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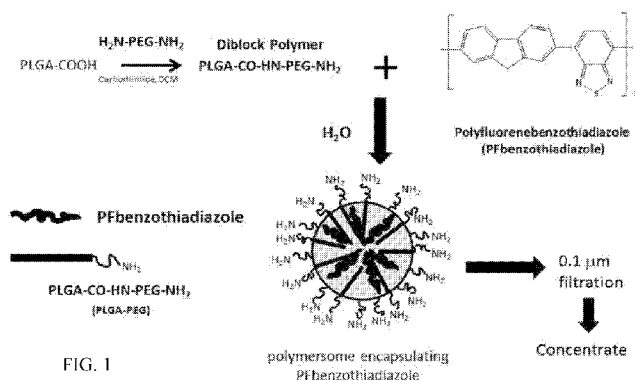
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(54) **Title:** POLYMERSOME ENCAPSULATION OF HYDROPHOBIC FLUORESCENT POLYMERS



(57) **Abstract:** Described herein are aqueous soluble polymersomes that encapsulate one or more hydrophobic fluorescent polymers and methods of their preparation and use. In one aspect, provided herein are aqueous soluble polymersomes comprising: (a) one or more hydrophobic fluorescent polymers; and (b) a plurality of amphiphilic copolymers encapsulating the one or more hydrophobic fluorescent polymers, the copolymers each comprising a hydrophobic block that forms a hydrophobic core structure and a hydrophilic block on the surface of the polymersome that is oriented toward an aqueous phase.

POLYMERSOME ENCAPSULATION OF HYDROPHOBIC FLUORESCENT POLYMERS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/885,932, filed October 2, 2013, which application is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] Fluorescent polymers increasingly play an important role in the field of biochemistry, protein and cell studies. The signals generated by fluorescent polymers can be monitored in real time and provide simple, rapid, and robust methods for the detection of biological targets and events. Dye-labeled antibodies are regularly used for the detection of target biomolecules in applications such as immunohistochemistry, protein arrays, ELISA tests, and flow cytometry. Integrating fluorescent polymers into such methodologies promise to provide a dramatic boost in the performance of such assays, enabling detection levels previously unattainable with conventional dyes. In addition, multiple fluorescent polymers which operate at different, discernible wavelengths can be used simultaneously to detect multiple analytes in the same test or to multiplex.

SUMMARY OF THE INVENTION

[0003] In one aspect, provided herein are aqueous soluble polymersomes comprising: (a) one or more hydrophobic fluorescent polymers; and (b) a plurality of amphiphilic copolymers encapsulating the one or more hydrophobic fluorescent polymers, the copolymers each comprising a hydrophobic block that forms a hydrophobic core structure and a hydrophilic block on the surface of the polymersome that is oriented toward an aqueous phase.

[0004] In some embodiments described above or below of an aqueous soluble polymersome, the amphiphilic block copolymers are diblock copolymers that comprise one hydrophobic polymer block and one hydrophilic polymer block. In some embodiments, the amphiphilic block copolymers are triblock copolymers that comprise one hydrophobic polymer block and two hydrophilic polymer blocks. In other embodiments, the amphiphilic block copolymers are triblock copolymers that comprise two hydrophobic polymer blocks and one hydrophilic polymer block. In other embodiments, the amphiphilic block copolymers are tetrablock copolymers that comprise one hydrophobic polymer block and three hydrophilic polymer blocks, or two hydrophobic polymer blocks and two hydrophilic polymer blocks, or three hydrophobic polymer blocks and one hydrophilic polymer block. In other embodiments, the

amphiphilic block copolymers are pentablock copolymers that comprise one hydrophobic polymer block and four hydrophilic polymer blocks, or two hydrophobic polymer blocks and three hydrophilic polymer blocks, or three hydrophobic polymer blocks and two hydrophilic polymer blocks, or four hydrophobic polymer blocks and one hydrophilic polymer block. In further embodiments, the amphiphilic block copolymers are hexablock copolymers that comprise of hydrophobic and hydrophilic polymer blocks that total six polymer blocks.

[0005] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophobic block is selected from a polymer group consisting of polyacrylonitrile, polycarbonate, aromatic polyether, poly(vinyl acetal), poly(alkyl vinyl ether), poly(alkyl vinyl ketone), polyurethane, polyamide, polyimide, polyester, polyfluorocarbon, polyolefin, polystyrene, polyvinylcyclohexane, polydioxanone, polymeric organosilicon, polysaccharide, poly(methyl methacrylate), or a copolymer combination thereof.

[0006] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophobic block is selected from a polymer group consisting of poly(lactide-co-glycolic acid (PLGA), polylactide (PLA), polyglycolide (PGA), polycaprolactone (PCL), poly(methyl methacrylate) (PMMA), polydimethylsiloxane (PDMS), polyethylene (PE) and polystyrene (PS).

[0007] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophobic block is poly(lactide-co-glycolic acid (PLGA).

[0008] In some embodiments described above or below of an aqueous soluble polymersome, the polymer ratio of PLGA is 50:50 (DL-lactide:glycolide).

[0009] In some embodiments described above or below of an aqueous soluble polymersome, the PLGA has a viscosity of about 0.15-0.25 dL/g.

[0010] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophobic block has a number average molecular weight of about 2,000 to 20,000 Daltons. In some embodiments described above or below of an aqueous soluble polymersome, the hydrophobic block has a number average molecular weight of about 10,000 Daltons. In some embodiments described above or below of an aqueous soluble polymersome, the hydrophobic block has a number average molecular weight of about 5,000 Daltons.

[0011] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophilic block is selected from a polymer group consisting of polyoxyalkylene, polymethacrylate, poly(methacrylic acid), polyacrylic acid, polyacrylate, poly(alkylacrylic

acid), poly(alkylacrylate), polyacrylamide, poly(N-isopropylacrylamide), poly(2-ethyl-2-oxazoline), polyethylenimine, poly(vinyl alcohol), poly(vinylpyrrolidone), poly(styrenesulfonate), poly(vinyl acid), poly(allylamine), poly(diallyldimethyl ammonium chloride), poly(methyl vinyl ether), poly(2-methyloxazoline), or copolymer combinations thereof.

[0012] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophilic block is polyether. In further embodiments described above or below of an aqueous soluble polymersome, the polyether is polypropylene glycol (PPG) or polyethylene glycol (PEG).

[0013] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophilic block is poly(2-methyloxazoline).

[0014] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophilic block has a number average molecular weight of about 1,000 to about 10,000 Daltons. In some embodiments described above or below of an aqueous soluble polymersome, the hydrophilic block has a number average molecular weight of about 5,000 Daltons. In some embodiments described above or below of an aqueous soluble polymersome, the hydrophilic block has a number average molecular weight of about 2,000 Daltons.

[0015] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophilic block further comprises an amine moiety. In some embodiments described above or below of an aqueous soluble polymersome, the amine moiety is further thiolated with 2-iminothiolane (2-IT).

[0016] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophilic block is attached to a biosensor molecule. In some embodiments described above or below of an aqueous soluble polymersome, the hydrophilic block is attached to a signaling chromophore for resonant energy transfer. In some embodiments described above or below of an aqueous soluble polymersome, the hydrophilic block is attached to an antigen-specific antibody.

[0017] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophobic block is PLGA and the hydrophilic block is PEG. In some embodiments described above or below of an aqueous soluble polymersome, the PLGA has a number average molecular weight of about 5,000 Daltons and the PEG has a number average molecular weight of about 2,000 Daltons.

[0018] In some embodiments described above or below of an aqueous soluble polymersome, the one or more hydrophobic fluorescent polymers are polyfluorene polymer, polyfluorene copolymer, or fluorescent homopolymer.

[0019] In some embodiments described above or below of an aqueous soluble polymersome, the one or more hydrophobic fluorescent polymers are selected from poly[(9,9-dioctyl-2,7-divinylene-fluorenylene)-alt-co-(9,10-anthracene)]; poly[(9,9-dioctyl-2,7-divinylene-fluorenylene)-alt-co-{2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene}]; poly[(9,9-dioctyl-2,7-divinylene-fluorenylene)-alt-co-(1,4-phenylene)]; poly[(9,9-dioctylfluorenyl-2,7-diyl)-alt-co-(1,4-benzo-{2,1',3'}-thiadiazole)]; poly[(9,9-dihexylfluorenyl-2,7-diyl)-alt-co-(2-methoxy-5-{2-ethylhexyloxy}-1,4-phenylene)]; poly[(9,9-dioctylfluorenyl-2,7-diyl)-co-(2,5-p-xylene)]; poly[(9,9-dioctylfluorenyl-2,7-diyl)-co-(N,N'-diphenyl)-N,N'-di(p-butylphenyl)-1,4-diamino-benzene)]; poly[(9,9-dioctylfluorenyl-2,7-diyl)-co-(1,4-benzo-{2,1',3'}-thiadiazole)]; poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene-vinylene], optionally end capped with dimethylphenyl; poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene-vinylene], optionally end capped with polysilsesquioxane; poly[2-methoxy-5-(3,7-dimethyloctyloxy)-1,4-phenylene-vinylene], optionally end capped with dimethylphenyl; poly[2-methoxy-5-(3,7-dimethyloctyloxy)-1,4-phenylene-vinylene], optionally end capped with polysilsesquioxane; poly[9,9-dioctylfluorenyl-2,7-diyl], optionally end capped with dimethylphenyl; poly[9,9-dioctylfluorenyl-2,7-diyl], optionally end capped with polysilsesquioxane; poly[9,9-dioctylfluorenyl-2,7-diyl], optionally end capped with N,N-bis(4-methylphenyl)-aniline; poly[9,9-dioctylfluorenyl-2,7-diyl], optionally end capped with 2,5-diphenyl-1,2,4-oxadiazole; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with dimethylphenyl; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with polysilsesquioxane; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with N,N-bis(4-methylphenyl)-aniline; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with 2,5-diphenyl-1,2,4-oxadiazole; poly[2-(5-cyano-5-methylhexyloxy)-1,4-phenylene], optionally end capped with dimethylphenyl; and poly[2,5-dioctyl-1,4-phenylene], optionally end capped with dimethylphenyl; poly[9,9-di(3,3'-N,N'-trimethyl-ammonium)propylfluorenyl-2,7-diyl]-alt-(9,9-dioctylfluorenyl-2,7-diyl)] diiodide salt, optionally end capped with dimethylphenyl; poly[2,5-bis(3,7-dimethyloctyloxy)-1,4-phenylene-vinylene]; poly(9,9-di{2-[2-(2-methoxy-ethoxy)ethoxy]ethyl} fluorenyl-2,7-diyl), optionally end capped with dimethylphenyl; and poly-BODIPY fluorescent polymers.

[0020] In some embodiments described above or below of an aqueous soluble polymersome, the one or more hydrophobic fluorescent polymers are selected from the optionally substituted polymer group consisting of poly(fluorene), poly(fluorene-alt-benzothiadiazole), poly(fluorene-alt-benzooxadiazole), poly(fluorene-alt-thiophene), and poly(fluorene-alt-phenylene).

[0021] In some embodiments described above or below of an aqueous soluble polymersome, the hydrophobic fluorescent polymer is optionally substituted poly(fluorene-alt-benzothiadiazole).

[0022] In some embodiments described above or below of an aqueous soluble polymersome, the polymersome comprises at least two hydrophobic fluorescent polymers. In some embodiments described above or below of an aqueous soluble polymersome, at least one hydrophobic fluorescent polymer acts as a light harvesting polymer and at least one hydrophobic fluorescent polymer acts as a signaling chromophore in a FRET process.

[0023] In some embodiments described above or below of an aqueous soluble polymersome, each of the one or more hydrophobic fluorescent polymers has a number average molecular weight of about 5,000 to about 30,000 Daltons. In some embodiments described above or below of an aqueous soluble polymersome, each of the one or more hydrophobic fluorescent polymers has a number average molecular weight of about 10,000 to about 20,000 Daltons.

[0024] In some embodiments described above or below of an aqueous soluble polymersome, the polymersome has an average particle size of about 200 nm. In some embodiments described above or below of an aqueous soluble polymersome, the polymersome has an average particle size of about 100 nm.

[0025] In some embodiments described above or below of an aqueous soluble polymersome, the polymersome further comprises one or more antioxidants. In some embodiments described above or below of an aqueous soluble polymersome, the one or more antioxidants are selected from butylated hydroxytoluene (BHT), 3-(3,5-ditert-butyl-4-hydroxyphenyl) propionic acid stearyl ester (PASE), triphenylamine (TPA), trans-stilbene (TSB), cyclooctatetraene (Cot), 4-nitrobenzyl alcohol (NBA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

[0026] In another aspect, provided herein are aqueous soluble polymersomes comprising: (a) one or more hydrophobic fluorescent polymers; and (b) a plurality of amphiphilic diblock copolymers encapsulating the one or more hydrophobic fluorescent polymers, the diblock copolymers each having a hydrophobic block that forms a hydrophobic core structure and a

hydrophilic block on the surface of the polymersome that is oriented toward an aqueous phase.

[0027] In another aspect, provided herein are methods of preparing an aqueous soluble polymersome comprising one or more hydrophobic fluorescent polymers, the method comprising: (a) forming a plurality of amphiphilic copolymers each comprising a hydrophobic block and a hydrophilic block; (b) adding one or more hydrophobic fluorescent polymers to the plurality of amphiphilic copolymers; and (c) placing the mixture of step (b) into an aqueous system so that the plurality of amphiphilic copolymers encapsulate the one or more hydrophobic fluorescent polymers, wherein the hydrophobic block forms a hydrophobic core structure and a hydrophilic block is oriented on the surface of the polymersome that is oriented toward an aqueous phase.

[0028] In some embodiments described above or below of a method of preparing an aqueous soluble polymersome, the amphiphilic block copolymers are diblock copolymers that comprise one hydrophobic polymer block and one hydrophilic polymer block. In some embodiments, the amphiphilic block copolymers are triblock copolymers that comprise one hydrophobic polymer block and two hydrophilic polymer blocks. In other embodiments, the amphiphilic block copolymers are triblock copolymers that comprise two hydrophobic polymer blocks and one hydrophilic polymer block. In other embodiments, the amphiphilic block copolymers are tetrablock copolymers that comprise one hydrophobic polymer block and three hydrophilic polymer blocks, or two hydrophobic polymer blocks and two hydrophilic polymer blocks, or three hydrophobic polymer blocks and one hydrophilic polymer block. In other embodiments, the amphiphilic block copolymers are pentablock copolymers that comprise one hydrophobic polymer block and four hydrophilic polymer blocks, or two hydrophobic polymer blocks and three hydrophilic polymer blocks, or three hydrophobic polymer blocks and two hydrophilic polymer blocks, or four hydrophobic polymer blocks and one hydrophilic polymer block. In further embodiments, the amphiphilic block copolymers are hexablock copolymers that comprise of hydrophobic and hydrophilic polymer blocks that total six polymer blocks.

[0029] In some embodiments described above or below of a method of preparing an aqueous soluble polymersome, the hydrophobic block of plurality of amphiphilic copolymers is PLGA.

[0030] In some embodiments described above or below of a method of preparing an aqueous soluble polymersome, the hydrophilic block of plurality of amphiphilic copolymers is PEG.

[0031] In some embodiments described above or below of a method of preparing an aqueous soluble polymersome, the formation of the amphiphilic copolymers comprises reacting PLGA with bis-aminopropyl-PEG in the presence of carbodiimide.

[0032] In some embodiments described above or below of a method of preparing an aqueous soluble polymersome, in step (c), the mixture and aqueous system is agitated. In some embodiments described above or below of a method of preparing an aqueous soluble polymersome, in step (c), the mixture and aqueous system is agitated by vigorous stirring. In other embodiments described above or below of a method of preparing an aqueous soluble polymersome, in step (c), the mixture and aqueous system is agitated by ultra-sonication.

[0033] In some embodiments described above or below of a method of preparing an aqueous soluble polymersome, the method further comprises filtering the polymersomes in the aqueous system.

[0034] In some embodiments described above or below of a method of preparing an aqueous soluble polymersome, the method further comprises concentrating the polymersomes in the aqueous system.

[0035] In some embodiments described above or below of a method of preparing an aqueous soluble polymersome, the method further comprises reacting the polymersomes with a thiolating reagent.

[0036] In some embodiments described above or below of a method of preparing an aqueous soluble polymersome, the method further comprises attaching an antigen-specific antibody to the polymersomes.

[0037] In another aspect, provided herein are methods of preparing an aqueous soluble polymersome comprising one or more hydrophobic fluorescent polymers, the method comprising: (a) forming a plurality of amphiphilic diblock copolymers each having a hydrophobic block and a hydrophilic block; (b) adding one or more hydrophobic fluorescent polymers to the plurality of amphiphilic diblock copolymers; and (c) placing the mixture of step (b) into an aqueous system so that the plurality of amphiphilic diblock copolymers encapsulate the one or more hydrophobic fluorescent polymers, wherein the hydrophobic block forms a hydrophobic core structure and a hydrophilic block is oriented on the surface of the polymersome that is oriented toward an aqueous phase.

[0038] In another aspect, provided herein are methods of solubilizing one or more hydrophobic fluorescent polymers, the method comprising: (a) forming a plurality of amphiphilic copolymers each comprising a hydrophobic block and a hydrophilic block; (b)

adding one or more hydrophobic fluorescent polymers to the plurality of amphiphilic copolymers; and (c) placing the mixture of step (b) into an aqueous system so that the plurality of amphiphilic copolymers encapsulate the one or more hydrophobic fluorescent polymers, wherein the hydrophobic block forms a hydrophobic core structure and a hydrophilic block is oriented on the surface of the polymersome that is oriented toward an aqueous phase.

[0039] In some embodiments described above or below of methods of solubilizing one or more hydrophobic fluorescent polymers, the amphiphilic block copolymers are diblock copolymers that comprise one hydrophobic polymer block and one hydrophilic polymer block. In some embodiments, the amphiphilic block copolymers are triblock copolymers that comprise one hydrophobic polymer block and two hydrophilic polymer blocks. In other embodiments, the amphiphilic block copolymers are triblock copolymers that comprise two hydrophobic polymer blocks and one hydrophilic polymer block. In other embodiments, the amphiphilic block copolymers are tetrablock copolymers that comprise one hydrophobic polymer block and three hydrophilic polymer blocks, or two hydrophobic polymer blocks and two hydrophilic polymer blocks, or three hydrophobic polymer blocks and one hydrophilic polymer block. In other embodiments, the amphiphilic block copolymers are pentablock copolymers that comprise one hydrophobic polymer block and four hydrophilic polymer blocks, or two hydrophobic polymer blocks and three hydrophilic polymer blocks, or three hydrophobic polymer blocks and two hydrophilic polymer blocks, or four hydrophobic polymer blocks and one hydrophilic polymer block. In further embodiments, the amphiphilic block copolymers are hexablock copolymers that comprise of hydrophobic and hydrophilic polymer blocks that total six polymer blocks.

[0040] In another aspect, provided herein are methods of solubilizing one or more hydrophobic fluorescent polymers, the method comprising: (a) forming a plurality of amphiphilic diblock copolymers each having a hydrophobic block and a hydrophilic block; (b) adding one or more hydrophobic fluorescent polymers to the plurality of amphiphilic diblock copolymers; and (c) placing the mixture of step (b) into an aqueous system so that the plurality of amphiphilic diblock copolymers encapsulate the one or more hydrophobic fluorescent polymers, wherein the hydrophobic block forms a hydrophobic core structure and a hydrophilic block is oriented on the surface of the polymersome that is oriented toward an aqueous phase.

[0041] In another aspect, provided herein are assay methods for detecting a target biomolecule in a sample comprising: (a) providing a sample that is suspected of containing a target biomolecule; (b) providing an aqueous soluble polymersome of claim 1 that is conjugated to a sensor biomolecule, wherein the sensor biomolecule is capable of interacting with the target biomolecule or a target-associated biomolecule and wherein the polymersome optionally comprises an acceptor dye; (c) contacting the sample with the sensor biomolecule and the aqueous soluble polymersome in a solution under conditions in which the sensor biomolecule can bind to the target biomolecule or a target-associated biomolecule if present; and (d) applying a light source to the sample that can excite the fluorescent polyfluorene polymer in the polymersome, and detecting whether light is emitted from the polymersome.

[0042] In some embodiments described above or below of an assay method, the sensor biomolecule is a protein, peptide, affinity ligand, antibody, antibody fragment, sugar, lipid, nucleic acid or an aptamer.

[0043] In some embodiments described above or below of an assay method, the sensor biomolecule is an antibody.

[0044] In some embodiments described above or below of an assay method, the target biomolecule is a target protein expressed on a cell surface.

[0045] In some embodiments described above or below of an assay method, the method is configured for flow cytometry, intracellular staining, immunohistochemistry or FISH.

[0046] In another aspect, provided herein are aqueous soluble polymersomes comprising: (a) one or more hydrophobic fluorescent polymers; (b) a plurality of amphiphilic copolymers encapsulating the one or more hydrophobic fluorescent polymers, the copolymers each comprising a hydrophobic block that forms a hydrophobic core structure and a hydrophilic block on the surface of the polymersome that is oriented toward an aqueous phase; and (c) an acceptor dye.

[0047] In another aspect, provided herein are aqueous soluble polymersomes comprising: (a) one or more hydrophobic fluorescent polymers; (b) a plurality of amphiphilic copolymers encapsulating the one or more hydrophobic fluorescent polymers, the copolymers each comprising a hydrophobic block that forms a hydrophobic core structure and a hydrophilic block on the surface of the polymersome that is oriented toward an aqueous phase; (c) an acceptor dye; and (d) one or more antioxidants.

INCORPORATION BY REFERENCE

[0048] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF FIGURES

[0049] **Fig. 1** illustrates a general scheme for the preparation of a non-limiting example of hydrophobic fluorescent polymer (polyfluorenebenzothiadiazole) encapsulated in aqueous soluble polymersomes, in which the carrier polymer is PLGA-CO-NH-PEG-NH₂.

[0050] **Fig. 2** illustrates a visual comparison of the polyfluorenebenzothiadiazole-encapsulated polymersomes of **Fig. 1** in water with polyfluorenebenzothiadiazole in water.

[0051] **Fig. 3** illustrates the size exclusion chromatography data for the polyfluorenebenzothia-diazole-encapsulated polymersomes of **Fig. 1** compared with molecular weight standards.

[0052] **Fig. 4** illustrates a comparison of the absorption properties of the polyfluorenebenzothia-diazole-encapsulated polymersomes of **Fig. 1** in buffer and the absorption properties of polyfluorenebenzothiadiazole in tetrahydrofuran.

[0053] **Fig. 5** illustrates a comparison of the fluorescence emission properties of the polyfluorene-benzothiadiazole-encapsulated polymersomes of **Fig. 1** in buffer and the fluorescence emission properties of polyfluorenebenzothiadiazole in tetrahydrofuran.

[0054] **Fig. 6** illustrates a standard curve for quantitation of MESF for polyfluorenebenzothia-diazole.

[0055] **Fig. 7** illustrates the general scheme for the preparation of a non-limiting example of antibody-polymersome conjugates; in this case, the polyfluorenebenzothiadiazole-encapsulated polymersomes of **Fig. 1** were conjugated to an SMCC-derivatized CD4 monoclonal antibody.

[0056] **Fig. 8** illustrates the size exclusion chromatography data for the fluorescent antibody-polymersome conjugates of **Fig. 7**.

[0057] **Fig. 9** illustrates the flow cytometric analysis of the fluorescent antibody-polymersome conjugates of **Fig. 7** after being captured on anti-kappa beads; in this case, “1” designates the signal from blank beads, “2” designates the signal from BV510-CD4 conjugate, “3” designates the signal from the unmodified fluorescent polymersomes of **Fig. 1**,

and “4” designates the signal from the fluorescent antibody-polymersome conjugates of **Fig. 7**.

DETAILED DESCRIPTION OF THE INVENTION

[0058] Provided here are water-soluble polymersomes that encapsulate water-insoluble, highly fluorescent polymers and methods of their preparation and use. The polymersomes described herein are useful as part of biological sensors in detecting target biomolecules in a system.

[0059] Disclosed herein, in certain embodiments, are aqueous soluble polymersomes comprising a hydrophobic fluorescent polymer and a plurality of amphiphilic copolymers encapsulating the fluorescent polyfluorene polymer, the copolymers each comprising a hydrophobic block that forms a hydrophobic core structure and a hydrophilic block on the surface of the polymersome that is oriented toward an aqueous phase. In some embodiments, the polymersomes self-assemble from amphiphilic block copolymers in the presence of one or more hydrophobic fluorescent polymers. In other embodiments, the polymersomes self-assemble from amphiphilic block copolymers in the presence of one or more hydrophobic fluorescent polymers and one or more hydrophobic antioxidants.

[0060] The amphiphilic block copolymers comprise at least one hydrophobic polymer block and at least one hydrophilic polymer block. Due to the hydrophobic nature of the fluorescent polymer(s), the antioxidant(s), and the hydrophobic polymer blocks, these polymers self-assemble together into a hydrophobic core structure, encapsulating the fluorescent polymer(s) and the antioxidant(s), when the amphiphilic block copolymers, the fluorescent polymer(s), and the antioxidant(s) are vigorously mixed together in the presence of water. The hydrophilic polymer blocks orient themselves towards the surface of the polymersome, thereby solubilizing the whole polymersome in an aqueous system.

[0061] In some embodiments, the hydrophilic polymer block further comprises a reactive chemical moiety for covalent attachment of an antigen-specific antibody to form an antibody-polymersome conjugate. In some embodiments, the antibody-polymersome conjugate is used as a specific labeling agent for cell staining, either for cell surface or intercellular markers. In other embodiments, the antibody-polymersome conjugate is used for the detection of analytes in a cell-free assay system.

Polymersomes

[0062] Polymersomes, as disclosed herein, are enclosures, self-assembled from amphiphilic block copolymers. These amphiphilic block copolymers are macromolecules comprising at least one hydrophobic polymer block and at least one hydrophilic polymer block. When hydrated, these amphiphilic block copolymers self-assemble into enclosures such that the hydrophobic blocks tend to associate with each other to minimize direct exposure to water and form the inner surface of the enclosure, and the hydrophilic blocks face outward, forming the outer surface of the enclosure. The hydrophobic core of these aqueous soluble polymersomes may provide an environment to solubilize additional hydrophobic molecules. As such, these aqueous soluble polymersomes may act as carrier polymers for hydrophobic molecules encapsulated within the polymersomes. Moreover, the self-assembly of the amphiphilic block polymers occurs in the absence of stabilizers, which would otherwise provide colloidal stability and prevent aggregation.

[0063] In some embodiments, these aqueous soluble polymersomes encapsulate one or more hydrophobic fluorescent polymers when the self-assembly occurs in the presence of one or more fluorescent polymers. In some embodiments, these aqueous soluble polymersomes encapsulate one or more hydrophobic fluorescent polymers and an acceptor dye when the self-assembly occurs in the presence of one or more fluorescent polymers and an acceptor dye. In other embodiments, these aqueous soluble polymersomes further comprise one or more hydrophobic antioxidants when the self-assembly occurs in the presence of one or more antioxidants. In some embodiments, the aqueous soluble polymersomes are self-assembled prior to the encapsulation of one or more fluorescent polymers. In some embodiments, the aqueous soluble polymersomes are self-assembled prior to the encapsulation of one or more fluorescent polymers and an acceptor dye. In other embodiments, the aqueous soluble polymersomes are self-assembled prior to the encapsulation of one or more fluorescent polymers and one or more antioxidants. In some embodiments, the aqueous soluble polymersomes are self-assembled prior to the encapsulation of one or more fluorescent polymers, an acceptor dye, and one or more antioxidants. In other embodiments, the aqueous soluble polymersomes are self-assembled prior to the encapsulation of one or more fluorescent polymers, an acceptor dye, and one or more antioxidants. In some embodiments, the aqueous soluble polymersomes are self-assembled in the presence of one or more fluorescent polymers prior to the addition of one or more antioxidants. In some embodiments, the aqueous soluble polymersomes are self-assembled in the presence of one or more

fluorescent polymers and an acceptor dye prior to the addition of one or more antioxidants. In other embodiments, the aqueous soluble polymersomes are self-assembled in the presence of one or more antioxidants prior to the addition of one or more fluorescent polymers.

[0064] In some embodiments, the one or more hydrophobic fluorescent polymers are polyfluorene polymers, polyfluorene copolymers, or fluorescent homopolymers. In some embodiments, the hydrophobic fluorescent polymers are devoid of any reactive functional groups. These hydrophobic fluorescent polymers may be used without further modification to generate fluorescently labeled water-soluble polymersomes, once the hydrophobic fluorescent polymers are encapsulated within the polymersome. These hydrophobic fluorescent polymers are not covalently attached to the carrier polymer (i.e., aqueous soluble polymersome).

[0065] In some embodiments, the aqueous soluble polymersome encapsulates at least one hydrophobic fluorescent polymer that acts as a light harvesting system (donor dye) and at least one acceptor dye that acts as the signaling chromophore in a FRET process. In some embodiments, the acceptor dye is a hydrophobic fluorescent polymer.

[0066] In some embodiments, the amphiphilic block copolymers are diblock copolymers that comprise one hydrophobic polymer block and one hydrophilic polymer block. In some embodiments, the amphiphilic block copolymers are triblock copolymers that comprise one hydrophobic polymer block and two hydrophilic polymer blocks. In other embodiments, the amphiphilic block copolymers are triblock copolymers that comprise two hydrophobic polymer blocks and one hydrophilic polymer block. In other embodiments, the amphiphilic block copolymers are tetrablock copolymers that comprise one hydrophobic polymer block and three hydrophilic polymer blocks, or two hydrophobic polymer blocks and two hydrophilic polymer blocks, or three hydrophobic polymer blocks and one hydrophilic polymer block. In other embodiments, the amphiphilic block copolymers are pentablock copolymers that comprise one hydrophobic polymer block and four hydrophilic polymer blocks, or two hydrophobic polymer blocks and three hydrophilic polymer blocks, or three hydrophobic polymer blocks and two hydrophilic polymer blocks, or four hydrophobic polymer blocks and one hydrophilic polymer block. In further embodiments, the amphiphilic block copolymers are hexablock copolymers that comprise of hydrophobic and hydrophilic polymer blocks that total six polymer blocks.

[0067] In some embodiments, the ratio of the hydrophobic polymer block to the hydrophilic polymer block is 20:80, 25:75, 30:70, 35:65, 40:60, 45:55, 50:50, 55:45, 60:40, 65:35, 70:30,

75:25, 80:20, or any ratio in between. In some embodiments, the ratio of the hydrophobic polymer block to the hydrophilic polymer block is 20:80 to 80:20. In some embodiments, the ratio of the hydrophobic polymer block to the hydrophilic polymer block is 30:70 to 70:30. In some embodiments, the ratio of the hydrophobic polymer block to the hydrophilic polymer block is 40:60 to 60:40.

[0068] The polymer blocks include, but are not limited to, polyglycolides (PGA), polylactides (LPLA and DPLA), polycaprolactone, polydioxanone (PDO or PDS), poly(lactide-co-glycolide) (PLGA), polyanhydrides, polyorthoesters, poly(amino acids) and “pseudo”-poly(amino acids), polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polycyanoacrylates, polyphosphazenes, polyphosphonates, polyiminocarbonates, polyamines, polyolefins, polystyrene, polyoxyalkylene, thermoset amino proteins, polysaccharides, poly(methyl methacrylate) (PMMA), polytetrafluoroethylene (PTFE), polyurethane, polyvinylchloride (PVC), polydimethylsiloxane (PDMS), polyesters, nylons, lignin-based biodegradable polymers, biodegradable polymers from soybeans, soy protein-based plastics, loose fill from corn, polymers based on synthetic genes, bacterially-produced polymers such as polyhydroxyalkanoates, and copolymer combinations thereof.

[0069] The hydrophilic block of the polymersome may be poly(lactic-co-glycolic acid) (PLGA), and the hydrophilic block of the polymersome may be polyethylene glycol (PEG). The PLGA may have a number average molecular weight of about 5,000 Daltons and the PEG may have a number average molecular weight of about 2,000 Daltons.

[0070] In various embodiments, the polymersome has an average particle size of about, for example, 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 11000, 12000, 13000, 14000, 15000, 16000, 17000, 18000, 20000 nm or more, including increments therein. In some embodiments, the polymersome has an average particle size of about 100 nm. In some embodiments, the polymersome has an average particle size of about 200 nm. In some embodiments, the polymersome has an average particle size of about 300 nm. In some embodiments, the polymersome has an average particle size of about 400 nm. In some embodiments, the polymersome has an average particle size of about 500 nm. In some embodiments, the polymersome has an average particle size of about 600 nm. In some embodiments, the polymersome has an average particle size of about 700 nm. In some embodiments, the polymersome has an average particle size of about 800 nm. In some embodiments, the polymersome has an average particle size of about 900 nm. In some embodiments, the polymersome has an

average particle size of about 1000 nm. In some embodiments, the polymersome has an average particle size of about 100-300 nm. In some embodiments, the polymersome has an average particle size of about 100-400 nm. In some embodiments, the polymersome has an average particle size of about 100-500 nm. In some embodiments, the polymersome has an average particle size of about 100-600 nm. In some embodiments, the polymersome has an average particle size of about 200-500 nm. In some embodiments, the polymersome has an average particle size of about 200-600 nm. In some embodiments, the polymersome has an average particle size of about 200-700 nm. In some embodiments, the polymersome has an average particle size of about 100-1000 nm. In some embodiments, the polymersome has an average particle size of about 25-100 nm. In some embodiments, the polymersome has an average particle size of about 25-200 nm. In some embodiments, the polymersome is considered to be a nanoparticle.

Hydrophobic polymer block

[0071] Hydrophobic polymer blocks are insoluble in water. In various embodiments, the hydrophobic polymer blocks have a number average molecular weight of about 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 11000, 12000, 13000, 14000, 15000, 16000, 17000, 18000, 19000, or 20000 Daltons, including increments therein. In some embodiments, the hydrophobic polymer blocks have a number average molecular weight of about 2,000 to 20,000 Daltons. In some embodiments, the hydrophobic polymer blocks have a number average molecular weight of about 2,000 to 5,000 Daltons. In some embodiments, the hydrophobic polymer blocks have a number average molecular weight of about 2,000 to 8,000 Daltons. In some embodiments, the hydrophobic polymer blocks have a number average molecular weight of about 2,000 to 10,000 Daltons. In some embodiments, the hydrophobic polymer blocks have a number average molecular weight of about 2,000 to 15,000 Daltons. In some embodiments, the hydrophobic polymer blocks have a number average molecular weight of about 5,000 to 10,000 Daltons. In some embodiments, the hydrophobic polymer blocks have a number average molecular weight of about 5,000 to 15,000 Daltons. In some embodiments, the hydrophobic polymer blocks have a number average molecular weight of about 10,000 Daltons. In some embodiments, the hydrophobic polymer blocks have a number average molecular weight of about 5,000 Daltons.

[0072] In some embodiments, the hydrophobic polymer block is polyacrylonitrile, polycarbonate, aromatic polyether, poly(vinyl acetal), poly(alkyl vinyl ether), poly(alkyl vinyl ketone), polyurethane, polyamide, polyimide, polyester, polyfluorocarbon, polyolefin,

polystyrene, polyvinylcyclohexane, polydioxanone, polymeric organosilicon, polysaccharide, poly(methyl methacrylate), or a copolymer combination thereof. In some embodiments, the hydrophobic polymer block is poly(lactic-co-glycolic acid) (PLGA). In some embodiments, the PLGA has a viscosity of about 0.15-0.25 dL/g.

[0073] In some embodiments, the hydrophobic polymer block is a polyamide. In some embodiments, the polyamide is an aliphatic polyamide. In some embodiments, the hydrophobic polymer block is a polyimide. In some embodiments, the polyimide is polyetherimide, polymaleimide, or a copolymer combination thereof.

[0074] In some embodiments, the hydrophobic polymer block is a polyester. In some embodiments, the polyester is unsaturated or saturated. In some embodiments, the polyester is a polyhydroxyalkanoate. In some embodiments, the polyester is polylactide, a polyglycolide, polycaprolactone, poly-3-hydroxybutyrate, poly-4-hydroxybutyrate a polyhydroxyvalerate, poly-hydroxyhexanoate, poly-hydroxyoctanoate, or a copolymer combination thereof.

[0075] In some embodiments, the hydrophobic polymer block is a polyfluorocarbon. In some embodiments, the polyfluorocarbon is polytetrafluoroethylene, poly(vinylidene fluoride), poly(chlorotrifluoroethylene), or a copolymer combination thereof.

[0076] In some embodiments, the hydrophobic polymer block is a polyolefin. In some embodiments, the polyolefin is polyethylene, polypropylene, polybutene, polyisobutylene, poly(4-methyl-1-pentene), or a copolymer combination thereof.

[0077] In some embodiments, the hydrophobic polymer block is a polystyrene. In some embodiments, the polystyrene is substituted or unsubstituted. In some embodiments, the hydrophobic polymer block is polyvinylcyclohexane.

[0078] In some embodiments, the hydrophobic polymer block is a polymeric organosilicon. In some embodiments, the polymeric organosilicon is polydimethylsiloxane.

[0079] In some embodiments, the hydrophobic polymer block is a hydrophobic polysaccharide. In some embodiments, the polysaccharide is a cellulosic polymer.

[0080] In some embodiments, the hydrophobic polymer block is polyacrylonitrile. In some embodiments, the hydrophobic polymer block is polycarbonate. In some embodiments, the hydrophobic polymer block is aromatic polyether. In some embodiments, the hydrophobic polymer block is poly(vinyl acetal). In some embodiments, the hydrophobic polymer block is poly(alkyl vinyl ether). In some embodiments, the hydrophobic polymer block is poly(alkyl vinyl ketone). In some embodiments, the hydrophobic polymer block is

polyurethane. In some embodiments, the hydrophobic polymer block is polydioxanone. In some embodiments, the hydrophobic polymer block is polycyanoacrylate. In some embodiments, the hydrophobic polymer block is poly(methyl methacrylate).

[0081] In some embodiments, the hydrophobic polymer block is selected from a polymer group consisting of poly(lactide-co-glycolic acid (PLGA)), polylactide (PLA), polyglycolide (PGA), polycaprolactone (PCL), poly(methyl methacrylate) (PMMA), polydimethylsiloxane (PDMS), polyethylene (PE) and polystyrene (PS).

[0082] In various embodiments, the ratio of two copolymers in the hydrophobic polymer block is 20:80, 25:75, 30:70, 35:65, 40:60, 45:55, 50:50, 55:45, 60:40, 65:35, 70:30, 75:25, 80:20, 85:15 or any ratio in between. In some embodiments, the ratio of two copolymers in the hydrophobic polymer block is 20:80 to 80:20. In some embodiments, the ratio of two copolymers in the hydrophobic polymer block is 30:70 to 70:30. In some embodiments, the ratio of two copolymers in the hydrophobic polymer block is 40:60 to 60:40. In some embodiments, the ratio of two copolymers in the hydrophobic polymer block is 50:50 to 85:15.

Hydrophilic Polymer Block

[0083] Hydrophilic polymer blocks contain polar or charged functional groups, rendering them soluble in water. In various embodiments, the hydrophilic polymer blocks have a number average molecular weight of about 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 11000, 12000, 13000, 14000, 15000, 16000, 17000, 18000, 19000, or 20000 Daltons, including increments therein. In some embodiments, the hydrophilic polymer block has a number average molecular weight of about 1,000 to about 2,000 Daltons. In some embodiments, the hydrophilic polymer block has a number average molecular weight of about 1,000 to about 3,000 Daltons. In some embodiments, the hydrophilic polymer block has a number average molecular weight of about 1,000 to about 5,000 Daltons. In some embodiments, the hydrophilic polymer block has a number average molecular weight of about 1,000 to about 8,000 Daltons. In some embodiments, the hydrophilic polymer block has a number average molecular weight of about 1,000 to about 10,000 Daltons. In some embodiments, the hydrophilic polymer block has a number average molecular weight of about 2,000 to about 5,000 Daltons. In some embodiments, the hydrophilic polymer block has a number average molecular weight of about 2,000 to about 8,000 Daltons. In some embodiments, the hydrophilic polymer block has a number average molecular weight of about 5,000 to about 15,000 Daltons. In some embodiments, the hydrophilic polymer block

has a number average molecular weight of about 2,000 to about 20,000 Daltons. In some embodiments, the hydrophilic polymer blocks have a number average molecular weight of about 5,000 Daltons. In some embodiments, the hydrophilic polymer blocks have a number average molecular weight of about 2,000 Daltons.

[0084] In some embodiments, the hydrophilic polymer block is polyoxyalkylene, polymethacrylate, poly(methacrylic acid), polyacrylic acid, polyacrylate, poly(alkylacrylic acid), poly(alkylacrylate), polyacrylamide, poly(*N*-isopropylacrylamide), poly(2-ethyl-2-oxazoline), polyethylenimine, poly(vinyl alcohol), poly(vinylpyrrolidone), poly(styrenesulfonate), poly(vinyl acid), poly(allylamine), poly(diallyldimethyl ammonium chloride), poly(methyl vinyl ether), poly(2-methyloxazoline), or copolymer combinations thereof.

[0085] In some embodiments, the hydrophilic polymer block is a polyether. In some embodiments, the polyether is a polyoxyalkylene polymer. In some embodiments, the polyoxyalkylene polymer is polyoxyethylene (or polyethylene glycol), polyoxypropylene (polypropylene glycol), polyoxybutylene, or copolymer combinations thereof. In some embodiments, the hydrophilic polymer block is derived from units of one or more alkyl oxide monomers. In some embodiments, the one or more alkyl oxide monomers are ethylene oxide, propylene oxide, or copolymer combinations thereof. In some embodiments, the hydrophilic polymer block is derived from units of one or more alkylene glycol monomers. In some embodiments, the one or more alkylene glycol monomers are ethylene glycol, polypropylene glycol, or combinations thereof.

[0086] In some embodiments, the hydrophilic polymer block is polymethacrylate salt, poly(methacrylic acid), derivatives of poly(methacrylic acid) or copolymer combinations thereof.

[0087] In some embodiments, the hydrophilic polymer block is polyacrylic acid, polyacrylate salt, a derivative of polyacrylic acid, or copolymer combinations thereof. In some embodiments, the hydrophilic polymer block may be poly(alkylacrylic acid), poly(alkylacrylate) salt, a derivative of poly(alkylacrylic acid), or copolymer combinations thereof. In some embodiments, the hydrophilic polymer block may be polyacrylamide (PAM). In some embodiments, the hydrophilic polymer block may be poly(*N*-isopropylacrylamide) (PNIPAM).

[0088] In some embodiments, the hydrophilic polymer block is a hydrophilic polysaccharide. In some embodiments, the polysaccharide may be dextrin or amylopectin. In some

embodiments, the polysaccharide may be dextrin. In other embodiments, the polysaccharide may be amylopectin.

[0089] In some embodiments, the hydrophilic polymer block is poly(2-ethyl-2-oxazoline). In some embodiments, the hydrophilic polymer block is polyethylenimine (PEI). In some embodiments, the hydrophilic polymer block is poly(vinyl alcohol) (PVA). In some embodiments, the hydrophilic polymer block is poly(vinylpyrrolidone) (PVP). In some embodiments, the hydrophilic polymer block is poly(styrenesulfonate) (PSS). In some embodiments, the hydrophilic polymer block is poly(vinyl acid) or their associated salts. The poly(vinyl acid) may be poly(vinylphosphonic acid), poly(vinyl sulfonic acid), their associated salts, or copolymer combinations thereof. In some embodiments, the hydrophilic polymer block is poly(allylamine). In some embodiments, the hydrophilic polymer block is poly(diallyldimethyl ammonium chloride). In some embodiments, the hydrophilic polymer block is poly(methyl vinyl ether). In some embodiments, the hydrophilic polymer block is maleic anhydride copolymer. In some embodiments, the hydrophilic polymer block is poly(2-methyloxazoline).

[0090] In various embodiments, the ratio of two copolymers in the hydrophilic polymer block is 20:80, 25:75, 30:70, 35:65, 40:60, 45:55, 50:50, 55:45, 60:40, 65:35, 70:30, 75:25, 80:20, 85:15 or any ratio in between. In some embodiments, the ratio of two copolymers in the hydrophilic polymer block is 20:80 to 80:20. In some embodiments, the ratio of two copolymers in the hydrophilic polymer block is 30:70 to 70:30. In some embodiments, the ratio of two copolymers in the hydrophilic polymer block is 40:60 to 60:40. In some embodiments, the ratio of two copolymers in the hydrophilic polymer block is 50:50 to 85:15.

[0091] In some embodiments, the hydrophilic polymer block further comprises a primary reactive chemical group. In some embodiments, the hydrophilic polymer block is terminally end capped with a primary reactive chemical group and the other terminus is covalently attached to either a hydrophobic polymer block or another hydrophilic polymer block. In some embodiments, the primary reactive chemical group is an amine moiety, a carboxylic acid moiety, an activated carboxylic acid moiety, an alcohol moiety, a halide moiety, a maleimide moiety, an azide moiety, an alkene, an alkyne, or a thiol moiety. In some embodiments, the primary reactive chemical group is reacted with to form a linker with a secondary reactive chemical group. In some embodiments, the secondary reactive chemical group is an amine moiety, a carboxylic acid moiety, an activated carboxylic acid moiety, an alcohol moiety, a halide moiety, a maleimide moiety, an azide moiety, or a thiol moiety. In

some embodiments, the primary reactive chemical group is used to directly attach a biosensor molecule to the polymersome. In other embodiments, the secondary reactive group is used to attach a biosensor molecule to the polymersome.

[0092] In some embodiments, the primary reactive chemical group is an amine moiety. In further embodiments, the amine moiety is further thiolated with 2-iminothiolane (2-IT). In still further embodiments, the thiol product of the reaction between the amine moiety and 2-IT is further reacted with a derivatized antibody. In some embodiments, the derivatized antibody reacts with a thiol moiety on the polymersome. In some embodiments, the derivatized antibody is an SMCC-derivatized CD4-monoclonal antibody. In some embodiments, the derivatized antibody is an SPDP-derivatized CD4 monoclonal antibody.

[0093] In some embodiments, the hydrophilic polymer block is attached to a biosensor molecule. It is envisioned that the biosensor molecule can be, but is not limited to, a dye, fluorescence protein, nanomaterial (e.g., Quantum Dot), chemiluminescence-generating molecule, a conjugate between dye and chemiluminescence-generating molecule, a conjugate between fluorescence protein and chemiluminescence-generating molecule, a conjugate between a nanomaterial (e.g., Quantum Dot) and chemiluminescence-generating molecule, streptavidin, avidin, enzyme, substrate for an enzyme, substrate analog for an enzyme, receptor, ligand for a receptor, ligand analog for a receptor, DNA, RNA, modified nucleic acid, DNA aptamer, RNA aptamer, modified nucleic aptamer, peptide aptamer, antibody, antigen, phage, bacterium or conjugate of any two of the items described above. In some embodiments, the biosensor molecule is a protein, peptide, affinity ligand, antibody, antibody fragment, sugar, lipid, nucleic acid or an aptamer. In some embodiments, the biosensor molecule is a substrate for an enzyme, a substrate analog for an enzyme, a ligand, or a ligand analog for a receptor. In some embodiments, the biosensor molecule is a DNA aptamer, a RNA aptamer, a modified nucleic aptamer, or a peptide aptamer. In some embodiments, the biosensor molecule is avidin. In some embodiments, the biosensor molecule is streptavidin. In some embodiments, the biosensor molecule is an antigen-specific antibody.

[0094] In some embodiments, the polymersome comprises an acceptor dye. In some embodiments, the acceptor dye is conjugated to the biosensor molecule. In some embodiments, the acceptor dye is encapsulated by the polymersome. In some embodiments, the acceptor dye is conjugated to the hydrophilic polymer block. It is envisioned that the acceptor dye can be, but is not limited to, rhodamine, coumarin, cyanine, xanthene, polymethine, pyrene, dipyrromethene borondifluoride, naphthalimide, a phycobiliprotein,

peridinium chlorophyll proteins, conjugates thereof, and combinations thereof. Non-limiting examples of acceptor dyes include, fluorescein, 6-FAM, rhodamine, Texas Red, California Red, iFluor594, tetramethylrhodamine, a carboxyrhodamine, carboxyrhodamine 6F, carboxyrhodol, carboxyrhodamine 110, Cascade Blue, Cascade Yellow, coumarin, Cy2®, Cy3®, Cy3.5®, Cy5®, Cy5.5®, Cy7®, Cy-Chrome, DyLight 350, DyLight 405, DyLight 488, DyLight 549, DyLight 594, DyLight 633, DyLight 649, DyLight 680, DyLight 750, DyLight 800, phycoerythrin, PerCP (peridinin chlorophyll-a Protein), PerCP-Cy5.5, JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein), NED, ROX (5-(and-6-)-carboxy-X-rhodamine), HEX, Lucifer Yellow, Marina Blue, Oregon Green 488, Oregon Green 500, Oregon Green 514, Alexa Fluor® 350, Alex Fluor® 430, Alexa Fluor® 488, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 633, Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, 7-amino-4-methylcoumarin-3-acetic acid, BODIPY® FL, BODIPY® FL-Br₂, BODIPY® 530/550, BODIPY® 558/568, BODIPY® 630/650, BODIPY® 650/665, BODIPY® R6G, BODIPY® TMR, BODIPY® TR, conjugates thereof, and combinations thereof.

[0095] In further embodiments, the acceptor dye contains a signaling chromophore for resonant energy transfer (Forster (or fluorescence) resonance energy transfer, FRET). Exemplary chromophores include, but are not limited to, 2,1,3-benzothiadiazole, benzoxadiazole, benzoselenadiazole, benzotellurodiazole, naphthoselenadiazole, 4,7-di(thien-2-yl)-2,1,3-benzothiadiazole, squaraine dyes, quinoxalines, perylene, perylene diimides, diketopyrrolopyrrole, thienopyrazine low bandgap commercial dyes, Nile Red, Coumarin 6, tetraphenylporphyrin, cyanine dyes (e.g., 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine), olefins, and cyano-substituted olefins and isomers thereof.

Hydrophobic fluorescent polymers

[0096] In some embodiments, the aqueous soluble polymersomes comprise one or more hydrophobic fluorescent polymers and a plurality of amphiphilic copolymers encapsulating the fluorescent polyfluorene polymer. The hydrophobic fluorescent polymer is an intensely fluorescent molecule. In some embodiments, the hydrophobic fluorescent polymer is a polyfluorene polymer, polyfluorene copolymer, or fluorescent homopolymer. In various embodiments, the hydrophobic fluorescent polymer has a number average molecular weight of about 5000, 10000, 15000, 20000, 25000, or 30000 Daltons, including increments therein. In some embodiments, the hydrophobic fluorescent polymer has a number average molecular

weight of about 5,000 to about 10,000 Daltons. In some embodiments, the hydrophobic fluorescent polymer has a number average molecular weight of about 5,000 to about 15,000 Daltons. In some embodiments, the hydrophobic fluorescent polymer has a number average molecular weight of about 5,000 to about 20,000 Daltons. In some embodiments, the hydrophobic fluorescent polymer has a number average molecular weight of about 5,000 to about 30,000 Daltons. In some embodiments, the hydrophobic fluorescent polymer has a number average molecular weight of about 10,000 to about 15,000 Daltons. In some embodiments, the hydrophobic fluorescent polymer has a number average molecular weight of about 10,000 to about 20,000 Daltons. In some embodiments, the hydrophobic fluorescent polymer has a number average molecular weight of about 10,000 to about 30,000 Daltons. In some embodiments, the hydrophobic fluorescent polymer has a number average molecular weight of about 20,000 to about 30,000 Daltons.

[0097] In some embodiments, the hydrophobic fluorescent polymer is selected from the optionally substituted polymer group consisting of poly(fluorene), poly(fluorene-alt-benzothiadiazole), poly(fluorene-alt-benzooxadiazole), poly(fluorene-alt-thiophene), and poly(fluorene-alt-phenylene). The polyfluorene polymer, polyfluorene copolymer, or fluorescent homopolymer may be optionally substituted poly(fluorene-alt-benzothiadiazole).

[0098] Polyfluorene polymers, polyfluorene copolymers, and fluorescent homopolymers may include, but are not limited to, poly[(9,9-dioctyl-2,7-divinylene fluorenylene)-alt-co-(9,10-anthracene)]; poly[(9,9-dioctyl-2,7-divinylene fluorenylene)-alt-co-{2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene}]; poly[(9,9-dioctyl-2,7-divinylene fluorenylene)-alt-co-(1,4-phenylene)]; poly[(9,9-dioctyl fluorenyl-2,7-diyl)-alt-co-(1,4-benzo-{2,1',3'}-thiadiazole)]; poly[(9,9-dihexyl fluorenyl-2,7-diyl)-alt-co-(2-methoxy-5-{2-ethylhexyloxy}-1,4-phenylene)]; poly[(9,9-dioctyl fluorenyl-2,7-diyl)-co-(2,5-p-xylene)]; poly[(9,9-dioctyl fluorenyl-2,7-diyl)-co-(*N,N'*-diphenyl)-*N,N'*-di(p-butylphenyl)-1,4-diaminobenzene)]; poly[(9,9-dioctyl fluorenyl-2,7-diyl)-co-(1,4-benzo-{2,1',3'}-thiadiazole)]; poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene-vinylene], optionally end capped with dimethylphenyl; poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene-vinylene], optionally end capped with polysilsesquioxane; poly[2-methoxy-5-(3,7-dimethyloctyloxy)-1,4-phenylene-vinylene], optionally end capped with dimethylphenyl; poly[2-methoxy-5-(3,7-dimethyloctyloxy)-1,4-phenylene-vinylene], optionally end capped with polysilsesquioxane; poly[9,9-dioctyl fluorenyl-2,7-diyl], optionally end capped with dimethylphenyl; poly[9,9-dioctyl fluorenyl-2,7-diyl], optionally end capped with polysilsesquioxane; poly[9,9-

dioctylfluorenyl-2,7-diyl], optionally end capped with *N,N*-bis(4-methylphenyl)-aniline; poly[9,9-dioctylfluorenyl-2,7-diyl], optionally end capped with 2,5-diphenyl-1,2,4-oxadiazole; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with dimethylphenyl; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with polysilsesquioxane; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with *N,N*-bis(4-methylphenyl)-aniline; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with 2,5-diphenyl-1,2,4-oxadiazole; poly[2-(5-cyano-5-methylhexyloxy)-1,4-phenylene], optionally end capped with dimethylphenyl; and poly[2,5-dioctyl-1,4-phenylene], optionally end capped with dimethylphenyl; poly[9,9-di(3,3'-*N,N'*-trimethyl-ammonium)propylfluorenyl-2,7-diyl]-alt-(9,9-dioctylfluorenyl-2,7-diyl)] diiodide salt, optionally end capped with dimethylphenyl; poly[2,5-bis(3,7-dimethyloctyloxy)-1,4-phenylene-vinylene]; poly(9,9-di{2-[2-(2-methoxy-ethoxy)ethoxy]ethyl} fluorenyl-2,7-diyl), optionally end capped with dimethylphenyl; and poly-BODIPY fluorescent polymers.

[0099] Polyfluorene polymers, polyfluorene copolymers, and fluorescent homopolymers may include, but are not limited to, ADS106RE, ADS108GE, ADS128GE, ADS133YE, ADS136BE, ADS145UV, ADS180BE, ADS232GE, ADS233YE, ADS100RE, ADS200RE, ADS104RE, ADS204RE, ADS300RE, ADS129BE, ADS229BE, ADS329BE, ADS429BE, ADS131BE, ADS231BE, ADS331BE, ADS431BE, ADS160BE, ADS120BE, and ADS121BE, which are commercially available from American Dye Sources.

[00100] In some embodiments, the aqueous soluble polymersome encapsulates at least two hydrophobic fluorescent polymers, at least one of which acts as a light harvesting polymer and at least one of which acts as a signaling chromophore in a FRET process. Signaling chromophores can include, but are not limited to, 2,1,3-benzothiadiazole, benzoxadiazole, benzoselenadiazole, benzotellurodiazole, naphthoselenadiazole, 4,7-di(thien-2-yl)-2,1,3-benzothiadiazole, squaraine dyes, quinoxalines, perylene, perylene diimides, diketopyrrolopyrrole, thienopyrazine low bandgap commercial dyes, Nile Red, Coumarin 6, tetraphenylporphyrin, cyanine dyes (e.g., 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine), olefins, and cyano-substituted olefins and isomers thereof.

Antioxidants

[00101] Molecular oxygen is involved in a number of processes which may lead to photodegradation of organic dyes, including formation of singlet oxygen, peroxides, and other reactive oxygen species (ROS). Triplet quenchers and ROS scavengers are often added

as antifade agents, to reduce the rate of photobleaching in fluorescence microscopy of biological samples. For the application of polyfluorene nanoparticles (PFNs) in fluorescence-based applications, such as flow cytometric analysis and imaging, photobleaching involving oxygen is expected to be a critical problem. The photostability of PFNs can be enhanced by incorporating hydrophobic antifade agents in PFNs during nanoparticle formation.

[00102] In some embodiments, the aqueous soluble polymersomes encapsulate one or more hydrophobic fluorescent polymers and one or more antioxidants. In some embodiments, the aqueous soluble polymersomes encapsulate one or more polyfluorene polymers, polyfluorene copolymers, or fluorescent homopolymers and one or more antioxidants. In some embodiments, the one or more antioxidants improve the photostability of the polymersomes.

[00103] In some embodiments, the antioxidants may be antifade or triple-state quencher (TSQ) compounds. Antifade and TSQ compounds include, but are not limited to, butylated hydroxytoluene (BHT), 3-(3,5-ditert-butyl-4-hydroxyphenyl) propionic acid stearyl ester (PASE), triphenylamine (TPA), trans-stilbene (TSB), cyclooctatetraene (Cot), 4-nitrobenzyl alcohol (NBA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

Preparation of Polymersomes

[00104] The amphiphilic multiblock copolymers that self-assemble into the enclosure structure of the polymersomes disclosed herein are prepared by covalent attachment of the individual blocks. In some embodiments, the hydrophobic polymer block precursor is terminated with a reactive chemical group which can react with a bifunctional hydrophilic polymer block precursor. This bifunctional hydrophilic polymer block precursor comprises two reactive chemical groups on either end of the precursor, one of which can react with the hydrophobic polymer block precursor or another bifunctional hydrophilic polymer block precursor and the other reactive group can optionally be tethered to a biosensor molecule after polymersome formation. The reactive chemical group can be, but is not limited to, an amine moiety, a carboxylic acid moiety, an activated carboxylic acid moiety, an alcohol moiety, a halide moiety, a maleimide moiety, an azide moiety, an alkene, an alkyne, or a thiol moiety. In some embodiments, the two reactive chemical groups of the bifunctional hydrophilic polymer block are identical. In some embodiments, the two reactive chemical groups of the bifunctional hydrophilic polymer block are different. A non-limiting example of an amphiphilic diblock polymer prepared in this manner is illustrated in **Fig. 1**.

[00105] In some embodiments, postpolymerization dispersions of a plurality of the amphiphilic multiblock copolymers leads to the formation of aqueous soluble polymersomes described herein. In some embodiments, postpolymerization dispersions of a plurality of the amphiphilic multiblock copolymers through a precipitation technique lead to the formation of aqueous soluble polymersomes. In other embodiments, postpolymerization dispersions of a plurality of the amphiphilic multiblock copolymers through an emulsion technique lead to the formation of aqueous soluble polymersomes.

[00106] In some embodiments, when a plurality of amphiphilic multiblock copolymers and one or more hydrophobic fluorescent polymers are agitated together in water, aqueous soluble polymersomes that encapsulate the one or more hydrophobic fluorescent polymers are formed. In further embodiments, the amphiphilic multiblock copolymers are diblock copolymers.

[00107] In some embodiments, when a plurality of amphiphilic multiblock copolymers, one or more hydrophobic fluorescent polymers, and one or more antioxidants are agitated together in water, aqueous soluble polymersomes that encapsulate the one or more hydrophobic fluorescent polymers and the one or more antioxidants are formed. In further embodiments, the amphiphilic multiblock copolymers are diblock copolymers.

[00108] In some embodiments, the fluorescent aqueous soluble polymersomes are further derivatized through the remaining reactive chemical groups on the terminal ends of the hydrophilic polymer blocks, these reactive chemical groups being localized on the surface of the polymersome. In some embodiments, the fluorescent aqueous soluble polymersomes are attached to biosensor molecules. In further embodiments, the biosensor molecules are antigen-specific antibodies. A non-limiting example of this antibody-polymersome conjugate is shown in **Fig. 9**.

[00109] In one aspect, disclosed herein are methods of preparing an aqueous soluble polymersome comprising one or more hydrophobic fluorescent polymers, the method comprising: (a) forming a plurality of amphiphilic copolymers each comprising a hydrophobic block and a hydrophilic block; (b) adding one or more hydrophobic fluorescent polymers to the plurality of amphiphilic copolymers; and (c) placing the mixture of step (b) into an aqueous system so that the plurality of amphiphilic copolymers encapsulate the one or more hydrophobic fluorescent polymers, wherein the hydrophobic block forms a hydrophobic core structure and a hydrophilic block is oriented on the surface of the polymersome that is oriented toward an aqueous phase. In some embodiments, the amphiphilic copolymers are

diblock copolymers. In some embodiments, the hydrophobic block of plurality of amphiphilic copolymers is PLGA. In some embodiments, the hydrophilic block of plurality of amphiphilic copolymers is PEG. In some embodiments, the formation of the amphiphilic copolymers comprises reacting PLGA with bis-aminopropyl-PEG in the presence of carbodiimide. In some embodiments, in step (c), the mixture and aqueous system is agitated. In some embodiments, the method further comprises filtering the polymersomes in the aqueous system. In some embodiments, the method further comprises concentrating the polymersomes in the aqueous system. In some embodiments, the method further comprises reacting the polymersomes with a thiolating reagent. In some embodiments, the method further comprises attaching an antigen-specific antibody to the polymersomes.

Uses of Polymersomes

[00110] Due to their low water-solubility, hydrophilic fluorescent polymers are not amenable for use in aqueous systems. However, encapsulation in aqueous soluble polymersomes enables the use of hydrophobic fluorescent polymers in water-containing media.

[00111] In one aspect, disclosed herein are methods of solubilizing one or more hydrophobic fluorescent polymers, the method comprising (a) forming a plurality of amphiphilic copolymers each comprising a hydrophobic block and a hydrophilic block; (b) adding one or more hydrophobic fluorescent polymers to the plurality of amphiphilic copolymers; and (c) placing the mixture of step (b) into an aqueous system so that the plurality of amphiphilic copolymers encapsulate the one or more hydrophobic fluorescent polymers, wherein the hydrophobic block forms a hydrophobic core structure and a hydrophilic block is oriented on the surface of the polymersome that is oriented toward an aqueous phase. In some embodiments, the amphiphilic copolymers are diblock copolymers.

[00112] In some embodiments, biosensor molecules, such as antigen-specific antibodies, are linked to the functionalizable chemical groups, e.g., amine moieties, on the surface of the polymersomes. These fluorescent biosensor-polymersome conjugates can be used in a number of applications, including, but not limited to, cell staining as well as analyte detection in cell-free assay environments. In some embodiments, the fluorescent biosensor-polymersome conjugates are used in flow cytometry applications.

[00113] Disclosed herein are assay methods for detecting a target biomolecule in a sample. In one aspect, an assay method for detecting a target biomolecule in a sample comprises (a) providing a sample that is suspected of containing a target biomolecule; (b)

providing an aqueous soluble polymersome disclosed herein that is conjugated to a sensor biomolecule, wherein the sensor biomolecule is capable of interacting with the target biomolecule or a target-associated biomolecule and wherein the polymersome optionally comprises an acceptor dye; (c) contacting the sample with the sensor biomolecule and the aqueous soluble polymersome in a solution under conditions in which the sensor biomolecule can bind to the target biomolecule or a target-associated biomolecule if present; and (d) applying a light source to the sample that can excite the fluorescent polyfluorene polymer in the polymersome, and detecting whether light is emitted from the polymersome. In some embodiments, the sensor biomolecule is a protein, peptide, affinity ligand, antibody, antibody fragment, sugar, lipid, nucleic acid or an aptamer. In some embodiments, the sensor biomolecule is an antibody. In some embodiments, the target biomolecule is a target protein expressed on a cell surface. In some embodiments, the method is configured for flow cytometry, intracellular staining, immunohistochemistry or FISH.

EXAMPLES

Example 1: Preparation of aqueous soluble polymersomes using PLGA₅₀₀₀-PEG₂₀₀₀-NH₂ encapsulating polyfluorenebenzothiadiazole.

[00114] The diblock copolymer of PLGA₅₀₀₀-PEG₂₀₀₀-NH₂ was prepared by attachment of a low molecular weight PLGA of about 5000 Dalton to bis-aminopropyl-PEG (H₂N-PEG-NH₂) of average molecular weight of about 2000 Dalton (**Fig. 1**). The desired amide formation between the terminal carboxylic acid of the PLGA with one of the amine moieties of bis-aminopropyl-PEG was performed under carbodiimide/dichloromethane conditions. This resultant diblock polymer was used as the amphiphilic carrier polymer to facilitate the encapsulation of polyfluorenebenzothia-diazole (MW = 10,000-20,000 Da; Ex (max) = 454 nm; Em = 545 nm; Sigma-Aldrich) into water-soluble polymersomes of core/shell structure. The formation of polyfluorenebenzothia-diazole-encapsulated polymersomes was performed using a precipitation technique in which the amphiphilic diblock polymer (60 mg, 15 μ mol) and polyfluorenebenzo-thiadiazole (440 μ g, 30nmol) were mixed vigorously under aqueous conditions. The resulting colloidal suspension (120 ml) was filtered, and solvent was removed until the desired concentration was obtained. As demonstrated in **Fig. 2**, polyfluorenebenzothiadiazole is insoluble in water, visible to the naked eye as clumps of material, but appears to be “dissolved” in the aqueous system once encapsulated in the aqueous soluble PLGA-PEG-NH₂ polymersome. Size exclusion

chromatography of these polyfluorenebenzothiadiazole-encapsulated polymersomes is shown in **Fig. 3**.

[00115] Notably, attempts to use a larger molecular weight PLGA of 46000 Dalton indicated that the encapsulation of polyfluorenebenzothiadiazole was not possible with the resultant PLGA-PEG copolymer using this precipitation technique.

Example 2: Characterization of aqueous soluble polymersomes using PLGA₅₀₀₀-PEG₂₀₀₀-NH₂ encapsulating polyfluorenebenzothiadiazole.

[00116] *Comparison with polyfluorene in organic solvent*

[00117] The polymersomes of Example 1 demonstrated absorption properties slightly different from those of polyfluorenebenzothiadiazole in organic solvent (**Fig. 4**). The absorption of the encapsulated polyfluorenebenzothiadiazole was about 10-14 nm red-shifted compared to that of polyfluorenebenzothiadiazole in an organic solvent, tetrahydrofuran (THF). Also, the ratio of two absorption peaks, 319 and 467 nm, was smaller than those of polyfluorenebenzothiadiazole in organic solvent that approaches unity. These spectral differences suggest that the microenvironment of the encapsulated polyfluorenebenzothiadiazole in the core of the polymersomes is different from that of solvated polyfluorenebenzothiadiazole in organic solvent. However, the fluorescence emission of the encapsulated polyfluorenebenzo-thiadiazole has virtually identical spectral properties to that of polyfluorenebenzothiadiazole dissolved in organic solvent (**Fig. 5**). Thus, encapsulation does not adversely impact the fluorescence emission of the encapsulated polyfluorenebenzothiadiazole in the core of the polymersomes.

[00118] *Quantitation of hydrophobic fluorescent polymer molecules in polymersome.*

[00119] Due to the partial quenching of the fluorescent polymer in the polymersome, quantitation of the total number of fluorochrome is not of significant value. However, the number of Molecules of Equivalent Soluble Fluorochrome (MESF) is more useful and can be obtained for any suspension of polymersomes, including antibody-polymersome conjugates. A standard curve for quantitation of MESF was obtained for the polyfluorenebenzothiadiazole (**Fig. 6**). Based on this curve, it was determined that 1.25 pmol fluorophore/ μ L of encapsulated polymersomes translated to 7.5×10^{11} MESF/ μ L of encapsulated polymersome suspension.

Example 3: Preparation of aqueous soluble antibody-polymersome conjugates using polymersomes of PLGA₅₀₀₀-PEG₂₀₀₀-NH₂ encapsulating polyfluorenebenzothiadiazole.

[00120] The general scheme for conjugation of antibodies to the surface of the polyfluorene-benzothiadiazole-encapsulated polymersomes of Example 1 is shown in **Fig. 7**. Amine functional groups from the PEG moiety on the surface of the particles were modified with Traut's reagent (2-Iminithiolane; 2-IT). The resulting free sulfhydryl groups were conjugated to an SMCC-derivatized antibody (anti-CD4) carrying reactive maleimide groups. Size exclusion chromatography for the purification of this antibody-polymersome conjugate is shown in **Fig. 8**.

Example 4: Characterization of aqueous soluble antibody-polymersome conjugates.

[00121] The CD4 antibody-polymersome conjugates of Example 3 were further evaluated for the presence of the antibody on the nanoparticles. **Fig. 9** illustrates the flow cytometric analysis of the fluorescent antibody-polymersome conjugates after being captured on anti-kappa beads. The beads were coated with anti-kappa antibodies that capture the kappa light chain of CD4 antibodies present on the antibody-polymersome conjugates. The experimental results clearly indicated that the captured antibody-polymersome conjugated on the beads were fluorescent (histogram #4) and that their fluorescent signal was sufficiently high enough to separate them from the non-specific background signal (histograms #1 and 3). As a positive control, BV510-CD4 conjugates (conjugates between CD4 antibody and BRILLIANT VIOLET™ 510 from BD Biosciences) were captured on the same beads (histogram #2).

[00122] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

WHAT IS CLAIMED IS:

1. An aqueous soluble polymersome comprising:
 - (a) one or more hydrophobic fluorescent polymers; and
 - (b) a plurality of amphiphilic copolymers encapsulating the one or more hydrophobic fluorescent polymers, the copolymers each comprising a hydrophobic block that forms a hydrophobic core structure and a hydrophilic block on the surface of the polymersome that is oriented toward an aqueous phase.
2. The aqueous soluble polymersome of claim 1 wherein the amphiphilic copolymers are diblock copolymers.
3. The aqueous soluble polymersome of claim 1 wherein the hydrophobic block is selected from a polymer group consisting of polyacrylonitrile, polycarbonate, aromatic polyether, poly(vinyl acetal), poly(alkyl vinyl ether), poly(alkyl vinyl ketone), polyurethane, polyamide, polyimide, polyester, polyfluorocarbon, polyolefin, polystyrene, polyvinylcyclohexane, polydioxanone, polymeric organosilicon, polysaccharide, poly(methyl methacrylate), or a copolymer combination thereof.
4. The aqueous soluble polymersome of claim 1 wherein the hydrophobic block is selected from a polymer group consisting of poly(lactide-co-glycolic acid (PLGA), polylactide (PLA), polyglycolide (PGA), polycaprolactone (PCL), poly(methyl methacrylate) (PMMA), polydimethylsiloxane (PDMS), polyethylene (PE) and polystyrene (PS).
5. The aqueous soluble polymersome of claim 1, wherein the hydrophobic block is poly(lactide-co-glycolic acid (PLGA)).
6. The aqueous soluble polymersome of claim 5, wherein the polymer ratio of PLGA is 50:50 (DL-lactide:glycolide).
7. The aqueous soluble polymersome of claim 5, wherein the PLGA has a viscosity of about 0.15-0.25 dL/g.
8. The aqueous soluble polymersome of claim 1, wherein the hydrophobic block has a number average molecular weight of about 2,000 to 20,000 Daltons.
9. The aqueous soluble polymersome of claim 1, wherein the hydrophobic block has a number average molecular weight of about 10,000 Daltons.
10. The aqueous soluble polymersome of claim 1, wherein the hydrophobic block has a number average molecular weight of about 5,000 Daltons.

11. The aqueous soluble polymersome of claim 1, wherein the hydrophilic block is selected from a polymer group consisting of polyoxyalkylene, polymethacrylate, poly(methacrylic acid), polyacrylic acid, polyacrylate, poly(alkylacrylic acid), poly(alkylacrylate), polyacrylamide, poly(*N*-isopropylacrylamide), poly(2-ethyl-2-oxazoline), polyethylenimine, poly(vinyl alcohol), poly(vinylpyrrolidone), poly(styrenesulfonate), poly(vinyl acid), poly(allylamine), poly(diallyldimethyl ammonium chloride), poly(methyl vinyl ether), poly(2-methyloxazoline), or copolymer combinations thereof.

12. The aqueous soluble polymersome of claim 1, wherein the hydrophilic block is polyether.

13. The aqueous soluble polymersome of claim 12, wherein the polyether is polypropylene glycol (PPG) or polyethylene glycol (PEG).

14. The aqueous soluble polymersome of claim 1, wherein the hydrophilic block is poly(2-methyloxazoline).

15. The aqueous soluble polymersome of claim 1, the hydrophilic block has a number average molecular weight of about 1,000 to about 10,000 Daltons.

16. The aqueous soluble polymersome of claim 1, the hydrophilic block has a number average molecular weight of about 5,000 Daltons.

17. The aqueous soluble polymersome of claim 1, the hydrophilic block has a number average molecular weight of about 2,000 Daltons.

18. The aqueous soluble polymersome of claim 1, wherein the hydrophilic block further comprises an amine moiety.

19. The aqueous soluble polymersome of claim 1, wherein the amine moiety is further thiolated with 2-iminothiolane (2-IT).

20. The aqueous soluble polymersome of claim 1, wherein the hydrophilic block is attached to a biosensor molecule.

21. The aqueous soluble polymersome of claim 1, wherein the hydrophilic block is attached to a signaling chromophore for resonant energy transfer.

22. The aqueous soluble polymersome of claim 1, wherein the hydrophilic block is attached to an antigen-specific antibody.

23. The aqueous soluble polymersome of claim 1, wherein the hydrophobic block is PLGA and the hydrophilic block is PEG.

24. The aqueous soluble polymersome of claim 23, wherein the PLGA has a number average molecular weight of about 5,000 Daltons and the PEG has a number average molecular weight of about 2,000 Daltons.

25. The aqueous soluble polymersome of claim 1, wherein the one or more hydrophobic fluorescent polymers are polyfluorene polymer, polyfluorene copolymer, or fluorescent homopolymer.

26. The aqueous soluble polymersome of claim 1, wherein the one or more hydrophobic fluorescent polymers are selected from poly[(9,9-dioctyl-2,7-divinylene fluorenylene)-alt-co-(9,10-anthracene)]; poly[(9,9-dioctyl-2,7-divinylene fluorenylene)-alt-co-{2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene}]; poly[(9,9-dioctyl-2,7-divinylene fluorenylene)-alt-co-(1,4-phenylene)]; poly[(9,9-dioctyl fluorenyl-2,7-diyl)-alt-co-(1,4-benzo-{2,1',3}-thiadiazole)]; poly[(9,9-dihexyl fluorenyl-2,7-diyl)-alt-co-(2-methoxy-5-{2-ethylhexyloxy}-1,4-phenylene)]; poly[(9,9-dioctyl fluorenyl-2,7-diyl)-co-(2,5-p-xylene)]; poly[(9,9-dioctyl fluorenyl-2,7-diyl)-co-(*N,N'*-diphenyl)-*N,N'*-di(*p*-butylphenyl)-1,4-diamino-benzene)]; poly[(9,9-dioctyl fluorenyl-2,7-diyl)-co-(1,4-benzo-{2,1',3}-thiadiazole)]; poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene-vinylene], optionally end capped with dimethylphenyl; poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene-vinylene], optionally end capped with polysilsesquioxane; poly[2-methoxy-5-(3,7-dimethyloctyloxy)-1,4-phenylene-vinylene], optionally end capped with dimethylphenyl; poly[2-methoxy-5-(3,7-dimethyloctyloxy)-1,4-phenylene-vinylene], optionally end capped with polysilsesquioxane; poly[9,9-dioctyl fluorenyl-2,7-diyl], optionally end capped with dimethylphenyl; poly[9,9-dioctyl fluorenyl-2,7-diyl], optionally end capped with polysilsesquioxane; poly[9,9-dioctyl fluorenyl-2,7-diyl], optionally end capped with *N,N*-bis(4-methylphenyl)-aniline; poly[9,9-dioctyl fluorenyl-2,7-diyl], optionally end capped with 2,5-diphenyl-1,2,4-oxadiazole; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with dimethylphenyl; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with polysilsesquioxane; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with *N,N*-bis(4-methylphenyl)-aniline; poly[9,9-di-(2-ethylhexyl)-fluorenyl-2,7-diyl], optionally end capped with 2,5-diphenyl-1,2,4-oxadiazole; poly[2-(5-cyano-5-methylhexyloxy)-1,4-phenylene], optionally end capped with dimethylphenyl; and poly[2,5-dioctyl-1,4-phenylene], optionally end capped with dimethylphenyl; poly[9,9-di(3,3'-*N,N'*-trimethyl-ammonium)propyl fluorenyl-2,7-diyl]-alt-(9,9-dioctyl fluorenyl-2,7-diyl)] diiodide salt, optionally end capped with

dimethylphenyl; poly[2,5-bis(3,7-dimethyloctyloxy)-1,4-phenylene-vinylene]; poly(9,9-di{2-[2-(2-methoxy-ethoxy)ethoxy]ethyl} fluorenyl-2,7-diyl), optionally end capped with dimethylphenyl; and poly-BODIPY fluorescent polymers.

27. The aqueous soluble polymersome of claim 1, wherein the one or more hydrophobic fluorescent polymers are selected from the optionally substituted polymer group consisting of poly(fluorene), poly(fluorene-alt-benzothiadiazole), poly(fluorene-alt-benzooxadiazole), poly(fluorene-alt-thiophene), and poly(fluorene-alt-phenylene).

28. The aqueous soluble polymersome of claim 1, wherein the hydrophobic fluorescent polymer is optionally substituted poly(fluorene-alt-benzothiadiazole).

29. The aqueous soluble polymersome of claim 1, wherein the polymersome comprises at least two hydrophobic fluorescent polymers.

30. The aqueous soluble polymersome of claim 29, wherein at least one hydrophobic fluorescent polymer acts as a light harvesting polymer and at least one hydrophobic fluorescent polymer acts as a signaling chromophore in a FRET process.

31. The aqueous soluble polymersome of claim 1, wherein each of the one or more hydrophobic fluorescent polymers has a number average molecular weight of about 5,000 to about 30,000 Daltons.

32. The aqueous soluble polymersome of claim 1, wherein each of the one or more hydrophobic fluorescent polymers has a number average molecular weight of about 10,000 to about 20,000 Daltons.

33. The aqueous soluble polymersome of claim 1, wherein the polymersome has an average particle size of about 200 nm.

34. The aqueous soluble polymersome of claim 1, wherein the polymersome has an average particle size of about 100 nm.

35. The aqueous soluble polymersome of claim 1, wherein the polymersome further comprises one or more antioxidants.

36. The aqueous soluble polymersome of claim 35, wherein the one or more antioxidants are selected from butylated hydroxytoluene (BHT), 3-(3,5-ditert-butyl-4-hydroxyphenyl) propionic acid stearyl ester (PASE), triphenylamine (TPA), trans-stilbene (TSB), cyclooctatetraene (Cot), 4-nitrobenzyl alcohol (NBA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

37. A method of preparing an aqueous soluble polymersome comprising one or more hydrophobic fluorescent polymers, the method comprising

- (a) forming a plurality of amphiphilic copolymers each comprising a hydrophobic block and a hydrophilic block;
- (b) adding one or more hydrophobic fluorescent polymers to the plurality of amphiphilic copolymers; and
- (c) placing the mixture of step (b) into an aqueous system so that the plurality of amphiphilic copolymers encapsulate the one or more hydrophobic fluorescent polymers, wherein the hydrophobic block forms a hydrophobic core structure and a hydrophilic block is oriented on the surface of the polymersome that is oriented toward an aqueous phase.

38. The method of claim 37, wherein the amphiphilic copolymers are diblock copolymers.

39. The method of claim 37, wherein the hydrophobic block of plurality of amphiphilic diblock copolymers is PLGA.

40. The method of claim 37, wherein the hydrophilic block of plurality of amphiphilic diblock copolymers is PEG.

41. The method of claim 37, wherein the formation of the amphiphilic diblock copolymers comprises reacting PLGA with bis-aminopropyl-PEG in the presence of carbodiimide.

42. The method of claim 37, wherein in step (c), the mixture and aqueous system is agitated.

43. The method of claim 37, wherein the method further comprises filtering the polymersomes in the aqueous system.

44. The method of claim 37, wherein the method further comprises concentrating the polymersomes in the aqueous system.

45. The method of claim 37, wherein the method further comprises reacting the polymersomes with a thiolating reagent.

46. The method of claim 37, wherein the method further comprises attaching an antigen-specific antibody to the polymersomes.

47. A method of solubilizing one or more hydrophobic fluorescent polymers, the method comprising

- (a) forming a plurality of amphiphilic copolymers each comprising a hydrophobic block and a hydrophilic block;

- (b) adding one or more hydrophobic fluorescent polymers to the plurality of amphiphilic copolymers; and
- (c) placing the mixture of step (b) into an aqueous system so that the plurality of amphiphilic copolymers encapsulate the one or more hydrophobic fluorescent polymers, wherein the hydrophobic block forms a hydrophobic core structure and a hydrophilic block is oriented on the surface of the polymersome that is oriented toward an aqueous phase.

48. The method of claim 47, wherein the amphiphilic copolymers are diblock copolymers.

49. An assay method for detecting a target biomolecule in a sample comprising:

- (a) providing a sample that is suspected of containing a target biomolecule;
- (b) providing an aqueous soluble polymersome of claim 1 that is conjugated to a sensor biomolecule,

wherein the sensor biomolecule is capable of interacting with the target biomolecule or a target-associated biomolecule and wherein the polymersome optionally comprises an acceptor dye;

- (c) contacting the sample with the sensor biomolecule and the aqueous soluble polymersome in a solution under conditions in which the sensor biomolecule can bind to the target biomolecule or a target-associated biomolecule if present; and
- (d) applying a light source to the sample that can excite the fluorescent polyfluorene polymer in the polymersome, and detecting whether light is emitted from the polymersome.

50. The assay method of claim 49, wherein the sensor biomolecule is a protein, peptide, affinity ligand, antibody, antibody fragment, sugar, lipid, nucleic acid or an aptamer.

51. The assay method of claim 49, wherein the sensor biomolecule is an antibody.

52. The assay method of claim 49, wherein the target biomolecule is a target protein expressed on a cell surface.

53. The assay method of claim 49, wherein the method is configured for flow cytometry, intracellular staining, immunohistochemistry or FISH.

54. An aqueous soluble polymersome comprising:

- (a) one or more hydrophobic fluorescent polymers;

- (b) a plurality of amphiphilic copolymers encapsulating the one or more hydrophobic fluorescent polymers, the copolymers each comprising a hydrophobic block that forms a hydrophobic core structure and a hydrophilic block on the surface of the polymersome that is oriented toward an aqueous phase; and
- (c) an acceptor dye.

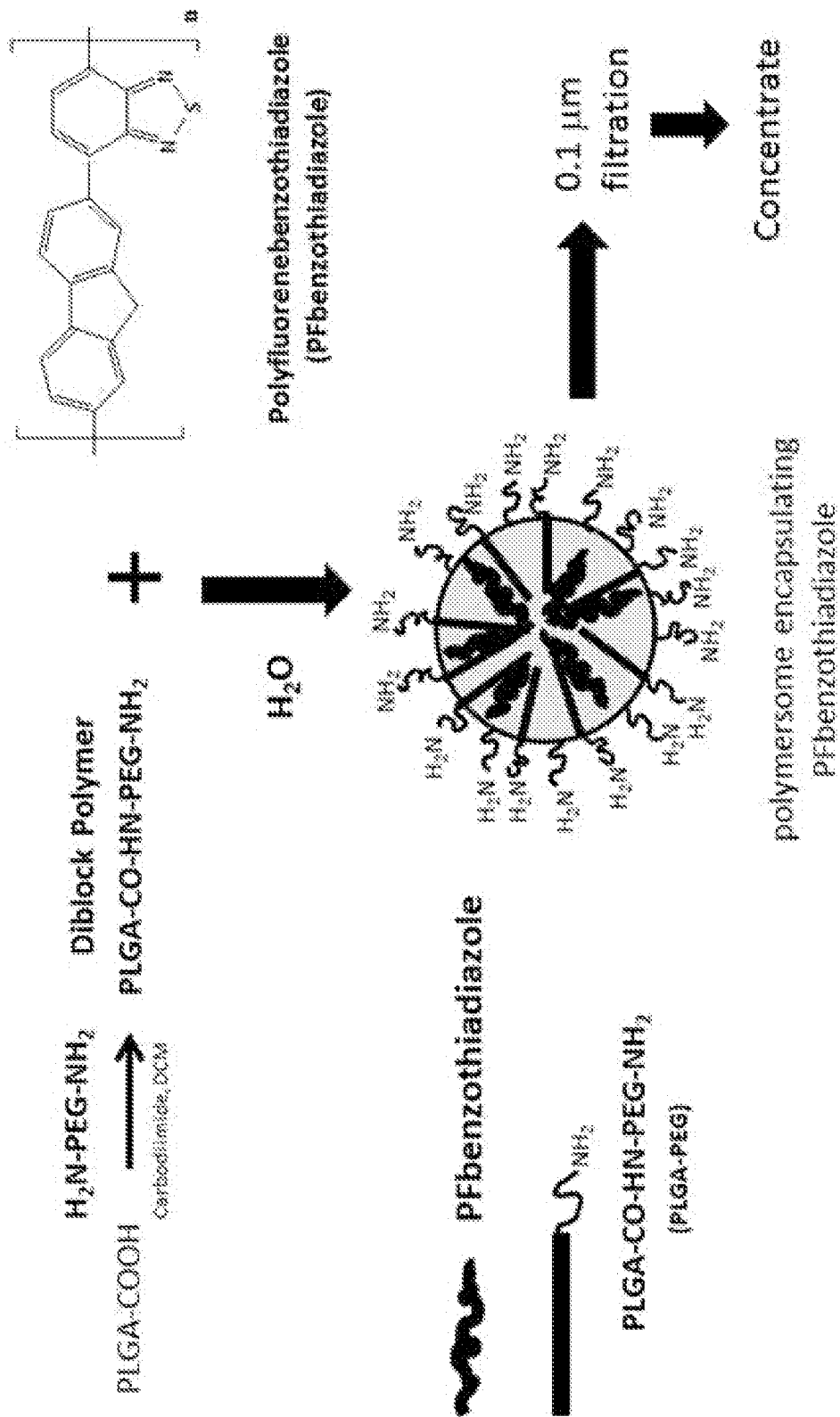
55. The polymersome of claim 54, wherein the amphiphilic copolymers are diblock copolymers.

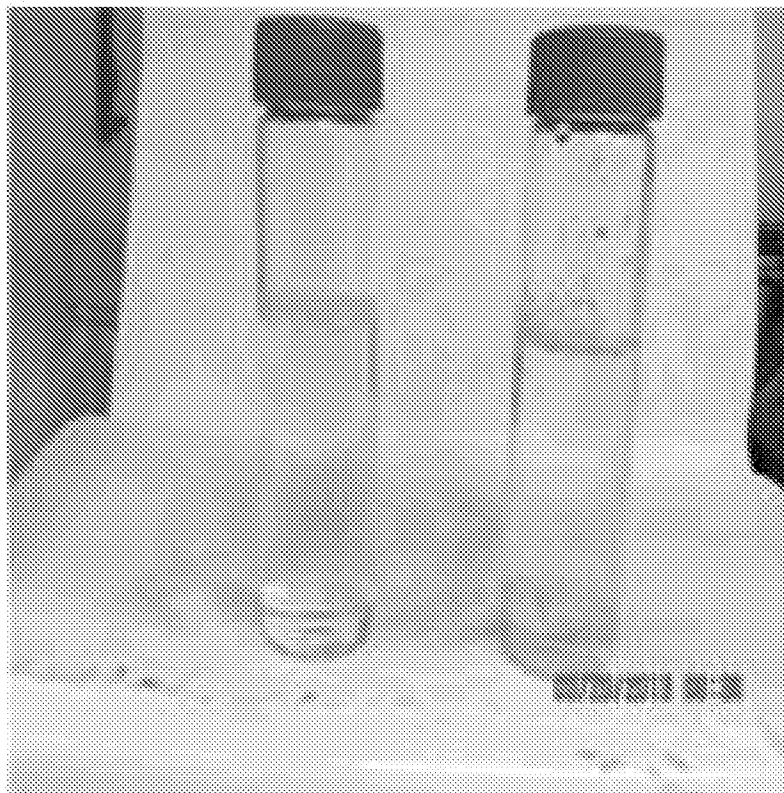
56. An aqueous soluble polymersome comprising:

- (a) one or more hydrophobic fluorescent polymers;
- (b) a plurality of amphiphilic copolymers encapsulating the one or more hydrophobic fluorescent polymers, the copolymers each comprising a hydrophobic block that forms a hydrophobic core structure and a hydrophilic block on the surface of the polymersome that is oriented toward an aqueous phase;
- (c) an acceptor dye; and
- (d) one or more antioxidants.

57. The polymersome of claim 56, wherein the amphiphilic copolymers are diblock copolymers.

FIG. 1





Left: PFbenzothiadiazole-encapsulated in
PLGA-PEG polymersomes in H₂O
Right: PFbenzothiadiazole in H₂O

FIG. 2

FIG. 3

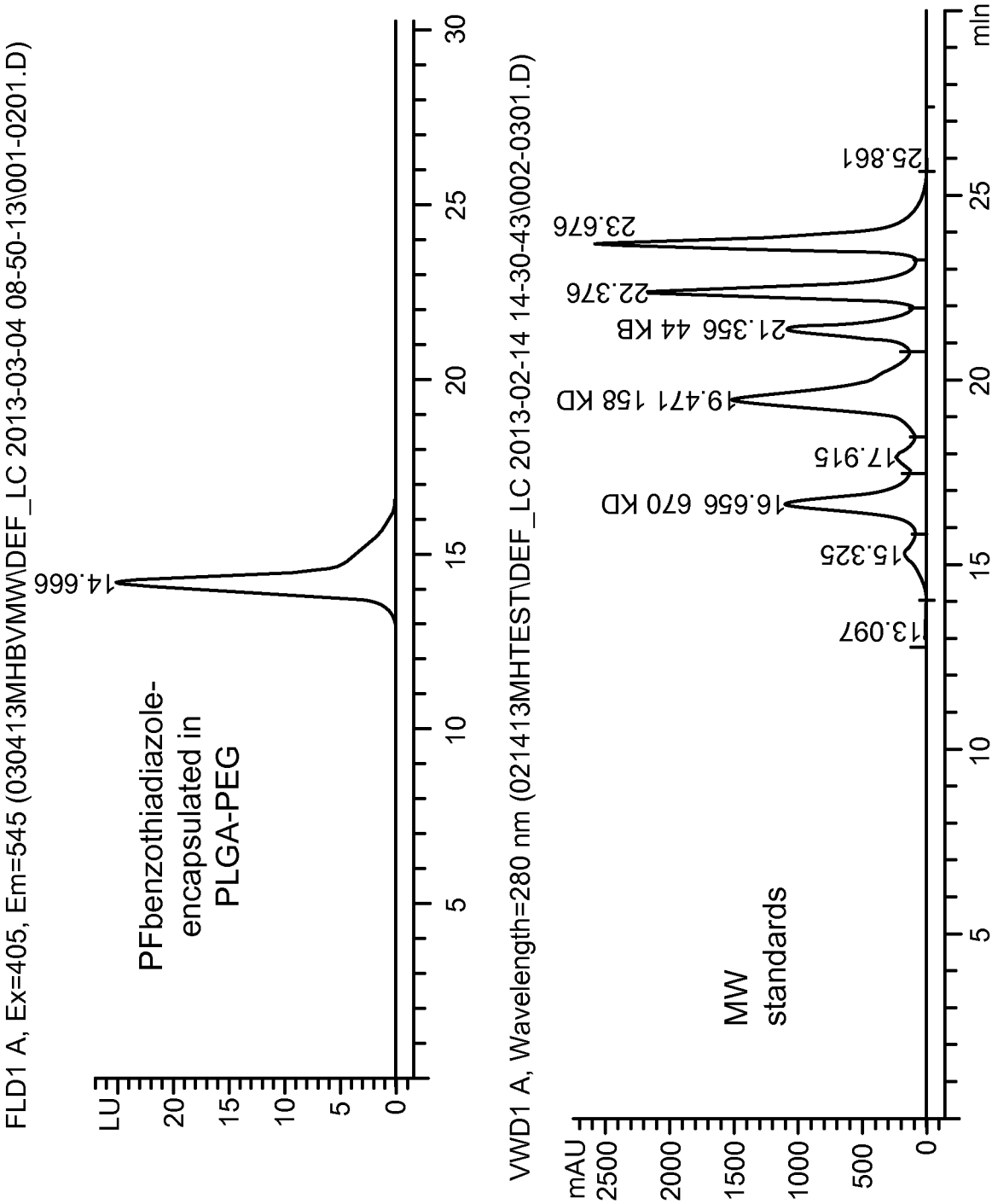


FIG. 4

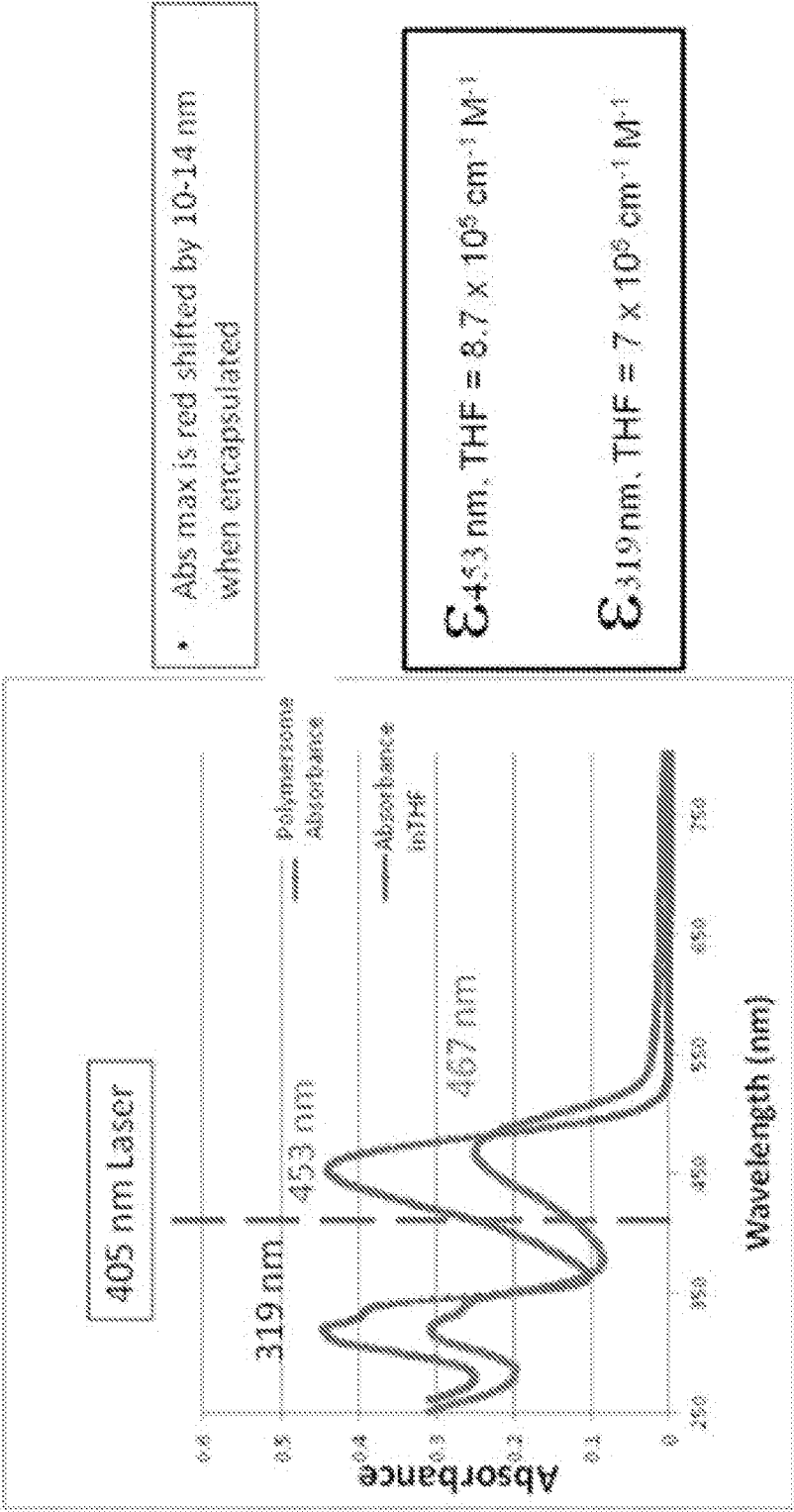
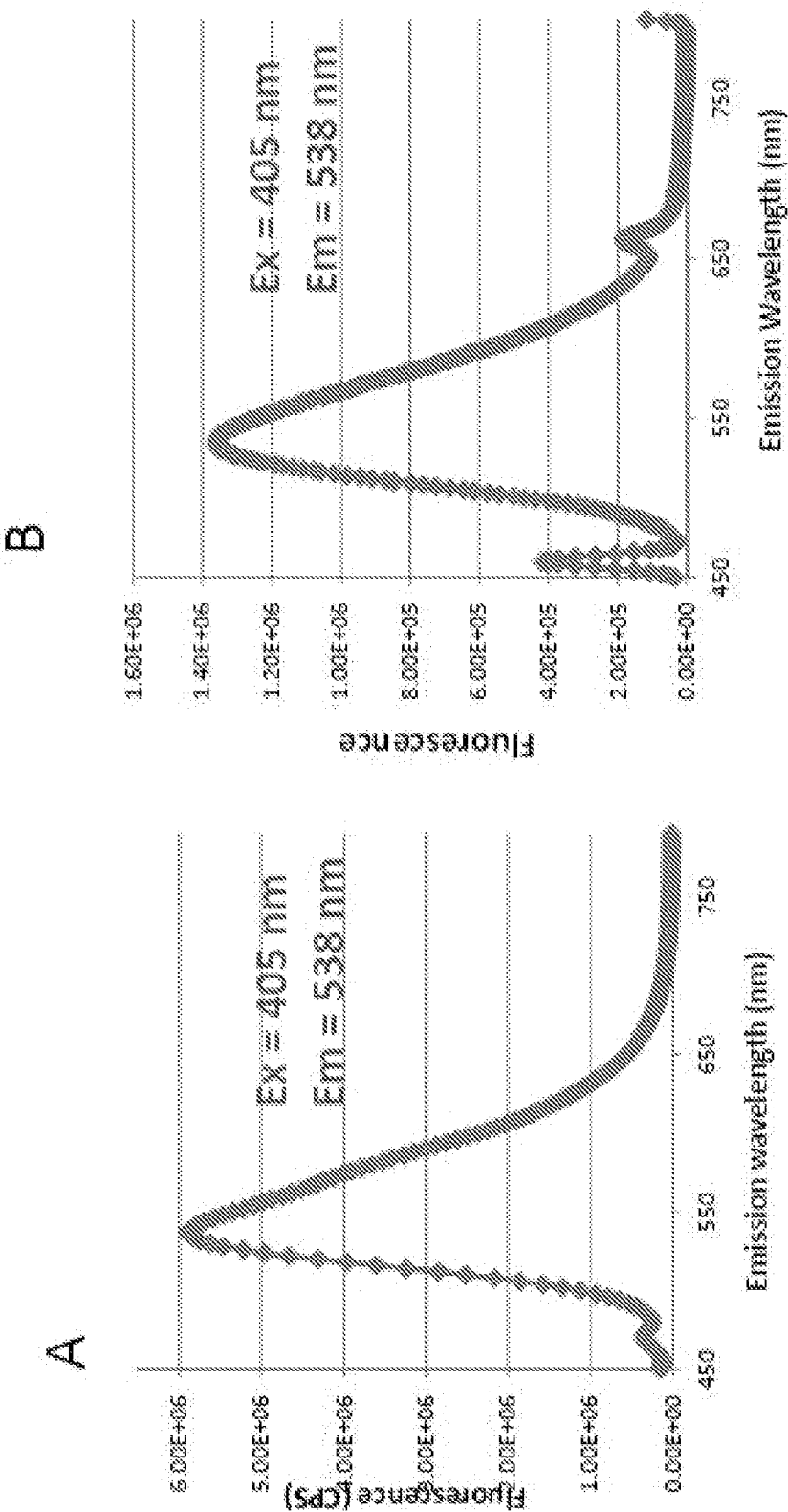


FIG. 5



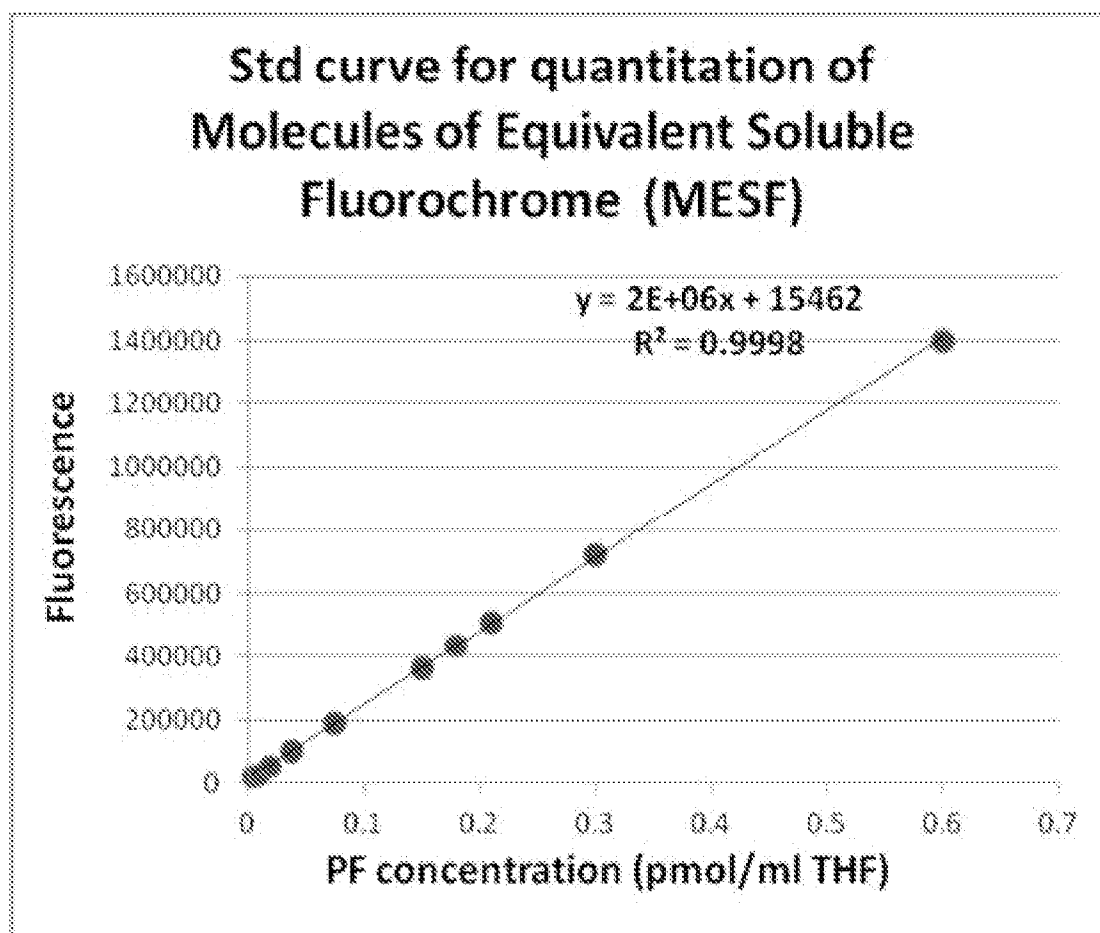


FIG. 6

FIG. 7

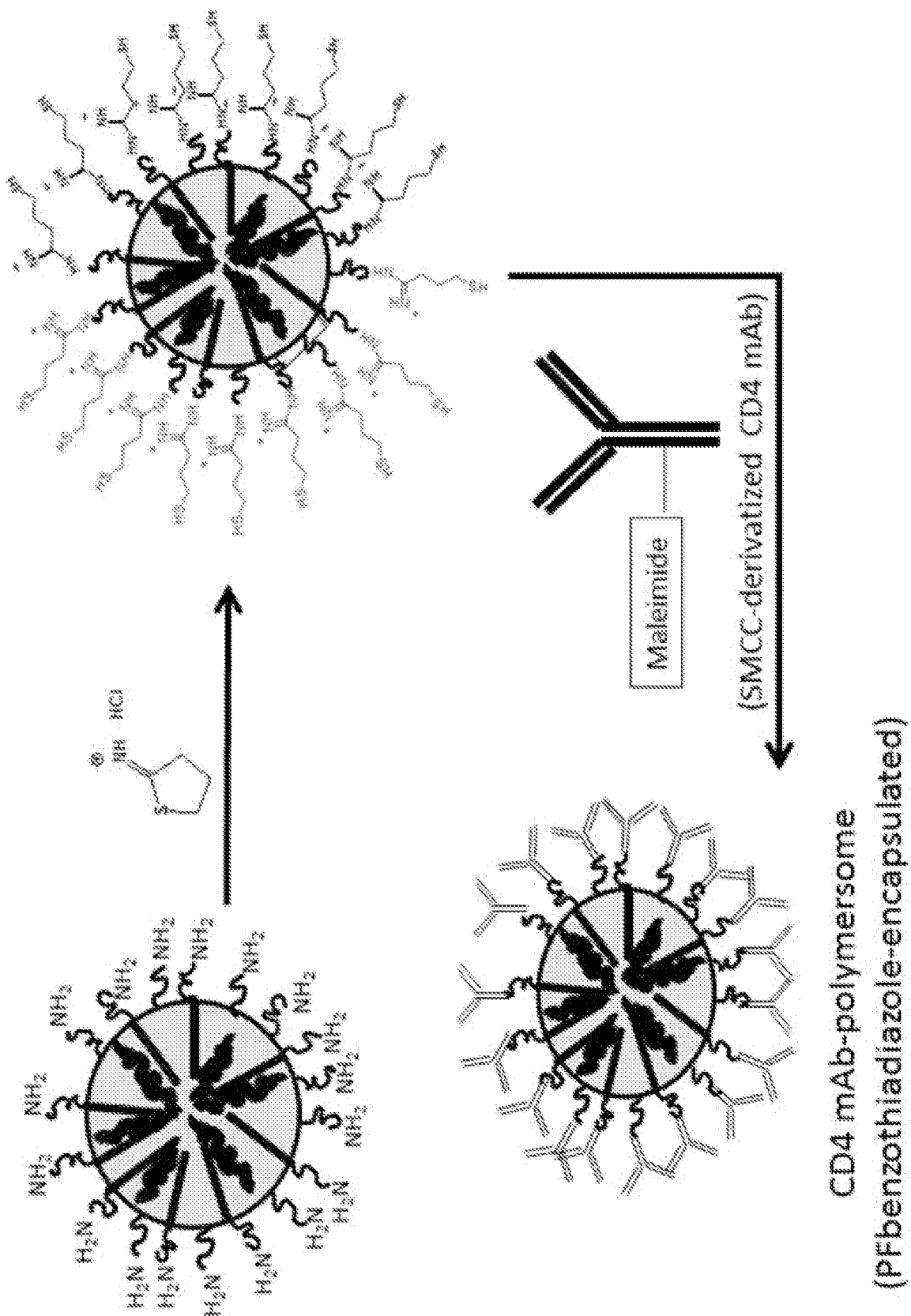
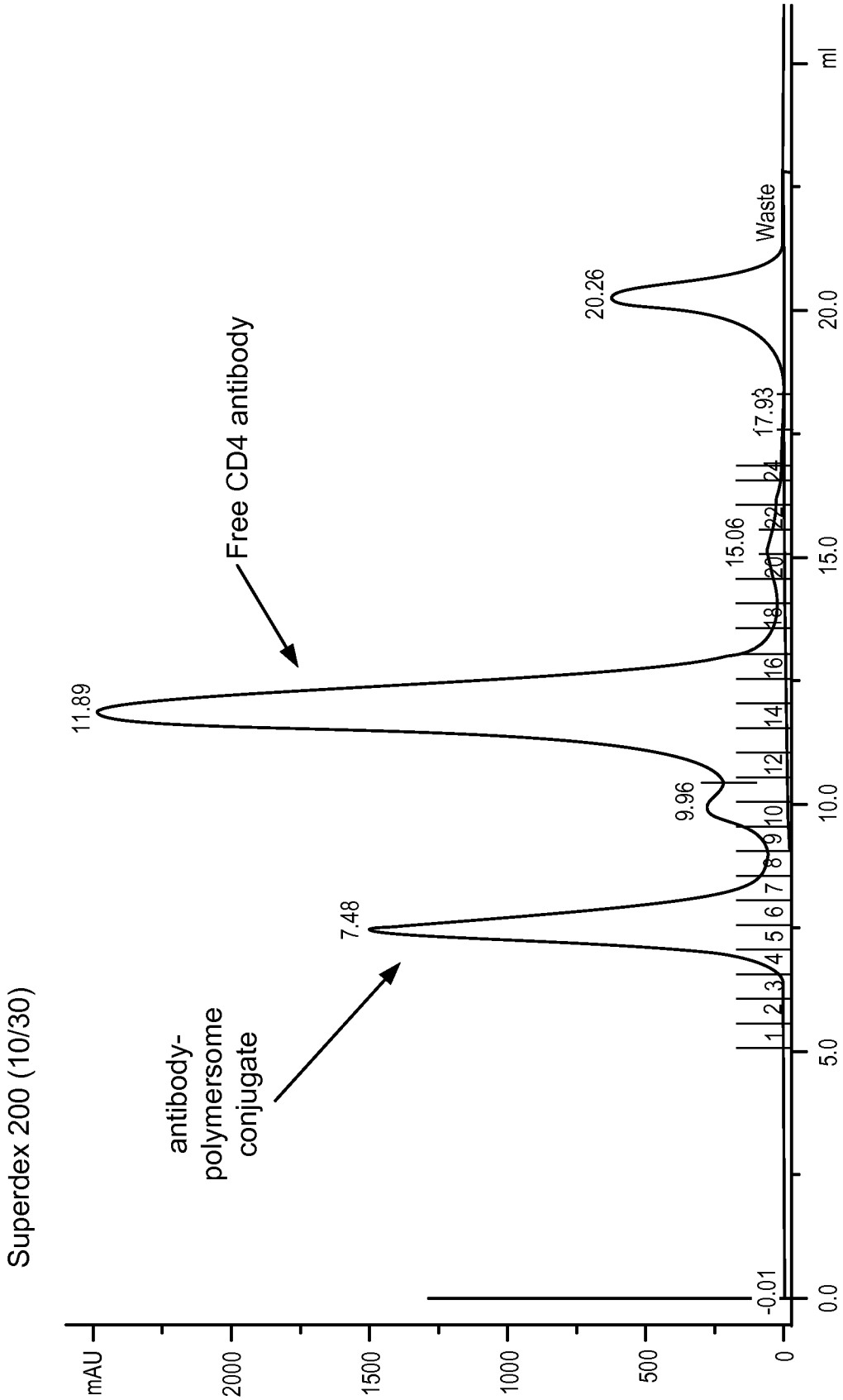
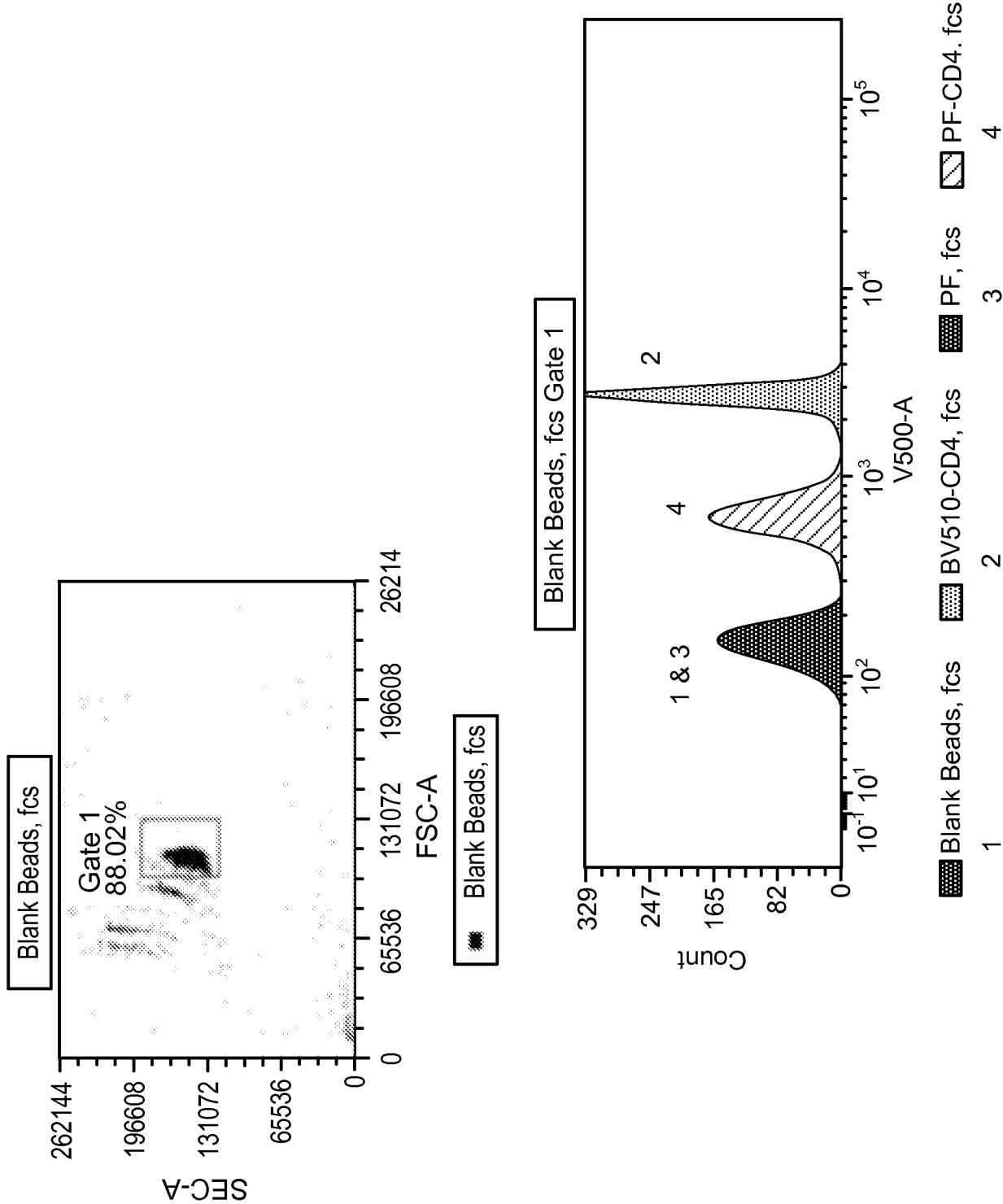


FIG. 8



FACSVerse analysis

FIG. 9



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/058309

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 9/127 (2015.01)

CPC - A61K 9/127 (2014.12)

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)

IPC(8) - A61K 9/127; C09K 11/00, 11/06; G01N 21/63, 21/64 (2015.01)

CPC - A61K 9/127; C09K 11/00, 11/06; G01N 21/63, 21/64 (2014.12) (keyword delimited)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 252/301.16, 301.35; 424/450; 436/164, 172 (keyword delimited)

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

Orbit, Google Patents, Google

Search terms used: amphiphilic polymersome hydrophobic hydrophilic amine iminothiolane light harvesting signaling micelle liposome

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2009/0220614 A1 (QIN et al) 03 September 2009 (03.09.2009) entire document	1-29, 31-36, 54-57
Y	US 2012/0282632 A1 (CHIU et al) 08 November 2012 (08.11.2012) entire document	1-29, 33-36, 54-57
Y	US 2013/0234067 A1 (CHIU et al) 12 September 2013 (12.09.2013) entire document	1, 31, 32
Y	US 2011/0223206 A1 (LEBOUILLE et al) 15 September 2011 (15.09.2011) entire document	3, 15-17, 23, 24, 33, 34
Y	NAM et al. New micelle-like polymer aggregates made from PEI-PLGA diblock copolymers: micellar characteristics and cellular uptake. Biomaterials. 24(12):2053-2059, 2003. [retrieved on 11 February 2015]. Retrieved from the Internet. <URL: http://www.researchgate.net/profile/Yoon_Sung_Nam/publication/10863104_New_micelle-like_polymer_aggregates_made_from_PEI-PLGA_diblock_copolymers_micellar_characteristics_and_cellular_uptake/links/00b7d52a4beb4d9c85000000.pdf > entire document	4-11
Y	WO 2009/139856 A1 (BIOCURE INC) 19 November 2009 (19.11.2009) entire document	14
Y	CHRISTIE et al. Targeted Polymeric Micelles for siRNA Treatment of Experimental Cancer by Intravenous Injection. ACS Nano. 6(6): 5174-5189, 2012. [retrieved on 11 February 2015]. Retrieved from the Internet. <URL: http://pubs.acs.org/doi/abs/10.1021/nn300942b > abstract	18, 19
Y	US 2008/0293164 A1 (GAYLORD et al) 27 November 2008 (27.11.2008) entire document	20-22

☒ 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

11 February 2015

Date of mailing of the international search report

03 MAR 2015

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/058309

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOOD et al. Nanocarriers for vascular delivery of antioxidants. Nanomedicine. 6(7):1257-1272, 2011. [retrieved on 11 February 2015]. Retrieved from the Internet. <URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3342819/ > entire document	35, 36, 56, 57
A	EVONIK INDUSTRIES AG. RESOMER RG 502 H. April 2013. [retrieved on 11 February 2015]. Retrieved from the Internet. <URL: http://www.resomer.com/product/biodegradable-polymers/Documents/evonik-specification-resomer-rg-502-h.pdf >. entire document	1-36, 54-57

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/058309

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.:
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:

See Extra Sheet

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.:
1-36 and 54-57

Remark on Protest

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

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/058309

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I: Claims 1-36 and 54-57 are drawn to an aqueous soluble polymersome.

Group II: Claims 37-48 are drawn to a method of preparing an aqueous soluble polymersome comprising one or more hydrophobic fluorescent polymers, and a method of solubilizing one or more hydrophobic fluorescent polymers.

Group III: Claims 49-53 are drawn to an assay method for detecting a target biomolecule in a sample.

The inventions listed in Groups I through III do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The special technical features of Group I, an aqueous soluble polymersome, are not present in Groups II and III; the special technical features of Group II, a method of preparing an aqueous soluble polymersome comprising one or more hydrophobic fluorescent polymers, a method of solubilizing one or more hydrophobic fluorescent polymers, are not present in Groups I and III; and the special technical features of Group III, an assay method for detecting a target biomolecule in a sample, are not present in Groups I and II.

Groups I through III share the technical features of an aqueous soluble polymersome comprising: (a) one or more hydrophobic fluorescent polymers; and (b) a plurality of amphiphilic copolymers encapsulating the one or more hydrophobic fluorescent polymers, the copolymers each comprising a hydrophobic block that forms a hydrophobic core structure and a hydrophilic block on the surface of the polymersome that is oriented toward an aqueous phase, and an acceptor dye. However, these features do not represent a contribution over the prior art.

Specifically, US 2009/0220614 A1 to Qin et al. teaches an aqueous soluble polymersome (Abstract, ...the hydrophilic block occupying the corona of the polymersome) comprising: (a) one or more hydrophobic agents (Para. [0008]; Para. [0010]); and (b) a plurality of amphiphilic copolymers encapsulating the one or more hydrophobic agents (Para. [0008]; Para. [0010]; Paras. [0006] and [0007], ...a polymersome comprising a thermo-responsive block copolymer that is capable of polymersome formation. One such block copolymer comprises a hydrophilic block comprising polyethylene glycol terminated with an alkyl ether, and a thermo-responsive block comprising a poly N-alkylacrylamide, poly N-alkylaminoacrylate...when the LCST is at, or slightly above physiological temperature, the thermo-responsive block displays hydrophobic properties, such that the block copolymer self-assembles in aqueous solution to form a polymersome with the thermo-responsive block occupying the core of the polymersome and the hydrophilic block occupying the corona of the polymersome), the copolymers each comprising a hydrophobic block that forms a hydrophobic core structure and a hydrophilic block on the surface of the polymersome that is oriented toward an aqueous phase (Para. [0008]; Para. [0010]; Paras. [0006] and [0007]).

Further, US 2012/0282632 A1 to Chiu et al. teaches a hydrophobic fluorescent polymer (Para. [0181], ...the chromophoric polymer dot comprises a blend of a polyfluorene polymer (PF) and fluorene-benzothiadiazole-thiophen copolymer (PF-0.1TBT); see Applicant's Specification, Para. [0096], ...the hydrophobic fluorescent polymer is a polyfluorene polymer, polyfluorene copolymer, or fluorescent homopolymer), and an acceptor dye (Para. [0185], many NIR fluorescent dyes are known in the art, for example cyanine dyes are the most commonly used near-IR fluorescent dyes. Other NIR dye families include oxazine, rhodamine, and phthalocyanine dyes; see Applicant's Specification, Para. [0094], ...the acceptor dye can be...rhodamine...cyanine...).

The inventions listed in Groups I through III therefore lack unity under Rule 13 because they do not share a same or corresponding special technical feature.