEI 40nM siRNA
PEG-PPS-PEI 80nM siRNA
PEG-PPS/PEI-PPS 40nM siRNA

FIGURE 24

Vehicle: AB/ABC blend 1:10

PEG_{44}-PPS_{20} 10mg +
$PEG_{44}PPS_{40}PEI_{120}$ 1mg





Average transfection efficiency = 60%

FIGURE 25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/65693

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 11/08 (2010.01)
USPC - 435/180
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 435/180

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 422/99; 424/1.21, 180; 435/7.9, 29; 436/518; 438/778, 780; 506/13 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic Databases Searched: USPTO WEST (PGPUB, EPAB, JPAB, USPT), Google. Search Terms Used: hydrophilic and hydrophobic, copolymer, co-polymer, encapsul$, pharmaceutical agent, synthesis or preparation, block copolymer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 4,242,474 A (Shinohara et al.) 30 December 1980 (30.12.1980) col 2, ln 40 to col 3, ln 64; col 6, ln 1-3; col 9, ln 55-60	1-3 --- 4-5, 11-22, 30-36, 41-43, 50, 60-62, 65-67, 72-74 47-49
X -- Y	US 2003/0153001 A1 (Soane et al.) 14 August 2003 (14.08.2003) para [0023], [0026], [0029], [0033], [0035], [0093], [0096], [0099], [0102], [0105], [0143]; [0153], [0224], [0280], [0292], [0294], [0324]	4-5, 11-22, 30-36, 41-43, 50, 60-62, 65-67, 72-74
Y	US 2003/0133963 A1 (Hubbell et al.) 17 July 2003 (17.07.2003) para [0009], [0029]	55-58, 67, 74
Y	US 5,502,102 A (Nazareth) 26 March 1996 (26.03.1996) col 9, ln 21-25	55-58

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 January 2010 (07.01.2010)	20 JAN 2010

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/65693

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-10, 23-29, 37-40, 44-46, 51-54, 59, 63-64, 68-71 and 75-78
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.