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(54) Title: METHODS OF SYNTHESIZING AND USING PEG-LIKE FLUOROCHROMES

(57) Abstract: Fluorescent compounds include near infrared fluorochromes that are covalently linked to polyethylene glycol (PEG). The compounds behave like PEG in biological systems. One fluorescent compound has the formula (I): wherein R¹ is a fluorescent moiety having an absorption wavelength maxima in the range of 450 to 1500 nanometers, R² is a non-reactive moiety, and n is an integer. Another fluorescent compound has the formula (II): wherein R¹ is a fluorescent moiety having an absorption wavelength maxima in the range of 450 to 1500 nanometers, R² is a non-reactive moiety, R³ is a scaffold including an amino acid group, and n is an integer. The scaffold can be attached to a chelate, protein, enzyme, peptide, antibody, or drug that can target a site in a subject.



Methods Of Synthesizing And Using PEG-Like Fluorochromes

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. Patent Application No. 61/709,424 filed October 4, 2012, which is incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

5 **[0002]** This invention was made with government support under grant number EB 009691 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

10 **[0003]** The invention relates to uses and compositions of near infrared (NIR) fluorochromes that are covalently linked to polyethylene glycol (PEG), and behave like PEG in biological systems, including synthetic methods, compositions and methods using these PEG-like fluorochromes. The NIR fluorochromes are improved by becoming PEG-like, or behaving like PEG in biological systems, by which is meant
15 they do not bind to cells, lipids or tissues unless through specific molecular interactions. In contrast, previously developed NIR fluorochromes, and materials made with them, interact strongly with cells, lipids and tissues.

2. Description of the Related Art

20 **[0004]** Fluorescent compounds play an essential role in molecular imaging both *in vitro* and *in vivo*. Of these fluorescent compounds, near infrared (NIR) fluorophores have ideal absorption/emission wavelengths between 550 and 1000 nanometers, which minimize autofluorescence interference from tissue and have minimal overlap with biological chromophores such as hemoglobin. Fluorophores in which NIR fluorochromes have been conjugated to peptides or nanoparticles have successfully
25 been applied to *in vivo* imaging of tumors.

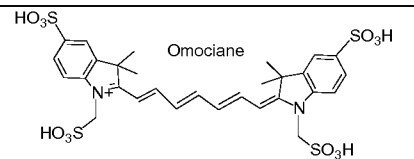
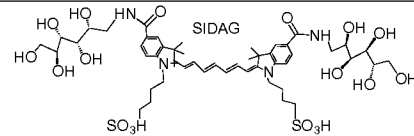
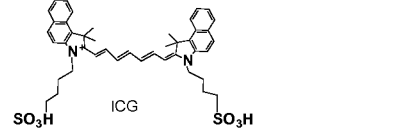
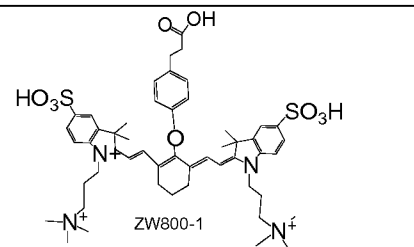
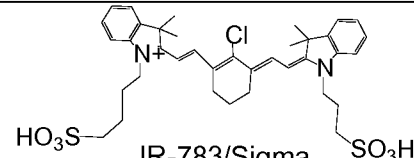
[0005] Existing NIR fluorochromes do have limitations. Though NIR fluorochromes are desirable for imaging in biological systems because of the tissue penetrating properties of their light, they are chemically complex structures involving multiple unsaturated double bonds linking multiple unsaturated rings. These features

lead to self-quenching due to fluorochrome /fluorochrome interactions, high non-specific binding to many cells, unwanted interactions with proteins and lipids *in vivo* (high non-specific binding), and enterohepatic circulation rather than renal elimination. Fluorescence dye quenching can take place by dye stacking, which
5 occurs when two or more fluorescence molecules are separated by a short-enough distance for their planar aromatic rings to interact to form aggregates. The absorbance spectra of dyes in a stacked state are substantially different from those of the same dye without stacking. For a description of the limitations of NIR fluorochromes, including the clinically used fluorochrome indocyanine green (ICG),
10 see Choi *et al.*, Synthesis and in vivo fate of zwitterionic near-infrared fluorophores. *Angewandte Chemie* 50, 6258-63 (2011).

[0006] Indocyanine green, a low molecular weight NIR fluorochrome that is currently widely used, binds to albumin, circulating lipoproteins and cell lipids, and is rapidly cleared to the liver by the hepatobiliary transport system of the liver. Though
15 cleared with a blood half of 2-4 minutes, and indicated for determining hepatic function and angiography of the eye, intraoperative ICG angiography (aneurysm repair, flap patency) have nevertheless exploited ICG's non-ideal, short lived period of vascular contrast. For intraoperative fluorescent imaging two major limitations of ICG are: (i) a short blood half-life which limits vascular phase contrast to a few
20 minutes post injection, and (ii) a high affinity for biomolecules that complicates efforts to use it as a probe of late phase (long time after injection), transcapillary passage/vascular permeability. This in turn limits the value of ICG in the key breast cancer application and is further discussed below.

[0007] Because the vast majority of ICG is tightly bound to biomolecules in vivo, ICG's transcapillary passage can occur as the free minority form of ICG, or as ICG
25 bound to the various molecules to which it binds (e.g. 5 nm. albumin, 20 nm. lipoprotein). Efforts to analyze ICG's levels and disposition in tissues are also frustrated by its intense binding to biomolecules. Thus, when tissue fluorescence (i.e. interstitial fluorescence resulting from transcapillary passage) increases, both the
30 mechanism of transcapillary transport and tissue levels of ICG cannot be ascertained. Others have recognized ICG's shortcomings and attempted to remedy

them by synthesizing low molecular weight (M_w), ICG-like fluorochromes, one of which has been used clinically. These are not ideal because they retain many of ICG's limitations, particular protein binding, albeit to a lesser extent. ICG-like NIR fluorochromes have often been synthesized using a medicinal chemistry/organic chemistry approach and are reviewed in Table 1. For a further review of fluorochromes generally, see Luo *et al.*, "A review of NIR dyes in cancer targeting and imaging", *Biomaterials* 32, 7127-38 (2011).

Table 1: NIR (ICG-like) Fluorochromes Derived Using A Medicinal Chemistry/Organic Chemistry Approach of Making Variations ICG		
Structure	References	Comment
 Omociane	(See 1-4 below.) Also called SF-74	Used clinically.
 SIDAG	(See 1-3 below.)	
 ICG	(See 1-3 below.)	Widely used, very short blood half-life & high retention
 ZW800-1	(See 5 below.) Research	
 IR-783/Sigma	Research use only.	

References for Table 1:

- (1) Ebert *et al.*, (2011) Cyanine dyes as contrast agents for near-infrared imaging in vivo: acute tolerance, pharmacokinetics, and fluorescence imaging. *Journal of biomedical optics* 16, 066003.
- (2) Perlitz *et al.*, (2005) Comparison of two tricarbo-cyanine-based dyes for fluorescence optical imaging. *Journal of fluorescence* 15, 443-54.
- (3) Licha *et al.*, (2000) Hydrophilic cyanine dyes as contrast agents for near-infrared tumor imaging: synthesis, photophysical properties and spectroscopic in vivo characterization. *Photochemistry and photobiology* 72, 392-8.
- (4) van de Ven, *et al.*, (2010) A novel fluorescent imaging agent for diffuse optical tomography of the breast: first clinical experience in patients. *Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging* 12, 343-8.
- (5) Choi *et al.*, (2011) Synthesis and in vivo fate of zwitterionic near-infrared fluorophores. *Angewandte Chemie* (International ed. in English) 50, 6258-63.

[0008] There have been uses of PEG linkers between targeting molecules and fluorochromes. Bifunctional PEG's have been used as a linkers or spacers between fluorochromes and targeting biomolecules. One end of the PEG is reacted with a fluorochrome and the other with the targeting biomolecule. These designs employ the PEG to achieve a distance between the fluorochrome and targeting biomolecule and preserve the activity of the biomolecule, to increase size, to increase water solubility, and to facilitate purification. See Basilion, "An Optical Probe for Non-invasive Molecular Imaging of Orthotopic Brain Tumors Overexpressing Epidermal Growth Factor Receptor", *Molecular cancer therapeutics* (2012); and Villaraza, "Improved speciation characteristics of PEGylated indocyanine green-labeled Panitumumab: revisiting the solution and spectroscopic properties of a near-infrared emitting anti-HER1 antibody for optical imaging of cancer", *Bioconjugate chemistry* 21, 2305-12 (2010).

[0009] There have been uses of fluorochromes and PEG for enzyme activatable probes. Fluorochromes and PEG's have been used in the design of enzyme activated fluorescence probes. Such probes feature multiple PEG's and multiple fluorochromes per mole of probe to generate strong fluorochrome-fluorochrome interactions. The PEG's are bifunctional, having two reactive ends. Interactions between multiple fluorochromes on the probe produce quenching, which is alleviated

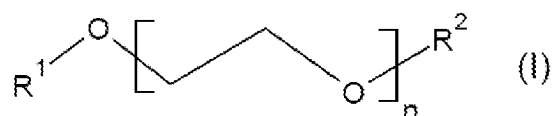
when an enzyme hydrolyzes the probe. This generates fragment(s) with smaller numbers of fluorochromes per mole and a higher fluorescence.

[0010] However, the vast potential of intraoperative fluorescent imaging can only be realized when near infrared fluorochromes are developed which behave as discrete small molecules, that is, they do not bind albumin, cell membranes or lipid. Therefore, there is a need for hydrophilic (water loving, non-biomolecule binding, near infrared fluorescent) fluorochromes that can be clinically translated (simple to synthesize/low cost/pharmaceutically acceptable reactions). .

SUMMARY OF THE INVENTION

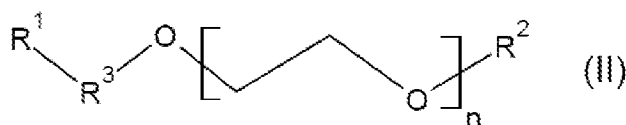
[0011] We have invented a new class of materials termed PEG-like NIR fluorochromes, and new methods of using PEG-like NIR fluorochromes, for diagnosis and treatment. The new materials can include a single PEG and a single fluorochrome that are covalently joined so that the fluorochrome is fully fluorescent (not quenched), and behaves like the PEG polymer in biological systems. PEG-like NIR fluorochromes can be used as untargeted, intravenous injected intraoperative diagnostic agents. PEG-like fluorochromes can also be used as targeted, locally administered therapeutic agents.

[0012] In one aspect, the invention provides a fluorescent compound having the formula (I):



wherein R^1 is a fluorescent moiety having an absorption wavelength maxima in the range of 450 to 1500 nanometers, R^2 is a non-reactive moiety, and n is an integer.

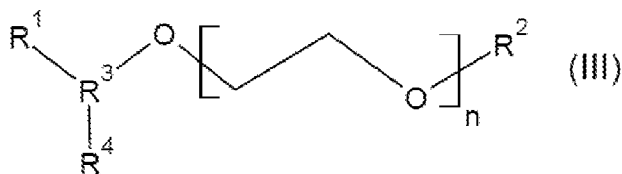
[0013] In another aspect, the invention provides a fluorescent compound having the formula (II):



wherein R^1 is a fluorescent moiety having an absorption wavelength maxima in the

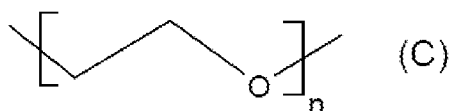
range of 450 to 1500 nanometers, R^2 is a non-reactive moiety, R^3 is a scaffold including an amino acid group, and n is an integer.

[0014] In yet another aspect, the invention provides a fluorescent compound having the formula (III):



wherein R^1 is a fluorescent moiety having an absorption wavelength maxima in the range of 450 to 1500 nanometers, R^2 is a non-reactive moiety, R^3 is a scaffold including an amino acid group, R^4 is selected from chelates, proteins, enzymes, peptides, antibodies, and drugs that can target a site in a subject, and n is an integer.

[0015] In any of compounds (I), (II) or (III), n can be selected such that chain (C) in the compound



has a molecular weight of 2,000 daltons or more, or a molecular weight of 2,000 to 10,000 daltons, or a molecular weight of 5,000 to 40,000 daltons, or a molecular weight of 2,000 to 50,000 daltons, or a molecular weight of 2,000 to 100,000 daltons. Preferably, chain (C) in the compound shields R^1 (i.e., the fluorescent moiety) from reaction with biological molecules. In any of compounds (I), (II) or (III), n can be selected such that after intravenous administration of the compound (I), (II) or (III) to a mammal, the compound undergoes renal elimination. In any of compounds (I), (II) or (III), n can be selected such that after intravenous administration of the compound (I), (II) or (III) to a mammal, clearance is by macrophages of the reticuloendothelial system of the mammal.

[0016] The fluorescent moiety in any of compounds (I), (II) or (III) may have an absorption wavelength maxima in the range of 550 to 850 nanometers or in the range of 650 to 850 nanometers. The fluorescent moiety can be a cyanine dye. The

fluorescent moiety can be a carbocyanine dye. The fluorescent moiety can be fluorescein. Any of the compounds (I), (II) or (III) can have a quantum yield of greater than 0.1.

[0017] Any of the compounds (I), (II) or (III) can have a molecular volume that correlates with an apparent molecular weight greater than about 10,000 daltons when analyzed by fast protein liquid chromatography and globular protein standards. Any of the compounds (I), (II) or (III) can have a molecular volume that correlates with an apparent molecular weight greater than about 20,000 daltons when analyzed by fast protein liquid chromatography and globular protein standards. Any of the compounds (I), (II) or (III) can have a molecular volume that correlates with an apparent molecular weight greater than about 30,000 daltons when analyzed by fast protein liquid chromatography and globular protein standards. Any of the compounds (I), (II) or (III) can have a molecular volume that correlates with an apparent molecular weight of about 10,000 daltons to about 30,000 daltons when analyzed by fast protein liquid chromatography and globular protein standards.

[0018] In any of compounds (I), (II) or (III), R^2 (i.e., the non-reactive moiety) can be selected from the group consisting of C_1 - C_{20} alkyl and aryl (e.g., phenyl). In any of compounds (I), (II) or (III), R^2 can be selected from the group consisting of C_1 - C_5 alkyl.

[0019] In compound (III), R^4 can be a chelate including a chelating agent and a chelated metal or metal ion. Example chelating agents are diethylene triamine pentaacetic acid (DTPA) or tetraazacyclododecane tetraacidic acid (DOTA) or desferoxamine (DFO). Preferably, the chelating agent is bifunctional, meaning that it possesses a metal binding moiety function and also possesses a separate chemically reactive functional group capable of covalently attaching to another moiety, such as a peptide. Non-limiting examples of bifunctional chelating agents that could be used include bifunctional DTPA, bifunctional DOTA, bifunctional DFO, bifunctional triazacyclononanetriacetic acid (NOTA), bifunctional tetraazabicyclopentadecatrienetriacetic acid (PCTA), and bifunctional oxatriazacyclododecanetriacetic acid (Oxo-DO3A). The chelated metal or metal ion in the chelate can be selected from Mn ions, Fe ions, gadolinium ions, ^{67}Ga , ^{68}Ga ,

⁸²Rb, ⁸⁹Zr, ⁹⁰Y, ^{99m}Tc, ¹¹¹In, ¹⁷⁷Lu, ²⁰¹Tl, ²¹³Bi, and ²²⁵Ac. In some embodiments, a non-metal halogen, such as ⁷⁵Br, ⁷⁶Br, ¹⁸F, ¹⁹F, ¹²³I, ¹²⁵I, or ¹³¹I, may be bound to the chelated metal or metal ion. The chelate can include a magnetic material, or a paramagnetic material, or a superparamagnetic material. In one non-limiting example, the chelating agent is desferoxamine (DFO) and the metal is ⁸⁹Zr.

[0020] In any of compounds (I), (II) or (III), the compound can have a hydrodynamic diameter in the range of 1 to 100 nanometers or in the range of 2 to 50 nanometers or in the range of 1 to 20 nanometers or in the range of 3 to 15 nanometers or in the range of 4 to 11 nanometers.

[0021] In any of the compounds (II) or (III), the scaffold can be a peptide including two or more residues selected from alanine, arginine, aspartate, cysteine, glycine, and lysine. The peptide scaffold can include any number of residues; however, for ease of synthesis and reproducibility in clinical trials, it is preferred to limit the residues in the peptide to 20 or less, more preferably, 10 or less, more preferred 5 or less, and most preferred 3 or less. The scaffold can be attached to pharmacologically active groups, immunoreactive haptens, polymers, nanoparticles, proteins, enzymes, drugs, and vitamins. In one example form, the scaffold is attached to a protein, enzyme, peptide, antibody, or drug that can target a specific site (e.g., tumor) in a subject (human or animal) undergoing a diagnostic medical procedure.

[0022] In still another aspect, the invention provides a method for imaging a region of interest of a subject. The method comprises administering to the subject any of the compounds (I), (II) or (III), wherein the compound enters the region of interest of the subject; directing light into the subject; detecting fluorescent light emitted from the subject; and processing the detected light to provide an image that corresponds to the region of interest of the subject. The light directed into the subject can have a wavelength in the range of 450 to 1500 nanometers. Use of a fluorescent moiety having an absorption wavelength maxima in the range of 450 to 1500 nanometers and light having a wavelength in the range of 450 to 1500 nanometers maximizes tissue penetration and minimizes absorption by physiologically abundant absorbers such as hemoglobin and water. The fluorescent light may be emitted via two-photon-

excited fluorescence. The method can further include imaging the subject with a second imaging method selected from positron emission tomography, single-photon emission computed tomography, magnetic resonance imaging, computerized tomography, optical imaging, and ultrasound. The region of interest of the subject may include a tumor. If the compound binds to the tumor, the method can further comprise administering to the subject a therapeutically effective amount of a cytotoxic material comprising any of the compounds (I), (II) or (III) associated with a cytotoxic agent.

[0023] In yet another aspect, the invention provides a method for treatment of a tumor in a subject. The method comprises administering to the subject a therapeutically effective amount of a cytotoxic material comprising any of the compounds (I), (II) or (III) associated with a cytotoxic agent. The cytotoxic material is targeted to the tumor in the subject.

[0024] In still another aspect, the invention provides a method for treatment of a tumor in a subject. The method comprises administering to the subject a therapeutically effective amount of a cytotoxic material comprising any of the compounds (I), (II) or (III) associated with a cytotoxic agent, wherein the cytotoxic material is targeted to the tumor in the subject. Preferably, the cytotoxic material is injected peritumorally, and at least a portion of the cytotoxic material is retained at or near the tumor by interactions between a scaffold of the compound and a receptor on a surface of a cell in the tumor.

[0025] In one version, the invention provides a composition of matter consisting (exclusively) of a NIR fluorochrome and a PEG.

[0026] In another version, the invention provides a composition of matter consisting of a single amino acid, a NIR fluorochrome and a PEG.

[0027] In yet another version, the invention provides a composition of matter consisting of a chelator, a PEG, and fluorochrome attached to a single amino acid.

[0028] In still another version, the invention provides a method of diagnostic imaging employing a passively targeted probe, PEG-like fluorochrome compound which is intravenously injected, and an image of the fluorescence in an animal or human is obtained, where the PEG-like fluorochrome consists of (i) a single NIR

fluorochrome per mole and (ii) a single PEG per mole, the PEG being larger than about 2 kDa, and which blocks fluorochrome-fluorochrome mediated interactions or fluorochrome-biomolecule interactions.

5 [0029] In yet another version, the invention provides a method of tumor therapy employing a probe consisting of a PEG-like fluorochrome, where the PEG-like fluorochrome consists of (i) a single NIR fluorochrome per mole and (ii) a single PEG per mole, the PEG being larger than about 2 kDa, and a targeting vehicle that is locally injected, allowed to diffuse through the interstitium, and retained at or near the tumor by interactions between the targeting vehicle component of the probe and a
10 receptor on the surface of cell in a tumor.

[0030] These and other features, aspects, and advantages of the present invention will become better understood upon consideration of the following detailed description, drawings, and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

15 [0031] Figure 1 shows a strategy that was used to synthesize peptides having a polyethylene glycol chain and a fluorochrome according to certain example embodiments of a fluorescent compound of the invention.

[0032] Figure 2 shows the synthesis of a linker peptide targeting vehicle suitable for use in certain example embodiments of a fluorescent compound of the invention.

20 [0033] Figure 3 shows the synthesis of trifunctional probes according to certain example embodiments of a fluorescent compound of the invention.

[0034] Figure 4A shows a comparative example (5a) with respect to a fluorescent compound of the invention.

25 [0035] Figure 4B shows an example embodiment (5b) of a fluorescent compound of the invention.

[0036] Figure 4C shows a comparative example (6a) with respect to a fluorescent compound of the invention.

[0037] Figure 4D shows a comparative example (6b) with respect to a fluorescent compound of the invention.

30 [0038] Figure 4E shows an example embodiment (7a) of a fluorescent compound of the invention.

[0039] Figure 4F shows an example embodiment (**7b**) of a fluorescent compound of the invention.

[0040] Figure 5 shows how a peptide scaffold, bearing PEG and a fluorochrome is attached to a targeting group which binds to a molecular target expressed by a cell within a tumor.

[0041] Figure 6 shows an example embodiment of a radioisotope labeled fluorescent compound (**9a, b**) of the invention.

[0042] Figure 7 depicts a comparison of intravenous administration and diffusion molecular retention according to an aspect of the invention.

[0043] Figure 8 shows tumor targeting in two animals by DMR by using the GFP expressing BT-20 breast carcinoma xenograft by surface fluorescence.

[0044] Figure 9 shows the efficiency of tumor targeting by DMR or IV methods.

[0045] Figure 10 shows SPECT/CT images after DMR and IV injections with an ¹¹¹In RGD probe using a BT-20 tumor model, and shows tissue radioactivity concentrations obtained with the RAD and RGD probes.

[0046] Figure 11 shows a general synthesis of PEG-like fluorochromes on a dipeptide scaffold.

[0047] Figure 12 shows general methods (a, b, c) of reacting a PEG and a fluorochrome with an amino acid.

[0048] Figure 13 shows two general strategies for reacting fluorochromes and polyethylene glycol according to an aspect of the invention.

[0049] Figure 14 shows three general methods of directly reacting a PEG with a fluorochrome.

[0050] Figure 15 shows a scheme for the synthesis of a (DOTA)-Lys-Cys peptide.

[0051] Figure 16 shows a scheme for the synthesis of a (DOTA)Lys-Cys(IR-783) peptide.

[0052] Figure 17 shows a scheme for the synthesis of a (DOTA)Lys-Cys(Cy3) peptide.

[0053] Figure 18 shows a scheme for the synthesis of a (DOTA)Lys-Cys(Fluorescein) peptide.

[0054] Figure 19 shows a scheme for the synthesis of a (DOTA)Lys(PEG)-Cys(IR-

783) peptide.

[0055] Figure 20 shows a scheme for the synthesis of a (DOTA)Lys(PEG)-Cys(Cy3) peptide.

[0056] Figure 21 shows a scheme for the synthesis of (DOTA)Lys(PEG)-Cys(Fluorescein) peptide.

[0057] Figure 22 shows the synthesis and design principles of PEG-like Nanoprobes (PN's). In Figure 22(a), a modular synthetic strategy is employed with one fixed component, the (DOTA)Lys-Cys peptide. A variable fluorochrome reacts with the cysteine side chain, followed reaction of an NHS ester of a PEG polymer, of variable length, with the lysine side chain. Figure 22(b) shows conferring "PEG-likeness" with a long PEG polymer and short peptide. PEG confers its size upon the resulting probe. Spectral tunability is generated by fluorochrome selection. Pharmacokinetic tunability is generated by PEG selection. In Figure 22(c), PEG-likeness enhances fluorochrome elimination: Surface fluorescence of mice after IV injections of the (DOTA)Lys-Cys(IR-783) peptide or PN(783)4.3, which is a PEGylated version of the same peptide. PEGylation leads to enhanced fluorochrome elimination, which is evident by the selective fluorescent bladder at 20 minutes.

[0058] Figure 23 shows tuning PN size, optical properties and the post-injection circulating form. In Figure 23(a), tuning PN size by varying PEG. FPLC chromatograms of size-variable PN's (constant IR-783 fluorochrome, variable PEG's, see Figure 22a) are shown. Volumes are given in Table 5. Figure 23(b,c) shows tuning size with different fluorochromes. The 5 kDa PEG yielded the magenta coded PN chromatograms with diameters of 4.3 nm regardless of the fluorochrome used. They are PN(783)4.3 (2a), PN(545)4.3 (2b), and PN(497)4.3 (2c). The 30 kDa PEG yielded the blue coded PN's of 10 nanometers. PN dimensions are determined by PEG and independent of the fluorochrome selected. In Figure 23(d,e,f), FPLC chromatograms of PN's are shown before (pre) and at various times after injection. PN's were PN(783)10 (Fig. 23d), PN(783) 6.1 (Fig. 23e), and PN(783)10.0 (Fig. 23f). After injection, PN's circulate at their PEG-determined and variable pre-injection sizes. In Figure 23(g), since PN's circulate at PEG-determined, pre-injection sizes, they cross capillaries at those sizes.

[0059] Figure 24 shows tuning PN Pharmacokinetics analyzed by the two-compartment pharmacokinetic model. Figure 24(a) shows a summary of the two compartment pharmacokinetic model showing three microscopic rate constants. Serum fluorescence for PN(783)10 (b) and PN(783)4.3 (c) after injection are shown. Data were fit to the two compartment model shown in Figure 24(a). In Figure 24(d), post injection time courses of blood fluorescence for PN(783)10 (g) and PN(783)4.3 (h) are shown. Lines are the fits to a two compartment pharmacokinetic model with constants provided in Table 5.

[0060] Figure 25 shows fluorescent imaging of three pharmacokinetic phases with PN's with diameters of 10 nm. Figure 25(a) shows vascular phase, two-photon microscopy of brain vasculature. Vessel intensity drops due to vascular escape, but there is no interstitial fluorescence in the brain due to the blood brain barrier. Scale marker = 50 microns. Figure 25 (b) shows intravital confocal microscopy of the vascular and interstitial phases of an mCherry expressing HT-29 xenograft. During the vascular phase (10 minutes post) injection, vessels are imaged, without interstitial fluorescence. During the interstitial phase (20 hours post), interstitial fluorescence is prominent. Scale marker = 20 microns. Figure 25 (c) shows confocal microscopy of the tumor retention phase of PN(497)10.0. Shown are a sectioned HT-29 mCherry expressing tumor with nuclei stained blue (DAPI), mCherry tumor cells (red), PN(497)10.0 (green) and a green/red overlay (yellow). Figure 25 (d) shows surface fluorescence/X-ray imaging of the tumor retention phase of PN(545)10.0. Shown are the HT-29/mCherry tumor with the skin removed as a white light image, mCherry tumor fluorescence (green), PN(545)10.0 fluorescence (purple) and the green/purple overlay (white).

[0061] Figure 26 shows multimodal imaging of tumor retention with PN(783)10.0, its biodistribution and elimination. Figure 26(a) shows SPECT/CT images of two mice bearing two HT-29 tumors as a function of time after injection. At two hours post injection, agent is in the blood and interstitium. By 24 hours post injection, tumors are becoming apparent as agent is being cleared. At 48 hours, labeling is highly tumor selective. Figure 26(b) shows surface fluorescence imaging of two additional mice bearing the same tumor. By surface fluorescence, as with SPECT,

labeling is highly tumor selective at 48 hours. Biodistribution as organ radioactivity concentrations Figure 26(c) and total organ radioactivity Figure 26(d) were obtained by dissection and ^{111}In counting at 24 hours and 48 hours post injection. Even at 48 hours post injection some 7.5% of injected dose is in the blood, with less than 5% in liver, even though the diameter of PN(783)10.0 exceeds that of albumin (6.7 nm). Data are means and standard deviations. Figure 26(e) shows a whole animal radioactivity elimination curve. By 72 hours, some 17% of injected dose was retained, approximately half of which was still in the blood based on Figure 26d.

[0062] Figure 27 shows the purity of PEG-like Nanoprobes (PN's) by FPLC and mass spectroscopy. Figure 27a) shows FPLC chromatogram of purification of PN(783)10.0 by removal of the low molecular weight (DOTA)Lys-Cys(IR-783) peptide which is used in PEG-like nanoprobe synthesis. FPLC's of pure PN's are shown in Figure 23. Figure 27b) shows MALDI-TOF Mass spectroscopy of pure PN(783)4.3 made by reaction of (DOTA)Lys-Cys(IR-783) with the 5 kDa PEG-NHS. Note the absence of species at 4800 to 5200 Da, expected if there was PEG contamination.

[0063] Figure 28 shows a scheme for the synthesis of (^{111}In -DOTA)Lys(PEG30kDa)-Cys(IR-783).

[0064] Figure 29 shows quantum yields of peptides and PEGylated PN's. In Figure 29a), quantum yields are shown for PN(783)'s made with different PEG's. In Figure 29b), quantum yields are shown for PN(545)'s made with different PEG's. In Figure 29c), quantum yields are shown for PN(497) made with different PEG's. As excitation maxima go up quantum yields go down. Quantum yields are always improved by PEGylation but the degree of improvement varies.

[0065] Figure 30 shows the effect of PEGylation on non-specific binding to cells. In Figure 30a), the (DOTA)Lys-Cys(IR-783) peptide binds cells but PEGylated versions have greatly reduced binding. The percent of cells with fluorescence higher than unstained cell is given in Table 5. In Figure 30b), the (DOTA)Lys-Cys(Cy3) peptide binds to cells but PEGylated versions have greatly reduced binding. In Figure 30c), the (DOTA)Lys-Cys(Fluorescein) peptide binds cells very weakly so PEG does not reduce binding. Arrows indicates the border of negative and positive binding.

[0066] Figure 31 shows a scheme for the synthesis of a (DFO)Lys-Cys peptide wherein DFO is desferoxamine.

[0067] Figure 32 shows a scheme for the synthesis of a (DFO)Lys-Cys(S-Mal-Cy5.5, Lumiprobe) peptide.

5 [0068] Figure 33 shows a scheme for the attachment of a 5 kDa PEG to the peptide of Figure 32.

[0069] Figure 34 shows a scheme for the attachment of a 30 kDa PEG to the peptide of Figure 32.

10 [0070] Figure 35 shows a scheme for the radiolabeling of (DFO)Lys(PEG 30kDa)-Cys(S-Mal-Cy5.5) with $^{89}\text{Zr}^{4+}$.

[0071] Figure 36 shows radioactive thin-layer chromatography monitoring for DFO-Lys(PEG5KDa)-Cys(S-Mal-Cy5.5)-NH₂.

[0072] Figure 37 shows radioactive thin-layer chromatography monitoring for DFO-Lys(PEG30KDa)-Cys(S-Mal-Cy5.5)-NH₂.

15 DETAILED DESCRIPTION OF THE INVENTION

[0073] We have discovered that the covalent linking of a PEG and NIR fluorochrome can lead to a loss of unwanted fluorochrome-fluorochrome interactions (which lead to quenching) and unwanted fluorochrome-biomolecule interactions which lead to non-specific binding to plasma proteins, lipoproteins, cell membranes. PEG covers the fluorochrome with an extended polymeric cloud, with entrapped water, shielding it from reaction with biological molecules. Hence PEG-like fluorochromes are PEG-fluorochrome shielded fluorochromes.

20 [0074] For diagnostic imaging, the intravenous administration of a passively targeted PEG-like fluorochrome is obtained. With low molecular weight PEG's (2-10 kDa by mass), the PEG-like fluorochrome undergoes renal elimination (small enough for glomerular filtration). With high molecular weight PEG's (>20 kDa), clearance is by macrophages of the reticuloendothelial system (too large for glomerular filtration). Passively targeted PEG-like fluorochromes are used as intraoperative diagnostic agents to determine blood vessel flow or permeability. Images are made with
25 fluorescent detection devices (cameras) such as those listed in Table 1 of Marshall, "Near-Infrared Fluorescence Imaging in Humans with Indocyanine Green: A Review

and Update", *The Open Surgical Oncology Journal* 2, 12-15 (2010). See also Alander, "A review of indocyanine green fluorescent imaging in surgery", *International journal of biomedical imaging* 2012, 940585 (2012).

[0075] Some advantageous features of PEG-like fluorochromes over the conventional low molecular weight fluorochromes, which are listed in Table 1 above, are summarized below.

[0076] 1. Because PEG-like fluorochromes exist as discrete species in biological systems (they do not interact with each other or biological molecules), their properties can be optimized for different intraoperative applications. PEG-like fluorochromes can be small (10 kDa) or large (e.g. 100 kDa), depending on the size of the PEG employed. The size of PEG-like fluorochromes can be varied to optimize their behavior as (i) angiographic agents (agents confined to the vasculature), (ii) as agents for visualizing transcapillary passage/vascular leak, and (iii) as agents for visualizing macrophages of the reticuloendothelial system.

[0077] 2. The PEG-like fluorochromes use clinically translatable chemistry. The three basic components of PEG-like fluorochromes (i.e., PEG, fluorochrome, and amino acid or peptide) are inexpensive. The synthesis of PEG-like fluorochromes on a large scale is practical and consistent with pharmaceutical practice.

[0078] 3. The PEG-like fluorochromes allow for detection by a second imaging modality. Our design allows the addition of a metal chelating functional group (e.g., a chelating agent such as diethylene triamine pentaacetic acid (DTPA), tetraazacyclododecane tetraacidic acid (DOTA), or desferoxamine (DFO)), and a chelated metal or metal ion (such as Mn ions, Fe ions, gadolinium ions, ⁶⁷Ga, ⁶⁸Ga, ⁸²Rb, ⁸⁹Zr, ⁹⁰Y, ^{99m}Tc, ¹¹¹In, ¹⁷⁷Lu, ²⁰¹Tl, ²¹³Bi, and ²²⁵Ac) to the PEG-like fluorochrome. Together, the chelating agent and the chelated metal or metal ion form a chelate. The presence of the chelate enables the PEG-like fluorochrome to be quantified by magnetic resonance imaging (MRI), positron emission tomography (PET), or single-photon emission computed tomography (SPECT), in addition to fluorescence. The multimodal capability can be used clinically or to accelerate the development of PEG-like fluorochromes for intraoperative applications by providing a method of measuring fluorochrome levels in tissues. To facilitate the incorporation of

the chelate into the PEG-like fluorochrome, the chelating agent is preferably bifunctional, meaning that it includes both a metal chelating function and a separate active functional group that can covalently bond to other groups, such as a peptide. Exemplary bifunctional chelating agents that could be used include without limitation bifunctional DTPA, bifunctional DOTA, bifunctional DFO, bifunctional NOTA, bifunctional PCTA, and bifunctional Oxo-DO3A. Further non-limiting examples of bifunctional chelating agents that could be used in the invention are provided by Brechbiel (see Brechbiel MW. Bifunctional chelates for metal nuclides. Q J Nucl Med Mol Imaging. 2008 Jun;52(2):166-73), which is incorporated by reference herein. Optionally, the invention can employ additional elements that are not metals by indirect chelation. After the metal or metal ion is chelated to the chelating agent, a non-metal halogen, such as ⁷⁵Br, ⁷⁶Br, ¹⁸F, ¹⁹F, ¹²³I, ¹²⁵I, ¹³¹I, may be bound to the chelated metal or metal ion, adding additional detection functionality. For example, McBride et al. have reported the binding of the halogen ¹⁸F to chelated aluminum ions to form a fluoride-aluminum-chelate complex (see McBride WJ, D'Souza CA, Sharkey RM, Karacay H, Rossi EA, Chang CH, Goldenberg DM. ¹⁸F labeling of peptides with a fluoride-aluminum-chelate complex. Bioconjug Chem. 2010 Jul 21;21(7):1331-40. doi: 10.1021/bc100137x).

[0079] 4. The invention provides the ability to use different fluorochromes. Since PEGylation enshrouds the fluorochrome, the fluorochrome can be varied while maintaining the PEG-like properties. This can allow the simultaneous use of two, spectrally distinct PEG-like fluorochromes (e.g., small and large PEG-like fluorochromes).

[0080] 5. The invention provides pharmacokinetic (PK) control by changing PEG molecular weight and size. Unlike the fluorochromes of Table 1, where PK is intrinsic to the fluorochrome, the PK of PEG-like fluorochromes can be altered through alterations in the molecular weight and size of PEG.

[0081] 6. The invention provides pharmacokinetic (PK) control by employing PEG to block proteolytic degradation. PEGylation can also PK control by blocking the degradation of fluorochrome bearing peptide by proteases. The degradation of

peptides often occurs when they then leave the vasculature and encounter proteases. PEGylation can provide PK control by blocking proteolytic degradation.

[0082] For cancer treatment, PEG-like probes are used with a molecular targeted delivery method called Diffusion Molecular Retention (DMR). DMR probes can use a short PEG linker as well as a larger PEG for fluorochrome shielding. Here PEG-like fluorochromes can be components of more complex probes that include molecular targeting groups and cytotoxic agents. Thus, a cytotoxic agent can be associated with a PEG-like fluorochrome.

[0083] A cytotoxic agent is “associated” with one of the PEG-like fluorochromes of the invention if the cytotoxic agent is directly or indirectly, physically or chemically bound to one of the PEG-like fluorochromes. Non-limiting examples of chemical bonds include covalent bonds, ionic bonds, coordinate bonds, and hydrogen bonds. Indirect bonding can include the use of a group of atoms (i.e., a linker) that chemically links the cytotoxic agent and the PEG-like fluorochrome. Non-limiting examples of physical bonding include physical adsorption and absorption. The cytotoxic agent can be a cytotoxin (e.g., ricin, pseudomonas exotoxin, diphtheria toxin). The cytotoxic agent can be a chemotherapeutic agent (e.g., alkylating agents, antagonists, plant alkaloids, intercalating antibiotics, enzyme inhibitors, antimetabolites, mitotic inhibitors, growth factor inhibitors, cell cycle inhibitors, enzymes, biological response modifiers). The cytotoxic agent can be a radiation-emitter (e.g., phosphorus-32, phosphorus-33, bromine-77, yttrium-88, yttrium-90, molybdenum-99m, technetium-99m, indium-111, indium-131, iodine-123, iodine-124, iodine-125, iodine-131, lutetium-177, rhenium-186, rhenium-188, bismuth-212, bismuth-213, astatine-211).

[0084] The molecularly targeted delivery of toxic “payloads” to tumors is limited by low tumor blood flow, capillary permeability barriers, high interstitial pressure, and kidney, liver and spleen uptake. The technique termed Diffusion Molecular Retention (DMR) comprises local administration of a fluorescent peptide probe, visualizing probe extensive probe diffusion through the interstitium by fluorescence, and obtaining retention if the probe encounters a molecular target. In this instance, PEG-like fluorochromes function as reporters of the interstitial diffusion of locally

administered, targeted therapeutic PEG-like fluorochromes. DMR employs peptide probes that by virtue of their PEGylation achieve a molecular volume of 25 kDa, and therefore have a slow vascular uptake, as well as an absence of non-specific binding to components of the interstitium. To demonstrate DMR, a trifunctional RGD probe bearing a DOTA, a 5 kDa PEG and a CyAl5.5 fluorochrome was synthesized and interstitial diffusion visualized by surface fluorescence. A control RAD probe was not retained, indicating retention of the RGD probe was due to integrin binding. By "local administration" is meant intratumorally, peritumorally or with subcutaneous or intramuscular injections that enable the probe to diffuse to and through the tumor with high efficiency (low uptake by normal organs like the liver, kidney and spleen).

[0085] Methods of using PEG-like fluorochromes as diagnostic imaging agents and for DMR cancer treatment are summarized in Table 2.

Table 2: Summary of Methods of Using PEG-like Fluorochromes for Diagnostic Imaging and DMR Tumor Therapy				
Use	Administration	Targeting	Roles of PEG	Benefit/Uses
Intraoperative diagnostic imaging	Intravenous (IV)	Passive, Size based	1. Shield fluorochrome, block unwanted non-specific interactions 2. Provide size-based pharmacokinetic control of fluorescence after injection. Renal elimination (low M_w) or reticuloendothelial system elimination (high M_w)	Intraoperative visualization of vessel fluorescence function and vessel permeability. See Example 3-5 below.
Therapeutic Targeting (DMR)	Intratumor (IT) Peritumor (PT) Intramuscular (IM) Subcutaneous (SC)	Active, (binding to molecular target)	1. Shield fluorochrome, block unwanted non-specific interactions 2. Provide a size (volume) optimal for high interstitial diffusion 3. Retention only due to molecular target binding, no non-specific binding	High efficiency tumor targeting (low normal organ uptake) of radioisotopes or other highly toxic "payloads." See Examples 1 and 2 below.

[0086] Two raw materials for the synthesis of PEG-like fluorochromes are monofunctional PEG's, preferably with molecular weights of 2000 daltons or greater, and NIR fluorochromes.

5 **[0087]** The PEG's used by invention are monoreactive, with one end connected to the fluorochrome (directly or indirectly) and the other non-reactive end of the PEG unmodified. (Hence, the PEG's used for fluorochrome shielding do not serve as linkers.) PEG's must be sufficiently long to block the chemical properties of the fluorochrome. Generally, they must have molecular weights of about 2000 Da or
10 greater and can be monodisperse (single molecular weight species) or polydisperse. Currently, PEG's of 2000 Da or greater are generally polydisperse. The NIR fluorochromes used have absorption wavelength maxima of 450 nanometers to 1500 nanometers, and must at least be site amenable to chemical modification.

[0088] Non-limiting examples of suitable NIR fluorochromes are Cy5.5, Cy5, CyAL-5, CyAL5.5, and IR-783. CyAL-5 and CyAL5.5 are carbocyanine dyes described in United States Patent Application Publication No. 2011/0286933, which is incorporated herein by reference. CyAL-5 and CyAL5.5 are available from Molecular Targeting Technologies, Inc., West Chester, Pennsylvania, USA. IR 783 is cyanine dye available from Sigma Aldrich, St. Louis, Missouri, USA. It is 2-[2-[2-
15 Chloro-3-[2-[1,3-dihydro-3,3-dimethyl-1-(4-sulfobutyl)-2H-indol-2-ylidene]-ethylidene]-1-cyclohexen-1-yl]-ethenyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-indolium hydroxide, inner salt sodium salt.
20

[0089] Preferably, PEG-like fluorochromes probes employ a single PEG per mole of probe to enshroud the fluorochrome and a single fluorochrome per mole. Thus,
25 the probes of the invention do not employ intramolecular quenching and are not activated by enzymes. In some cases a second, short PEG can be employed as a linker between a targeting peptide and the PEG used to enshroud the NIR fluorochrome.

[0090] PEG-like fluorochromes of the invention (both actively or passively
30 targeted) have one or more of the following chemical properties: (i) they have one fluorochrome per mole; (ii) they have one PEG per mole; (iii) the PEG has a

molecular weight greater than about 2000 Da; (iv) the PEG is monofunctional (has only one chemically reactive end); (v) they have molecular volumes greater than about 10 kDa when analyzed by fast protein liquid chromatography (FPLC) and globular protein standards, and their volume is comprised mostly of the volume of the PEG rather than the fluorochrome, i.e., without PEG the fluorochrome has a volume of less than about 2 kilodaltons; (vi) they have characteristic, unstacked absorption spectra; and (vii) they have improved quantum yields (in PBS) over non-PEGylated fluorochrome.

[0091] Preferably, PEG-like fluorochromes of the invention have one or more of the properties below when interacting with biological systems: (i) they have low non-specific bindings with cultured cells; (ii) they can undergo clearance (by surface fluorescence) from a local intramuscular (IM) injection site within 24 hours; and (iii) when probe volumes are below about 30 kDa, they undergo predominant renal elimination after intravenous injection.

[0092] PEG and an NIR fluorochrome can be combined by direct attachment. Alternatively, PEG and the NIR fluorochrome can be attached to a low molecular weight scaffold (e.g., an amino acid or a peptide), yielding compositions comprising a fluorochrome, scaffold and PEG. Some amino acids (e.g., lysine, cysteine, aspartate) can accommodate a PEG, a chelate and a fluorochrome.

[0093] For targeted uses for PEG-like fluorochromes, an example embodiment employs a probe made according to the methods of U.S. Patent Application Publication No. 2011/0159566, which is incorporated herein by reference. To obtain a therapeutic method of treating a tumor, the multifunctional probe is locally administered (peritumorally or subcutaneously) and bears a cytotoxic agent such as a radiation emitter.

[0094] The invention is further illustrated in the following Examples which are presented for purposes of illustration and not of limitation.

EXAMPLES

Example 1

[0095] Fluorochromes were attached to peptides both with and without PEG. Table 3A below provides a summary of the Example 1 compounds.

Tetrapeptide Probe Synthesis

Overall strategy

[0096] A strategy that was used to synthesize trifunctional RGD and RAD is shown in Figure 1. In Figure 1(a), a multifunctional reagent module was first synthesized and attached to a linker-targeting vehicle module via a copperless click reaction, to yield a multifunctional probe. PEGylation was conferred by the 5 kDa PEG at the F3 position. Figure 1(b) shows the synthesis of linker-targeting vehicles bearing RGD (arginine, glycine, aspartate) or RAD (arginine, alanine, aspartate) peptides. Figure 1(c) shows the synthesis of multifunctional probes with functional groups of F1= DOTA, F2= CyAL5.5 fluorochrome, and F3 = 5 kDa PEG. The reaction conditions were: a: 1) NH_2NH_2 , DMF; 2) CyAL5.5 Acid/ PyBOP/ DMF/ DIPEA; 3) TFA; b: PEG-5K-NHS, DMSO; c: 5a, cRGD-PEG4-DBCO (3a) or RAD-PEG4-DBCO (3b), DMSO; d: PEG-5K-NHS. Complete structures are given in Figures 4A to 4F.

Materials and Methods

[0097] Protected L-amino acids, PyBOP and Rink Amide MBHA resin were from Novabiochem (EMD Biosciences). Other special chemicals were from other sources: DOTA(CO_2Bu^t)₃ (Macrocyclics), mPEG-NHS ester (5 kDa) (Creative PEGworks), Fmoc-Lys(N_3)-OH (AnaSpec), and DBCO-PEG₄-NHS (Click Chemistry Tools). The fluorescent dye CyAL5.5 was synthesized as described in United States Patent Application Publication No. 2011/0286933. CyAL5.5 is also available from Molecular Targeting Technologies, Inc., West Chester, Pennsylvania, USA. All the other solvents and chemicals were from Sigma-Aldrich. Molecular weights were obtained by MS-ESI Micromass (Waters) and MALDI-TOF analyses at the Tufts University Core Facility. RP-HPLC (Varian ProStar detector and delivery modules) employed an eluant A (0.1% TFA /water) and eluant B (0.1% TFA in 9.9% water in acetonitrile). RGD and RAD peptides were cRGDfK and cRADfK from Peptides International.

[0098] Figure 2 shows the synthesis of linker RGD (or RAD) targeting vehicles. For (3a): A stock solution of DBCO-PEG₄-NHS ester (2) (containing 7.5 mg, 10.8 μmol) in anhydrous DMSO was added to the solution of the RGD peptide, cRGDfK (3a) (5.6 mg, 9.28 μmol from Peptides International) in anhydrous DMSO (0.4 ml).

After DiPEA (9 μ l) was added, the mixture was incubated at room temperature overnight. After diluted with buffer A, the mixture was purified by HPLC with gradient of 20%B-100%B in 15 minutes, then back to 20% B in 5 minutes and isocratic for 5 minutes; flow: 12ml/min; λ_{max} : 226nm; column: Higgins Analytical Inc. Clipseus C18, 10 μ m, 250x20mm, P/N: CS-2520-C181, S/N: 186532. A white powder (**5a**) was obtained. Yield: >90% C₅₉H₇₉N₁₁O₁₅, MW: 1182.32, MS: Cal. 1181.58, Observed: 1182.11. For (**3b**), the procedure was followed **3a** by using the RAD peptide cRADfK with similar results. C₆₀H₈₁N₁₁O₁₅, MW: 1196.35, MS: Cal. 1195.59, Observed: 1196.30.

[0099] Figure 3 shows the synthesis of trifunctional probes. a: NH₂NH₂, DMF; b: CyAL5.5 Acid, PyBOP, DMF, DIPEA; c: TFA; d: PEG-5K-NHS, DMSO; e: **5a**, cRGD-PEG4-DBCO (**3a**) or RAD-PEG4-DBCO (**3b**), DMSO; f: PEG-5K-NHS.

[00100] Synthesis of **5a** (Comparative Example): The DOTA(CO₂-Bu^t)₃-Lys(ivDde)-Lys(Boc)- β -Ala-Lys(N₃) peptide (**4a**) was manually synthesized on Rink Amide MBHA resin (0.15 mmol) with an Fmoc/*t*-Bu strategy using a polypropylene 5-mL disposable syringe fitted with a sintered frit. Coupling reactions employed 2 equiv. (relative to resin) of *N*- α -Fmoc-protected amino acid activated in situ with 2 equiv. of PyBOP and 4 equiv. of DiPEA in DMF (10 mL/g resin) for 1-2 hrs. Coupling efficiency was assessed with picrylsulfonic acid. *N*- α -Fmoc groups were removed with a piperidine/DMF solution (1:4) for 4x10 minutes (10 mL/g resin). The coupling of DOTA was overnight with same equivalent of other reagents. After intermediate (**4b**) was obtained by *N*- ϵ -ivDde group removal with 2% hydrazine in DMF for 5 minutes (10 mL/g resin), the attachment of CyAL5.5 (for intermediate **4c**) was carried out on the solid phase for overnight by using CyAL5.5 acid (2 equiv.) under the in situ activation of PyBOP (2 equiv.) and DiPEA (8 equiv.). Intermediate DOTA-Lys(CyAL5.5)-Lys(NH₂)- β -Ala-Lys(N₃) (**5a**) was released from the solid support with TFA/H₂O/TIS/EDT 88:2:5:5 (twice, 4 h, 20 mL/g resin). After the solvent was evaporated, the residue was precipitated and triturated with cold ether. A blue solid could be obtained by centrifuge. The solid was purified further by preparative HPLC with a gradient of 20% - 80%B in 15 minutes, back to 20%B in 3 minutes, and isocratic for 3 minutes; λ_{max} : 670 nm; flow: 21 ml/min; column: Higgins Analytical Inc., Clipseus C18 10 μ m, 250x20 mm. A blue powder of compound (**5a** - see Fig. 4A) was obtained after lyophilization with a yield of 40%.

$C_{81}H_{117}N_{16}O_{18}S_2^+$; MW: 1667.02; MS: cal. 1665.82; found (m/z): 1666.2 and 833.8.

[00101] Synthesis of **5b**: To a solution of DOTA-Lys(CyAL5.5)-Lys(NH₂)- β -Ala-Lys(N₃)-NH₂ (**5a**) (1.0 mg, 0.6 μ mol) in anhydrous DMSO (0.4ml), was added the solution of m-PEG-5K-NHS (13.8mg, 2.76 μ mol) in anhydrous DMSO (0.5ml). After DiPEA (10 μ L) was added, the reaction mixture was incubated at room temperature for 3 days. The mixture was diluted by acetonitrile and water (0.1% TFA, 1:1 v/v) and purified by HPLC with a gradient of 20%-100%B in 20 minutes, then back to 20% B in 5 minutes and isocratic for 5 minutes; flow: 5ml/min; λ_{max} : 670 nm; Varian Pursuit XRs 5 C18, 250x10 mm column, P/N: A6000250X100, S/N: 1007962. A blue powder (**5b** - see Fig. 4B) was obtained after lyophilization. Yield: >90%. Mass was in a wide range from 6200 to 7100 due to PEG.

[00102] Synthesis of unPEGylated RGD and RAD probes, **6a**, **6b** (Comparative Examples): For **6a**, a mixture of the solution of DOTA-Lys(CyAL5.5)-Lys(NH₂)- β -Ala-Lys(N₃)-NH₂ (**5a**) (3.2 mg, 1.92 μ mol) in DMSO (0.4 ml) with the solution of DBCO-PEG₄-cRGD (**3a**) (2.5 mg, 2.11 μ mol) in DMSO (0.4ml) was incubated for 2 hours at room temperature. The product was purified by HPLC with a gradient of 20%-100%B in 20 minutes, then back to 20% B in 5 minutes and isocratic for 5 minutes; flow: 5 ml/min; λ_{max} : 670 nm; column: Varian Pursuit XRs 5 C18, 250x10 mm, P/N: A6000250X100, S/N: 1007962. A blue powder (**6a** - see Fig. 4C) was obtained. Yield: ~99%. $C_{140}H_{196}N_{27}O_{33}S_2^+$, MW: 2849.34, MS: cal. 2847.39, found: 2848.29. For (**6b** - see Fig. 4D), the procedure was followed **6a** by using **3b** with similar results. $C_{141}H_{198}N_{27}O_{33}S_2^+$, MW: 2863.37, MS: cal. 2861.41, found: 2862.22

[00103] Synthesis of multifunctional PEGylated RGD and RAD probes, **7a** and **7b**: To a solution of DOTA-Lys(CyAL5.5)-Lys(NH₂)- β -Ala-Lys(N₃-DBCO-PEG₄-cRGD)-NH₂ (**6a**) (1.54 mg, 0.54 μ mol) in DMSO (0.9 ml), was added the solution of m-PEG-5K-NHS (18 mg, 3.6 μ mol). After DiPEA (10 μ L) was added, the reaction mixture was incubated at room temperature for 3 days. The mixture was diluted by acetonitrile and water (0.1% TFA, 1:1 v/v) and purified by HPLC purification with a gradient of 20%-100%B in 20 minutes, then back to 20% B in 5 minutes and isocratic for 5 minutes; flow: 5ml/min. λ_{max} : 670 nm; column: Varian Pursuit XRs 5 C18,

250x10mm, P/N: A6000250X100, S/N: 1007962. A blue powder (**7a** - see Fig. 4E) was obtained. Yield: >90%. Mass was observed in a wide range from 7300 to 8300 due to PEG. For (**7b** - see Fig. 4F), the procedure was followed **7a** by using **6b** with similar results. Masses ranged from 7300 to 8300 Da due to PEG polydispersity.

5 **[00104]** The structures of compounds **5a** to **7b** are given in Figures 4A to 4F. The most prominent species in the polydisperse 5 kDa PEG is $n = 115$, and is shown for **5b**, **7a** and **7b**.

[00105] Figure 5 shows how the tetrapeptide scaffold, bearing the PEG and fluorochrome is attached to a "targeting moiety" or "targeting group" which binds to a
10 molecular target expressed by a cell within the tumor. Looking at Figure 5, the targeting group is the smaller oval. PEG shields the fluorochrome, providing a diffuse cloud (the larger oval in Fig. 5). A linker is shown between the scaffold, bearing the PEG and fluorochrome, and the targeting group.

[00106] Synthesis of 111 Indium labeled probes RGD and RAD probes, **9a** and

15 **9b**: The synthesis of 111 -indium labeled probes RGD and RAD probes (**9a**,**9b**) is shown in Figure 6. For the ^{111}In labeling of **7a** or **7b**. a: $^{111}\text{InCl}_3$, 1M HEPES, pH 5, 70°C. To the solution of **7a** or **7b** (20 nmoles), reconstituted with 1M HEPES buffer (pH 5.0) (0.7ml) in a 5 ml conic react vial, was added the solution of $^{111}\text{InCl}_3$ in 0.05N HCl (150 μl , 333MBq, 9mCi). After 45 minutes at 70°C, the mixture was cooled in a
20 water bath for 2 minutes. A stock solution of EDTA (70mM, 50 μl) was added and the solution was allowed to stay at room temperature for 15 minutes. After the mixture was diluted with ammonium acetate buffer (0.5ml, 1M, pH 6), and loaded onto a C18 cartridge preconditioned by ethanol (0.5ml, 0.1% acetic acid) and water (1ml, 0.1% acetic acid) sequentially. The labeled tracers (**9a**,**9b**) were purified by washing the
25 cartridge with water (1ml, 0.1% acetic acid) and collected by eluting with acetonitrile (200 μl , 0.1% TFA). The acetonitrile and TFA were removed by co-evaporation with ethanol (3 x 200 μl) together with Ar flow and reconstituted with 0.9% saline for injection. Radioactive products were identified by their co-chromatography with the corresponding nonradioactive indium labeled compounds. Radiochemical yield
30 (RCY) was 50-70%.

[00107] Probe volume determinations: Size (volume) was determined by FPLC

using an ÄKTA Purifier 10 and Superdex™ 75 10/300GL column (GE Healthcare Lifesciences) with a running buffer of 0.05 M sodium phosphate, 0.15 M NaCl (0.1% Tween, pH 7.2) and flow rate of 0.5ml/min. The protein standards (Gel Filtration Calibration Kit LMW, code no. 28-4038-41, GE Healthcare) (0.3mg/ml, mixture of Aprotinin, Ribonuclease A, Ovalbumin, and Conalbumin) and Blue Dextran 2000 were used. To obtain volumes, Mr (apparent molecular weight based on size exclusion retention) was plotted versus Kav. $K_{av} = (V_e - V_o) / (V_t - V_e)$, Vt = total volume, Ve = elution volume, Vo = void volume.

[00108] BT-20 transfection for GFP expression: Day 1, BT-20 cells were planted into 24-well plate at 750,000 cells/well in culture medium (EMEM with 10% FBS). Day 2, the old culture medium was replaced with new culture medium containing lenti-virus (1×10^8 particles/mL) and protamine sulphate (American Pharmaceutical Partners, Los Angeles, California; 10mg/mL; 1:500 of total volume of medium). Day 3, after 16 hours of transfection, medium was changed to the culture medium (EMEM with 10% FBS). 8 hours later, green cells could be seen via fluorescence microscope.

[00109] BT-20 tumor model: All animal experiments were approved by the Institutional Review Committee of Massachusetts General Hospital. Female nude mice (25-30g; 6-8 weeks old; nu/nu; Cox 7, Massachusetts General Hospital, Boston, MA) were anesthetized with isoflurane/O₂. Tumor cell implantation was performed both sides around the shoulder. 200 µl of cell suspension containing 10^6 cells in Matrigel (BD) was injected subcutaneously. Tumor cells were inoculated for 7 to 10 days.

[00110] Biodistribution of indium-111 labeled 7a or 7b: 150 µl of Indium-111 (with 300µCi) labeled compounds 7a or 7b were injected to BT-20 tumor-bearing animals intravenously. 24 hours later, animals were sacrificed, organs, such as, tumors, blood, liver, spleen, stomach, kidneys, small intestine, lung, heart, tail, fat, and muscle, were collected. The radioactivities of those organs were measured by gamma counter (Perkin Elmer, Wizard² 2480).

[00111] SPECT/CT imaging: 150 µl of Indium-111 (with 300µCi radioactivity) labeled compound **7a** was injected to BT-20 tumor-bearing animals intravenously. 24

hours later, SPECT/CT images were taken with a triple modality microPET-SPECT-CT imaging device (Triumph, GE Healthcare).

Example 2

Diffusion Molecular Retention (DMR) Technique

5 **[00112]** The molecularly targeted delivery of toxic “payloads” to tumors is limited by low tumor blood flow, capillary permeability barriers, high interstitial pressure, and kidney, liver and spleen uptake. We demonstrated a technique termed Diffusion Molecular Retention (DMR) that comprises peritumorally injecting a fluorescent peptide probe, visualizing probe extensive probe diffusion through the interstitium by fluorescence, and obtaining retention if the probe encounters a molecular target. 10 DMR employs peptide probes that by virtue of their PEGylation achieve a volume of 25 kDa, and therefore have a slow vascular uptake, as well as an absence of non-specific binding to components of the interstitium. To demonstrate DMR, a trifunctional RGD probe bearing a DOTA, a 5 kDa PEG and fluorochrome was 15 synthesized and interstitial diffusion visualized by surface fluorescence. A control RAD probe was not retained, indicating retention of the RGD probe was due to integrin binding. With DMR and a [¹¹¹In] RGD probe, SPECT-CT indicated a highly specific tumor uptake, with tumor concentrations of 390% ID/gm (percentage injected dose per gram) compared to only 4% ID/gm by intravenous administration. DMR 20 could be used to visualize tumor margins by intraoperative fluorescence or to deliver high doses of radiotoxic metals to invasive but non-metastatic tumors. Given the difficulties encountered with high efficiency molecular delivery of diagnostic or therapeutic “payloads” to solid tumors by intravenous administration, the DMR technique might be evaluated in a variety of settings.

25 **[00113]** Figure 7a summarizes the results of an intravenous injection of a peptide or antibody probe binding a molecular target expressed on a tumor and normal tissues where high probe concentrations occur in the liver, kidney or other organs (and resulting in dose-limiting toxicities). Normal organ accumulation can be a target mediated (e.g. RGD probes binding integrins expressed in normal tissues) or 30 a non-target mediated (non-specific) accumulation.

[00114] Here we present an alternative to intravenous administration called

Diffusion Molecular Retention (DMR), that increases the fraction of an injected probe retained by a tumor due to molecular interactions. DMR comprises (see Figure 7b) of a peritumoral (PT) injection of a PEGylated fluorochrome and chelate bearing probe, observing probe diffusion through the interstitium by fluorescence, and
5 obtaining probe retention if the probe encounters a molecular target to which it binds. Potential applications of DMR include the delivery of NIR fluorochromes to tumors for intraoperative margin delineation and the delivery of radioisotopes (e.g. toxic, short range alpha emitters) to tumors for radiotherapy.

[00115] To illustrate DMR, we synthesized multifunctional integrin binding RGD
10 and control RAD probes as in Example 1 above. Integrin specificity of the RGD probe cells or tissues was taken as the difference in binding of RGD and RAD probes, which differ in a 15 dalton methyl group out of total mass of about 8000 daltons, see Table A below. Synthesis employed a multifunctional reagent module consisting of a peptide scaffold, and DOTA, CyAL5.5 fluorochrome and 5 kDa PEG
15 functional groups. The multifunctional reagent module in peptide notation can be written as (DOTA)Lys(CyAL5.5)-Lys(5 kDa PEG)- β ala-Lys(N₃). The reagent module was the reacted with RGD or RAD peptides bearing a short PEG spacer and terminal dibenzylcyclooctyne (DBCO) group, using a copperless click reaction. DOTA was used to chelate ¹¹¹In³⁺ for SPECT-CT and quantitative biodistribution studies, while
20 the CyAL5.5 fluorochrome was used to visualize diffusion from the peritumoral injection site by surface fluorochrome. The 5 kDa PEG endowed the RGD or RAD probes with a volume of 25 kDa, similar to that of small proteins (e.g. Fv =12 kDa, scFV = 25 kDa), since PEG's assume far greater volumes in solution than suggested by their molecular weight. The 5 kDa PEG creates a diffuse cloud that blocks
25 RGD/integrin mediated interactions. Physical properties of RGD and RAD probes are summarized in Table A.

Table A: Physical Properties of Integrin Targeted and Control Probes

	Target Peptide	MW (Da)	MS Obs. (Da)	Equiv. Vol. (kDa)
RGD Probe	RGD	7987.4	7980 (peak)	25
RAD probe	RAD	8001.4	8000 (peak)	25

[00116] The diffusion and elimination of the non-integrin binding RAD probe after an intramuscular administration in the front extremity of a nude mouse was visualized. Using surface fluorescence, the probe rapidly diffused through the extremity and shoulder of the mouse, with vascular uptake and renal elimination evident from bladder fluorescence at 4 hours post injection. By 24 hours post injection, detectable fluorescence was not found, indicating clearance from the injection site.

[00117] The diffusion and molecular retention required of the DMR technique with a tumor bearing model were investigated. We peritumorally injected the RGD probe into an animal bearing two GFP expressing BT-20 breast carcinomas and monitored tumor GFP fluorescence and probe fluorescence as a function of time after injection. The non-integrin binding RAD probe was injected into a second animal also bearing two tumors. Overlaying purple probe fluorescence over green GFP yields white. The BT-20 cell line binds RGD peptides and antibodies to the $\alpha_v\beta_3$ integrin. Both probes rapidly diffused from their injection sites, surrounding the tumor within 10 minutes of the injection. The RGD probe was retained by the tumor while the RAD probe was cleared by 24 hours post injection. Tumor surface fluorescence from both probes was quantified by the use of solution standards. With the RAD probe tumor fluorescence at 24 hours post injection was not observed, indicating that the fluorescence retained at 24 hours with RGD was due to molecular interactions with RGD binding integrins.

[00118] Figure 8 shows tumor targeting by DMR by using the GFP expressing BT-20 breast carcinoma xenograft by surface fluorescence. In Figure 8a), two animals bearing two tumors were PT injected with the RGD probe or RAD probe as indicated and surface fluorescence images were obtained. With the RAD injected animal, tumors were more sagittal so two views of the same animal are provided.

Green equaled GFP. Purple equaled probe. White equaled green + purple overlay. The RGD probe diffused around the tumor and is retained while the RAD probe was eliminated. In Figure 8b), quantitation of tumor surface fluorescence after injections of the RGD or RAD probes as above. Surface fluorescence was quantified through the use of standard solutions. Only the RGD probe was retained by the tumor. n = 4, values are mean $\pm$ 1 SEM.

[00119] To compare the DMR and intravenous (IV) methods, surface fluorescence images of tumor GFP and RGD probe fluorescence were obtained with skin removed, and overlays from the two signals obtained. With both DMR and IV administration, probe fluorescence extended beyond tumor GFP margins to a stromal area beyond the tumor. However, tumor fluorescence was far higher with DMR than IV injection, even though dose was far lower (50 pmoles/mouse by DMR versus 2000 pmoles/mouse by IV).

[00120] Figure 9 shows the efficiency of tumor targeting by DMR or IV methods. In Figure 9a), skin covering GFP-BT-20 tumor was removed. Shown are visible GFP fluorescence, probe (CyAL5.5) fluorescence, and the overlay of GFP and probe fluorescence plus an X-ray image. Green GFP plus purple CyAL5.5 fluorescence yielded a white overlaid image. In Figure 9b), with DMR or IV, probe fluorescence included a stromal zone of integrin binding surrounding the tumor was seen. In Figure 9c), a comparison of tumor surface fluorescence by DMR versus the IV methods is shown. Doses were 50 pmoles (DMR) and 2 nmoles (IV).

[00121] SPECT-CT images were obtained with the ^{111}In labeled RGD probe by the DMR and IV methods. With IV administration, radioactivity was predominant in the liver, kidney and small intestine, with a small tumor radioactivity seen at 2 hours post injection. Radioactivity in the lower abdomen was from the stomach and small intestine based dissection studies. With a single DMR administration, radioactivity was concentrated in the tumor at 2 hours post injection and exclusively in the tumor at 24 hours post injection.

[00122] Figure 10 shows SPECT/CT images after DMR and IV injections with the ^{111}In RGD probe using the BT-20 tumor model. Images after single injections of the ^{111}In -RGD probe by the IV (Fig. 10a) or DMR (Fig. 10b) methods are shown.

Radioactivity is shown with a green to red color scale, while CT bone density is yellow. White arrows show single or double tumors. In Figure 10c), images after dual DMR injections are shown at 24 hours and 48 hours post injection. In Figure 10d), tissue radioactivity concentrations obtained with the ^{111}In -RGD and ^{111}In -RAD probes by DMR. In Figure 10e), radioactivity per organ with the ^{111}In -RGD and ^{111}In -RAD probes by IV is shown. Radioactivity was 0.3 mCi per injection IV and single and dual DMR injection.

[00123] Tissue concentrations were then obtained with the IV and DMR methods using an ^{111}In labeled RGD probe and an ^{111}In RAD probe. With the RGD probe tumor radioactivity was 390% ID/gm by DMR versus 4% ID/gm by IV administration. With both DMR and IV, tumor radioactivity was highly dependent on molecular interactions with integrins. Markedly higher tumor probe concentrations with DMR relative to IV was seen with both fluorescence and radioactive measurements. Tumor fluorescence was 15.0 au (absorbance units) with DMR compared to 1.5 au with IV.

[00124] DMR employs a peritumoral administration, followed by visualizing the high interstitial diffusion that follows with fluorescence, to deliver high levels of an RGD probe to integrins expressed by the BT-20 tumor. To obtain the extensive interstitial diffusion needed for molecular targeting with the peritumoral administration, two conditions must be obtained. First, transport from the interstitial space to the vascular compartment (blood) must be slow, providing the time needed for extensive interstitial diffusion. The 5 kDa PEG increased probe volume to that of a small protein, and conferred a highly hydrophilic character on the probe, both of which slow the rate of interstitial to vascular compartment transport. Second, the probe must not adhere to components of the interstitium, so that complete clearance from the injection site is obtained in the absence of molecular interactions. Probes had blood half-lives (blood fluorescence after IV injection) of 10.7 minutes and underwent predominant renal elimination.

[00125] A variety of minimally invasive injection or local injection techniques might permit peritumoral injection with tumors in a variety of anatomical settings. Local injection techniques are used for sentinel lymph node determination, treating

benign prostatic hyperplasia, treating urinary incontinence, and for stem cell delivery.

[00126] The use of fluorescence to observe probe diffusion after a PT injection is a key feature of DMR. This may permit a determination of probe diffusion with human tumors, which will be larger and which will occur in a wider variety of anatomical locations than those seen with our mouse xenografts. Multiple PT injection sites and modest volumes (0.1 to 0.5 mL) may be employed to enable probes to diffuse through larger and more varied human tumors. Our DMR method in the mouse employed 50 pmoles of probe (3.0 ng as RGD peptide) corresponding to 8.4 µg of peptide for a 70 kg human. With human tumors multiple injection sites with 10 µg of peptide per site might be used with minimal systemic chemotoxicity from the targeting peptide. Dosage could vary from 0.001 µg/kg to 10 µg/kg.

[00127] The modular synthetic strategy (see Example 1) used to obtain DMR probes allows two types of substitutions. First, using this principle we have shown that a variety of fluorochromes, chelates and PEG functional groups can be attached to scaffold peptides for subsequent reactions with a targeting peptide. Second, using the multifunctional reagent employed here, other receptor targeted peptides bearing a single amino functional group might be used as targeting vehicles. A personalized selection of targeting peptide might be based on a histochemical method of determining peptide/receptor in tumor section.

[00128] We have demonstrated the use of DMR as attractive drug delivery alternative to IV injection. These principles are peritumoral injection, visualization of the extensive interstitial diffusion by fluorescence, and molecular retention. A second important goal was to employ a modular synthetic approach that may permit peptides binding various molecular targets, and delivering a wide variety of “payloads”, to be used. We do suggest that an attractive class of applications for DMR lies with the molecularly targeted delivery of fluorochromes to invasive tumors that are operable only with a high functional loss. Here the greatly reduced amounts of probe used with DMR may reduce costs, particularly prominent with the use of NIR fluorochromes, and reduce the risks of systemic chemotoxicity. With human tissue microarrays, an RGD peptide bound ductal carcinomas (22 of 25) but not normal breast (2 of 10), suggesting a PEGylated, NIR probe bearing an RGD targeting

peptide might be useful in this setting. See, Montet *et al.*, (2006) Enzyme-based visualization of receptor-ligand binding in tissues, *Lab Invest.* 86(5):517-25. A second attractive class of DMR applications is the delivery of therapeutic radioisotopes to invasive, pre-metastatic tumors. Here DMR offers the delivery of high radiation doses to tumors and greatly reduced radiation burdens to normal organs. The DOTA functional group can chelate a range of metals for SPECT or PET (^{111}In , ^{68}Ga) or radiotherapy (e.g. ^{213}Bi , ^{177}Lu , ^{90}Y , or ^{225}Ac . DMR maybe particularly well suited to the delivery of alpha particle emitters, with their high toxicity and short range of action.

[00129] Given the frustrating difficulties encountered with efficient molecular delivery of toxic “payloads” to solid tumors by the IV administration, the DMR technique may find use in selected settings.

Example 3

[00130] A general synthesis of PEG-like fluorochromes on a dipeptide scaffold is shown in the Figure 11, where a Lys-Cys dipeptide is employed. The DOTA-Lys-Cys-NH₂ peptide was made by solid phase synthesis as described in Garanger (2010) “Divergent oriented synthesis for the design of reagents for protein conjugation,” *J Comb Chem.* 12(1):57-64. The DOTA-dipeptide was reacted with IR-783, and then with an NHS (N-hydroxysuccinimide) ester of a PEG (MW’s variable) to yield the compounds shown in Table 3B.

[00131] The reaction of DOTA-Lys-Cys-NH₂ with the thiol reactive IR-783 followed procedures in Garanger above or with slight modifications. A solvent of dry DMSO was employed with 1.5 to 2.5 equivalents of IR-783 per equivalent of dipeptide and DIEPA added (2-4 equivalents DIEPA per equivalent of dipeptide). Typically DOTA-dipeptide amounts were 2-20 μmoles in 0.2 to 2 mL of DMSO. Reaction was overnight at room temperature. The reaction mixture was purified by reverse phase HPLC, which separated unreacted IR-783 from DOTA-Lys-Cys(IR-783)-NH₂. Further purification or determination of volume was by size exclusion FPLC.

[00132] DOTA-Lys-Cys(IR783)-NH₂ is then dissolved in DMSO, with 2 equivalents of an NHS ester of PEG and 6 equivalents of DIPEA. The reaction was

at room temperature for 5 days. Product was purified by reverse phase HPLC separation. Product molecular weight was by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), with product molecular volume by size exclusion FPLC using globular protein standards (GE Healthcare life science AKTApurifier 10).

[00133] Using these procedures and PEG's of different molecular weights, PEG-like NIR fluorochromes of different molecular weights, volumes and pharmacokinetics were synthesized, with results summarized in Table 3B. Blood half-lives were determined by tail injection into mice and measuring serum fluorescence as a function of time. Data (fluorescence versus time) were fit to a single decay constant equation to yield blood half-lives. Human pharmacokinetics will be far slower than that of mice.

Example 4

[00134] Three proposed, general methods (a, b, c) of reacting a PEG and a fluorochrome with an amino acid are shown in Figure 12.

[00135] For Figure 12 a), a mixture of Cy5.5-NHS ester (**1**, 1mmol), 6-azidolysine (**2**, 1mmol), and DIPEA (5 mmol) will be incubated under room temperature in anhydrous DMSO for overnight. TFA (5mmol) will be added to neutralize the DIPEA. After the mixture is diluted with water:acetonitrile (1:1, v/v), the product (**3**) can be isolated by HPLC.

[00136] The powder of **3** (1mmol) and 20KDa-PEG-DBCO (**4**, 1mmol) will be mixed and incubated together in DMSO for overnight. After the mixture will be diluted with water:acetonitrile (1:1, v/v), the product (**5**) will be isolated by HPLC. Additional purification might be obtained by FPLC SEC purification.

[00137] For Figure 12 b), a mixture of Cy5.5-NHS ester (**1**, 1mmol), 6-aminohexanoic acid (**2**, 1mmol), and DIPEA (5 mmol) will be incubated under room temperature in anhydrous DMSO for overnight. TFA (5mmol) will be added to neutralize the DIPEA. After the mixture will be diluted with water:acetonitrile (1:1, v/v), the product (**3**) will be isolated by HPLC.

[00138] The powder of **3** (1mmol) and PEG-amine (1mmol) will be mixed and incubated together in the presence of EDC (2mmol) in DMSO for overnight. After the

mixture is diluted with water:acetonitrile (1:1, v/v), the product (**4**) will be isolated by HPLC. More pure product might be obtained by FPLC SEC purification.

[00139] For Figure 12 c), Fmoc-Lys(Boc)-OH will be attached to Rink Amide MBHA resin (0.15 mmol) with an Fmoc/*t*-Bu strategy using a polypropylene 5-mL disposable syringe fitted with a sintered frit. Coupling reactions will employ 2 equiv. (relative to resin) of *N*- α -Fmoc-protected lysine activated in situ with 2 equiv. of PyBOP and 4 eq. of DIPEA in DMF (10 mL/g resin) for 1-2 hrs. Coupling efficiency will be assessed with trinitrobenzylsulfonyl. *N*- α -Fmoc groups can be removed with a piperidine/DMF solution (1:4) for 4x10 min (10 mL/g resin). The coupling of CyAL5.5 will be overnight with same equivalents as with the other reagents. The deprotected CyAL5.5-Ly(NH₂)-NH₂ intermediate will be released from the solid support with TFA/H₂O/TIS/EDT 88:2:5:5 (twice, 4h, 20 mL/g resin). After the solvent will be evaporated, the residue will be precipitated and triturated with ether. The blue solid will be obtained by centrifugation. The solid will be purified further by preparative HPLC with a C18 column. The PEGylation will be carried out by the incubation for 2 days in anhydrous DMSO in the presence of PEG-NHS (1.5 eq.) and DIPEA (2 eq.). The mixture will be diluted with water:acetonitrile (1:1, v/v), and the product will be isolated by HPLC.

[00140] Table 3C below provides a summary of other proposed Example 4 compounds.

Example 5 - Direct Linkage of PEG and Fluorochrome

[00141] Table 3D below provides a summary of the proposed Example 5 compounds.

[00142] Figure 13 gives two general strategies for reacting fluorochromes and PEG's. One could synthesize a fluorescent compound (**8a**) having a direct linkage of PEG and fluorochrome by reacting an amine reactive methoxy-PEG (mPEG - M_w = 5000) available from Nanocs, Boston, Massachusetts, USA with a Cy5 fluorochrome NHS ester (available from Lumiprobe). Still referring to Figure 13, one could synthesize another fluorescent compound (**8b**) having a direct linkage of PEG and fluorochrome by reacting an alkyne reactive methoxy-PEG (M_w = 5000) available from IRIS Biotech GmbH, Marktreidwitz, Germany with a Cy5.5 fluorochrome azide

(available from Lumiprobe).

[00143] Three proposed, general methods of directly reacting a PEG with a fluorochrome are shown in Figure 14. For Figure 14(a), a mixture of PEG-SH (1 mmol), IR-783 (3 mmol), and DIPEA (3 mmol) will be incubated under room temperature in anhydrous DMSO with the protection by Ar for 3 days. TFA (3mmol) will be added to neutralize the DIPEA. After dilution with water:acetonitrile (1:1, v/v), the product will be isolated by HPLC. A more pure product will be obtained by FPLC SEC purification, if necessary. For Figure 14 (b), a mixture of a fluorochrome-NHS ester (2 mmol), a PEG-NH₂ (1mmol), with DIPEA (2 mmol) will be incubated in anhydrous DMSO for overnight at room temperature. TFA (2 mmol) will be added to neutralize the DIPEA. After the mixture will be diluted with water, acetonitrile (1:1, v/v) and the product will be isolated by HPLC. For Figure 14(c), to a solution of PEG-alkyne (3mmol) and fluorochrome azide (3mmol) in water, will be added to sodium ascorbate (0.3 mmol, 300 μ L of freshly prepared 1 M solution in water), followed by copper(II) sulfate pentahydrate (7.5 mg, 0.03 mmol, in 100 μ L of water). The mixture will be stirred vigorously overnight and purified with HPLC with C18 column.

[00144] Tables 3A to 3D provide a summary of passively targeted PEG-like fluorochromes relevant to the intravenous diagnostic method.

Table 3A: PEG-like Fluorochromes for Passive Targeting Intravenous Diagnostic Method PEG and Fluorochromes attached to tetrapeptides					
Compound	M _w , (kDa)	Molecular Volume (kDa)	Quantum Yield	Wavelength Absorption/ Emission (nm.)	Comment
CyAl5.5 Fluorochrome			0.08	675/695	Fluorochrome Reference
DOTA-Lys(CyAL5.5)-Lys-βAla- Lys(N ₃)-NH ₂ (See 5a of Example 1)	1.667	<1	0.061	675/695	No PEG Control
DOTA-Lys(CyAL5.5)- Lys(5 kDa PEG)- βAla - Lys(N ₃)-NH ₂ (See 5b of Example 1)	6.805	<1	0.17	675/695	Passive Targeting, Fluorochrome PEG Shielded
DOTA-Lys(CyAL5.5)-Lys- βAla - Lys(sPEG-RGD), (See 6a of Example 1)	2.848	<1	0.082	675/695	No PEG Control
DOTA-Lys(CyAL5.5)-Lys- βAla - Lys(sPEG-RAD), (See 6b of Example 1)	2.862	<1	0.081	675/695	No PEG Control
DOTA-Lys(CyAL5.5)- Lys(5 kDa PEG)- βAla - Lys(sPEG-RAD), (See 7b of Example 1)	8.002	20	0.20	675/695	Passive Targeting, Fluorochrome PEG Shielded

Table 3B: PEG-like Fluorochromes for Passive Targeting Intravenous Diagnostic Method PEG and Fluorochromes attached to dipeptides					
Compound	M _w , (kDa)	Molecular Volume FPLC (kDa)	Quantum Yield	Wavelength Absorption/ Emission (nm.)	Serum half- life, min (mouse, IV)
ICG Fluorochrome (Fluorochrome reference)	0.775		0.041		
IR 783 Fluorochrome (Fluorochrome reference)	0.748		0.043	782/795	
DOTA-Lys-Cys(IR783)-NH ₂ (Example 3) (No PEG Control)	1.329	0.600	0.053	792/807	
DOTA-Lys(PEG 2kDa)- Cys(IR783)-NH ₂ (Example 3)	3.341	11.2	0.15	789/810	1.4
DOTA-Lys(PEG 5kDa)- Cys(IR783)-NH ₂ (Example 3) (Passive Targeting, Fluorochrome PEG Shielded)	6.290	35.2	0.17	789/810	13.1
DOTA-Lys(PEG 10kDa)- Cys(IR783)-NH ₂ (Example 3)	11.405	100.7	0.17	789/810	
DOTA-Lys(PEG 20kDa)- Cys(IR783)-NH ₂ (Example 3)	21.283	262.5	0.17	789/810	17.6
DOTA-Lys(PEG 40 kDa)- Cys(IR783)-NH ₂ (Example 3) (Passive Targeting, PEG Shield)	44.995	690	0.18	789/809	

5

Table 3C: PEG-like Fluorochromes for Passive Targeting Intravenous Diagnostic Method PEG and Fluorochromes attached to Amino Acids					
Compound	M _w , (kDa)	Molecular Volume (kDa)	Quantum Yield	Wavelength Absorption/ Emission (nm.)	Comment
DOTA-Lys(PEG 40 kDa)- Cys(FI)-NH ₂ (Example 4)	43.581	690	0.60	490/510	Passive, Fluorochrome PEG Shielded
NH ₂ -Cys(IR-783)-OH (Example 4)		<2	<0.10	780/800	No PEG Control
5 kDa PEG-Cys (IR-783)-OH (Example 4)		>20	> 10	780/800	Passive, Fluorochrome PEG Shielded

FI = fluorescein

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Table 3D: PEG-like Fluorochromes for Passive Targeting Intravenous Diagnostic Method Direct linkage PEG and Fluorochrome					
Compound	M _w , (kDa)	Molecular Volume (kDa)	Quantum Yield	Wavelength Absorption/ Emission (nm.)	Comment
8a in Figure 13 (Example 5)	2-40	20-400	>0.10	NIR Range	
8b in Figure 13 (Example 5)	2-40	20-400	>0.10	NIR Range	

5 **[00145]** Table 4 provides a summary of actively targeted PEG-like fluorochromes relevant to the Diffusion Molecular Retention (DMR) method.

Table 4: PEG-like Fluorochromes Active Targeting DMR method					
Compound	M _w , (kDa)	Molecular Volume (kDa)	Quantum Yield	Wavelength Absorption/ Emission (nm.)	Comment
DOTA-Lys(CyAL5.5)- Lys(5 kDa PEG)- βAla - Lys(sPEG-RGD), (See 7a of Example 1)	7.987	20-30	0.20	675/695	Active Targeting, Fluorochrome PEG Shielded
DOTA-Lys(CyAL5.5)- Lys(5 kDa PEG)- βAla - Lys(sPEG-RAD), (See 7b of Example 1)	8.002	20-30	0.20	675/695	Targeting control, Fluorochrome PEG Shielded

Example 6 - Synthesis of PEG-like Nanoprobes

10 **[00146]** In this Example, we prepared multimodal, pharmacokinetically and optically tunable nanomaterials.

Materials and Methods

15 **[00147]** Protected L-amino acids, PyBOP and Rink Amide MBHA resin were from Novabiochem (EMD Biosciences). Other special chemicals were from other sources: DOTA(CO₂Bu^t)₃ (Macrocylics), mPEG-NHS ester (2-30 kDa from Creative PEGworks; 40 kDa from NOF corporation, Japan). The fluorescent dye IR-783 was purchased from Sigma-Aldrich, fluorescein-5-maleimide was from Thermo Scientific, and Cy3-maleimide was from Lumiprobe. All the other solvents and chemicals were from Sigma-Aldrich.

[00148] The synthesis of PEG-like nanoprobe (PN's) involves three steps: (i) synthesis of the (DOTA)Lys-Cys peptide (see Figure 15), (ii) reaction of thiol reactive fluorochrome to the cysteine thiol (see Figures 16, 17, and 18) and, (iii) reaction of an NHS-ester of PEG with variable molecular weight to the lysine side chain (see Figures 19, 20 and 21).

[00149] (i) Synthesis of the (DOTA)Lys-Cys peptide (see Figure 15): The DOTA(CO₂Bu^t)₃-Lys(Boc)-Cys(Trt) peptide was manually synthesized on Rink Amide MBHA resin (0.15 mmol) with an Fmoc/t-Bu strategy using a polypropylene 5 mL disposable syringe fitted with a sintered frit. Coupling reactions employed 2 equiv. (relative to resin) of Fmoc-protected amino acid activated in situ with 2 equiv. of PyBOP and 4 equiv. of DIPEA in DMF (10 mL/g resin) for 1-2 hrs. Coupling efficiency was assessed with picrylsulfonic acid. Fmoc groups were removed with a piperidine/DMF solution (1:4) for 4x10 min (10 mL/g resin). The coupling of DOTA was overnight with same equivalent of other reagents. (DOTA)Lys-Cys was released from the solid support with TFA/H₂O/TIS/EDT 88:2:5:5 (twice, 4 h, 20 mL/g resin). The residue was precipitated and triturated with cold ether. A white solid could be obtained by centrifuge. The solid was purified further by HPLC with column: Higgins Analytical Inc., Cliepus C18 10μm, 250x20 mm; gradient: 20% - 100% B (0.1% TFA and 9.9% water in acetonitrile) in 15 minutes, back to 20%B in 5 minutes, and isocratic for 5 minutes. A white powder of compound (DOTA)Lys-Cys was obtained after lyophilization with a yield of 40%. For (DOTA)Lys-Cys, theoretical MW = 634.75, found MW (M+1) = 635.57.

[00150] (ii) Synthesis of (DOTA)Lys-Cys(FL) peptides where FL can be IR-783, Cy3 or Fluorescein. See Figures 16-18: With all three fluorochromes, the molar ratio of (DOTA)Lys-Cys to fluorochrome 1:1.2. Reaction with IR783 was in DMF, under argon, at room temperature for 15 hours, with 6 equiv of DIPEA (see Figure 16). The reaction with Cy3-maleimide (see Figure 17) or Fluorescein-maleimide (see Figure 18) was in DMSO at room temperature for overnight. Products were purified with reverse phase HPLC with a C18 column. The yield of each was around 45% with respect to the starting quantity of fluorochrome. For (DOTA)Lys-Cys(IR-783), theoretical MW = 1325.6, found MW = 1325.8. For (DOTA)Lys-Cys(Cy3), theoretical

MW = 1213.6, found MW = 1213.8. For (DOTA)Lys-Cys(Fluorescein), theoretical MW = 1061.4, found (M+1) = 1062.6.

[00151] (iii) Reaction of the (DOTA)Lys-Cys(FL) with NHS esters of PEG, see Figures 19-21. To a solution of (DOTA)Lys-Cys(fluorochrome) in anhydrous DMSO, was added the solution of PEG-NHS in anhydrous DMSO. The molar ratio of (DOTA)Lys-Cys(fluorochrome) and PEG-NHS was 1:2. After about 6 equiv. of DiPEA was added, the reaction mixture was incubated at room temperature for 7 days. Purification was first by a reverse phase HPLC (C18 column) with gradients as described in (i) to remove low molecular weight impurities from the synthesis and to obtain an exchange to an aqueous solvent. After lyophilization a second purification was by FPLC, which removed traces of non-PEGylated peptides, see Figure 27. PN's were concentrated and desalted with a C18 cartridge (Sep-Pak cartridge, Waters, Milford, MA, USA), eluting with acetonitrile and drying by lyophilization. The yield based on starting (DOTA)Lys-Cys(FL), was about 50%.

[00152] PN and peptide characterization: The mass spec of low molecular weights (MW) were obtained by MS-ESI Micromass (Waters) and high MW molecules were determined through MALDI-TOF analyses at the Tufts University Core Facility. RP-HPLC (Varian ProStar detector and delivery modules) employed an eluant A (0.1% TFA /water) and eluant B (0.1% TFA and 9.9% water in acetonitrile). Probe size (volume) was determined by FPLC using an ÄKTA Purifier 10 and SuperdexTM 200 10/300GL column (GE Healthcare) with a running buffer of 0.05 M sodium phosphate, 0.15 M NaCl (0.1% Tween, pH 7.2) and flow rate of 0.8 ml/min. Standards (GE Healthcare) were Ferritin, Ribonuclease A, Carbonic Anhydrase, and Conalbumin and Blue Dextran 2000. To obtain probe volumes, Mr (apparent molecular weight based on size exclusion retention) was plotted versus Kav. $K_{av} = (V_e - V_o) / (V_t - V_o)$, V_t = total volume, V_e = elution volume, V_o = void volume.

[00153] Purity of materials made: The four peptides used (see Table 5) were characterized by mass spectroscopy. The use of FPLC to remove low molecular weight peptide is shown in Figure 27a. FPLC's of the purified PN's are shown in Figure 23. The mass spec of PN(783)4.3, the peptide (DOTA)Lys(PEG 5 kDa)-

Cys(IR-783), is shown in Figure 27b.

[00154] Radiolabelling of PN(783)10.0. See Figure 28: $^{111}\text{InCl}_3$ (9.43 mCi)

(Nordion, Canada) was diluted with HCl (50 μl , 0.05N) into a total volume of 80 μl and was transferred into a conic reaction vial which contained PN(783)/10.0 (20 nmol) in HEPES buffer (1 M, pH 5, 1 ml). The reaction vial was incubated on a preheated heating blot under 70°C for 45 minutes while it was shaken every 10 minutes. Then the vial was cooled down to room temperature in ice water for 5 minutes. EDTA (70 mM, 100 μl , 7 mmol) was added and well mixed. The solution was stayed at room temperature for 15 minutes. After the solution was diluted with water (0.1% TFA) (1:10 v/v), the compound was loaded on a C18 cartridge preconditioned with ethanol (1 ml, 0.1% TFA) and water (3 ml, 0.1% TFA) (Strata-X 33u, Polymeric reverse Phase Phenomenex, 30 mg/3 ml, 8B-S100-TBL). The cartridge was washed with water (Millipore, 0.1% HOAc) (2ml) and purged by air with a syringe. The labeled compound was collected by eluting with acetonitrile (0.1% TFA) (0.4 ml) into a new reaction vial. The acetonitrile and TFA were removed by evaporation under N_2 flow. The final product (4.06 mCi) was reconstituted with PBS buffer for mouse injection. The radioactive product was confirmed to be free of low molecular weight forms of indium by HPLC with cold internal standard with C18 column. (Gradient: 10% B to 100% B in 20 minutes, back to 10% in 5 minutes, and isocratic for 5 minutes; Abs: 783 nm; flow: 5 ml/min; Column: Higgins Analytical Inc. Proto 300 C18 5 μm , 250X10 mm, P/N: CS-2520-C185). RCY: 43%; specific activity: 0.4 Ci / μmole .

[00155] Quantum yield: Quantum yields were determined as described in Demas *et al.*, "Measurement of photoluminescence quantum yields. Review", *Journal of Physical Chemistry* 75, 991-1024 (1971), and Shao *et al.*, "Facile Synthesis of Monofunctional Pentamethine Carbocyanine Fluorophores. *Dyes and pigments : an international journal* 90, 119-122 (2011). For IR-783 a reference quantum yield of 0.043 was used (see Li *et al.*, "Synthesis and characterization of glucosamine-bound near-infrared probes for optical imaging", *Organic letters* 8, 3623-3626, 2006); for fluorescein a reference quantum yield was 0.18 (see Sjoback *et al.*, "Absorption and florescence properties of fluorescein", *Spectrochimica Acta Part A* 51, L7-L21, 1995). For Cy3 a reference quantum yield of 0.31 was used (Luminprobe Inc). For PN(783)'s,

excitation was at 730 nm and emission spectra were recorded from 765 nm to 870 nm in PBS and maximum emission used. For PN(545)'s, excitation was at 515 nm and emission spectra were recorded from 538 nm to 700 nm in PBS and maximum emission used. For PN(497)'s, excitation was at 450 nm and emission spectra were recorded from 475 nm to 620 nm in PBS and maximum emission used. Absorbance of each probe was adjusted less than 0.1. Measurements were made in triplicate and are expressed as mean $\pm$ SD.

[00156] PN and peptide binding to cells (effect of PEGylation on NSB): HT-29, a human colon carcinoma cell line, was from the American Tissue Culture Collection and maintained according to their instructions. Cells were seeded on 24-well plates at 5×10^5 cells/well in culture medium (RPMI 1640 with 10% FBS) the day before the assay. The day of assay, medium was removed, wells rinsed twice with DPBS (+Ca, +Mg), and 100 μ L of 2% FBS / DPBS (+Ca, +Mg) added. 100 μ L of Nanoprobes (2 μ M) in DPBS (+Ca, +Mg) was added to cells and incubated for 30min at 37°C. (Probe concentrations were determined spectrophotometrically (783 nm, extinction coefficient of $314\,471\text{ cm}^{-1}\text{ M}^{-1}$ for IR-783; 497 nm, extinction coefficient of $68\,000\text{ cm}^{-1}\text{ M}^{-1}$ for Fluorescein; 545 nm, extinction coefficient of $150\,000\text{ cm}^{-1}\text{ M}^{-1}$ for Cy3). Cells were detached by Trypsin/EDTA and assayed for fluorescence by FACS (BD 7 laser LSR2 for nanoprobes with IR-783; BD 3 laser LSR2 for nanoprobes with Fluorescein or Cy3).

[00157] Circulating form of PN's: 20 nmoles of PN(783)4.3, PN(783)6.1, or PN(783)10.0 was injected (IV, tail vein) into nude mice (female; 25-30 g; 6-8 weeks old; nu/nu). At the indicated time, 50 μ L of blood was collected with microhematocrit capillary tube (Fisher Scientific) from the tail, and transferred to Eppendorf microcentrifuge tube with anticoagulant (EDTA) coating (Fisher Scientific). Tubes were centrifuged (5000 rpm for 5 minutes), and the supernatant was injected to the FPLC, a ÄKTA Purifier 10 with SuperdexTM 200 10/300GL column.

[00158] PN pharmacokinetics: Groups of 5 nude mice (female; 25-30g; 6-8 weeks old; nu/nu) were injected (tail vein, IV) with 10 nmole of PN(783)4.3 or PN(783)10.0. 50 μ L of blood was collected from tail tip at the indicated times. The blood was processed as above, and diluted (25 μ L plasma, 700 μ L of PBS).

Fluorescence was measured with Cary Eclipse Fluorescence Spectrophotometer, excitation at 765 nm and emission from 790 to 880 nm. The fluorescence intensity at 806 nm was plotted over time, and the data was fit with two-phase decay curve. The fast and slow distribution half-life was given by the two-phase decay fit with Graphpad Prism software.

[00159] Two compartment model: From the two-phase decay fit, a biexponential equation for blood concentration as a function of time, $C_p = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$, was obtained. By the relation of macro constants and micro constants, $k_{\text{distribution}} = \frac{A\beta + B\alpha}{A+B}$, $k_{\text{elim}} = \frac{\alpha\beta}{k_{\text{distribution}}}$, $k_{\text{system}} = \alpha + \beta - k_{\text{distribution}} = k_{\text{distribution}} - k_{\text{elim}}$, micro constants k 's can be

obtained, and the half-life was calculated by $t_{1/2} = 0.693/k$, as described in

Rosenbaum, S.E. *Basic Pharmacokinetics and Pharmacodynamics: An Integrated Textbook and Computer Simulations*, (John Wiley and Sons, Hoboken, New Jersey, 2011). The curve for interstitium concentration vs. time was fit with MATLAB based on the curve of blood concentration vs. time.

[00160] Whole animal surface fluorescence imaging: A Kodak FX multispectral imaging system was used (Carestream Molecular Imaging, Rochester, NY).

Excitation at multiple wavelengths (620, 650, 690, 710, 720, 730, 750 and 760 nm) with the emission at 830 nm was setup for IR-783 spectrum; Excitation at multiple wavelengths (420, 440, 460, 480, 510, 520, 530, and 540 nm) with the emission at 600 nm was setup for Cy3 spectrum; Excitation at multiple wavelengths (450, 470, 510, 520, 530, 540, 550, 570, and 590 nm) with the emission at 700 nm was setup for mCherry; with manufacturer's software to separate (unmix) the IR-783 spectrum, Cy3 spectrum, or mCherry spectrum from skin autofluorescence and chlorophyll fluorescence from food. X-ray images were taken after fluorescence images.

Animals were anesthetized with 2% isoflurane with O₂ flow (2 l/min) during imaging.

[00161] Tumor surface fluorescence (skin removed): The PN(783)10.0 or PN(545)10.0 (10 nmoles, 100 μ L) was injected (IV, tail vein), the skin around tumor was removed at 48 hours post injection, with tumor visualized as mCherry fluorescence using the Kodak FX.

[00162] HT-29 or mCherry-HT-29 tumor model: Female nude mice (25-30g; 6-8

weeks old; nu/nu) were anesthetized with 2% isoflurane/O₂. HT-29 or mCherry-HT-29 cells were detached, pelleted and 200µl of cell suspension containing 10⁶ cells in Matrigel (BD Bioscience) was injected subcutaneously into right and left shoulders. Tumors were allowed to grow 5-7 days before experiments.

5 **[00163]** SPECT/CT: The imaging was performed by Triumph II multimodality imaging system (Gamma Medica Ideas, LLC) comprising XSPECT with four CZT (Cadmium Zinc Telluride) detectors and X-O CT with CMOS detector. SPECT data of the ¹¹¹In-labeled compound was acquired for 60 minutes using 5-pinhole collimators and processed with 3D-OSEM algorithm using 4 subsets and 5 iterations.

10 3-dimensional CT data was processed with modified Feldkamp software. The processed 3D-images were fused and displayed with VIVID software package installed to the Triumph data management. Animals were under isoflurane anesthesia (1.5%) with O₂ flow (1.5 l/min) and kept warm during the imaging with a heated animal bed.

15 **[00164]** Organ biodistribution of ¹¹¹In-PN(783)10.0: 150 µl of ¹¹¹In-labeled PN(783)10 (400 µCi, ~2 nmole) were injected to tumor-bearing animals by tail vein (IV). 24 hours or 48 hours later, animals were sacrificed, and tumors, blood, liver, spleen, stomach, kidneys, small intestine, lung, heart, tail, fat, and muscle, were collected. Radioactivity was measured with Perkin Elmer, Wizard2 2480 gamma
20 counter.

[00165] Confocal imaging: The mCherry-HT-29 tumor sample was collected at 48 hours post IV injection with PN(497)10.0, and then cryosectioned with thickness of 5 µm. The tumor section was fixed with 4% PFA, mounted with 90% glycerol/10% PBS (at pH 8.5 for best fluorescein fluorescence), and stained with DAPI. Confocal
25 imaging was performed on a Zeiss LSM510 laser scanning confocal microscope (Zeiss Axiophot, Carl Zeiss, Jena, Germany). A 405 nm diode Laser, 488 nm argon laser, and 561 nm diode laser were used for the excitation of DAPI, fluorescein, and mCherry, respectively. A primary dichroic HFT 405/488/561 was used in combination with an LP420 emission filter for DAPI, BP505-530 for fluorescein, and LP575 for
30 mCherry. Images were analyzed with ImageJ64.

[00166] Brain vascular phase imaging (angiography): Craniotomies in C57Bl/6J

wildtype mice (from Jackson Laboratory, Bar Harbor, Maine, USA, 3-4 months old) were performed with minor modifications (see Skoch *et al.*, "In vivo imaging of amyloid-beta deposits in mouse brain with multiphoton microscopy", *Methods in molecular biology (Clifton, N.J.)* **299**, 349-363, 2005). To summarize, animals were anesthetized using 2% isoflurane in balanced oxygen, and then a 5 mm diameter skull flap was removed. A craniotomy was performed, and the exposed brain area was covered by a 8 mm round glass coverslip, which was sealed to the skull with dental cement (see Spires-Jones *et al.*, "Monitoring protein aggregation and toxicity in Alzheimer's disease mouse models using in vivo imaging", *Methods (San Diego, Calif.)* **53**, 201-207, 2011; and Fukumura, *et al.* "Tumor induction of VEGF promoter activity in stromal cells", *Cell* **94**, 715-725, 1998). This procedure allowed a transparent window into the mouse brain for use with in vivo microscopy of the cerebrovasculature. Mice were allowed 2-3 weeks for complete recovery after the craniotomy prior to imaging.

[00167] For imaging, mice were anesthetized with 2% isoflurane in balanced oxygen and secured in a custom stereotaxic frame, which fit into the microscope stage. The cerebrovasculature was imaged using the Olympus FluoView FV1000MPE multiphoton laser-scanning system mounted on an Olympus BX61WI microscope (Olympus, Tokyo, Japan). A DeepSee Mai Tai Ti:sapphire mode-locked laser (Mai Tai; Spectra-Physics, Fremont, CA) produced two-photon fluorescence with 800 nm excitation. The vessels were imaged at depth of 45 to 100µm from the surface of the brain.

[00168] 2 nmole of PN(497)/10.0 probe (300-400µl) was injected retro-orbital into the anesthetized mouse. A time course was taken for up to 70 minutes post injection. Images were acquired using the Fluoview software and analyzed using ImageJ.

[00169] Imaging tumor interstium: Dorsal skinfold chamber (DSFC) tumors were grown in female nude mice (nu/nu; 25-30g; 6-8 weeks old) with modifications from previously published techniques (see Fukumura *et al.*, "Tumor induction of VEGF promoter activity in stromal cells", *Cell* **94**, 715-725, 1998; and Marangoni *et al.*, "The transcription factor NFAT exhibits signal memory during serial T cell

interactions with antigen-presenting cells", *Immunity* 38, 237-249, 2013). 10^6 mCherry-HT-29 tumor cells in matrixgel (BD) were subcutaneously injected in the back of mice ~ 1.5 cm left of the dorsal midline approximately halfway from the neck to the tail base. 4 days later, DSFCs were installed in a way that the tumors were centered in the imaging window of the chamber and accessible to longitudinal investigation by MP-IVM. On days 2, 3, and 4 days after tumor DSFC implantation, when tumors were typically 3 mm in diameter, image stacks of tumor tissue were recorded under general anesthesia with Ketamine and Xylazine. 100 μ l (10 nmole) of PN(497)10.0 was injected (IV, tail vein).

[00170] Multiphoton excitation was obtained through DeepSee and MaiTai Ti:sapphire lasers (Newport/Spectra-Physics) tuned to 920 and 1000 nm to excite all fluorescent probes used. Stacks of 11 square optical sections with 4 μ m z-spacing were acquired every 20 seconds on an Ultima IV multiphoton microscope (Prairie Technologies) using a 20X/0.95 NA lens with optical zoom of up to 1x to provide image volumes 30 μ m in depth and 200 μ m in width. Emitted fluorescence was detected through 460/50, 525/50, 595/50, 660/40 band-pass filters and non-descanned detectors to generate four-color images. Sequences of image stacks were transformed into volume-rendered, time-lapse movies with Imaris software (Bitplane).

[00171] Two compartment model, see Figure 24. Serum fluorescence data from Figure 24b and Figure 24c was analyzed using the two-compartment model as described in Rosenbaum, S.E. *Basic Pharmacokinetics and Pharmacodynamics: An Integrated Textbook and Computer Simulations*, (John Wiley and Sons, Hoboken, New Jersey, 2011). Data were first fit to biexponential equation, yielding values of Alpha, Beta (apparent decay constants) and values of A and B as shown in equations 1 and 2 below.

General Biexponential Equation: $C_p = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$

Equation 1, PN(783)10: $C_p = 1039.5 \exp(-1.361 t) + 595.2 \exp(-0.09903 t)$

Equation 2, PN(783)4.3: $c_p = 112.8 \exp(-5.301 t) + 161 \exp(-0.425 t)$

By the relation of macro constants and micro constants, $k_{vas return} = \frac{\alpha + \beta}{2}$,

$k_{elim} = \frac{\alpha \beta}{k_{vas return}}$, $k_{perm} = \alpha + \beta - k_{vas return} - k_{elim}$, micro constants k 's can be obtained,

and the half-life was calculated by $t_{1/2} = 0.693/k$, see above

Table S1: Summary of constants for PN(783)4.3 and PN(783)10.0 obtained with The two-compartment model				
Constant	PN(783)4.3 rate	PN(783)4.3 half-life	PN(783)10.0 rate	PN(783)10.0 half-life
Alpha	5.30 h ⁻¹	0.13 h	1.36 h ⁻¹	0.51 h
Beta	0.42 h ⁻¹	1.64	0.099 h ⁻¹	7.0 h
¹¹¹ In-PN(783)10 elimination*				7.8 h
k perm	1.74 h ⁻¹	0.40 h	0.67 h ⁻¹	1.02 h
k vas return	3.30 h ⁻¹	0.20h	0.56 h ⁻¹	1.23 h
k elim	0.68 h ⁻¹	1.01h	0.24 h ⁻¹	4.2 h

*See figure 26e.

Overview of Example 6

[00172] PEG-like Nanoprobes (PN's) are pharmacokinetically and optically tunable materials whose disposition in biological systems can be determined by fluorescent or radioactive imaging modalities. PN's are synthesized by attaching different fluorochromes and PEG polymers of different molecular weights to a (DOTA)Lys-Cys dipeptide scaffold, yielding PN's with different sizes, pharmacokinetics, and excitation and emission maxima. PN's exploit the PEG-fluorochrome shielding effect, where PEG polymers are used to block the interactions of fluorochromes with each other or biomolecules. PN's were used to image brain capillaries (2-photon microscopy), tumor capillary permeability (intravital microscopy), and the tumor EPR effect (¹¹¹In-PN) by SPECT imaging. DOTA provides a radiolabeling option that not only allows SPECT imaging, but allows ready determination of PN biodistribution and elimination. ¹¹¹In-PN with a diameter of 10 nanometers exhibited a combination of a long circulation time and low whole body

retention, with a low hepatic uptake (despite being nearly double the 5.4 nm of albumin), and virtually no kidney retention (despite employing dipeptide scaffold). PN's provide a unique combination of pharmacokinetic tunability (through PEG selection), spectral tunability (through fluorochrome selection) and easy radiolabeling (DOTA chelation). PN's offer a simple and superior chemistry for obtaining passively targeted, pharmacokinetically tunable fluorochromes and/or radiometals.

[00173] In Example 6, we introduce passively targeted, fluorescent and/or radioactive nanomaterials with PEG-determined sizes in the nanometer range and termed "PEG-like Nanoprobes" (PN's). PN's are synthesized by attaching different fluorochromes and different PEG polymers to a (DOTA)Lys-Cys dipeptide scaffold, yielding PN's with different sizes, pharmacokinetics, and excitation and emission maxima. PN's are based on the discovery that PEG's (MW>5 kDa), when covalently linked to fluorochromes, block the interactions of fluorochromes with each other and blocking their interactions with biomolecules and cells. (See Guo *et al.* (2012) "PEG-Fluorochrome Shielding Approach for Targeted Probe Design," JACS). PN's achieve spectral flexibility by endowing different fluorochromes with PEG-like rather than fluorochrome-like behavior in vitro and in vivo. In Example 6, we show how PN's can employ a modular design approach, with a fixed scaffold adorned by a variable fluorochrome and a variable PEG, an approach which yields pharmacokinetic and spectral flexibility, a large number of potential uses (fluorescent and radioactive imaging), and a high potential for clinical safety.

[00174] Although a wide range of approaches has been explored for obtaining passive, non-receptor mediated fluorochromes or radiometals, all have important limitations. Novel nanomaterials (e.g. nanoshells, carbon nanotubes, dendrimers, quantum dots) suffer from a lack of knowledge about their toxicity and/or elimination and a lack of clinical history. Fluorescent dextrans have been widely used. However, dextrans induce histamine release in rodents, altering capillary permeability. Clinical use of dextrans is complicated by anti-dextran antibodies and dextran induced anaphylaxis. In contrast, PEG polymers employed by PN's have little if any immunogenicity and are widely recognized as safe due to their extensive use in parenteral pharmaceuticals.

[00175] As a carrier for the passive delivery of diagnostic agents albumin is not ideal because of an albumin receptor, and because modified albumins can be recognized as abnormal versions of normal albumin and cleared by scavenger receptors. Reversible complexation with albumin provides another general technique for obtaining passively targeted, long-circulating diagnostic agents. Albumin complexes with (ICG) or dyes (Evans Blue) before or after injection. However, the reversibility means transcapillary passage and interstitial accumulation can be due to the slow transport of the major albumin-bound form or a fast passage of the minor, low molecular weight species. Albumin-based approaches, whether covalent or reversible complexation, cannot be used to understand the size dependence of biological processes preclinically, or permit optimization of size and pharmacokinetics for clinical uses.

Example 6 Results

[00176] PEG-like Nanoprobes (PN's) employ a modular synthetic strategy (see Figure 22a) where a variable, fluorochrome is reacted with the cysteine thiol of a (DOTA)Lys-Cys peptide (see Figures 15-18). (All peptides are C-terminal amides with the final $-NH_2$ omitted.) Peptides are denoted (DOTA)Lys-Cys(FL), where FL is a fluorochrome: IR-783, Cy3 or fluorescein. (DOTA)Lys-Cys(FL) peptides are then reacted with NHS esters of a PEG polymer of different molecular weights (see Figures 19-21). PN's and their properties are summarized in Figure 22a and Table 5. DOTA provides a radiolabeling option the value of which is explained below.

[00177] Two nomenclatures are employed, a PN nomenclature and a peptide nomenclature. PN(783)4.3, (column 1 of Table 5) indicates a PEG-like Nanoprobe with an absorption maxima of 783 nm and hydrodynamic diameter of 4.3 nm. With peptide nomenclature PN(783)4.3 is (DOTA)Lys(PEG 5 kDa)-Cys(IR783), see column 2 of Table 5.

[00178] Key features of PN's are summarized in Figure 22b. Since PEG's are extended, water infiltrated structures with solution diameters are far larger than their molecular weights, the PEG polymer confers nanometer sizes upon PN's. For example, reaction of the (DOTA)Lys-Cys(IR-783) peptide (diameter = 1.1 nm), with a 5 kDa PEG yielded PN(783)4.3 (diameter = 4.3 nm). Hence the PEG-based

expansion of volume of was 59 fold, (4.3/1.1).

[00179] Attachment of PEG increased the quantum yields of fluorochromes as shown in Figure 29 and summarized in Table 5. The attachment of a 2 kDa PEG to (DOTA)Lys-Cys(IR-783) (i.e. yielding PN(783)3.0), resulted in significantly less improvement in quantum yield than larger PEG's (see Figure 29); this was scored as incomplete PEG fluorochrome shielding and only PN's made with larger PEG's (PEG = 5 kDa or greater) were studied further. Previous studies have shown that attachment of a 5 kDa PEG's results a loss absorption spectra PBS that are similar to unstacked spectra obtained in methanol, suggesting quantum yield improvement reflects a blockage of fluorochrome/fluorochrome stacking (see Guo *et al.*, "The PEG-Fluorochrome Shielding Approach for Targeted Probe Design," *J Am Chem Soc* 2012, **134**(47): 19338-19341). PEG decreased the non-specific binding to HT29 cells, scored as the percent of cells above the cutoff for unstained cells seen with FACS (Table 5, Figure 30). PEG reduction of binding was not detectable when the (DOTA)Lys-Cys(Fluorescein) peptide was PEGylated, reflecting a lack of nonspecific binding with this peptide and/or a higher intrinsic cell fluorescence at lower wavelengths.

[00180] A dramatic illustration of the effect of attaching a 5 kDa PEG to the (DOTA)Lys-Cys(IR-783) peptide is its ability enhance elimination following an IV injection. As shown in Figure 22c, the resulting PN, PN(783)4.3 PEGylation with a 5 kDa PEG also enhanced the elimination of IM administered peptides. "PEG-like Nanoprobes" employ PEG to decrease fluorochrome/fluorochrome interactions nonspecific interactions with cells and to enhance the elimination of fluorochrome bearing peptides.

[00181] To demonstrate the ability of PEG to tune (vary) PN size, the diameters of PN's synthesized using PEG's of different molecular weights (Figure 22a) and IR-783 were determined by FPLC gel-filtration chromatography (Figure 23a). The column was calibrated with globular protein standards, and globular protein equivalent molecular weights (in kDa) were obtained (Table 5, column 5). These were converted to PN diameters in nm using the relationship: Radius in nm = $0.066M^{1/3}$ where M is the molecular weight of a globular protein expressed in daltons

(see Erickson, "Size and Shape of Protein Molecules at the Nanometer Level Determined by Sedimentation, Gel Filtration, and Electron Microscopy", *Biological Procedures Online* 2009, **11**(1): 32-50). PN's diameters ranged from 3.0 to 11.8 nm (see Figure 23a). Using PEG's of 5 kDa and 30 kDa, selected for their low polydispersity, PN's were synthesized using IR-783, Cy3 and Fluorescein (see Figure 22a) and their sizes determined (see Figures 23a, 23b, 23c). With the 5 kDa PEG and these three fluorochromes (see the magenta chromatograms in Figures 22a, 23b and 23c) PN diameters were 4.3 nm. With the 30 kDa PEG and these three fluorochromes (blue chromatograms, 23a, 23b, 23c), PN diameters were now 10.0 nm. For reference, the diameter of a 67kDa albumin determined by this method was 5.4 nm.

[00182] To see if the tunable size of PN's could be translated into tunable pharmacokinetics, it was essential to first establish that PN's circulated at their variable, PEG-determined pre-injection sizes. FPLC chromatograms of PN(783)10.0, PN(783)6.1 and PN(783)4.3, at their pre-injection sizes and at varying times post injection, are shown in Figures 23d, 23e and 23f. By using PN's absorbing at 783 nm, chromatograms before and after injection (i.e. serum of injected mice) can be compared, since there are no compounds in serum that absorb at this wavelength. With the FPLC chromatograms of PN(783)10.0 (2d), peaks from a pre-injection sample or serum at 21 or 40 minutes were at identical elution volumes, indicating that this PN circulates at its pre-injection size. Similarly, peaks for PN(783)6.1 (see Figure 23e) and PN(783)4.3 (see Figure 23f) were identical for pre-injection samples and for sera from injected mice. Thus molecular weight of the PEG chosen determines PN dimensions and those dimensions are maintained after injection.

[00183] Since PN's circulate at variable PEG-determined sizes, they undergo transcapillary passage as their injected form as shown in Figure 23g. In contrast, albumin-binding compounds used in the determination transcapillary passage exist as albumin bound and free forms. Examples include fluorophores (ICG), chromophores (Evans Blue), and Gd chelates (gadofosveset). PN's are size variable, multimodal nanomaterials for the determination capillary permeability without the uncertainties by presented by albumin bound and free forms in

circulation.

[00184] To assess the relationship between PN dimensions and transcapillary passage, the classic two-compartment pharmacokinetic model (see Rosenbaum, *Basic Pharmacokinetics and Pharmacodynamics: An Integrated Textbook and Computer Simulations*, (John Wiley and Sons, Hoboken, New Jersey, 2011) (see Figure 24a) was applied to PN(783)4.3 and PN(783)10.0, see Figures 24b and 24c. After injection, blood concentrations exhibit an initial fast exponential decay (vascular escape), followed by a slow exponential decay (whole body clearance). The three microscopic rate constants for the two-compartment model are given in Figures 24b and 24c. Further details on the two-compartment model are provided above with summary of all pharmacokinetic constants (see Table S1). The rapid initial fall of PN blood concentration is due to vascular escape, as occurs with fluorescent dextrans of a similar size. Although the circulation times of PN's in mice appear modest, our values are consistent with studies using fluorescent dextrans in mice. Since a 40 kDa dextran has plasma half-life of 10 hours, considerably longer half-lives of PN's are expected if used clinically.

[00185] The concentrations of PN(783)10.0 in the blood and interstitial compartments using the values from Figure 24b are shown in Figure 24d. Three pharmacokinetic phases shown are a vascular phase (approximately for 1 h post injection), an interstitial phase at (at 10-25 h), and an enhanced permeability retention (EPR) based uptake by a tumor at 48 hours. These phases were examined with SPECT imaging and radioactive biodistribution studies in Figure 25 and with fluorescence imaging techniques in Figure 26.

[00186] To demonstrate a multimodal imaging capability, the ability of ¹¹¹In-PN(783)10.0 (or PN(783)10.0) to image the EPR effect of an HT29 tumor was determined by SPECT/CT (see Figure 25a) and surface fluorescence (see Figure 25b). Initially (2 hours post injection) by SPECT or surface fluorescence, PN(783)10.0 was broadly distributed, consistent with PN(783)10's vascular and interstitial distribution seen with the two-compartment model (see Figures 24b, 24d). At 48 hours, PN(783)10.0 was retained in the tumor with SPECT and surface fluorescence, reflecting the high tumor concentrations (7.28±0.93%ID/gm) and the proximity of the

tumor to the surface (see Figure 25c).

[00187] Biodistribution studies with ^{111}In -PN(783)10.0 are shown in Figure 25c and 25d. At 48 hours post injection, $4.71 \pm 0.38\%$ of dose was in the liver (see Figure 25d), despite the fact that with a diameter of 10 nm this PN is considerably larger than albumin (diameter = 5.4 nm). In addition only $0.44 \pm 0.02\%$ of the injected dose was in the kidney, which typically accumulates high levels of radiolabeled peptides due renal peptide transporters. Whole body radioactivity decreased with a half-life of 7.8 hours and was $13.75 \pm 0.74\%$ of injected dose by 48 hours (see Figure 25e).

[00188] To image the vascular phase, PN(497)10.0 was used for two-photon intravital microscopy of brain capillaries (see Figure 26a), since the blood brain barrier blocks interstitial accumulation. Vessel fluorescence decreased as imaging time increased from 10 to 70 minutes, reflecting a decrease in the blood concentration of PN(497)10.0 from transcapillary passage (see Figure 24b). To further examine the vascular and interstitial phases, a dorsal skinfold chamber was used for the intravital, two photon microscopy of an mCherry expressing HT29 tumor (Figure 25b). At ten minutes post injection, PN(497)10.0 (green) was confined to the vasculature at the periphery of the mCherry tumor (red). At 20 hours post injection, the PN was seen in the interstitium at the tumor periphery.

[00189] To determine the cells responsible for the EPR accumulation of PN(783)10.0 seen with SPECT and surface fluorescence (see Figure 25), we employed confocal microscopy of mCherry/HT29 tumor sections (see Figure 26c). PN(545)10.0 was seen in mCherry/HT29 cells, presumably by fluid phase pinocytosis, since PEG does not bind known receptors. The retention of PN(545)10.0 was also evident from surface fluorescence measurements as shown in Figure 26d. With skin removed, an overlay of tumor mCherry (green) and PN(545)10.0 (purple) gave a white superimposition.

Example 6 Discussion

[00190] By using a single PEG polymer of sufficient length (5 kDa or greater), PN's achieve the properties of PEG in vitro and in vivo that enable them to be described as "PEG-like Nanoprobes." In vitro, PEG-like properties include an increased quantum yield, a decreased binding to cultured cells (see Table 5), and the

attainment of sizes in the nanometer size range. In vivo, PEG-like properties include extended circulation times, low hepatic uptake and excellent whole body elimination.

[00191] PN's provide a unique combination of pharmacokinetic tunability and low whole body retention. ^{111}In -PN(783)10.0 had only $4.71 \pm 0.38\%$ of the injected dose in the liver (48 hours post), in spite of the fact that its diameter is nearly twice that of albumin (5.4 nm). ^{111}In -PN(783)10.0 is therefore unlike high molecular weight dextrans, which have extended blood half-lives but undergo eventual hepatic uptake, principally by Kupffer cells. ^{111}In -PN(783)10.0 is unlike the low molecular weight near infrared fluorochrome ICG, which undergoes rapid hepatic clearance as the albumin bound complex. Finally, ^{111}In -PN(783)10.0 is unlike many radiolabeled peptides that are excellent substrates for renal peptide transporters, and which make the kidney the organ of highest tracer concentration and organ of dose limiting toxicity. In contrast, ^{111}In -PN(783)10.0 exhibited a renal retention of only $0.44 \pm 0.02\%$ (48 hours post). Based on their low renal and hepatic accumulation, the model for PN's where PEG shields both the Lys-Cys peptide and the attached fluorochrome (see Figure 22b) is supported.

[00192] At least three potential applications of PN's can be considered. First, the blood half-life control provided by fluorochromes PN's could enable a long duration fluorescent angiography in neurosurgery or reconstructive surgery. ICG, with a blood half-life of 4 minutes, is now used. Here PN's were used for fluorescent angiography in normal brain and the HT-29 tumor (see Figures 25a, 25b). Second, PN's might be used to image tumor the EPR effect (see Figure 26a), resolving the issue of its existence and magnitude with human tumors. Long-circulating nanomedicine therapeutics, liposomes and polymer conjugates, approved or in clinical trials, may utilize the EPR effect in part for their efficacy.

[00193] However, a broad and important class of uses for PN's lies in the determination capillary permeability and endothelial function that can be aberrant in relatively common conditions including diabetes, sepsis and ischemic insult. PN's are ideal for the determination of capillary permeability (by SPECT or fluorescence) because they exist post injection as PEG-determined, size variable, pharmacokinetically tunable materials (see Figure 23). Here the radiolabeling option

may lead to a unique path to clinical development of PN's as fluorescent or radioactive capillary permeability agents, permitting microdose pharmacokinetic studies as a function of PN size (see Figure 24). This would enable selection of a PN with optimal pharmacokinetics for fluorescent capillary permeability imaging.

- 5 Thus PN's exhibit pharmacokinetic tunability, spectral tunability and a radiolabeling option, a combination that has not been achieved with previous nanomaterials used for passive pharmacokinetic targeting. This unique combination of properties and capabilities may lead to their use in various areas of clinical practice.

TABLE 5: SUMMARY OF PROPERTIES OF PEG-LIKE NANOPROBES

PEG-like Nanoprobe "PN" Nomenclature	Peptide Nomenclature	Abs/em (nm)	MW, (kDa) Observed	Volume FPLC, (Fig. 23a)	Quant. Yield (Fig. 29)	Cell NSB (Fig. 30)
Not Applicable	(DOTA)Lys-Cys	Not Applicable	0.6356	Not Applicable	Not Applicable	Not Applicable
Not Applicable	(DOTA)Lys-Cys(IR-783)	789/807	1.3258	0.600 kDa 1.10 nm	0.053	95%
PN(783)3.0	(DOTA)Lys(PEG2K)-Cys(IR-783)	789/810	3.34	11.2 kDa 3.0 nm	0.13	50%
PN(783)4.3	(DOTA)Lys(PEG5K)-Cys(IR-783)	789/810	6.29	35.2 kDa 4.3 nm	0.16	20%
PN(783)6.1	(DOTA)Lys(PEG10K)-Cys(IR-783)	789/810	11.41	100.7 kDa 6.1 nm	0.16	14%
PN(783)8.4	(DOTA)Lys(PEG20K)-Cys(IR-783)	789/810	21.28	262.5 kDa 8.4 nm	0.16	14%
PN(783)10.0	(DOTA)Lys(PEG30K)-Cys(IR-783)	789/810	30.62	435.4 kDa 10.0 nm	0.16	18%
PN(783)11.7	(DOTA)Lys(PEG40K)-Cys(IR-783)	789/809	45.00	690 kDa 11.7 nm	0.16	
Not Applicable	(DOTA)Lys-Cys(Cy3)	544/559	1.2138	0.6 kDa	0.32	98%
PN(545)4.3	(DOTA)Lys(PEG5K)-Cys(Cy3)	545/560	6.18	35 kDa	0.59	31%
PN(545)10.0	(DOTA)Lys(PEG30K)-Cys(Cy3)	545/560	30.51	435 kDa	0.61	15%
Not Applicable	(DOTA)Lys-Cys(FI)	497/521	1.0626	0.6 kDa	0.50	0.4%
PN(497)4.3	(DOTA)Lys(PEG5K)-Cys(FI)	497/521	6.04	35 kDa	0.59	0.2%
PN(497)10.0	(DOTA)Lys(PEG30K)-Cys(FI)	497/521	30.36	435 kDa	0.60	0.1%

Example 7 - Synthesis of PEG-like Nanoprobes

[00194] In this Example, we prepared multimodal, pharmacokinetically and optically tunable nanomaterials using desferoxamine (DFO) and $^{89}\text{Zr}^{4+}$.

5 **[00195]** In Example 6 above, we show how PEGylated fluorochromes can be made with different PEG's and different fluorochromes. In Example 6, we demonstrated the synthesis of PEGylated fluorochromes of the general formula (DOTA)Lys(PEG)-Cys(FL), where "FL" is a fluorochrome like IR-783 or Cy3 or Fluorescein and PEG is polymer PEG chain with molecular weight between about 2
10 kDa and 40 kDa. By varying the PEG molecular weight, pharmacokinetic variation and tunability is obtained. By varying the fluorochrome (FL), optical properties are varied and spectral tunability is obtained.

[00196] Unfortunately, there are no long-lived, positron emitting metal ions for which DOTA has a high affinity. DOTA has a high affinity for $^{111}\text{In}^{3+}$ (half-life = 2.7
15 days, good for SPECT imaging) and $^{68}\text{Ga}^{3+}$ (half-life= 68 minutes, good for PET imaging).

[00197] To remedy the inability of DOTA to chelate long-lived, positron emitting isotopes, we have now replaced the DOTA with desferoxamine (DFO), to obtain materials with the general formula (DFO)Lys(PEG)-Cys(FL). The conjugation of
20 desferoxamine (DFO) to proteins (e.g. antibodies and albumin), followed by chelation of the positron emitting $^{89}\text{Zr}^{4+}$ (half-life 78 hours), and imaging protein disposition by PET is now a widely accepted approach to imaging long circulating proteins. We have shown that these yield ($^{89}\text{Zr}^{4+}$:DFO)Lys-Cys(FL) where FL = a fluorochrome, which can be used for PET imaging.

Synthesis of the DFO-Lys(NH₂)-Cys(SH) peptide (see Figure 31)

25 **[00198]** The DFO-Lys(Boc)-Cys(Trt) peptide was manually synthesized on Rink Amide MBHA resin (0.15 mmol) with an Fmoc/t-Bu strategy using a polypropylene 5 mL disposable syringe fitted with a sintered frit. Coupling reactions employed 2 equiv. (relative to resin) of Fmoc-protected amino acid activated in situ with 2 equiv.
30 of PyBOP and 4 equiv. of DIPEA in DMF (10 mL/g resin) for 1-2 hours. Coupling efficiency was assessed with picrylsulfonic acid. Fmoc groups were removed with a

piperidine/DMF solution (1:4) for 4x10 min (10 mL/g resin). The N-terminal of the peptide was succinylated by succinic anhydride(8eq) with the presence of DIPEA (8eq) in DMF, while a carboxylic acid was generated for the attachment of DFO . PyBop (4eq) and DIPEA (16 eq) in DMSO (1ml) was pulled into the syringe and stayed in room temperature for 20 minutes. Then the solution of DFO-mesylate salt (4eq) in DMSO (3ml) was mixed with the PyBOP solution in the syringe and incubated under room temperature for overnight. DFO-Lys-Cys was released from the solid support with TFA/H₂O/TIS/EDT 88:2:5:5 (2 h, 20 mL/g resin). The residue was precipitated and triturated with cold diethyl ether. A white solid could be obtained by centrifuge. The solid was purified further by HPLC with buffer B from 15% to 65% in 10 minutes, back to 15% B in 2 minutes, and isocratic for 3 minutes with a flow of 12ml/min at λ_{max} =226nm on a column of Agilent, PLRP-S 100A, 15-20 μ m, P/N: PL1812-6200. A double charged peak with MS 446.5 was found. Overall yield: 37.5%.

Synthesis of DFO-Lys(NH₂)-Cys(S-Mal-Cy5.5, Lumiprobe) (see Figure 32)

[00199] DFO-Lys(NH₂)-Cys(SH)-NH₂ (5.7mg, 6.4 μ mol) and Cy5.5-Maleimide (4.73mg, 6.4 μ mol) was mixed in DMSO(0.7ml) in the presence of DIPEA (7 μ l, 40.3 μ mol) for overnight in room temperature under N₂. The product was purified by HPLC separation (a gradient of 20-100% buffer B in 10 minutes, back to 20% in 5 minutes and isocratic for 5 minutes, flow: 21ml/min, 275 nm; column: Agilent, PLRP-S 100A, 15-20 μ m, P/N: PL1812-6200). A blue powder was obtained after lyophilization with a yield: >90%; MS: C₈₄H₁₁₉N₁₄O₁₅S⁺, calculated: 1595.87, found: 798.7 [M+1]²⁺, 533 [M+2]³⁺.

Synthesis of DFO-Lys(PEG5KDa)-Cys(S-Mal-Cy5.5)-NH₂ (see Figure 33)

[00200] To a solution of DFO-Lys(NH₂)-Cys(S-Mal-Cy5.5)-NH₂ (1.28mg, 0.8 μ mol in DMSO), was added the solution of m-PEG-5K-NHS (12mg, 2.4 μ mol, 3eq). After DiPEA (7.37 μ L, 42.4 μ mol, 53eq to MSAP) was added, the reaction mixture was incubated for 3 days at room temperature. The product was purified by HPLC (gradients: 20-100%B in 10 minutes, back to 20% buffer B in 5 minutes, then isocratic for 5 minutes; flow, 21ml/min, 675nm, column: Agilent, PLRP-S 100A, 15-20 μ m, P/N: PL1812-6200). Yield: >90%, MS: 6689.73 (multiple dispersed).

Synthesis of DFO-Lys(PEG30KDa)-Cys(S-Mal-Cy5.5)-NH₂ (see Figure 34)

[00201] To a solution of DFO-Lys(NH₂)-Cys(S-Mal-Cy5.5)-NH₂ (0.8μmol, 1.28mg, in DMSO), was added the solution of m-PEG-30K-NHS (72mg, 2.4μmol, 3eq in DMSO 1ml) and incubated for 3 days at room temperature in the presence of DiPEA (7.37μL, 42.4μmol, 53eq to MSAP). The product was purified by HPLC (20-100% buffer B in 10 minutes, back to 20%B in 5 minutes, then isocratic for 5 minutes; flow, 21ml/min, 675nm, column: Agilent, PLRP-S 100A, 15-20μm, P/N: PL1812-6200). Yield: >90%, MS: 30,000 (multiple dispersed).

⁸⁹Zr labeling of compounds DFO-Lys(PEG5KDa)-Cys(S-Mal-Cy5.5)-NH₂ and DFO-Lys(PEG30KDa)-Cys(S-Mal-Cy5.5)-NH₂ (see Figures 35-37)

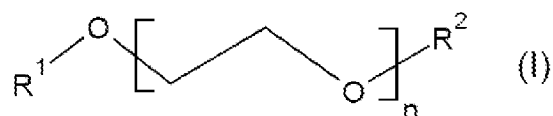
[00202] A solution of ⁸⁹Zr-oxalate (250μl, 287μCi) was neutralized by Na₂CO₃ (1M, in chelexed water, 170μl) until the up to pH 8.5. Two aliquots were made for the labeling of 2nmol DFO-Lys(PEG5KDa)-Cys(S-Mal-Cy5.5)-NH₂ and DFO-Lys(PEG30KDa)-Cys(S-Mal-Cy5.5)-NH₂ by adding their stock solutions in chelexed water respectively. They were incubated under room temperature for 2 hours with radioactive TLC monitoring. The labeling yield were 40% for DFO-Lys(PEG5KDa)-Cys(S-Mal-Cy5.5)-NH₂ and 75-80% for DFO-Lys(PEG30KDa)-Cys(S-Mal-Cy5.5)-NH₂. The labeled compounds were purified by PD-10 column with fraction collection.

[00203] Although the present invention has been described in detail with reference to certain embodiments, one skilled in the art will appreciate that the present invention can be practiced by other than the described embodiments, which have been presented for purposes of illustration and not of limitation. Therefore, the scope of the appended claims should not be limited to the description of the embodiments contained herein.

CLAIMS

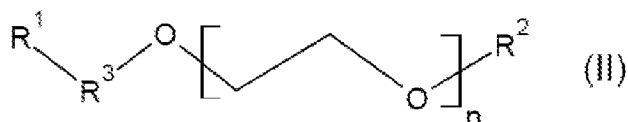
What is claimed is:

1. A fluorescent compound having the formula (I):



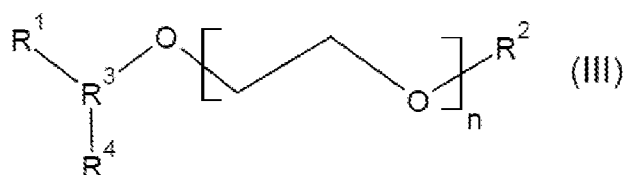
wherein R^1 is a fluorescent moiety having an absorption wavelength maxima in the range of 450 to 1500 nanometers, and
 wherein R^2 is a non-reactive moiety, and
 wherein n is an integer.

2. A fluorescent compound having the formula (II):



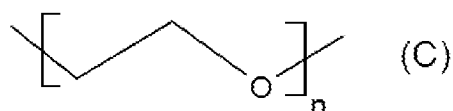
wherein R^1 is a fluorescent moiety having an absorption wavelength maxima in the range of 450 to 1500 nanometers, and
 wherein R^2 is a non-reactive moiety, and
 wherein R^3 is a scaffold including an amino acid group, and
 wherein n is an integer.

3. A fluorescent compound having the formula (III):



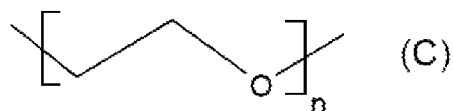
wherein R¹ is a fluorescent moiety having an absorption wavelength maxima in the range of 450 to 1500 nanometers, and
 wherein R² is a non-reactive moiety, and
 wherein R³ is a scaffold including an amino acid group, and
 wherein R⁴ is selected from chelates, proteins, enzymes, peptides, antibodies, and drugs that can target a site in a subject, and
 wherein n is an integer.

4. The compound of any of claims 1 to 3 wherein:
 n is selected such that chain (C) in the compound



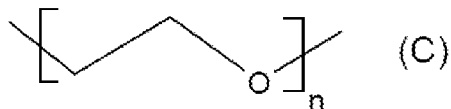
has a molecular weight of 2,000 daltons or more.

5. The compound of any of claims 1 to 3 wherein:
 n is selected such that chain (C) in the compound



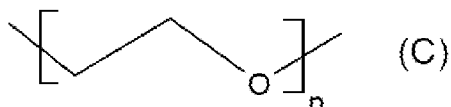
has a molecular weight of 2,000 to 10,000 daltons.

6. The compound of any of claims 1 to 3 wherein:
n is selected such that chain (C) in the compound



has a molecular weight of 5,000 to 40,000 daltons.

7. The compound of any of claims 1 to 3 wherein:
n is selected such that chain (C) in the compound

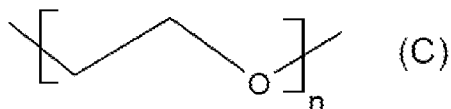


has a molecular weight of 2,000 to 100,000 daltons.

8. The compound of any of claims 1 to 3 wherein:
n is selected such that after intravenous administration of the compound to a mammal, the compound undergoes renal elimination.

9. The compound of any of claims 1 to 3 wherein:
n is selected such that after intravenous administration of the compound to a mammal, clearance is by macrophages of the reticuloendothelial system of the mammal.

10. The compound of any of claims 1 to 3 wherein:
chain (C) in the compound



shields R¹ from reaction with biological molecules.

11. The compound of any of claims 1 to 3 wherein:
the fluorescent moiety has an absorption wavelength maxima in the range of 550 to 850 nanometers.
12. The compound of any of claims 1 to 3 wherein:
the fluorescent moiety has an absorption wavelength maxima in the range of 650 to 850 nanometers.
13. The compound of any of claims 1 to 3 wherein:
the compound has a quantum yield of greater than 0.1.
14. The compound of any of claims 1 to 3 wherein:
the compound has a molecular volume that correlates with an apparent molecular weight greater than about 10,000 daltons when analyzed by fast protein liquid chromatography and globular protein standards.
15. The compound of any of claims 1 to 3 wherein:
 R^2 is selected from the group consisting of C_1 - C_{20} alkyl and aryl.
16. The compound of any of claims 1 to 3 wherein:
 R^2 is selected from the group consisting of C_1 - C_5 alkyl.
17. The compound of any of claims 1 to 3 wherein:
the fluorescent moiety is a cyanine dye.
18. The compound of any of claims 1 to 3 wherein:
the fluorescent moiety is a carbocyanine dye.
19. The compound of any of claims 1 to 3 wherein:
the fluorescent moiety is selected from CyAL dyes.

20. The compound of any of claims 1 to 3 wherein:
the fluorescent moiety is fluorescein.
21. The compound of claim 3 wherein:
 R^4 is a chelate.
22. The compound of claim 21 wherein:
the chelate includes a chelated metal or metal ion selected from the group consisting of Mn ions, Fe ions, gadolinium ions, ^{67}Ga , ^{68}Ga , ^{82}Rb , ^{89}Zr , ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{111}In , ^{177}Lu , ^{201}Tl , ^{213}Bi , and ^{225}Ac .
23. The compound of claim 22, wherein the chelate further includes a non-metal halogen selected from the group consisting of ^{75}Br , ^{76}Br , ^{18}F , ^{19}F , ^{123}I , ^{125}I , and ^{131}I that is bound to the chelated metal or metal ion.
24. The compound of claim 21 wherein:
the chelate includes a magnetic material.
25. The compound of claim 21 wherein:
the chelate includes diethylene triamine pentaacetic acid (DTPA) or tetraazacyclododecane tetraacidic acid (DOTA) or desferoxamine (DFO).
26. The compound of claim 21 wherein:
the chelate includes desferoxamine (DFO) and ^{89}Zr .
27. The compound of claim 21, wherein the chelate comprises a bifunctional chelating agent.

28. The compound of claim 27, wherein the bifunctional chelating agent is selected from the group consisting of a bifunctional DTPA, a bifunctional DOTA, a bifunctional DFO, a bifunctional triazacyclononanetriacetic acid (NOTA), a bifunctional tetraazabicyclopentadecatrienetriacetic acid (PCTA), and a bifunctional oxatriazacyclododecanetriacetic acid (Oxo-DO3A).

29. The compound of any of claims 1 to 3 wherein:
the compound has a hydrodynamic diameter in the range of 1 to 100 nanometers.

30. The compound of claim 2 or claim 3 wherein:
the scaffold is a peptide including two or more residues selected from alanine, arginine, aspartate, cysteine, glycine, and lysine.

31. A method for imaging a region of interest of a subject, the method comprising:
administering to the subject a compound of any of claims 1 to 3, wherein the compound enters the region of interest of the subject;
directing light into the subject;
detecting fluorescent light emitted from the subject; and
processing the detected light to provide an image that corresponds to the region of interest of the subject.

32. The method of claim 31 wherein:
the light directed into the subject has a wavelength in the range of 450 to 1500 nanometers.

33. The method of claim 31 wherein:
the fluorescent light is emitted via two-photon-excited fluorescence.

34. The method of claim 31 further comprising:

imaging the subject with a second imaging method selected from positron emission tomography, single-photon emission computed tomography, magnetic resonance imaging, computerized tomography, optical imaging, and ultrasound.

35. The method of claim 31 wherein:

the region of interest of the subject includes a tumor.

36. The method of claim 35 wherein:

if the compound binds to the tumor, the method further comprises administering to the subject a therapeutically effective amount of a cytotoxic material comprising a compound of any of claims 1 to 3 associated with a cytotoxic agent.

37. A method for treatment of a tumor in a subject, the method comprising:

administering to the subject a therapeutically effective amount of a cytotoxic material comprising a compound of any of claims 1 to 3 associated with a cytotoxic agent,

wherein the cytotoxic material is targeted to the tumor in the subject.

38. A method for treatment of a tumor in a subject, the method comprising:

administering to the subject a therapeutically effective amount of a cytotoxic material comprising a compound of any of claims 1 to 3 associated with a cytotoxic agent,

wherein the cytotoxic material is targeted to the tumor in the subject.

39. The method of claim 38 wherein:

the cytotoxic material is injected peritumorally, and

at least a portion of the cytotoxic material is retained at or near the tumor by interactions between the scaffold and a receptor on a surface of a cell in the tumor.

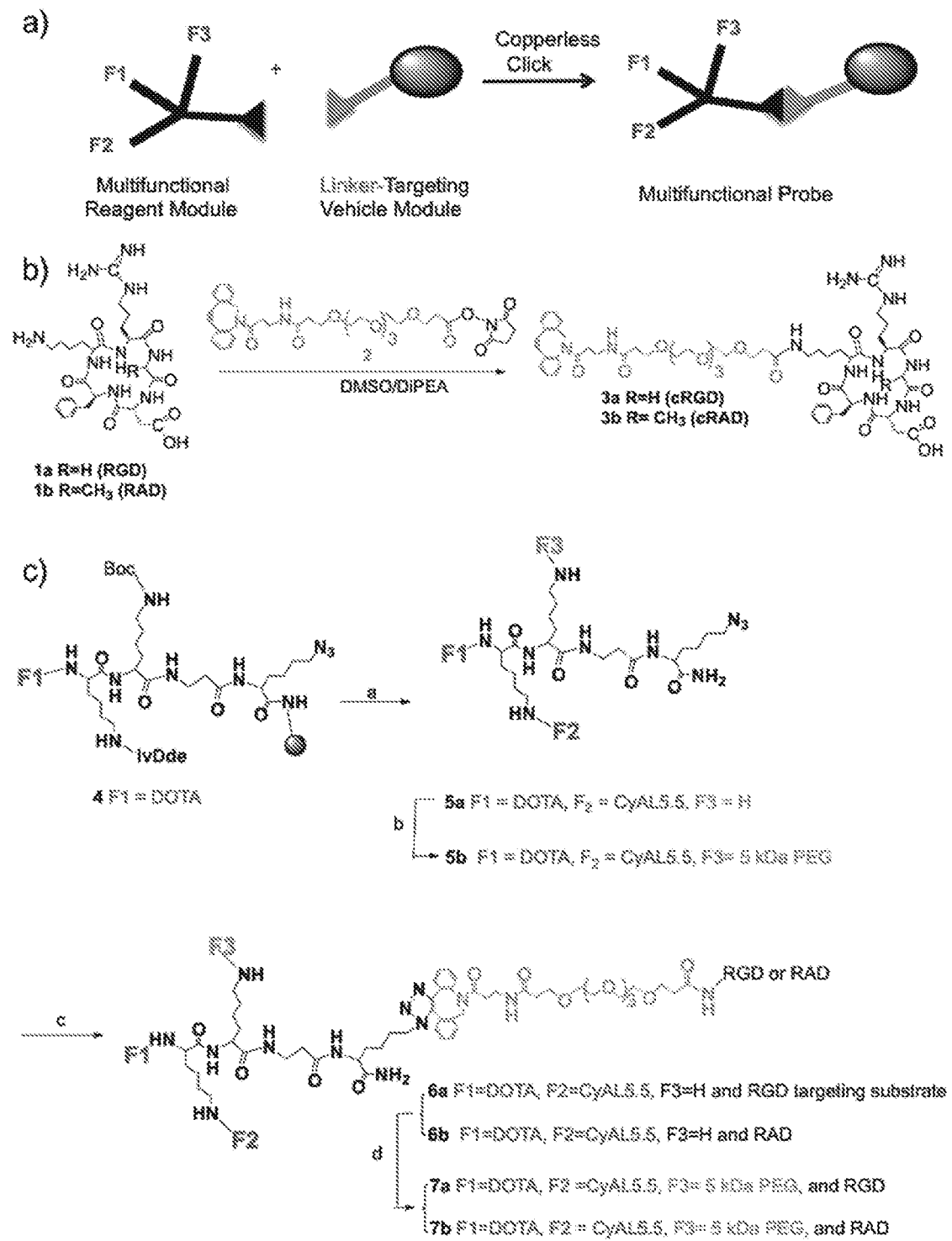


Fig. 1

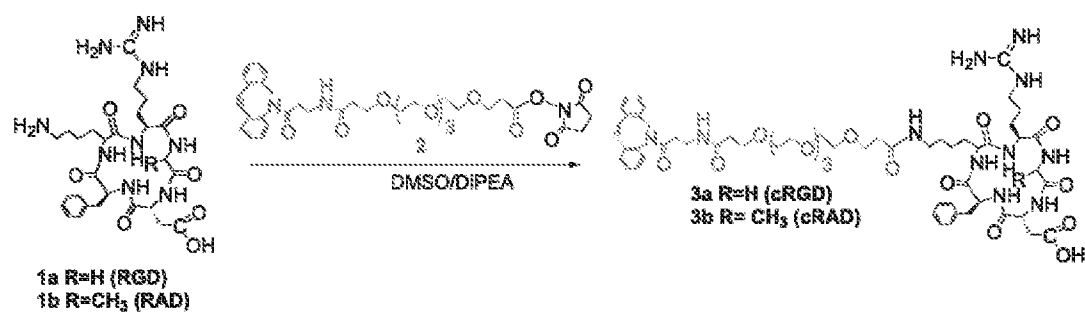


Fig. 2

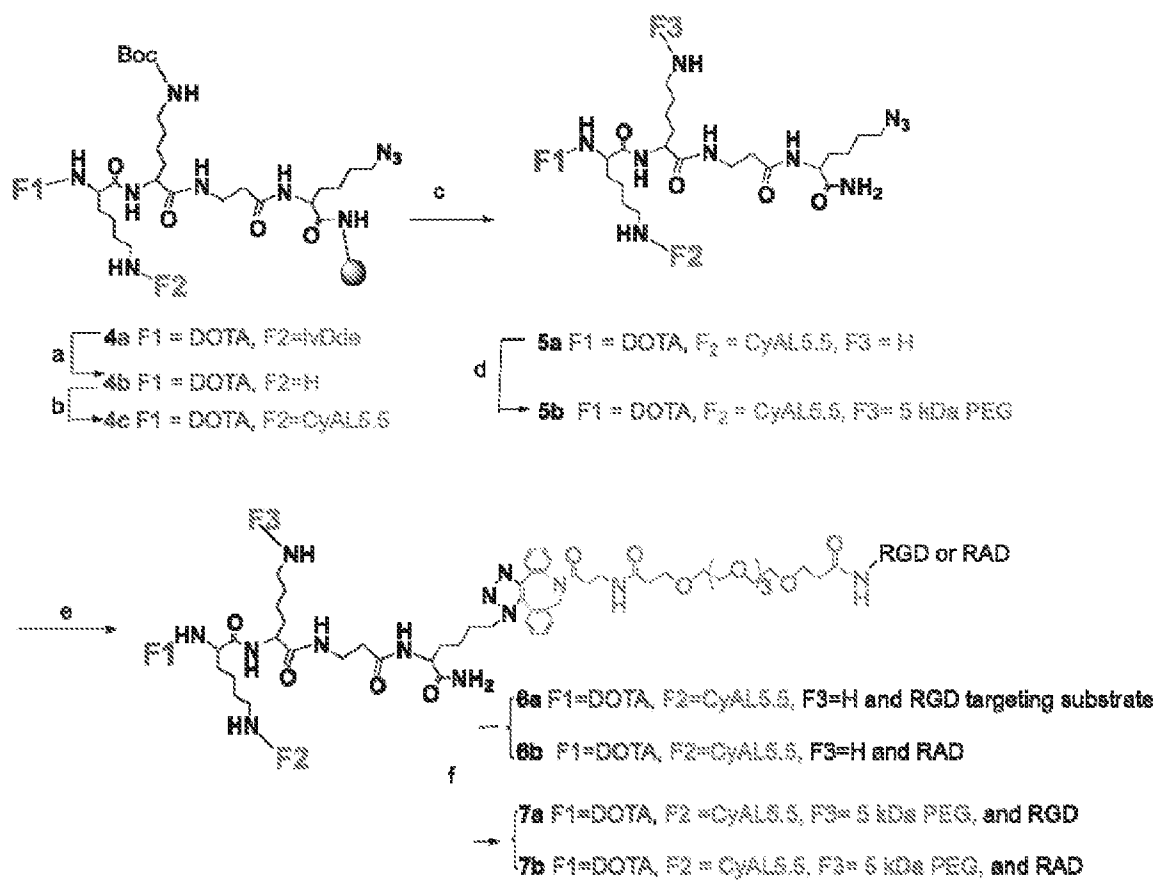
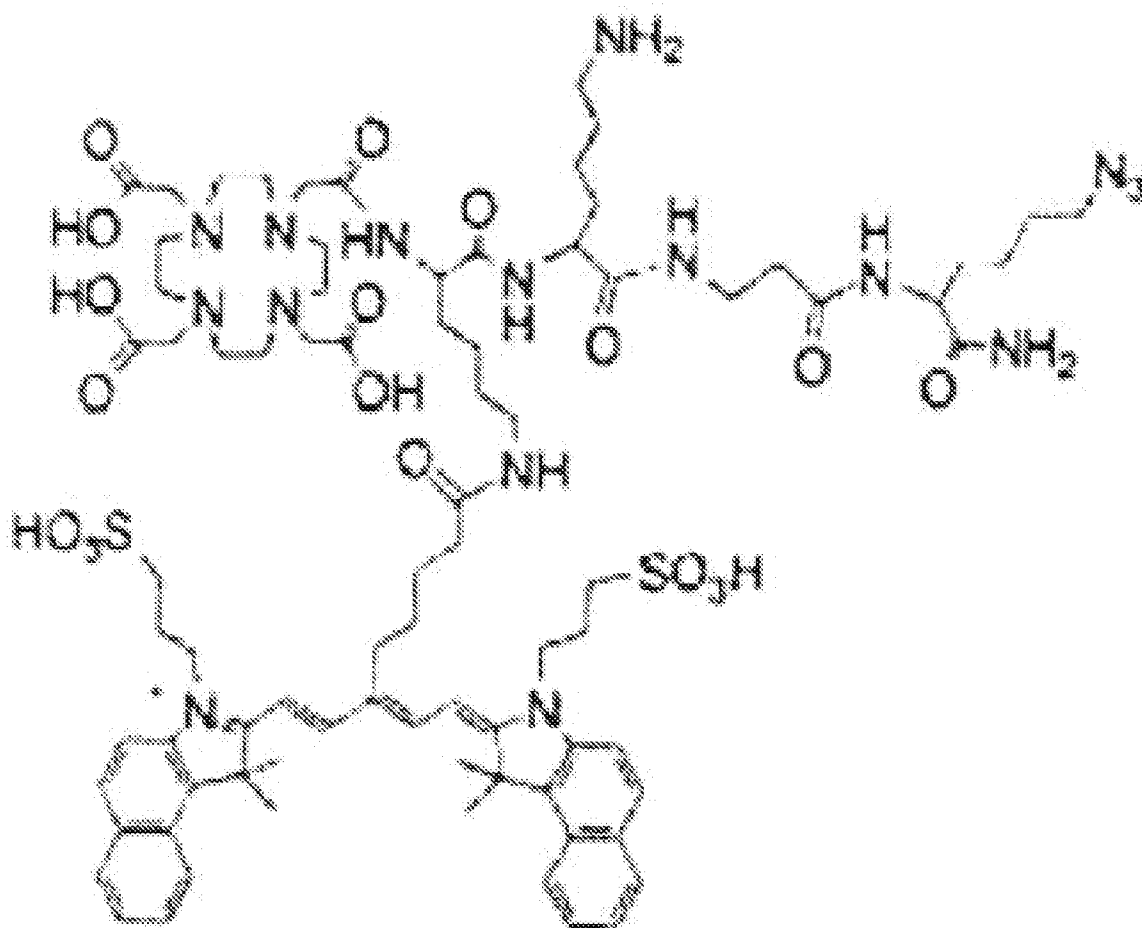


Fig. 3



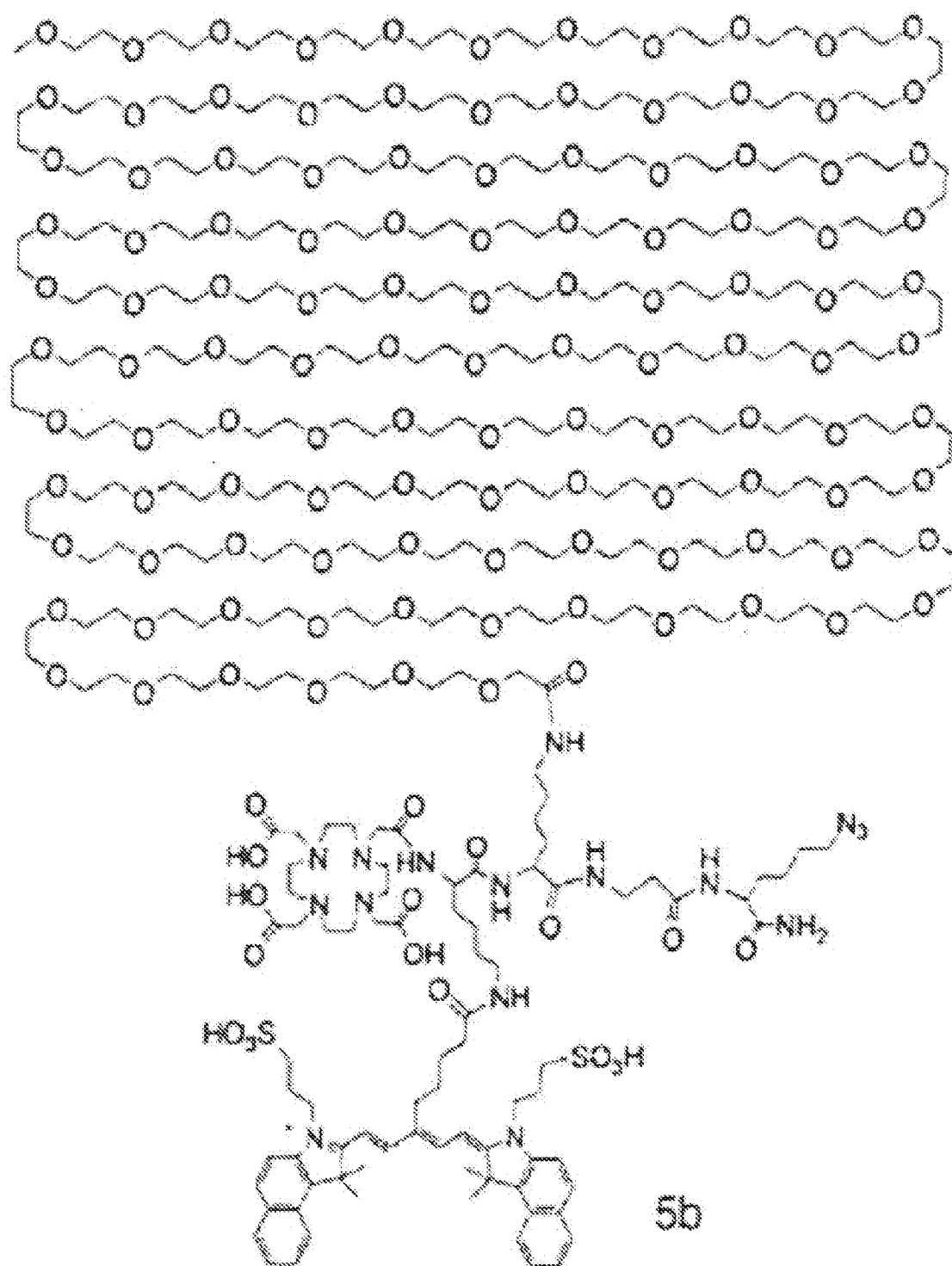
Chemical Formula: C₈₁H₁₁₁N₁₆O₁₈S₂⁺

Exact Mass: 1665.82

Molecular Weight: 1667.02

5a

Figure 4A



Chemical Formula: $C_{314}H_{521}N_{13}O_{135}S_7$
Exact Mass: 6800.85
Molecular Weight: 6800.13

Figure 4B

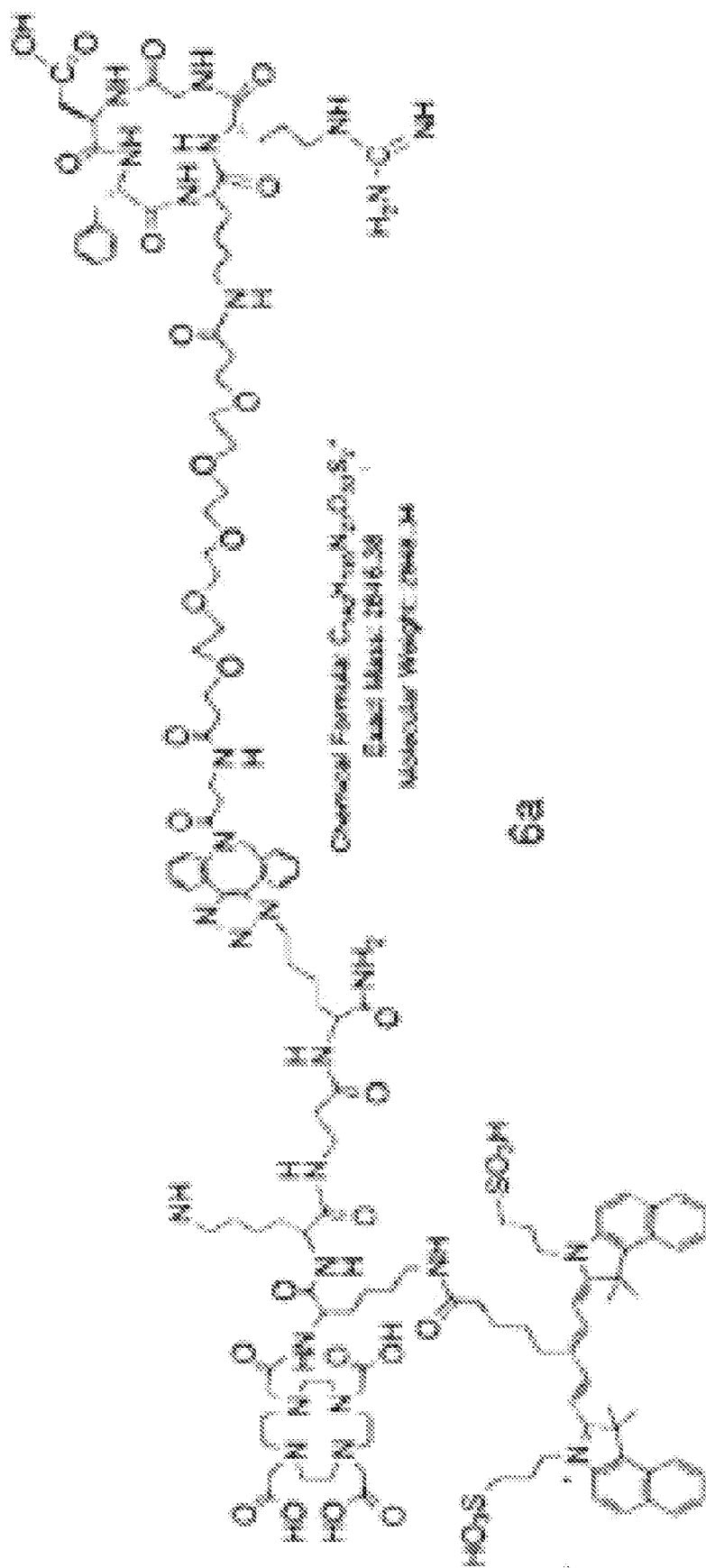


Figure 4C

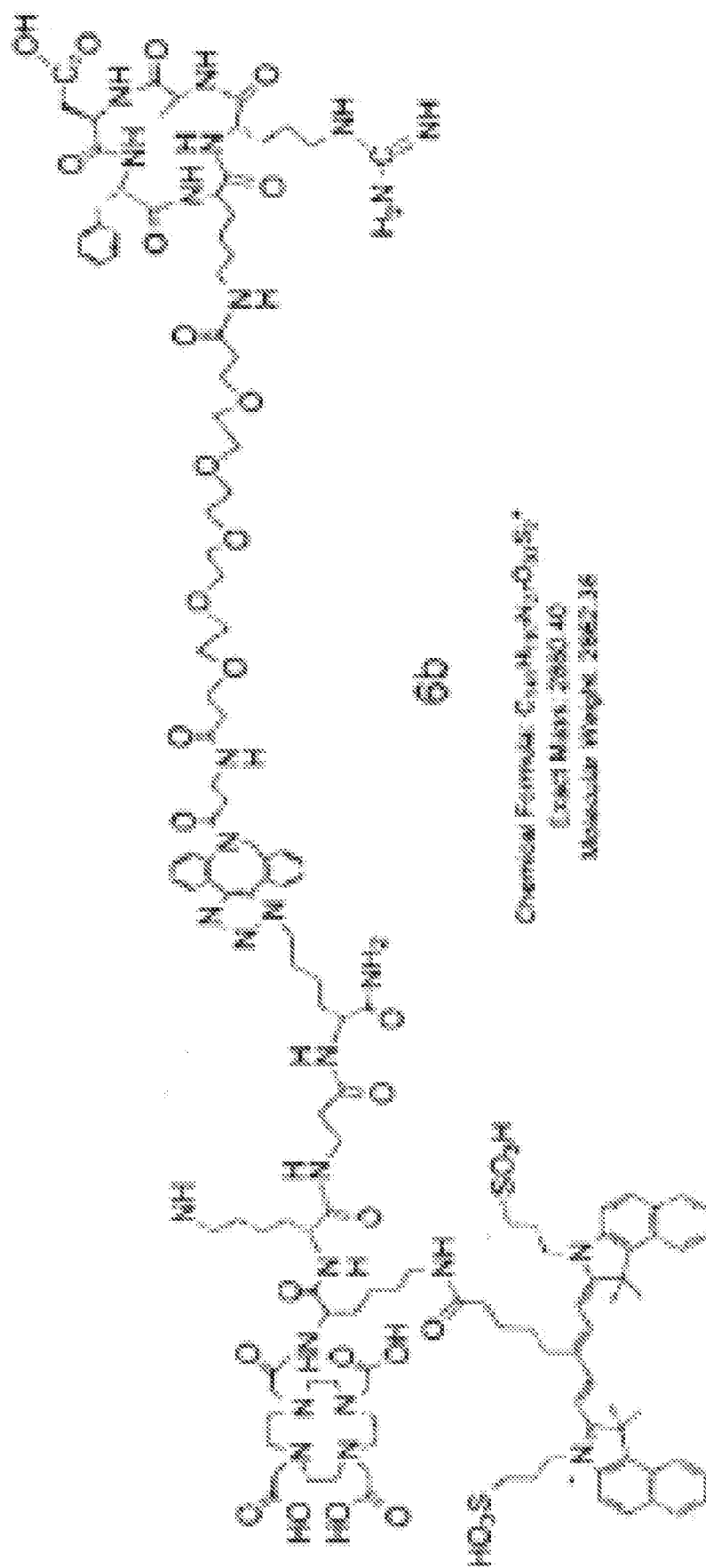


Figure 4D

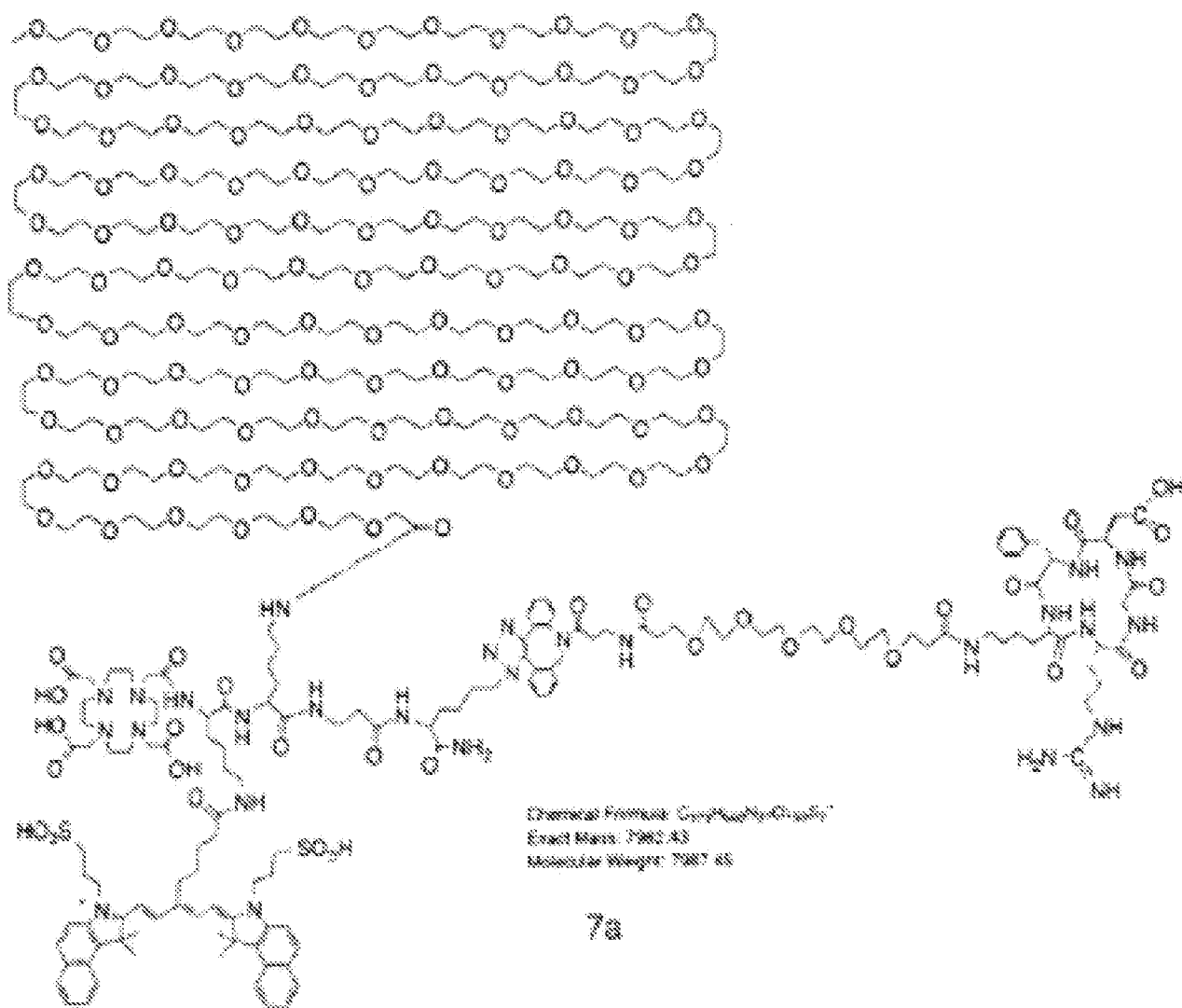


Figure 4E

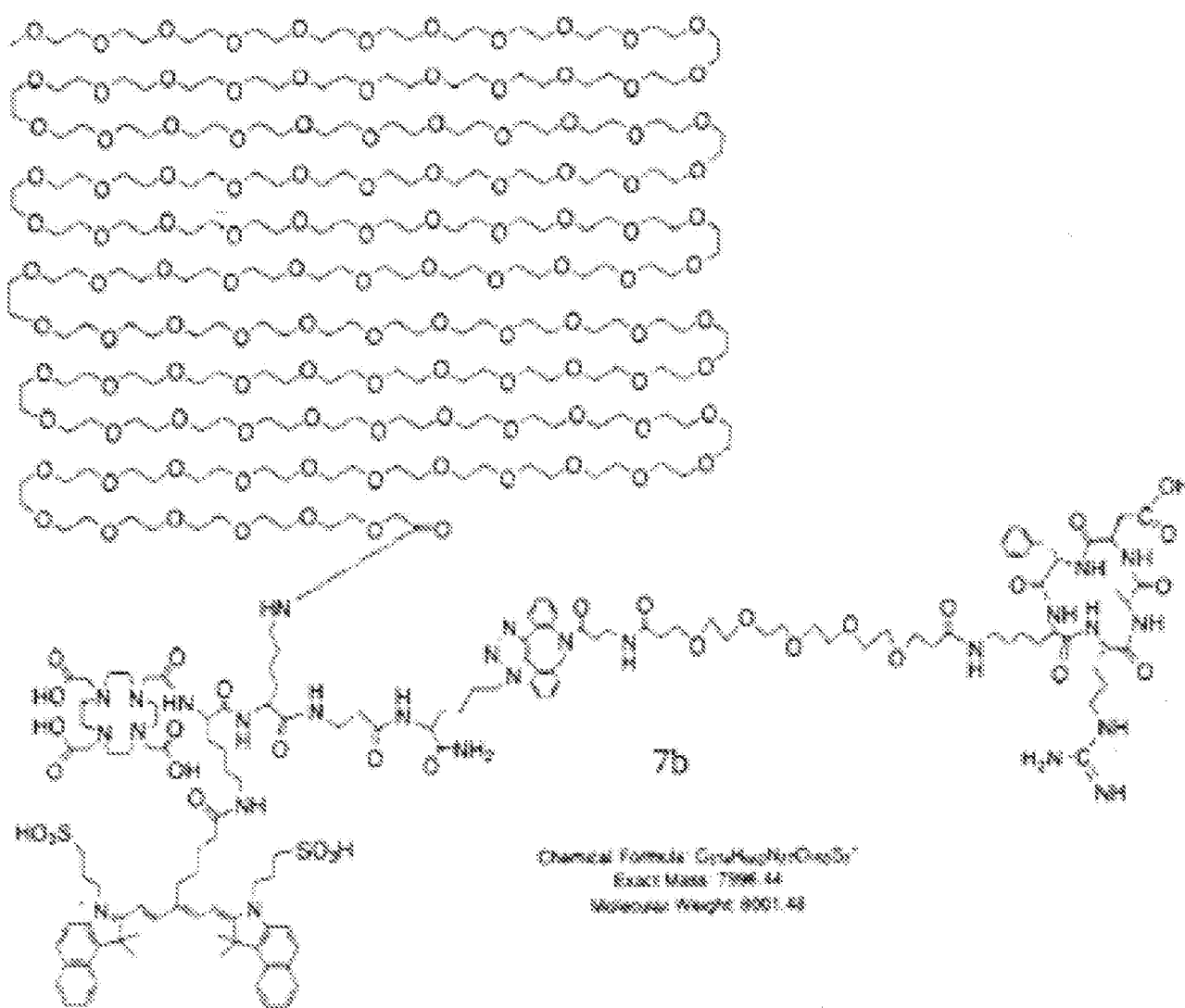


Figure 4F

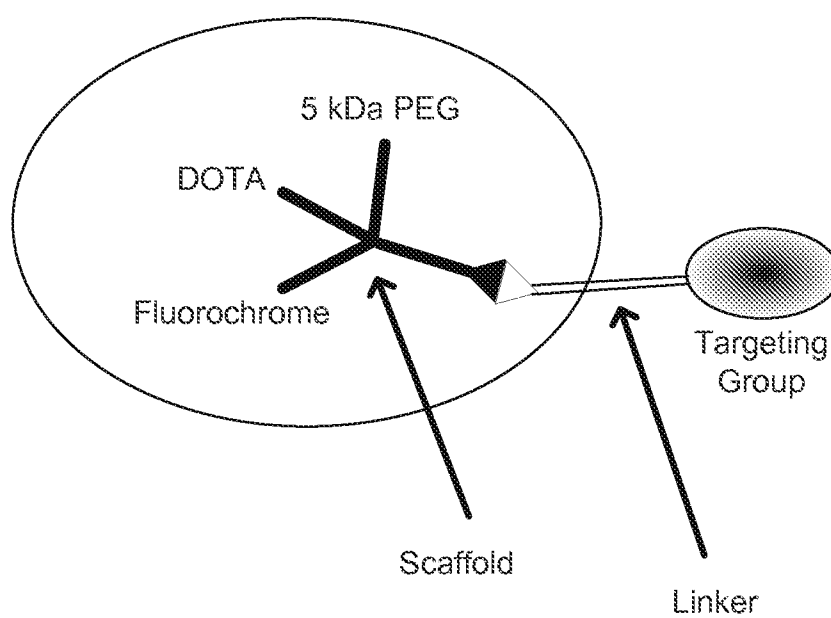


Figure 5

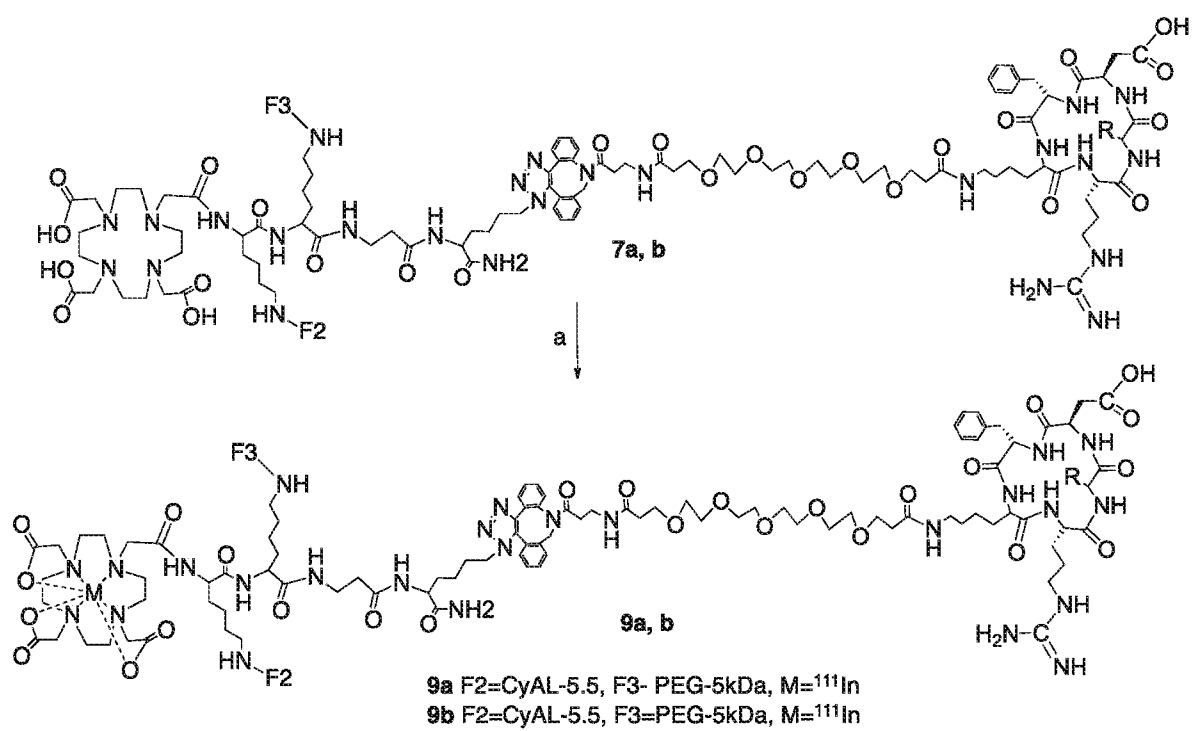


Fig. 6

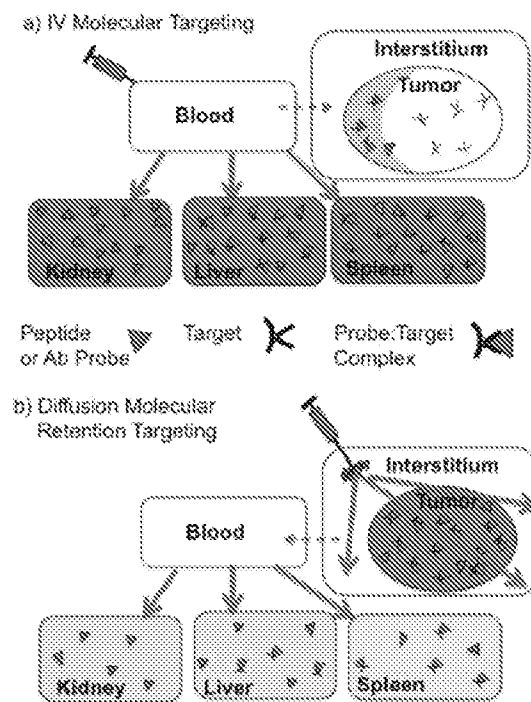


Fig. 7

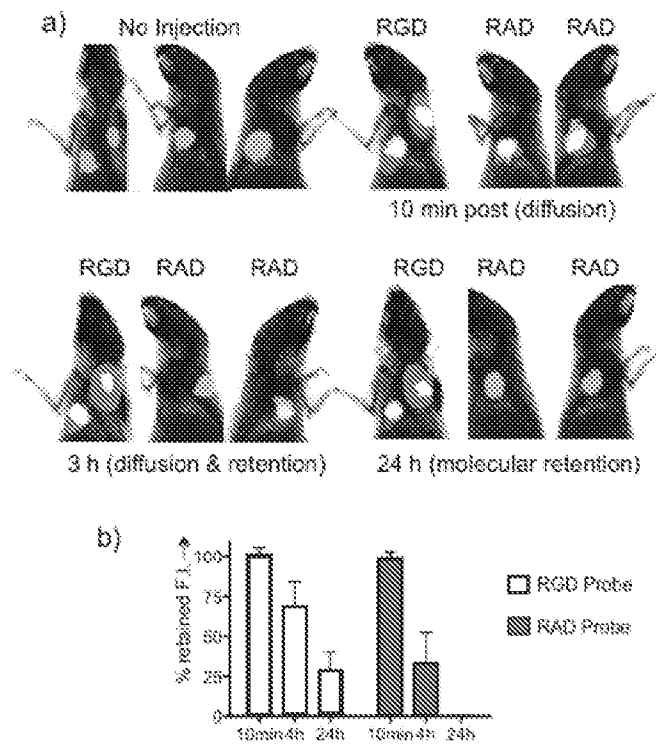


Fig. 8

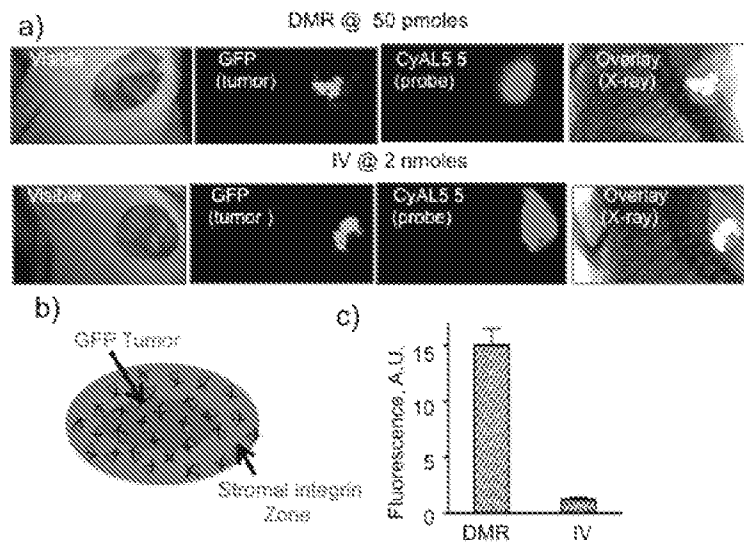


Fig. 9

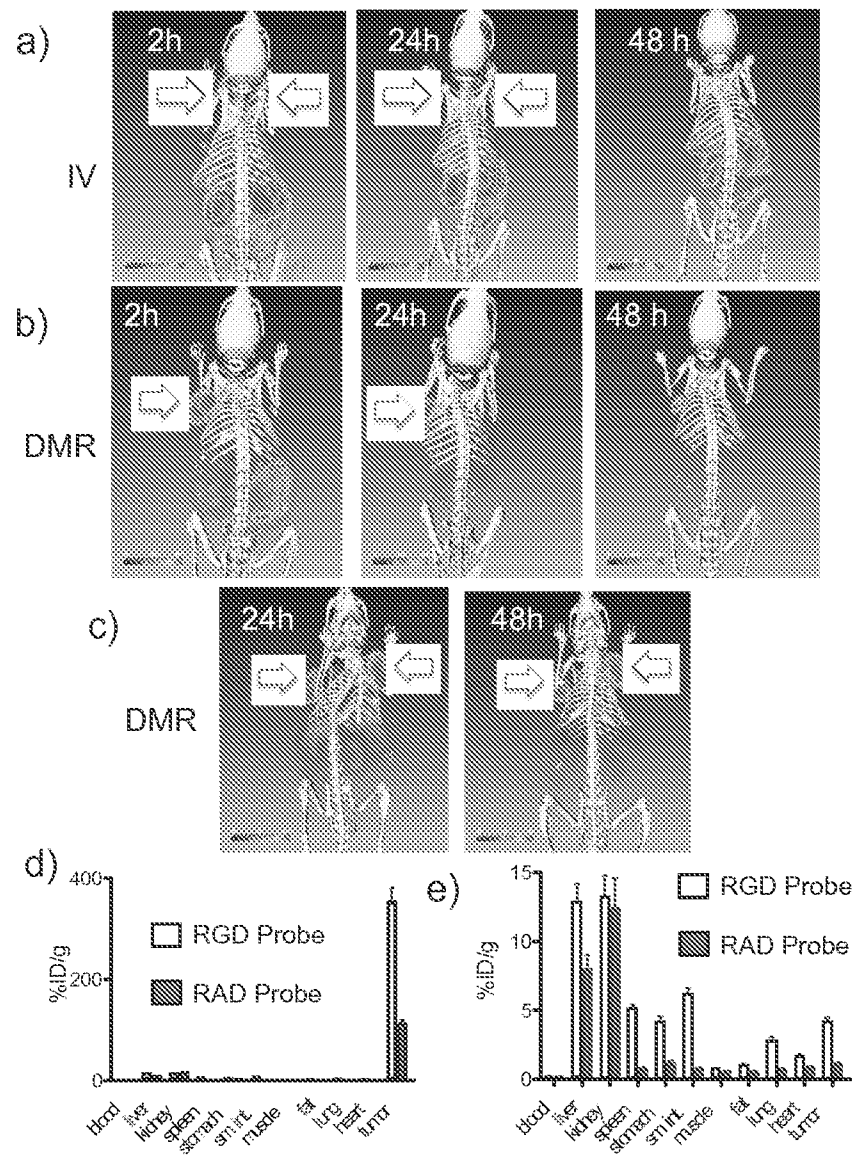


Fig. 10

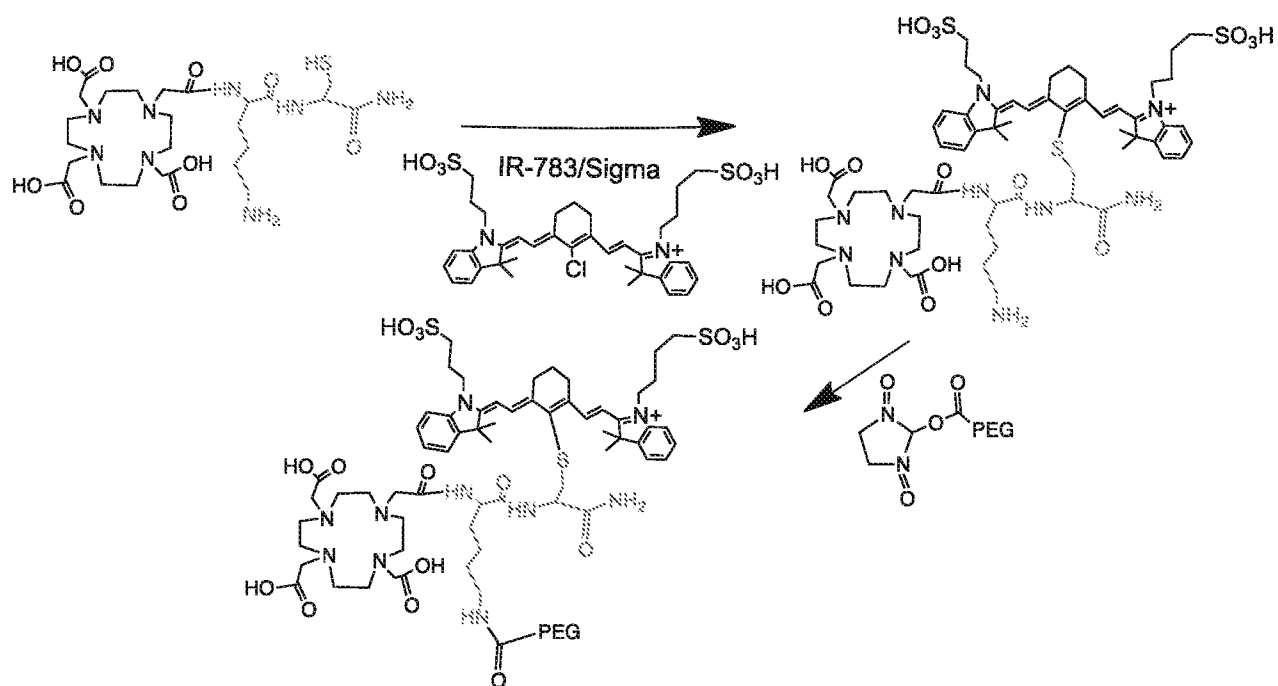


Fig. 11

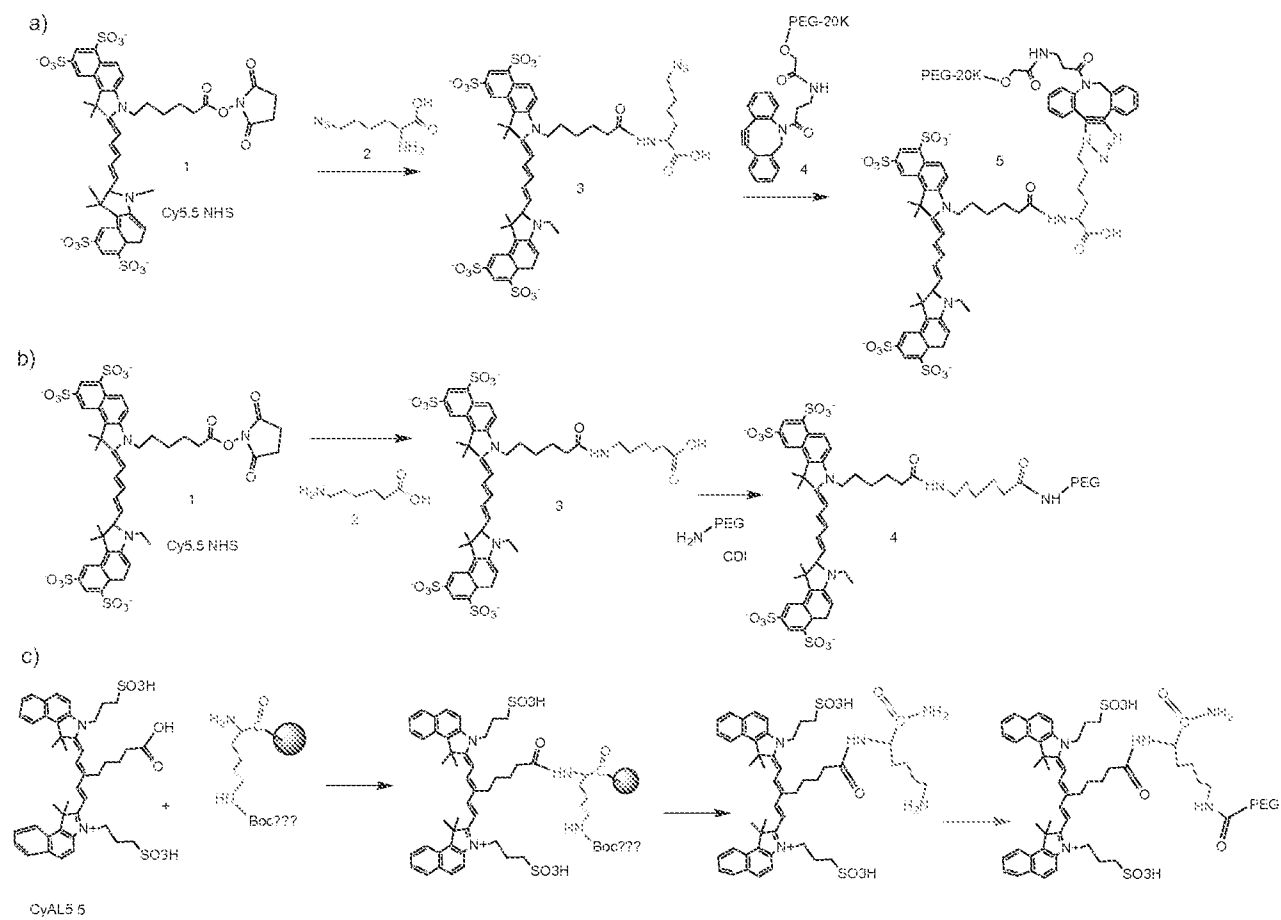
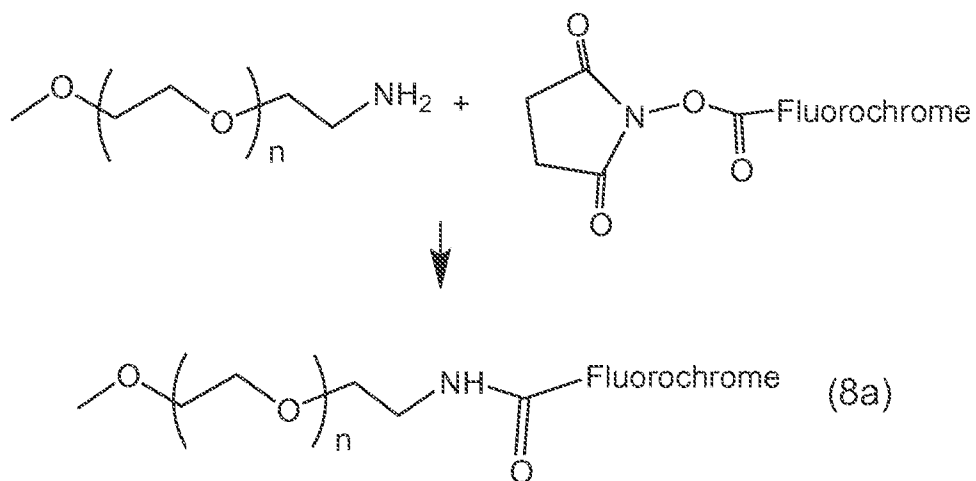


Fig. 12

Reaction of PEG-amine and N-Hydroxysuccinimide Ester of a Fluorochrome



Reaction of PEG-alkyne and Azide of a Fluorochrome

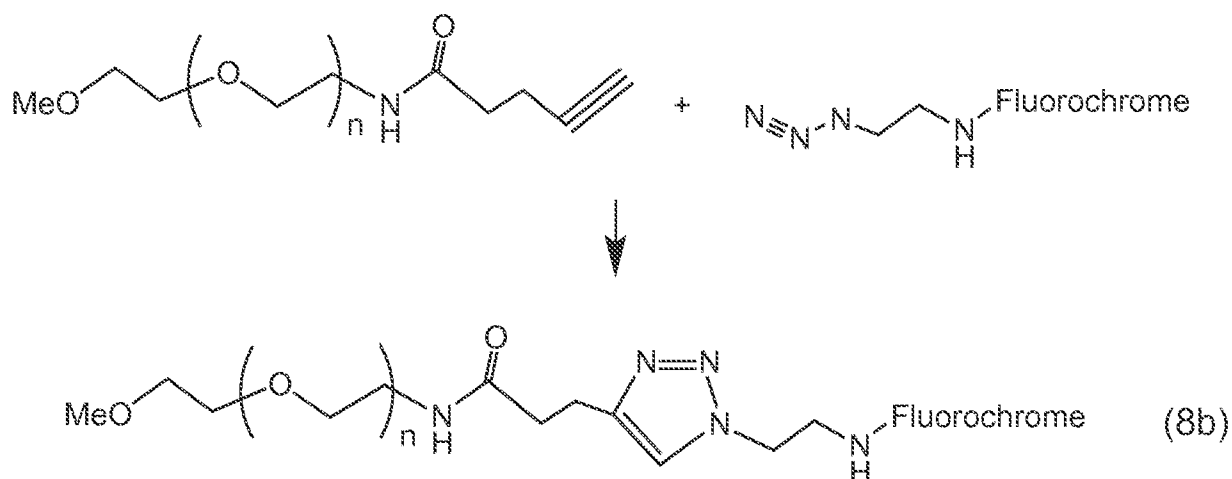


Fig. 13

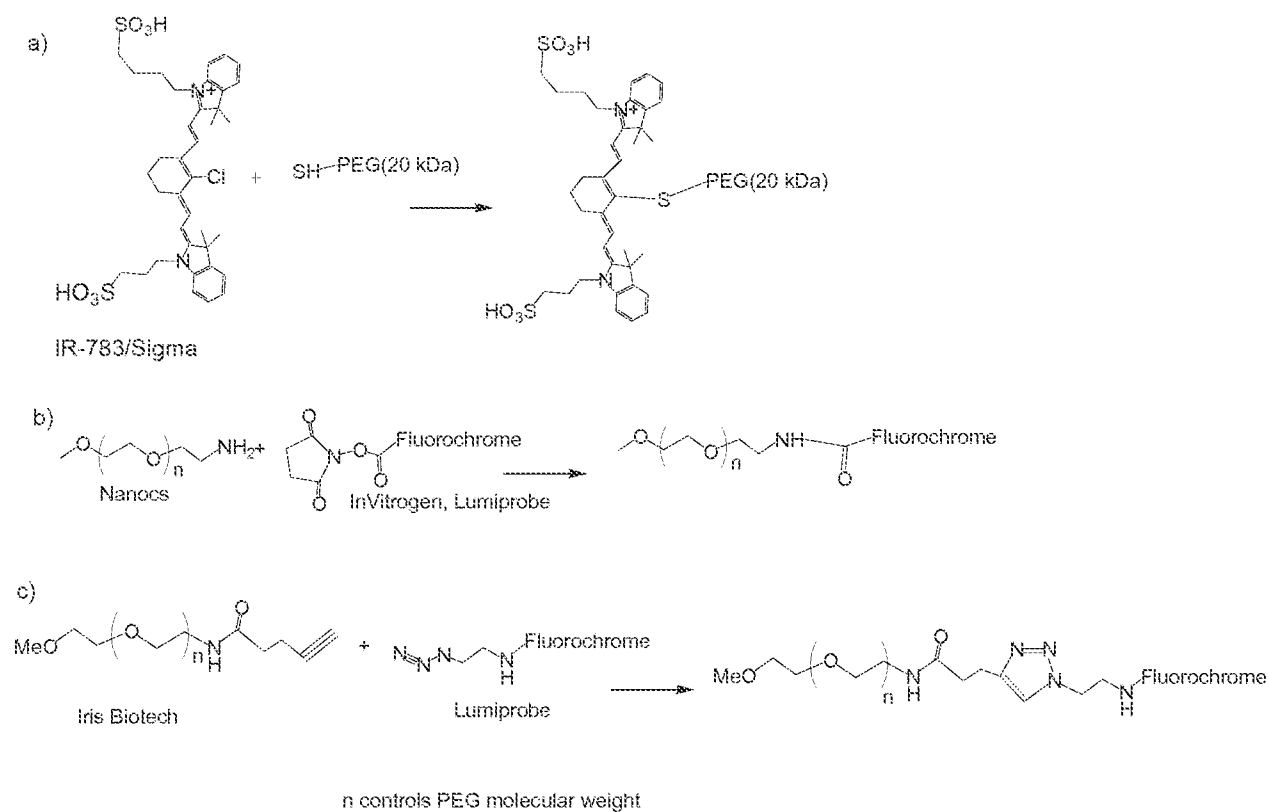
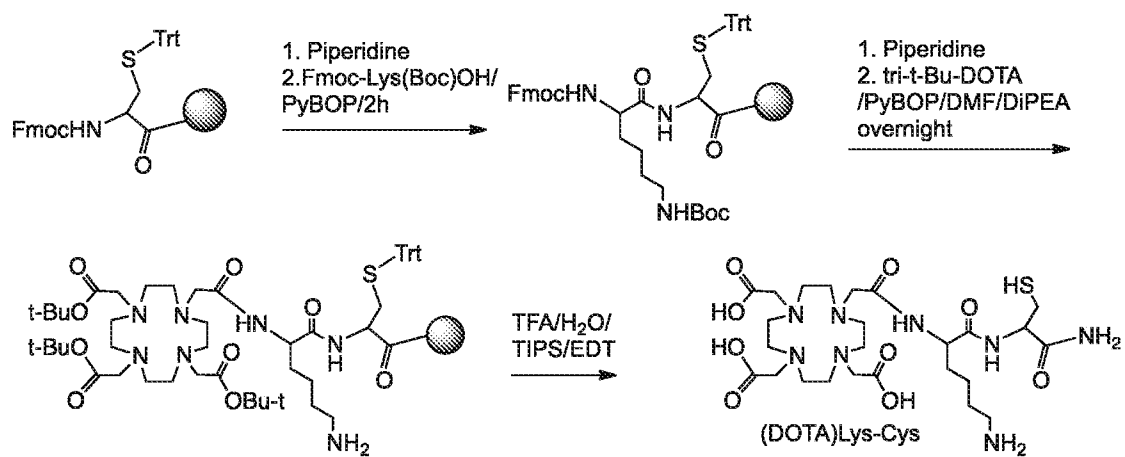


Fig. 14

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Synthesis of (DOTA)Lys-Cys

Figure 15

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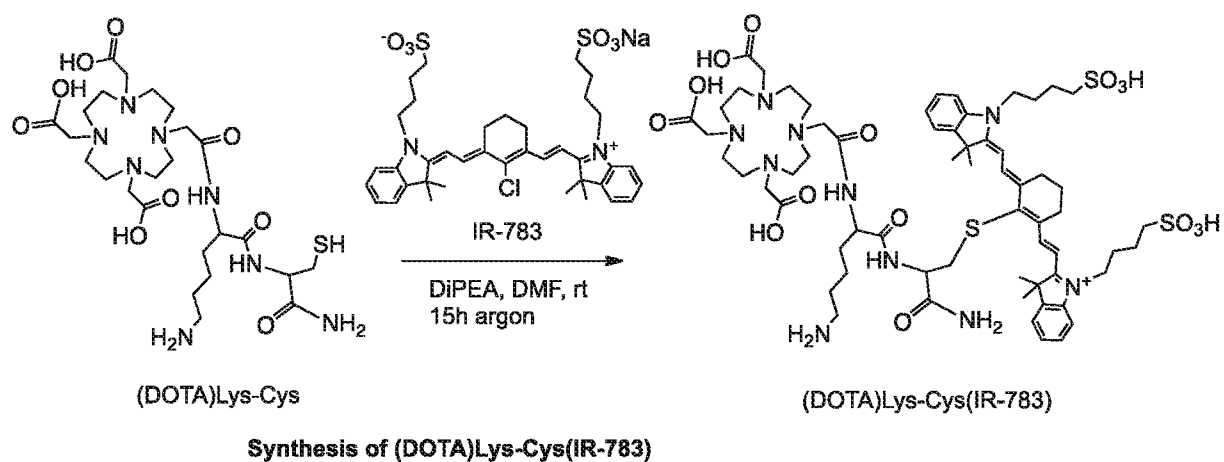


Figure 16

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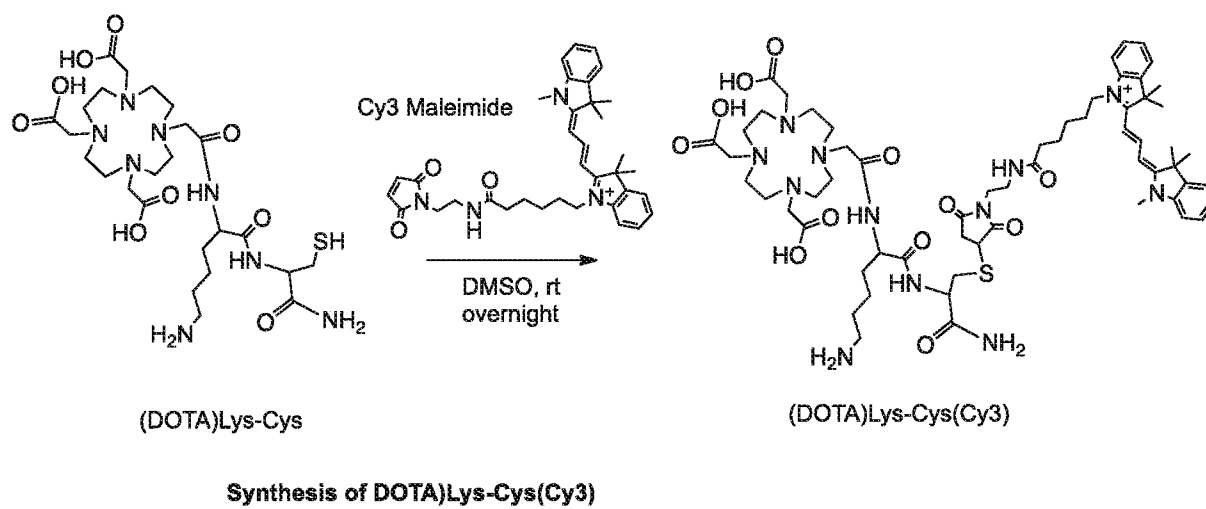
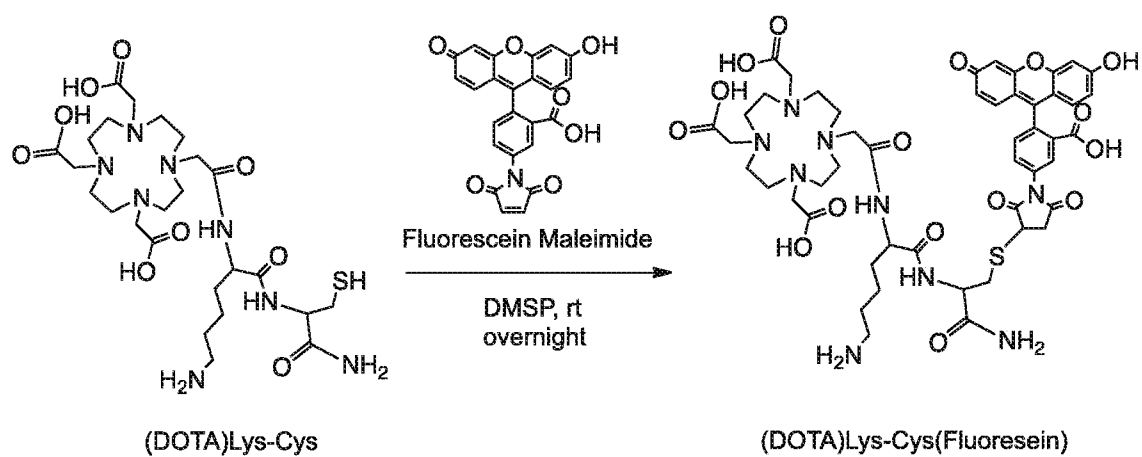


Figure 17

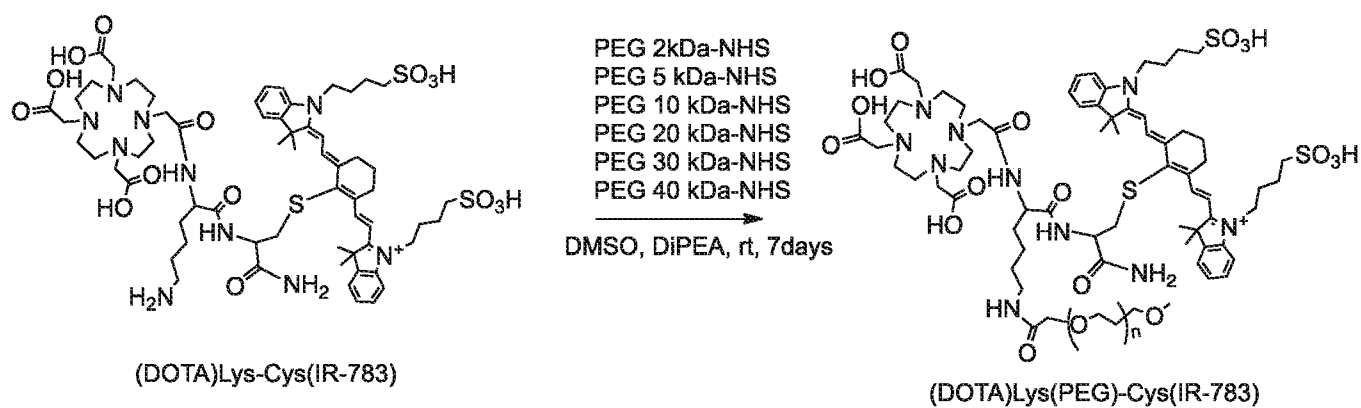
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Synthesis of (DOTA)Lys-Cys(Fluorescein)

Figure 18

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Synthesis of PN(783)'s, (DOTA)Lys(PEG)-Cys(IR-783)

Figure 19

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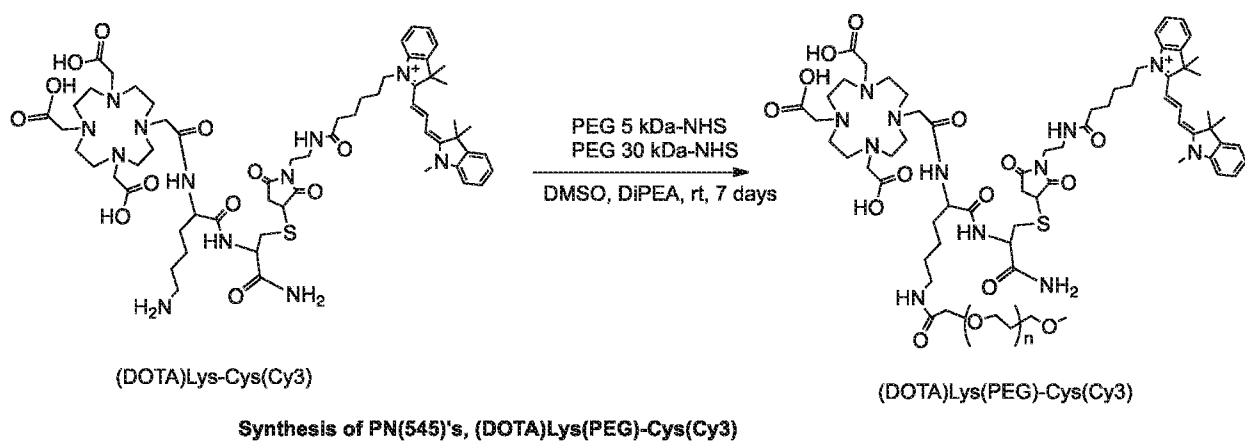


Figure 20

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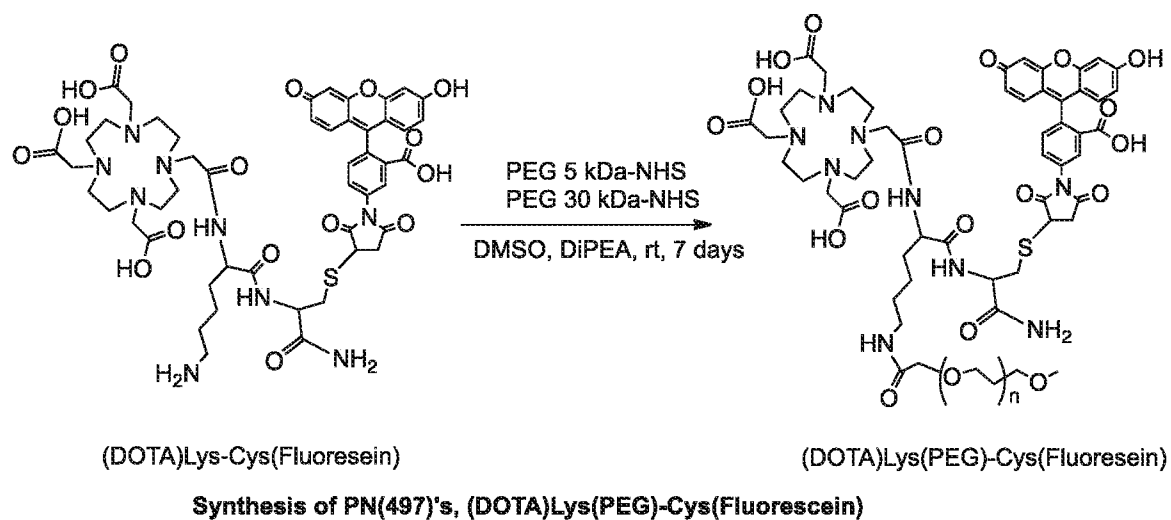


Figure 21

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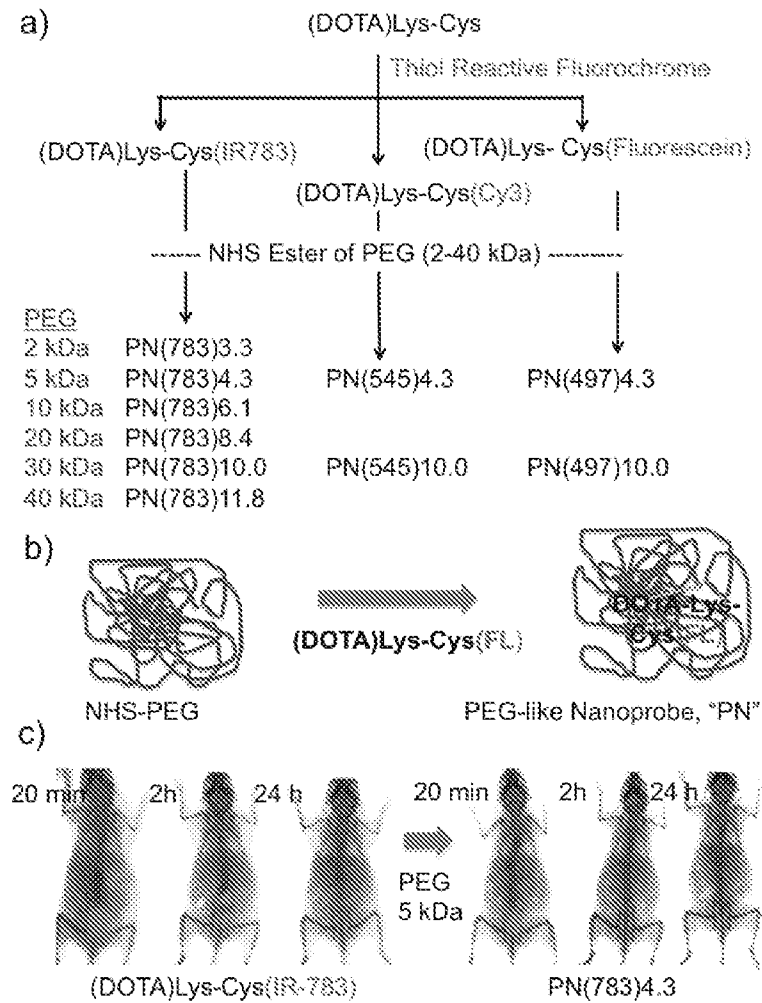


Figure 22

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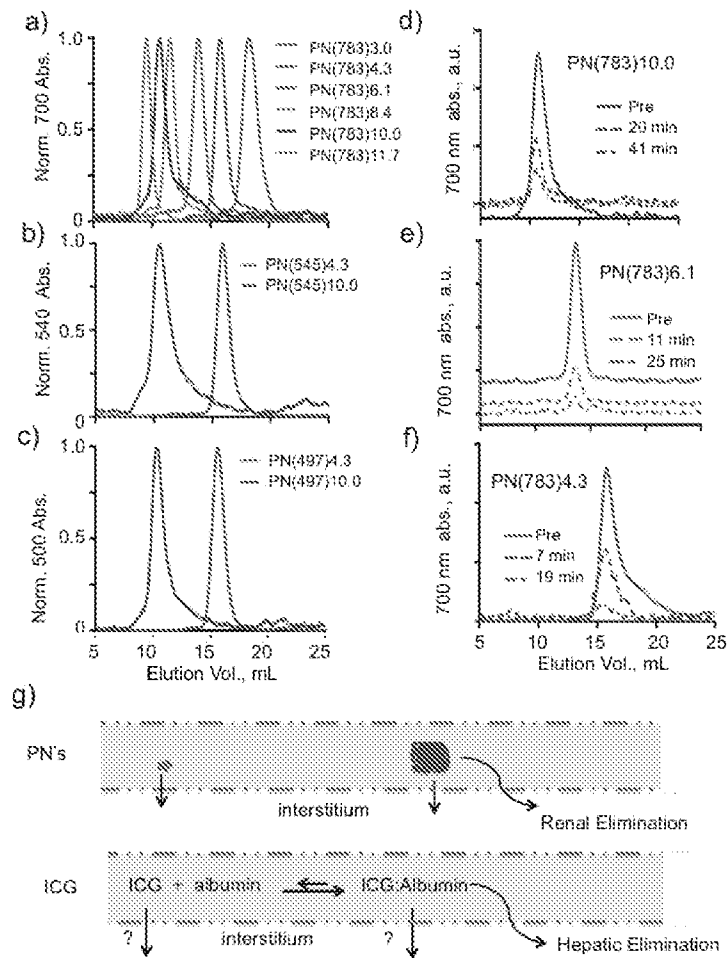


Figure 23

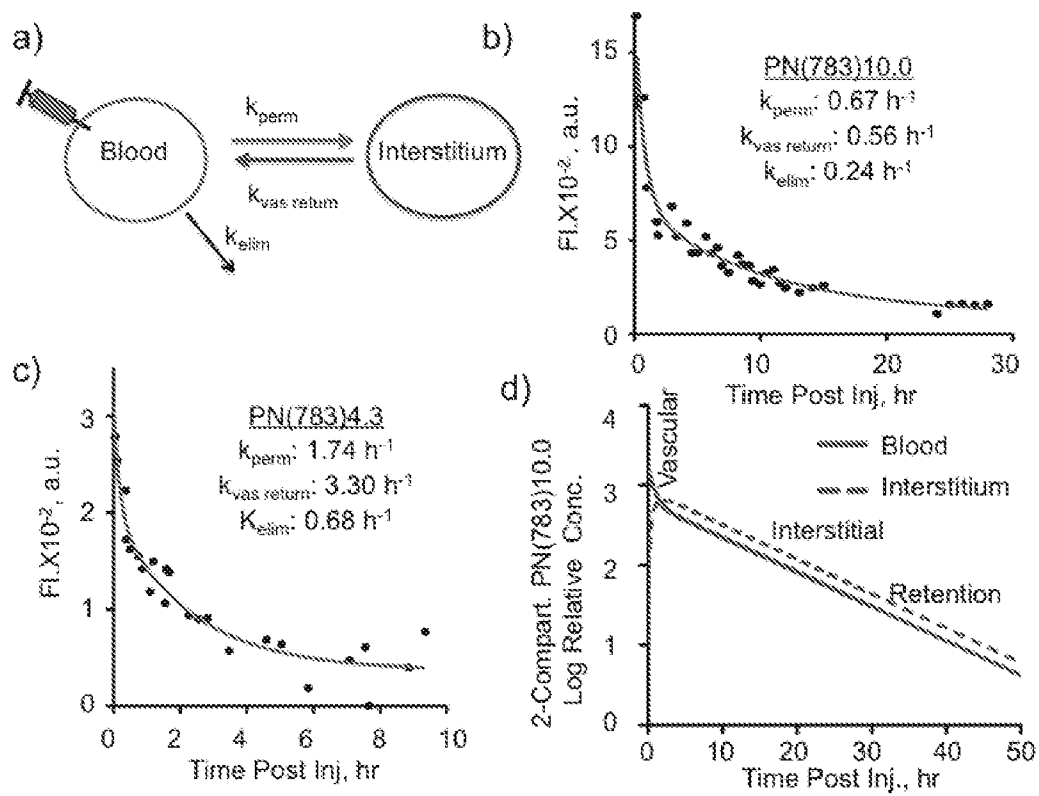


Figure 24

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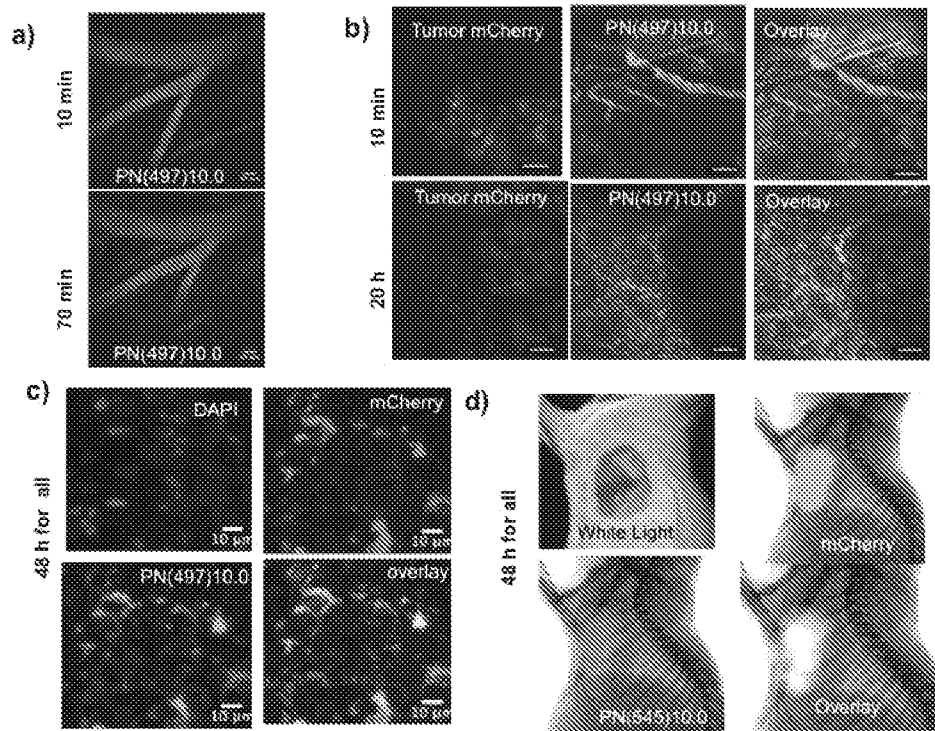


Figure 25

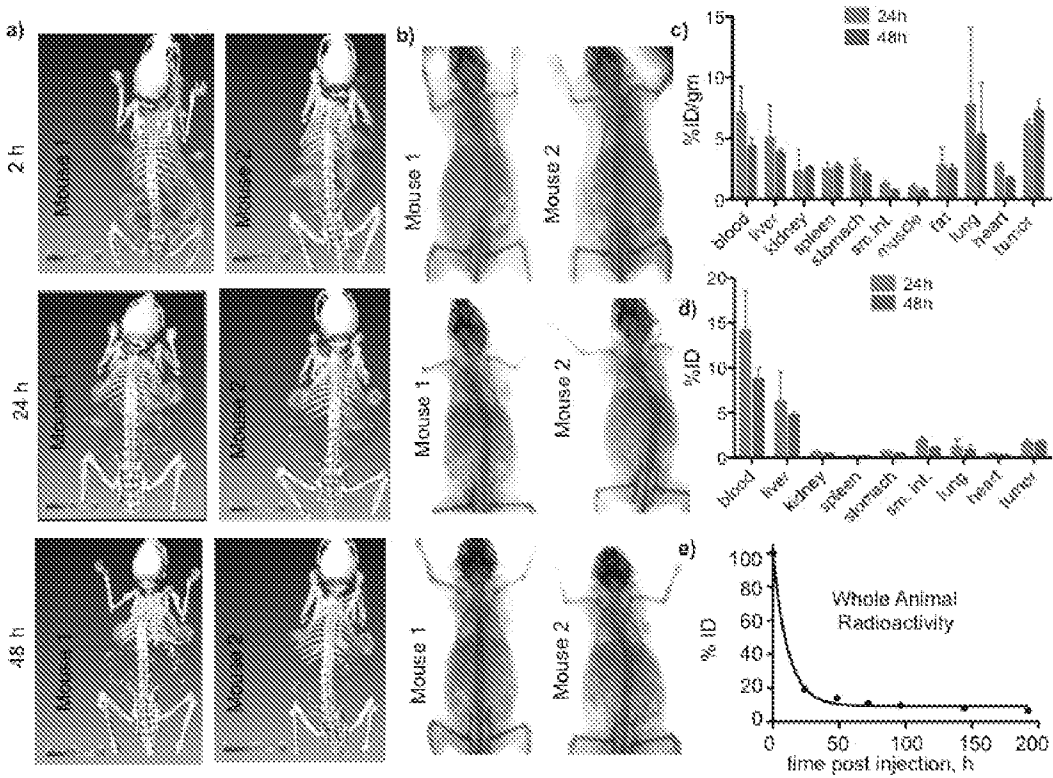


Figure 26

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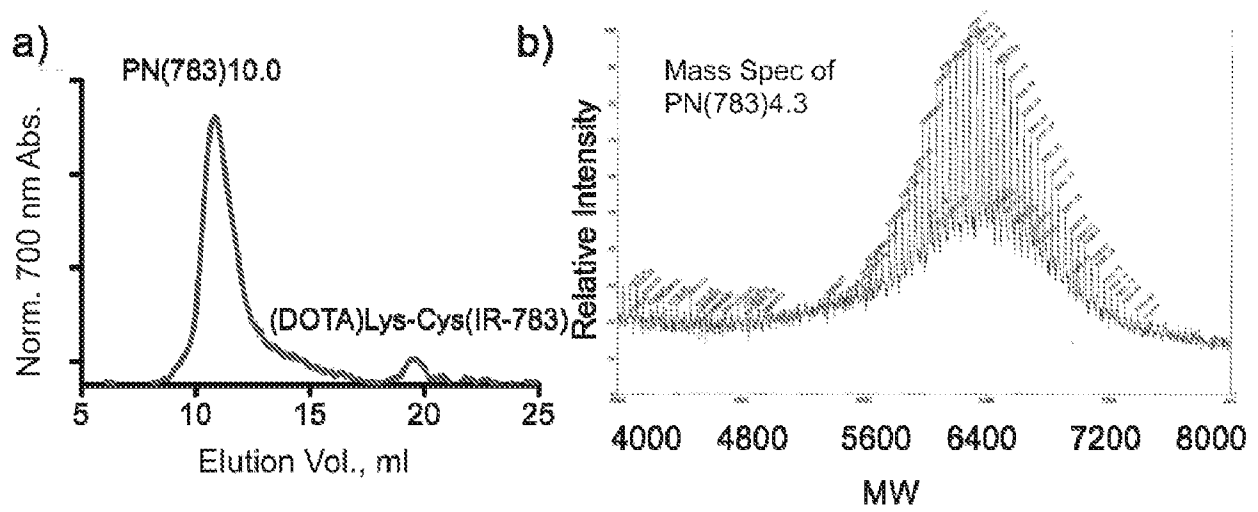


Figure 27

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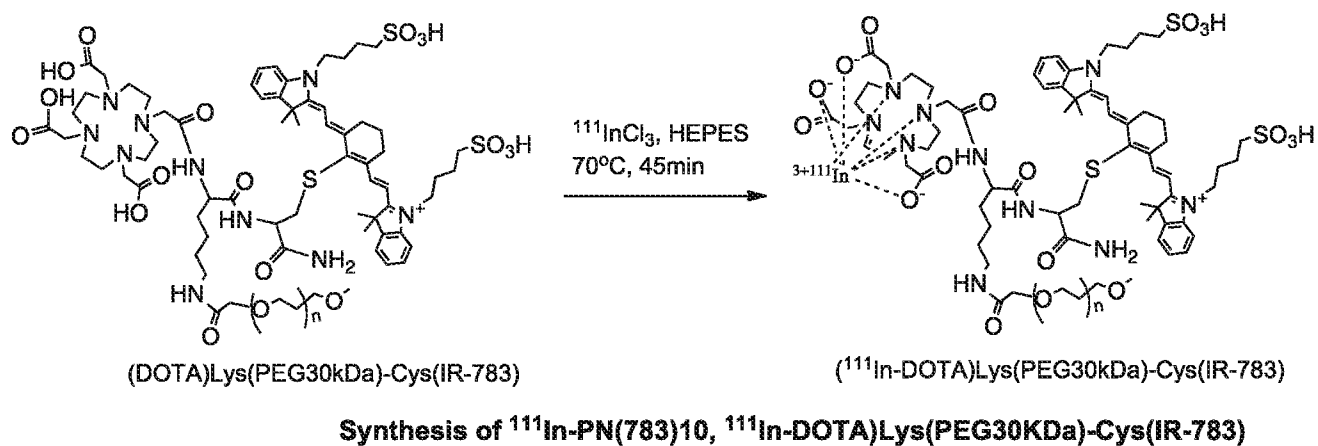


Figure 28

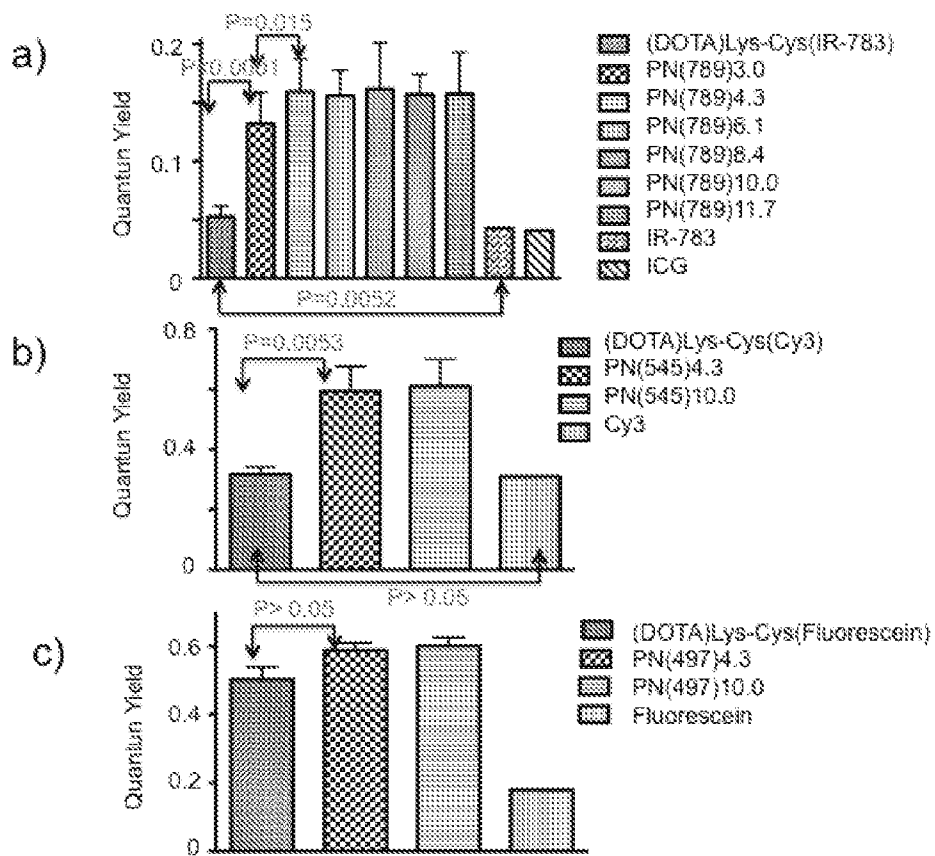


Figure 29

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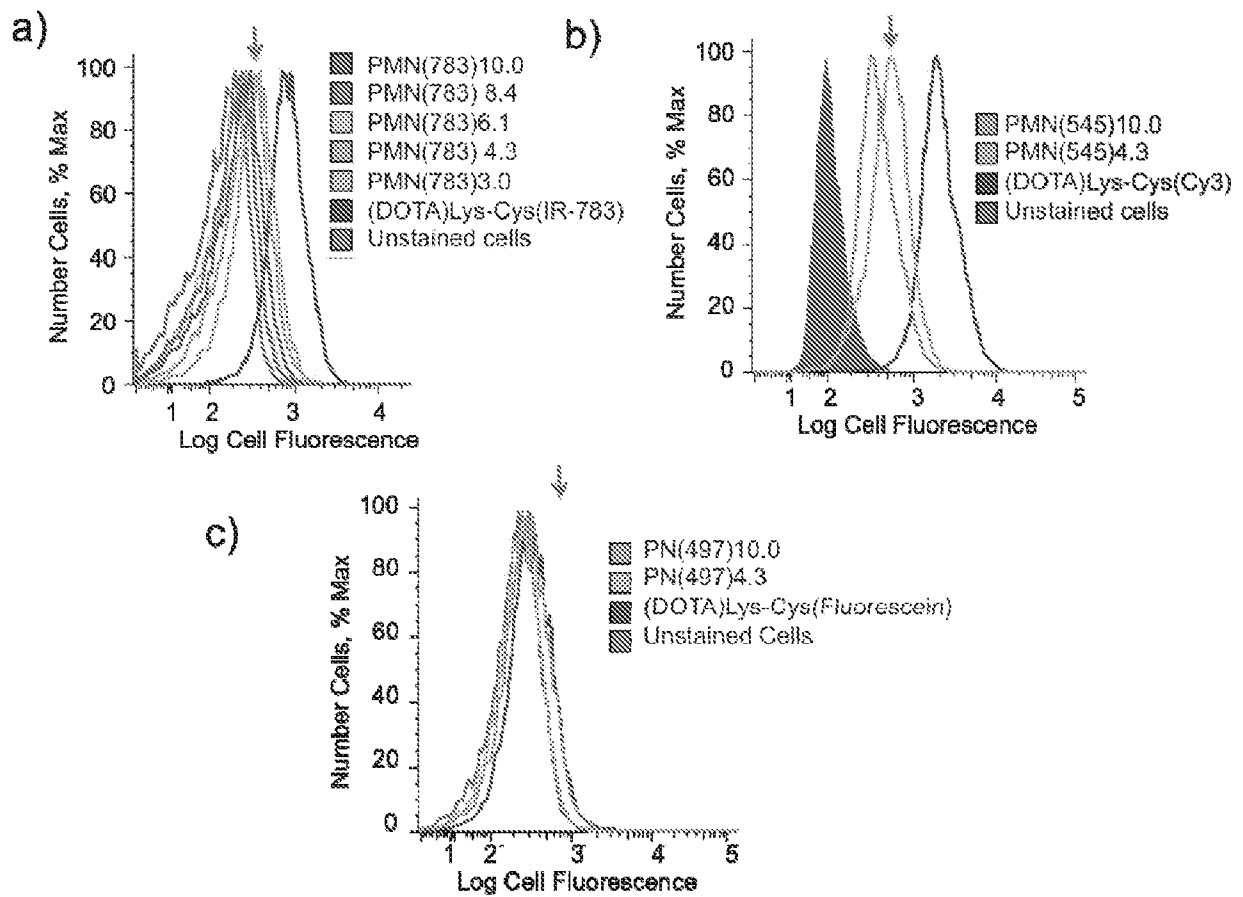


Figure 30

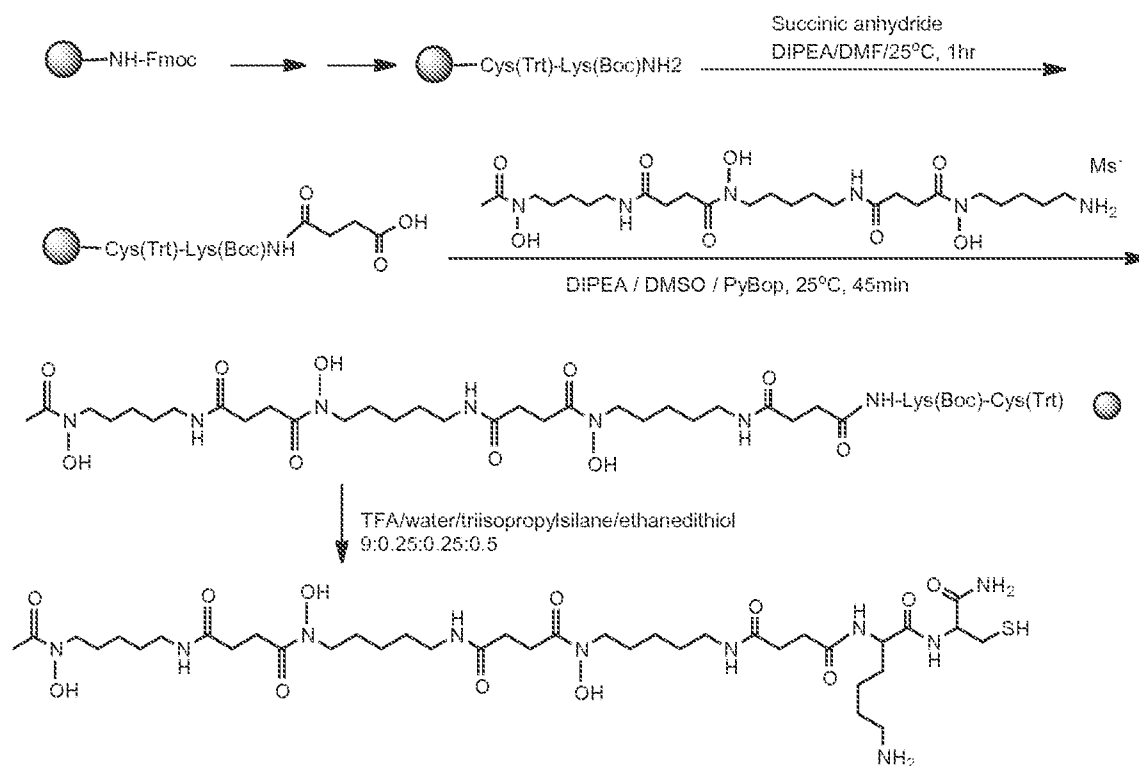


Figure 31

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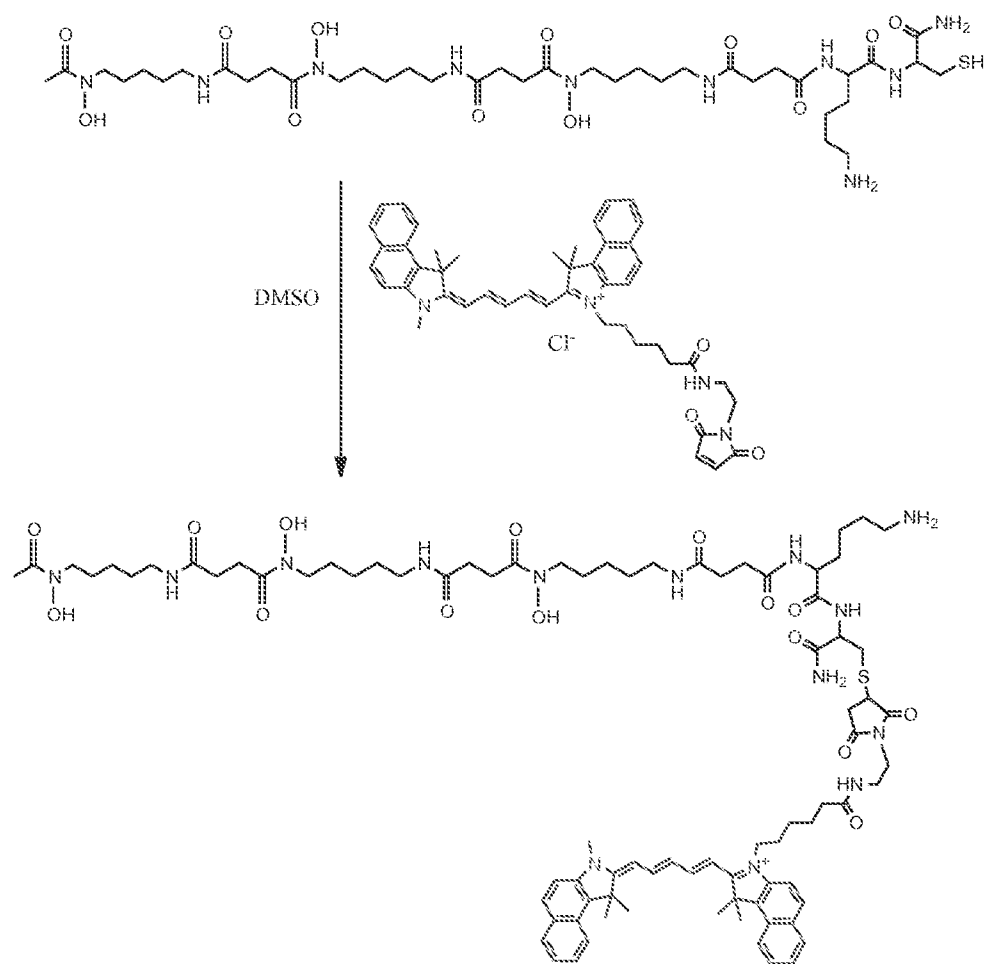


Figure 32

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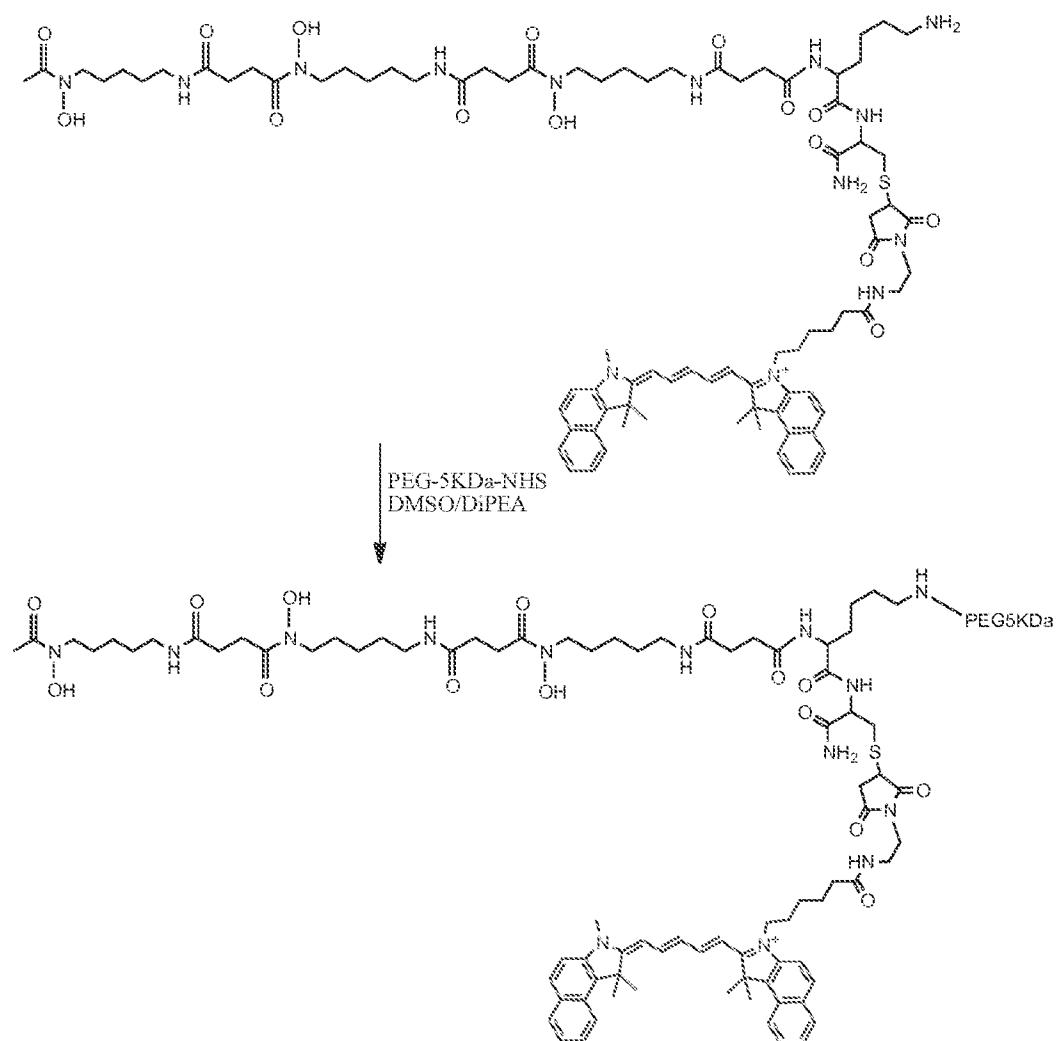


Figure 33

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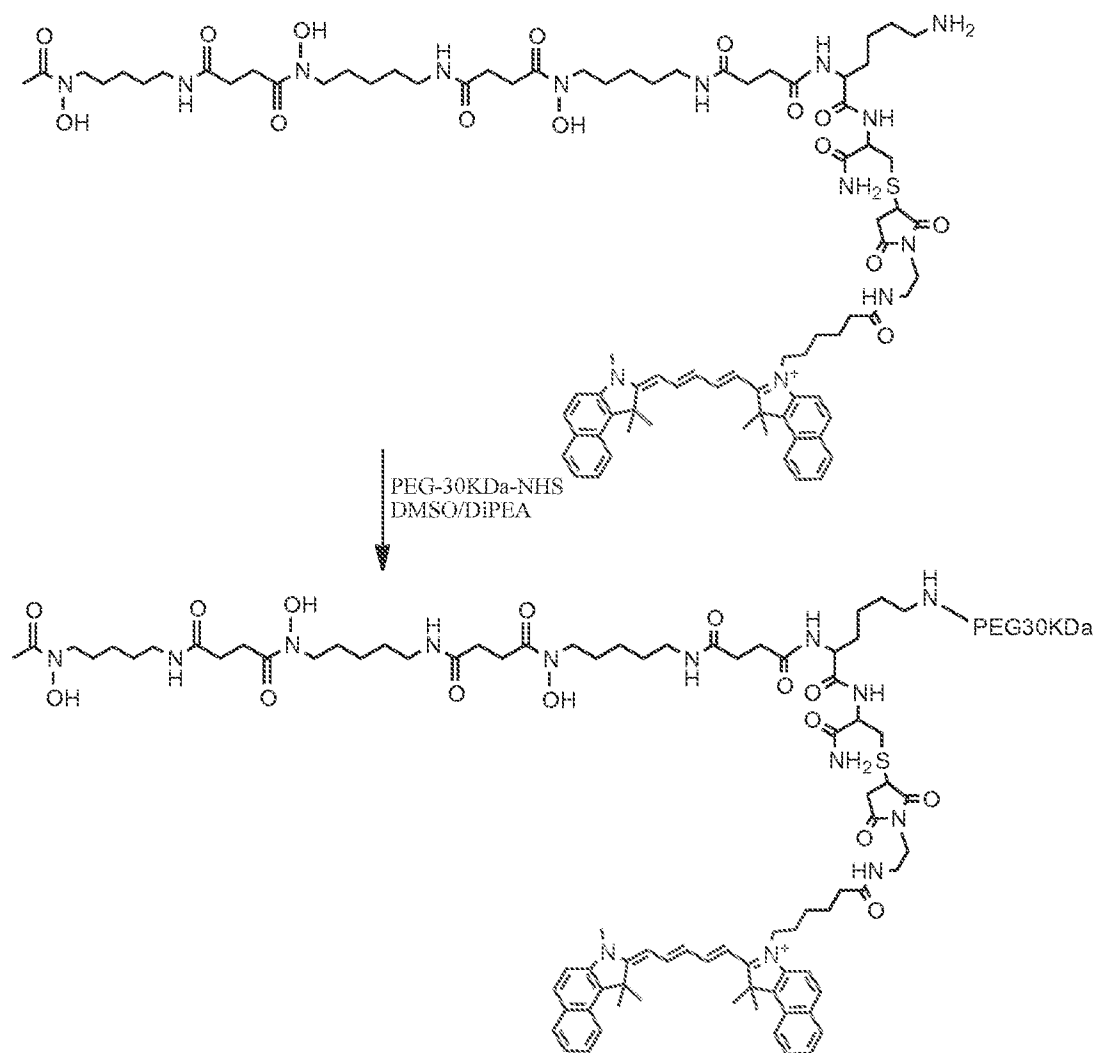


Figure 34

Figure 35

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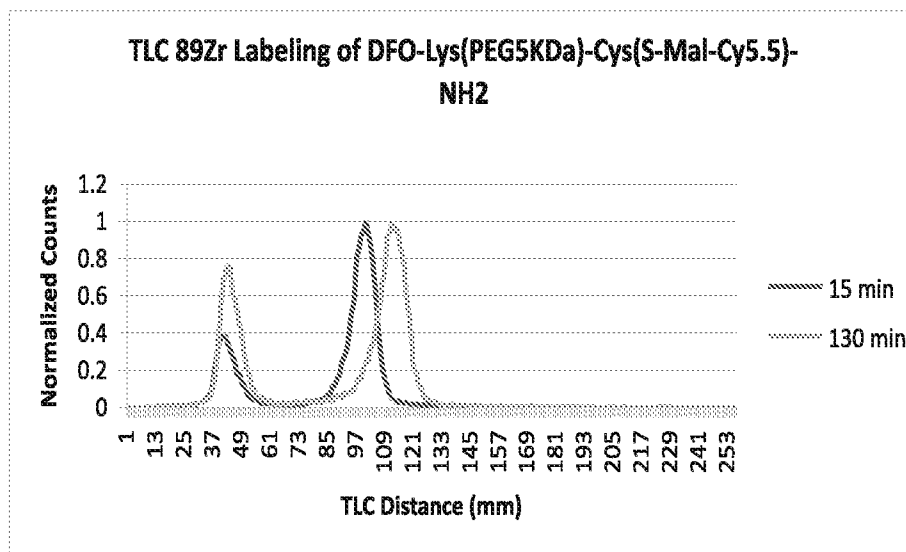


Figure 36

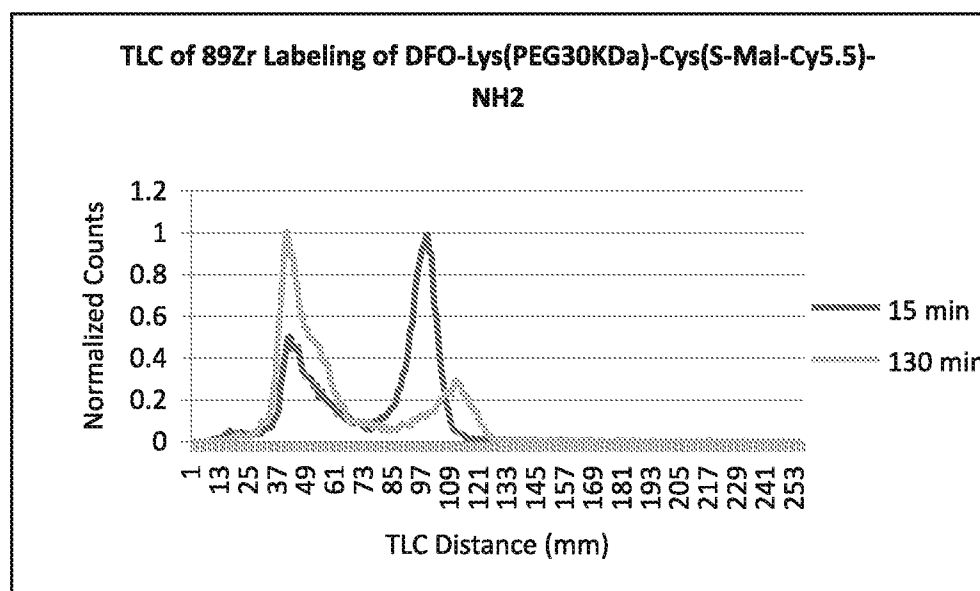


Figure 37

INTERNATIONAL SEARCH REPORT	International application No. PCT/US 2013/060565												
A. CLASSIFICATION OF SUBJECT MATTER <i>C08G 63/66 (2006.01)</i> <i>C08G 63/91 (2006.01)</i> <i>A61K 38/02 (2006.01)</i> <i>A61K 47/30 (2006.01)</i> <i>A61K 49/12 (2006.01)</i> According to International Patent Classification (IPC) or to both national classification and IPC													
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C08G 63/66, 63/88, 63/91, A61K 38/02, 38/03, 38/04, 47/30, 47/34, 47/42, 49/00, 49/12 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Espacenet, PAJ, CIPO, SIPO, DWPI, USPTO, Patsearch (RUPTO internal), EAPATIS													
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category*</th> <th style="width: 70%;">Citation of document, with indication, where appropriate, of the relevant passages</th> <th style="width: 20%;">Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X Y</td> <td>WO 2010/106169 A1 (GENERAL ELECTRIC COMPANY et al.) 23.09.2010, p. 7, line 10, p. 8, line 8, p. 9, line 5 - p. 12, line 9 - p. 17, line 13, p. 20, line 1 - p. 25, line 29, examples 2, 3, 4, claims 1-19</td> <td style="text-align: center; vertical-align: top;">1-20, 31-39 21-30</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X Y</td> <td>US 2010/0297017 A1 (MARK SAVELLANO) 25.11.2010, abstract, fig. 1A, 1B, paragraphs [0004] - [0018], [0022] - [0036], example 1, claims 1-14</td> <td style="text-align: center; vertical-align: top;">2-20 21-30</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X Y</td> <td>US 6083486 A (THE GENERAL HOSPITAL CORPORATION) 04.07.2000, abstract, fig. 1A, 1B, col. 1, line 45 - col. 5, line 45, col. 7, line 60 - col. 10, line 31, claims 1-19</td> <td style="text-align: center; vertical-align: top;">2-20, 31-39 21-30</td> </tr> </tbody> </table>		Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X Y	WO 2010/106169 A1 (GENERAL ELECTRIC COMPANY et al.) 23.09.2010, p. 7, line 10, p. 8, line 8, p. 9, line 5 - p. 12, line 9 - p. 17, line 13, p. 20, line 1 - p. 25, line 29, examples 2, 3, 4, claims 1-19	1-20, 31-39 21-30	X Y	US 2010/0297017 A1 (MARK SAVELLANO) 25.11.2010, abstract, fig. 1A, 1B, paragraphs [0004] - [0018], [0022] - [0036], example 1, claims 1-14	2-20 21-30	X Y	US 6083486 A (THE GENERAL HOSPITAL CORPORATION) 04.07.2000, abstract, fig. 1A, 1B, col. 1, line 45 - col. 5, line 45, col. 7, line 60 - col. 10, line 31, claims 1-19	2-20, 31-39 21-30
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.											
X Y	WO 2010/106169 A1 (GENERAL ELECTRIC COMPANY et al.) 23.09.2010, p. 7, line 10, p. 8, line 8, p. 9, line 5 - p. 12, line 9 - p. 17, line 13, p. 20, line 1 - p. 25, line 29, examples 2, 3, 4, claims 1-19	1-20, 31-39 21-30											
X Y	US 2010/0297017 A1 (MARK SAVELLANO) 25.11.2010, abstract, fig. 1A, 1B, paragraphs [0004] - [0018], [0022] - [0036], example 1, claims 1-14	2-20 21-30											
X Y	US 6083486 A (THE GENERAL HOSPITAL CORPORATION) 04.07.2000, abstract, fig. 1A, 1B, col. 1, line 45 - col. 5, line 45, col. 7, line 60 - col. 10, line 31, claims 1-19	2-20, 31-39 21-30											
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.													
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Date of the actual completion of the international search 26 November 2013 (26.11.2013)	Date of mailing of the international search report 06 March 2014 (06.03.2014)												
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Authorized officer E. Malashkova Telephone No. (495)531-64-81												

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2013/060565

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 6592847 B1 (THE GENERAL HOSPITAL CORPORATION) 15.07.2003, abstract, fig. 1A, 1B, col. 1, line 50 - col. 3, line 24, col. 4, line 25 - col. 5, line 55, col. 10, line 32 - col. 12, line 60, claims 1-6, 24-28	2-20, 31-39 21-30
X Y	RU 2393167 C2 (DZHI-I KHELTKE AS) 27.06.2010, p. 5, line 1 - p. 10, line 40, p. 16, line 34 - p. 18, line 53	2-20, 31-39 21-30
X Y	WO 2008/139206 A2 (GE HEALTHCARE AS et al.) 20.11.2008, p. 19, line 14 - p. 20, p. 6, line 14 - p. 7, line 10, p. 10, lines 1-5, p. 25, line 1 - p. 28, line 23	1, 4-20, 31-39 21-30
Y	RU 2441668 C2 (DZHI-I KHELTER LIMITED et al.) 10.02.2012, p. 11, lines 19-49, p. 14, line 26 - p. 16, line 16, p. 23, line 13 - p. 37, line 21	21-30

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US 2013/060565

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2013/060565

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:

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

Remark on Protest

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