



(51) International Patent Classification:

A61K 49/14 (2006.01) *A61K 9/16* (2006.01)
A61K 31/7042 (2006.01) *A61P 35/00* (2006.01)
A61K 47/48 (2006.01)

(21) International Application Number:

PCT/US2013/021434

(22) International Filing Date:

14 January 2013 (14.01.2013)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/586,282 13 January 2012 (13.01.2012) US
61/666,474 29 June 2012 (29.06.2012) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: EPHERIN RECEPTOR TARGETING AGENTS

(57) Abstract: Peptide targeting agents are provided that may be used as imaging and/or therapeutic agents for tumor cells overexpressing Ephrin receptors. In particular, the agents can be used for targeting cells expressing EphB4 and/or EphA2 receptors. Methods for non-invasive imaging and anticancer therapy using the targeting agents are likewise provided.



WO 2013/106824 A1

DESCRIPTION

EPHERIN RECEPTOR TARGETING AGENTS

BACKGROUND OF THE INVENTION

[0001] This application claims the benefit of United States Provisional Patent
5 Application Nos. 61/586,282, filed January 13, 2012, and 61/666,474, filed June 29, 2012,
the entirety of which are incorporated herein by reference.

[0002] The invention was made in part with government support under Grant No.
RC2 GM092599 awarded by the National Institutes of Health. The government has certain
rights in the invention.

10 **1. Field of the Invention**

[0003] The present invention relates generally to the field of molecular imaging and
nanomedicine. More particularly, it concerns cancer targeting agents and methods for
targeted imaging and therapy of cancer cells.

2. Description of Related Art

15 [0004] Over the past two decades, various radiolabeled peptides have been developed
as nuclear imaging agents for tumor detection and noninvasive assessment of receptor
expression in solid tumors. For example, small radiolabeled somatostatin peptidyl analogs
with a molecular weight of ~1.5 kDa have been successfully utilized in the clinic for
localizing neuroendocrine tumors expressing somatostatin receptors (Bakker *et al.*, 1991;
20 Hammond *et al.*, 1993). Cyclic Arg-Gly-Asp (RGD) peptide that strongly binds to integrin
 $\alpha v \beta 3$ receptors is currently under clinical investigation (Haubner *et al.*, 2009). Several other
peptide-based imaging agents, including melanocyte-stimulating hormone (MSH) analog
(Chen *et al.*, 2000), substance P (Hargreaves, 2002), calcitonin (Blower *et al.*, 1998), atrial
natriuretic peptide (ANP) (Liu *et al.*, 2010), bombesin/gastrin-releasing peptide (GRP),
25 cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and neuropeptide-Y (NPY), have
also been identified and characterized for tumor receptor imaging (Schottelius and Wester,
2009). In spite of these efforts, peptides that can be used for the noninvasive detection of
prostate, ovarian, melanoma, and colon cancer remain elusive (Schottelius and Wester, 2009).

[0005] Many solid tumors, including prostate, ovarian, melanoma, and colon cancer, overexpress EphB4 receptor, a member of the Ephrin receptor tyrosine kinase family. Noninvasive imaging of EphB4 using peptide-based imaging agents could potentially increase early detection rates, monitor response to therapy directed again EphB4, and improve patient outcomes. Likewise, specific targeting of therapeutic agents to EphB4 expressing cancers could greatly improve the efficacy and specificity of cancer therapy.

SUMMARY OF THE INVENTION

[0006] In a first embodiment there is provided an Ephrin receptor targeting agent comprising at least a first peptide comprising the amino acid sequence TNYLFSPNGPIA (SEQ ID NO: 2) conjugated to a therapeutic agent or an imaging agent. In some aspects, the first peptide is defined as a peptide that binds specifically or preferentially to Eph receptor EphB4. For example, the peptide can comprise a sequence TNYLFSPNGPIARAW (SEQ ID NO: 1) or a sequence identical to SEQ ID NO:1, but comprising 1 or 2 amino acid substitutions, insertions or deletions. In still a further aspect, the first peptide is a cyclic peptide, such as a peptide comprising a lactam bridge (*e.g.*, a cyclic peptide comprising the structure shown in FIG. 25).

[0007] In a further embodiment, a targeting agent of the embodiments further comprises a second peptide targeting agent. For example, the second peptide can bind to the same target as the first peptide or to a second target. In some aspects, the second peptide binds to an EphA2 receptor. Accordingly, in certain aspects, a targeting agent is provided that binds to EphA2 and EphB4 receptors. For instance, in some cases, the second peptide comprises an amino acid sequence of SEQ ID NO: 4. In certain aspects, a first and second peptide of the embodiments are comprised in a fusion protein. In still further aspects, the first and second peptides are connected by a linker. Examples of linkers include, without limitation, linker peptides, carbohydrate polymers and fatty acids. In some cases, the linker is a polyethylene glycol (PEG) moiety.

[0008] In further aspects of the embodiments, the first peptide of a targeting agent is conjugated to a radioisotope, a nanoparticle, a toxin, a chemotherapeutic agent, a fluorescent dye or a combination thereof. For example, the conjugate may be through covalent linkage or a non-covalent association, such as via chelator moiety (*e.g.*, 1,4,7,10-tetraazadodecane-N, N',N'',N'''-tetraacetic acid (DOTA)). In certain aspects, the first peptide is conjugated to an

imaging agent, such as a SPECT imaging agent, PET imaging agent an MRI contrast agent, or a fluorescent dye.

[0009] In still further aspects, the first peptide is conjugated to a radioisotope, such as a gamma emitter, a positron emitter or a beta-emitter. In certain aspects, the radioisotope is conjugated to the first peptide through a chelating moiety (*e.g.*, DOTA). In other aspects, the radioisotope is conjugated to the first peptide as part of a molecule that comprises the radioisotope. Example, radioisotopes for use in accordance with the invention include, without limitation, astatine-211, chromium-51, cobalt-57, cobalt-58, copper-60, copper-61, copper-62, copper-64, copper-66, copper-67, Eu-152, gallium-67, gallium-68, indium-111, iron-59, lutetium-177m, rhenium-186, rhenium-188, selenium-75, strontium-89, technetium-99m, thorium-227, and/or yttrium-90.

[0010] In still further aspects, a first peptide of the embodiments is conjugated to a therapeutic agent, such as chemotherapeutic agent, a radioisotope, or a therapeutic nanoparticle. For example, in some aspects, the chemotherapeutic agent is an anthracycline antibiotic, such as doxorubicin (Dox). Examples of therapeutic nanoparticles include, without limitation, nanoparticles that may be used to apply a photothermal ablation therapy, such as a hollow gold nanosphere (HAuNS). In some aspects, a nanoparticle of the embodiments is further loaded with or conjugated to a chemotherapeutic agent, such as Dox.

[0011] In a further embodiment a method of imaging a subject is provided comprising: (a) administering an effective amount of a targeting agent of the embodiments to the subject (*e.g.*, a EphB4 and/or EphA2 targeting agent); and (b) imaging the subject to detect the presence of the targeting agent. For example, imaging the subject can comprise positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), or photoacoustic imaging.

[0012] In still a further embodiment a method of treating a subject is provided comprising administering an effective amount of a targeting agent of the embodiments to the subject. In certain aspects, a method of the embodiment can further comprise administering one or more secondary therapy to the patient. For example, the secondary therapy can be a surgical therapy, chemotherapy, radiation therapy or hormonal therapy. In some aspects, the secondary therapy can facilitate the activity the targeting agent. For example, in the cases, a targeting agent that comprises a nanoparticle, such as a HAuNS, and a photothermal ablation

therapy can be applied to heat the tissues surrounding the targeting agent. Likewise, in some cases, a nanoparticle can be loaded with a chemotherapy agent (*e.g.*, Dox) such that heating of the nanoparticle activates local release of the chemotherapy agent.

5 **[0013]** In still a further embodiment a method for dual imaging and therapy of a subject is provided comprising administering an effective amount of a targeting agent of the embodiments to a subject (*i.e.*, wherein the targeting agent comprises a therapeutic and imaging agent); and imaging the subject.

10 **[0014]** As used here a subject refers to an animal, such a human, canine or feline subject. In some aspects, the subject has or is suspected of a cancer, such as an EphB4- or EphA2-overexpressing cancer.

15 **[0015]** Thus, in some embodiments, there is provided an imaging probe for noninvasive imaging of EphB4 receptors comprising a heterodimer peptide, wherein said peptide has motifs which target both EphA2 and EphB4. For example, the heterodimer bispecific polypeptide can comprise the amino acid sequence of SEQ ID NO: 1 or 2 and 4 (*e.g.*, the amino acid sequence of SEQ ID NO: 1 and 4). Thus, in some aspects, the heterodimer, bispecific polypeptide comprises an EphA2-targeting motif and an EphB4-targeting motif separated by a linker, such as PEG linker. In some very specific aspects, the heterodimer polypeptide comprises from amino- to carboxy-terminus the amino acid sequence of SEQ ID NO: 1; a PEG linker; and the amino acid sequence of SEQ ID NO: 4.

20 **[0016]** In a further embodiment a method of monitoring a cancer patient is provided comprising the steps of (i) injecting the patient with a heterodimer peptide of the embodiments; (ii) imaging the patient using a PET/CT scan imaging device wherein the device produces a PET image; (iii) acquiring the PET image of a tumor in the patient; (iv) determining tumor-to-muscle ratio in the patient; and (v) administering a cancer treatment to
25 the patient based on the tumor-to-muscle ratio.

[0017] In still a further embodiment, a method is provided of identifying EphB4 receptor expression in a subject in need thereof comprising administering to the subject a therapeutic amount of the heterodimer peptide of the embodiments, and determining uptake or binding of the peptide in said subject.

[0018] In another aspect, embodiments of the present invention contemplate the use of targeting nanoparticles in combination with physiological and physical approaches, such as tumor priming, vascular disruption, degradation of the extracellular matrix, and vessel normalization.

5 [0019] In a still a further embodiment a method of reducing renal uptake of a radio-labeled agent in a subject is provided comprising administering the radio-labeled agent in conjunction with metformin. For example, the metformin can be administered before, after or essentially simultaneously with the radio-labeled agent. In some aspects, the radio-labeled agent is a radio-labeled therapeutic or imaging agent (*e.g.*, an Eph Receptor targeting agent of
10 the embodiments). Thus, in some aspects, there is provided a pharmaceutical composition comprising a radio-labeled therapeutic or imaging agent and metformin formulated together in a pharmaceutically acceptable carrier. Such a composition may be used for instance to reduce renal uptake of a radio-labeled agent in subject (*e.g.*, a subject being treated or imaged with the agent).

15 [0020] As used herein the specification, “a” or “an” may mean one or more. As used herein in the claim(s), when used in conjunction with the word “comprising”, the words “a” or “an” may mean one or more than one.

[0021] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive,
20 although the disclosure supports a definition that refers to only alternatives and “and/or.” As used herein “another” may mean at least a second or more.

[0022] Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

25 [0023] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art
30 from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0025] **FIG. 1.** Structure of ^{64}Cu -DOTA-TNYL-RAW and $^{\text{nat}}\text{Cu}$ -DOTA-TNYL-RAW peptides.

[0026] **FIG. 2.** SPR sensorgrams of $^{\text{nat}}\text{Cu}$ -DOTA-TNYL-RAW and a scrambled peptide on sensor chips coated with EphB4. The peptides were injected as ten two-fold concentration series from 1.6 nM to 800 nM and were analyzed in duplicate binding cycles. Data sets (shown in black) are overlaid with curves fit to a 1:1 mass transfer interaction model (gray lines). The vertical axes in response units represent binding of each peptide to immobilized EphB4.

[0027] **FIG. 3.** Uptake of ^{64}Cu -DOTA-TNYL-RAW in EphB4-expressing PC-3M and CT26 cells and EphB4-negative A549 cells. The cell-to-medium uptake ratio is expressed as [cpm/ μg protein in pellet]/[cpm/ μg medium]. ^{64}Cu -DOTA-TNYL-RAW exhibited increased uptake over time in PC-3M and CT26 cells. This uptake was blocked by the parent TNYL-RAW peptide. FIG. 3A: CT26 cells. FIG. 3B: PC-3M cells. FIG. 3C: A549 cells.

[0028] **FIG. 4.** Representative $\mu\text{PET/CT}$ images of mice bearing CT26, PC-3M, and A549 tumors after intravenous administration of ^{64}Cu -DOTA-TNYL-RAW ($n = 4$).

[0029] **FIG. 5.** Representative $\mu\text{PET/CT}$ images showing blocking of ^{64}Cu -DOTA-TNYL-RAW uptake in CT26 tumors at 4 h and in PC-3M tumors at 24 h after radiotracer injection. For the blocking experiment, ^{64}Cu -DOTA-TNYL-RAW was co-injected with cold TNYL-RAW (50 $\mu\text{g}/\text{mouse}$).

[0030] **FIG. 6.** FIG. 6A. Biodistribution of ^{64}Cu -DOTA-TNYL-RAW in mice bearing PC-3M and A549 tumors at 24 h after injection. Data are presented as percent of injected dose per gram of tissue (%ID/g) and are expressed as mean $\pm$ standard deviation ($n = 4$). FIG. 6B. Tumor-to-muscle-uptake ratio. In the blocking group, mice were injected with ^{64}Cu -

DOTA-TNYL-RAW (200 μ Ci) and cold TNYL-RAW peptide (20 μ g/mouse). Data are presented as mean $\pm$ standard deviation (n = 6). Data were obtained at 4 h after injection in mice with CT26 tumors and at 24 h after injection in mice with PC-3M tumors.

[0031] FIG. 7. FIG. 7A. The reaction scheme for the synthesis of $^{nat}\text{Cu}/^{64}\text{Cu}$ -DOTA-TNYL-RAW. FIG. 7B. Mass spectrometric confirmation of the formation of the $^{nat}\text{Cu(II)}$ -labeled complex ^{nat}Cu -DOTA-TNYL-RAW. The m/z for $[\text{M}+\text{Cu}]^{2+} = 1076.9957$ ($\text{C}_{96}\text{H}_{143}\text{CuN}_{26}\text{O}_{27}$, calculated $[\text{M}+\text{Cu}]^{2+}$: 1076.9917). FIG. 7C. Under the same HPLC conditions, ^{nat}Cu -DOTA-TNYL-RAW (UV detector) and ^{64}Cu -DOTA-TNYL-RAW (radiodetector) had almost identical retention times (12.5 min and 12.6 min, respectively).

[0032] FIG. 8. SPR sensorgrams of TNYL-RAW and DOTA-TNYL-RAW peptides on sensor chips coated with EphB4. The peptides were injected as ten two-fold concentration series from 1.6 nM to 800 nM and were analyzed in duplicate binding cycles. Data sets (shown in black) are overlaid with curves fit to a 1:1 mass transfer interaction model (gray lines). The vertical axes in response units represent binding of each peptide to immobilized EphB4.

[0033] FIG. 9. Reaction scheme for the synthesis of SH-PEG-c(TNYL-RAW) and its conjugation to HAU NS.

[0034] FIG. 10. Cellular uptake of T-DOX@HAU NS. FIG. 10A. Representative photomicrographs of Hey cells after incubation with T-DOX@HAU NS for 2 h. The scattering signal from the HAU NS was visualized using a dark-field condenser, and fluorescence was from DOX. Cell nuclei were counterstained with DAPI. Bar, 20 μ m. FIG. 10B. Quantitative cellular uptake of nanoparticles in Hey and A549 cells after 3 h incubation with ^{111}In -labeled T-DOX@HAU NS and T-DOX@HAU NS plus free c(TNYL-RAW) (blocking). **P < 0.005; *P < 0.05.

[0035] FIG. 11. T-DOX@HAU NS pharmacokinetics, biodistribution, and tumor uptake. FIG. 11A. Activity-time profiles of ^{111}In -labeled T-DOX@HAU NS and DOX@HAU NS in Swiss mice. The data are expressed as percentage of the injected dose per gram of blood (%ID/g) and are presented as mean $\pm$ standard deviation (n = 8). FIG. 11B. Biodistribution of ^{111}In -labeled T-DOX@HAU NS, T-DOX@HAU NS with blocking, and DOX@HAU NS in nude mice at 24 h after injection. FIG. 11C. Comparison of nanoparticle uptake in Hey, A2780, and MDA-MB-231 tumors at 24 h postinjection. The data are

expressed as percentage of the injected dose per gram of tissue (%ID/g) and are presented as mean $\pm$ standard deviation (n = 6). **P < 0.005; *P < 0.05.

[0036] FIG. 12. NIR-induced temperature change in tumors injected with H AuNS.

[0037] FIG. 13. Antitumor activity of various treatments against Hey tumors. FIG.

5 13A. Tumor growth curves for mice treated with saline plus laser (n = 6), H AuNS plus laser (n = 6), DOX@H AuNS plus laser (n = 7), and T-DOX@H AuNS plus laser (n = 8). NIR laser irradiation (2.0 W/cm² for 3 min) was commenced at 24 h after injection. FIG. 13B. Average tumor weights (left panel) and photographs (right panel) of tumors from different treatment groups. Tumors were removed on day 22 for all groups except the saline-plus-laser group, in
10 which tumors were removed on day 9. Six tumors in the T-DOX@H AuNS group regressed completely by day 22 after treatment, and only the scar tissues at the site of tumor inoculation were removed and photographed (circle, right panel). FIG. 13C. Representative photomicrographs of hematoxylin and eosin-stained slides from scar tissue from a mouse
15 treated with T-DOX@H AuNS-plus-laser or tumors from mice treated with saline or DOX@H AuNS-plus-laser on day 22 after treatment.

[0038] FIG. 14. Physical properties of H AuNS. FIG. 14A. Absorption spectrum of H AuNS. FIG. 14B. TEM image of H AuNS. FIG. 14C. High-resolution TEM of a single H AuNS.

[0039] FIG. 15. FIG. 15A. SPR sensorgrams of c(TNYL-RAW) peptide on sensor
20 chips coated with EphB4. Peptides were injected as ten two-fold concentration series from 1.6 nM to 800 nM and were analyzed in duplicate binding cycles. Data sets (shown in black) are overlaid with curves fit to a 1:1 mass transfer interaction model (gray lines). The vertical axes in response units represent binding of each peptide to immobilized EphB4. c(TNYL-RAW) peptide bound to EphB4 with an association rate (k_{on}) of 1.09×10^6 [M⁻¹S⁻¹], a
25 dissociation rate (k_{off}) of 4.82×10^{-3} [s⁻¹], and an equilibrium dissociation constant ($K_d = k_{off}/k_{on}$) of 4.4×10^{-9} [M]. FIG. 15B. Comparison of peptide stability in mouse plasma. ⁶⁴Cu-DOTA-TNYL-RAW or ⁶⁴Cu-DOTA-c(TNYL-RAW) was incubated in mouse plasma at 37°C. A 100 μ L of plasma was removed from the incubation solution at 0, 2, 4, 8, 12, and 24
h time points and subjected to solid phase extraction on a C18 cartridge SPE column (Waters,
30 Milford, MA). Then 20 μ L of the extract was analyzed by reversed phase-high-performance liquid chromatography (RP-HPLC) on an Agilent 1100 system (C-18, Vydac, 4.6×250 mm,

10 μm) equipped with a radiodetector. The system was eluted with a linear gradient of 10%–90% acetonitrile in a 0.1% aqueous trifluoroacetic acid solution over 35 min at a flow rate of 1.0 mL/min.

5 [0040] **FIG. 16.** Western blot analysis of EphB4 expression in MDA-MB-231, A549, A2780, and Hey cell lines.

[0041] **FIG. 17.** Cell viability as a function of equivalent DOX concentration. A549 (FIG. 17A) and A2780 (FIG. 17B) cells were treated with T-DOX@HAuNS, DOX@HAuNS, or free DOX. The viability of cells was determined using MTT assay. Circles are T-DOX@HAuNS; Squares are DOX@HAuNA; Triangles are Free DOX.

10 [0042] **FIG. 18.** Percentage of body weight change after various treatments. All data are presented as mean $\pm$ standard deviation. Diamonds are Saline + Laser; Squares are HAuNS + Laser; Triangles are DOX@HAuNS + Laser; X is T-DOX@HAuNS + Laser.

15 [0043] **FIG. 19.** FIG. 19A. Structure of heterodimer peptides with motifs targeting both EphA2 and EphB4 receptors. FIG. 19B. Characterization of heterodimer peptides by ESI-MS. FIG. 19C. Characterization of heterodimer peptides by HPLC.

[0044] **FIG. 20.** Surface competition assays. FIG. 20A. Surface competition assays were performed with YSA-TNYL-RAW dimer and ephrinB2 with the surface coated with EphB4 using an increasing concentration of heterodimer and a constant concentration of ephrinB2, the natural ligand of EphB4. FIG. 20B. Surface competition assays were also performed with YSA-TNYL-RAW dimer and EphA2 with the surface coated with ephrinA1, the natural ligand of EphA2, using an increasing concentration of heterodimer and a constant concentration of EphA2. The highest concentration corresponds to the bottom line on the sensorgrams while the lowest corresponds to the top line.

25 [0045] **FIG. 21.** Cell binding and inhibition in ovarian cancer Hey cells. FIG. 21A. Cell binding of ^{64}Cu -DOTA-YSA-TNYL-RAW dimer over time. FIG. 21B. Cell binding at a single time point with various blocking agents. The bars, from left to right, are as follows: No blocking, Block w/ Dimer, Block w/ TNYL-RAW, Block w/ YSA.

[0046] **FIG. 22.** Biodistribution of ^{64}Cu -labeled YSA-TNYL-RAW bispecific heterodimer targeting both EphA2 and EphB4 receptors in ovarian cancer xenografts.

[0047] **FIG. 23.** Structure of the dual-labeled EphB4-binding peptide DOTA-TNYL-Cy5.5.

[0048] **FIG. 24.** Immunohistochemistry of tumor sections confirming the binding of ^{64}Cu -DOTA-TNYL-Cy5.5 to tumor cells and tumor microvessels. U251 and U87 tumors were stained and imaged for CD31 and EphB4.

[0049] **FIG. 25.** Structure of EphB4-targeting cyclic peptide c(TNYL-RAW).

[0050] **FIG. 26.** Surface plasmon resonance curves for binding of c(TNYL-RAW) to EphB4.

[0051] **FIG. 27.** Stability of peptides in mouse serum as assayed by LC-MS. Cyclic peptide c(TNYL-RAW) was more stable in mouse serum than its corresponding linear peptide. Top line is c(TNYL-RAW); bottom line is TNYL-RAW.

[0052] **FIG. 28.** FIG. 28A. $\mu\text{PET/CT}$ images of nude mice bearing subcutaneous human melanoma A375SM xenografts using 7.4 MBq (200 μCi) of ^{68}Ga -NOTA-c(TNYL-RAW) peptide. Reduced retention in the liver and the spleen, as well as minimal retention in the lungs and GI tract, were shown by $\mu\text{PET/CT}$ imaging. FIG. 28B. Uptake of ^{68}Ga -NOTA-c(TNYL-RAW) in different organs. The peptide was labeled with ^{68}Ga through radiometal chelator 1,4,7-tetraazacyclododecane-N,N',N''-tetraacetic acid (NOTA). Data presented were obtained at 4 h postinjection by quantifying radioactivity in the region-of-interest from $\mu\text{PET/CT}$ images. M1 = mouse #1; M2 = mouse #2.

[0053] **FIG. 29.** Biodistribution of ^{68}Ga -NOTA-c(TNYL-RAW) injected with and without co-injection with metformin. The data presented were obtained from $\mu\text{PET/CT}$ imaged of melanoma A375SM xenografts. Images were acquired 1 h after intravenous injection of ^{68}Ga -NOTA-c(TNYL-RAW) together with metformin at a dose of 40 mg metformin/kg. Metformin is a clinically used drug. Co-injection with metformin reduced kidney uptake while in the mean time increased tumor uptake of the radiotracer.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

I. The Present Invention

[0054] Provided herein are a new class of peptides for imaging cells and patients
5 expressing EphB4 receptors. Radionuclide- and fluorescent dye-labeled peptides and a
method of using the peptides in imaging EphB4 receptors and Ephrin family of receptors with
the radionuclide and/or fluorescent dye labeled peptides are likewise provided. The disclosed
peptides can be potentially used for early detection of cancer and for monitoring response to
treatment directed at EphB4 receptors. The imaging properties in disease models include, but
10 are not limited to, dosimetry and toxicity.

[0055] Many solid tumors overexpress EphB4 receptor, a member of the Ephrin
receptor tyrosine kinase family. Noninvasive imaging of EphB4 could potentially increase
early detection rates, monitor response to therapy directed against EphB4, and improve patient
outcomes. A series of peptide-based imaging agents are disclosed with high receptor binding
15 affinity for nuclear imaging of EphB4 receptors.

[0056] In the first generation imaging probes, the EphB4-binding peptide
TNYLFSPNGPIARAW (TNYL-RAW) was conjugated with 1,4,7,10-tetraazadodecane-N,
N',N'',N'''-tetraacetic acid (DOTA). DOTA-TNYL-RAW was labeled with ^{64}Cu with high
labeling efficiency. In vitro, ^{64}Cu -DOTA-TNYL-RAW displayed high binding affinity to
20 EphB4 (Kd- 2 nM), was selectively taken up by CT26 and PC-3M cells, but not by A549
cells. Binding of FITC-TNYL-RAW and ^{64}Cu -DOTA-TNYL-RAW to CT26 and PC-3M
cells could be blocked by an excess amount of TNYL-RAW. *In vivo*, ^{64}Cu -DOTA-TNYL-
RAW showed significantly higher uptake in PC-3M tumors than in A549 tumor, with
percentages of injected dose per gram of tumor (%ID/g) values of 0.84 ± 0.09 and 0.44 ± 0.09 at
25 24 hr after radiotracer injection, respectively $\mu\text{PET/CT}$ imaging clearly revealed deposition of
 ^{64}Cu -DOTA-TNYL-RAW in CT26 and PC-3M tumors but not in A549 tumors. Furthermore,
uptake of ^{64}Cu -DOTA-TNYL-RAW in both CT26 and PC-3M tumors could be blocked by
cold TNYL-RAW. Thus, it was demonstrated that the expression of EphB4 receptors can be
noninvasively interrogated by $\mu\text{PET/CT}$ using ^{64}Cu -DOTA-TNYLRAW.

30 [0057] In the second generation imaging probes, the stability and the
pharmacokinetics of the 1st generation peptides were further improved, leading to imaging
probes with improved imaging properties

[0058] Both EphA2 and EphB4 receptors are over-expressed in a variety of solid tumors including cancers in the ovarian, breast, colorectal, brain, and prostate. These receptors are also expressed in angiogenic blood vessels. Therefore, members of the Ephrin receptor family are attractive targets for cancer imaging and therapy (Pasquale, *Nat Rev. Cancer*, 10:165, 2010). A series of peptidyl heterodimers as disclosed here with high receptor binding affinity to EphA2 and EphB4 suitable for molecular imaging of Ephrin receptors. It is demonstrated that the heterodimers exemplified by YSA-TNYL-DOTA-⁶⁴Cu had significantly higher target-to-background ratio than its corresponding monomeric imaging probes ⁶⁴Cu-DOTA-TNYL-RAW and ⁶⁴Cu-DOTAYSA targeting EphB4 and EphA2, respectively. Thus, imaging probes that simultaneously bind to both EphA2 and EphB4 can potentially increase early detection rates and be used to monitor response to therapy directed against EphA2 and EphB4. Moreover, a dual labeling approach is provided by introducing both a radionuclide and a near-infrared dye to EphB4-targeting peptide that allow dual modal imaging of the receptors. Such imaging probes can provide increased information content and are useful for both diagnostic imaging and guiding surgery intraoperatively.

[0059] A peptidyl heterodimer, YSAYPDSVPMMS(SEQ ID NO: 4)-PEG-TNYLFSPNGPIARAW (SEQ ID NO: 1) (YSA-TNYL), was synthesized by linking the two peptides YSAYPDSVPMMS (YSA; SEQ ID N: 4) targeting EphA2 and TNYLFSPNGPIARAW (TNYL; SEQ ID NO: 1) targeting EphB4 together with a polyethylene glycol (PEG) linker. YSA-TNYL was then labeled with the positron emitter ⁶⁴Cu through 1,4,7,10-tetraazacyclododecane-N, N', N'', N'''-tetraacetic acid (DOTA) chelator. The receptor-binding characteristics and tumor-targeting efficacy of heterodimer YSA-TNYL was evaluated *in vitro* with Surface Plasmon Resonance (SPR) sensor chip technology and *in vivo* using μ PET imaging. YSA-TNYL-DOTA peptide had comparable binding affinity to EphB4 compared with TNYL peptide and higher binding affinity to EphA2 than YSA peptide.

[0060] In human ovarian tumor HEY xenografts, YSA-TNYL-DOTA-⁶⁴Cu dimer showed significantly higher tumor uptake value compared with monomeric ⁶⁴Cu-DOTA-TNYL and monomeric ⁶⁴Cu-DOTA-YSA monomer analogs at all time points examined. The tumor uptake of YSA-TNYL-DOTA-⁶⁴Cu could be partially blocked with an excess amount of cold TNYL, YSA, or mixture of TNYLRAW and YSA peptides. Compared with ⁶⁴Cu-DOTATNYL and ⁶⁴Cu-DOTA-YSA monomeric tracers, the heterodimer YSA-TNYL-

DOTA-⁶⁴Cu also showed improved pharmacokinetics, resulting in a significantly higher target-to-background ratio. Thus, this class of radiotracers directed at both EphA2 and EphB4 should be useful imaging probes for early tumor detection and noninvasive characterization of Ephrin receptors.

5 **[0061]** ⁶⁴Cu-DOTA and Cy5.5 dye were introduced to TNYL peptide to synthesize dual-tracer imaging probe. In vitro, dual labeled TNYL displayed significantly higher binding to U251 glioma cells over-expressing EphB4 than to U87 cells that express low level of EphB4. The binding of dual labeled TNYL-RAW to U251 cells could be blocked by a large excess of unlabeled peptide. In vivo, μ PET/CT and near-infrared fluorescence optical
10 imaging clearly showed the uptake of the dual labeled TNYL-RAW peptide in both U251 and U87 tumors in the brains of nude mice after intravenous injection. The specific uptake of dual labeled peptide in both tumors was confirmed by blocking experiments. In U87 tumors, Cy5.5-labeled peptide was found co-localized only with CD31- and EphB4-expressing tumor blood vessels. In U251 tumors, ⁶⁴Cu-DOTA-TNYLCy5.5 was found to bind to both brain
15 tumor cells and angiogenic blood vessels expressing EphB4 receptors.

[0062] Thus, in certain embodiments, this invention discloses new classes of peptide imaging agents suitable for noninvasive detection of tumor cells overexpressing EphB4 receptors (and in some cases EphA2 receptors). The disclosed peptides can be potentially used for early cancer detection and monitoring of treatment response. The concept can be
20 potentially used for guiding cancer surgery under fluorescent imaging. The same concept can be applied to the detection of other receptors in the Ephrin receptor family.

[0063] In some aspects, this invention discloses a novel ⁶⁴Cu-labeled peptide with high receptor binding affinity (i.e., low nanomolar K_D values) for PET imaging of EphB4 receptors. The expression of EphB4 receptors can be noninvasively interrogated by μ PET/CT
25 using the disclosed peptide, ⁶⁴Cu-DOTA-TNYL-RAW.

[0064] Photothermal ablation (PTA) is an emerging technique that uses near-infrared laser light-generated heat to destroy tumor cells. However, complete tumor eradication by PTA therapy alone is difficult because heterogeneous heat distribution can lead to sub-lethal thermal dose in some areas of the tumor. Successful PTA therapy requires selective delivery
30 of photothermal conducting nanoparticles to mediate effective PTA of tumor cells, and the ability to combine PTA with other therapy modalities. Multifunctional doxorubicin (DOX)-

loaded hollow gold nanospheres (DOX@HAuNS) were synthesized that target EphB4, a member of the Eph family of receptor tyrosine kinases overexpressed on the cell membrane of multiple tumors and angiogenic blood vessels. The tumors in six of eight mice treated with T-DOX@HAuNS where T represents a cyclic peptide targeting EphB4 plus laser regressed completely with only residual scar tissue by 22 days following injection, and none of the treatment groups experienced a loss in body weight. Concerted chemo-photothermal therapy with a single nanodevice capable of mediating simultaneous PTA and local drug release may have promise as a new anticancer therapy.

[0065] In some other aspects, this invention discloses an optimized cyclic peptide with high stability and low background suitable for noninvasive detection of tumor cells overexpressing EphB4 receptors. The disclosed peptides can be potentially used for early cancer detection and monitoring of treatment response. The same peptide can also be used for targeted delivery nanoparticles and therapeutic agents. The cyclic peptide can be labeled with any positron emitter or gamma emitter for PET and SPECT imaging, respectively. The peptide can also be labeled with a fluorescent dye to guide surgery.

[0066] In some aspects, this invention discloses a method of nuclear imaging for reducing renal uptake of radiotracers. The method can be potentially used to reduce renal toxicity of radiolabeled compounds used in radionuclide therapy and imaging.

II. Ephrin receptors

[0067] The Eph receptors are the largest family of receptor tyrosine kinases (Pasquale, 2005). Eph receptor tyrosine kinases and their ligands (ephrin) regulate a wide range of cell contact-dependent signaling that can effect cell proliferation, migration, morphology, adhesion, and invasion (Pitulescu *et al. Genes & Dev.*, 24:2480-2492, 2010). This can occur through Eph signaling which alters the actin cytoskeleton organization and integrins and intercellular adhesion molecules processes. Eph-ephrin interactions are important for many biological roles including axon growth and maturation, cell positioning in the gastrointestinal tract, blood vessel morphogenesis and angiogenic sprouting, insulin secretion, bone remodeling, and immune function.

[0068] Eph receptors are divided into an EphA and an EphB class that bind to glycosylphosphatidylinositol-linked ephrin-A ligands and the transmembrane ephrin-B ligands, respectively. EphB4 receptors play important roles in a variety of biological

processes, including cell aggregation and migration, neural development, embryogenesis and angiogenesis, and vascular development (Dodelet and Pasquale, 2000; Noren *et al.*, 2004; Erber *et al.*, 2006; Wang *et al.*, 1998). EphB4 selectively binds to its endogenous ligand, ephrin-B2, to promote cell signaling required for cancer progression and angiogenesis and
5 has been shown to be profoundly upregulated in numerous cancer types, such as prostate, colon, lung, gastric, bladder, ovarian, and breast cancers (Xia *et al.*, 2006; Davalos *et al.*, 2006; Xia *et al.*, 2005; Takai *et al.*, 2001; Kumar *et al.*, 2009; Stephenson *et al.*, 2001; Kumar *et al.*, 2006). Overexpression of EphB4 in cancer cells is associated with tumorigenesis and angiogenesis by stimulating reverse signaling through ephrin-B2. EphB4 forward signaling,
10 on the other hand, has been shown to inhibit cellular proliferation (Noren *et al.*, 2004).

[0069] Eph receptors are typically divided into a globular ligand-binding domain, a cysteine-rich region, and two fibronectin type III repeats in the extracellular region, a short transmembrane region with several conserved tyrosine residues and the tyrosine kinase domain, a sterile α motif (SAM) protein-protein interaction domain, and a C-terminal PDZ-
15 binding motif in the intracellular region. Eph receptors are divided into two classes, EphA and EphB. Likewise, Eph ligands (ephrins), which are also cell-surface associated proteins, divided into two classes, GPI-anchored ephrin-A and transmembrane ephrin-B. Ephrin-B molecules contain a cytoplasmic domain with several highly conserved tyrosine phosphorylation sites and a C-terminal PDZ motif. Generally, EphA receptors bind to ephrin-
20 A, and EphB receptors bind to ephrin-B, but cross-signaling can occur. Eph and ephrins are capable of bi-directional signaling through trans interactions, though interactions in cis (*i.e.*, between molecules expressed in the same cell) appear to inhibit receptor activation.

III. Targeting agents and Conjugates

Radioisotopes

25 [0070] In certain aspects, a targeting peptide of the embodiments is conjugated to a radioisotope. In some aspects, the targeting agent comprises a chelating moiety and a radionuclide chelate. For example, the peptide can be chelated to a radionuclide, such as a technetium ion, a copper ion, an indium ion, a thallium ion, a gallium ion, an arsenic ion, a rhenium ion, a holmium ion, a yttrium ion, a samarium ion, a selenium ion, a strontium ion, a
30 gadolinium ion, a bismuth ion, an iron ion, a manganese ion, a lutetium ion, a cobalt ion, a platinum ion, a calcium ion, and/or a rhodium ion. Examples of radionuclides include, but are

not limited to, ^{99m}Tc , ^{188}Re , ^{186}Re , ^{153}Sm , ^{166}Ho , ^{90}Y , ^{89}Sr , ^{67}Ga , ^{68}Ga , ^{111}In , ^{183}Gd , ^{59}Fe , ^{225}Ac , ^{212}Bi , ^{211}At , ^{45}Ti , ^{177}Lu , ^{60}Cu , ^{61}Cu , ^{67}Cu , and ^{64}Cu .

[0071] Chelating moieties for use according to the invention include, but are not limited to, a acyclic polyamioncarboxylate, a diposphine, a Schiff base, a bis(thiosemicarbazone), a cyclic polyamine, a cyclic polyaminocarboxylate, a cross-bridged cyclic polyamine, a cross-bridged cyclicpolyamioncarboxylate, a 1,3,5-cis,cis-triamioncyclohexane derivative, a sarcophagine, or a sepulchrate. For example, the chelating moiety can be 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), diethylenetriaminetetraacetic acid (DTTA), Diethylenetriaminopentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA), 1,4,8,11-tetraazacyclotetradecane-1,8-diacetic acid (TE2A) Mercaptoacetyltriglycine (MAG_3) or 4,5-bis(2-mercaptoacetamido)pentanoic acid, metformin, or phenformine.

Chemotherapeutic agents

[0072] In some aspects, a targeting peptide of the embodiments is conjugated to or associated with a chemotherapeutic agent. Examples of chemotherapeutic agents of use as conjugates (or for loading in peptide-conjugated nanoparticles) include alkylating agents such as thiotepa and cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramidate, triethylenethiophosphoramidate and trimethylolomelamine; acetogenins (*e.g.*, bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammall and

calicheamicin omegaII; dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores, aclacinomysins, actinomycin, aithrarnycin, azaserine, bleomycins, cactinomycin, carabacin, carminomycin, carzinophilin, 5 chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalarnycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, 10 tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, 15 dromostanolone propionate, epitiostanol, mepitiothane, testolactone; anti-adrenals such as aminogluthethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziqune; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; 20 lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and 25 anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.*, paclitaxel and doxetaxel; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum coordination complexes such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; 30 novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (*e.g.*, CPT-11); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, busulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin,

mitomycin, etoposide (VP16), tamoxifen, raloxifene, estrogen receptor binding agents, taxol, paclitaxel, docetaxel, gemcitabine, navelbine, farnesyl-protein transferase inhibitors, transplatinum, 5-fluorouracil, vincristine, vinblastine and methotrexate; toll-like receptor agonists such as CpG; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

Nanoparticles

[0073] As used herein, the term “nanoparticle” (NP) refers to any particles having dimensions in the 1-1,000 nm range. In some embodiments, nanoparticles have dimensions in the 2-200 nm range, preferably in the 5-150 nm range, and even more preferably in the 10-100 nm range. In certain aspects, the nanoparticles may be conjugated to a targeting peptide of the embodiments.

[0074] Example nanoparticles include for example polymeric micelles, polyethylene glycol, carbon nanotubes, gold nanostructures, such as gold nanoshells, gold nanorods, gold nanocages, and hollow gold nanospheres, as detailed herein, and copper sulfide nanoparticles (Haram *et al.*, 1996; Xu *et al.*, 2009; Huang *et al.*, 2010). For example, CuS NPs have been synthesized and demonstrated for photothermal destruction of tumor cells *in vitro* using a NIR laser beam centered at 808 nm.

IV. Methods of Using Targeting Agents

[0075] Targeting agents of the embodiments may be used in an imaging or detection method for diagnosis or localization of tumor or angiogenic tissues. Any optical or nuclear imaging method may be contemplated, such as PET, SPECT, CT, or photoacoustic tomography. The integrated radioactive isotope in the nanoparticle may exert a radiotherapeutic effect on the tissue incorporating such nanoparticle. In addition, a photothermal ablation therapy may be administered to the tissue having the targeting agents to enhance the therapeutic effect.

[0076] Targeting agents may be used in PET. Positron emission tomography (PET) is a powerful and widely used diagnostic tool that has the advantages of high sensitivity (down to the picomolar level) and ability to provide quantitative imaging analyses of *in vivo* abnormalities (Scheinin *et al.*, 1999; Eckelman, 2003; Welch *et al.*, 2009). ^{64}Cu ($T_{1/2} = 12.7$ h; β^+ , 0.653 MeV [17.8%]; β^- , 0.579 MeV [38.4%]) has decay characteristics that allow for both PET imaging and targeted radiotherapy for cancer (Shokeen and Anderson, 2009). It has

been investigated as a promising radiotracer for real-time PET monitoring of regional drug concentration, pharmacokinetics, and dosimetry during radiotherapy (Shokeen and Anderson, 2009; Lu *et al.*, 2010). PET may be used in certain aspects to trace nanoparticles *in vivo*.

[0077] Certain targeting agents may also be used in SPET. Single photon emission
5 computed tomography (SPECT, or less commonly, SPET) is a nuclear medicine tomographic imaging technique using gamma rays. It is very similar to conventional nuclear medicine planar imaging using a gamma camera. However, it is able to provide true 3D information. This information is typically presented as cross-sectional slices through the patient, but can be freely reformatted or manipulated as required.

10 [0078] The SPET basic technique requires injection of a gamma-emitting radioisotope called radionuclide) into the bloodstream of the patient. In certain aspects, a radioisotope is conjugated to a targeting agent, which allow it to be concentrated in ways of medical interest for disease detection. In other aspects, a targeting agent comprising a marker
15 radioisotope, which is of interest for its radioactive properties, has been attached to a targeting ligand, which is of interest for its chemical binding properties to certain types of tissues. This marriage allows the combination of ligand and radioisotope (the radiopharmaceutical) to be carried and bound to a place of interest in the body, which then (due to the gamma-emission of the isotope) allows the ligand concentration to be seen by a gamma-camera.

20 [0079] Targeting agents may also be used in MRI. Magnetic resonance imaging (MRI) is a medical imaging method routinely used in the clinic. Sometimes contrast materials such as intravenous DOTA-Gd contrast are used. This is useful to highlight structures such as blood vessels that otherwise would be difficult to delineate from their surroundings. Using contrast material can also help to obtain functional information about tissues.

25 [0080] Certain targeting agents may also be used in photoacoustic tomography. Photoacoustic tomography (PAT), or photoacoustic computed tomography (PACT), is a materials analysis technique based on the reconstruction of an internal photoacoustic source distribution from measurements acquired by scanning ultrasound detectors over a surface that
30 encloses the source under study. The PA source is produced inside the object by the thermal expansion that results from a small temperature rise, which is caused by the absorption of externally applied radiation of pulsed electromagnetic (EM) waves. This technique has great

potential for applications in the biomedical field because of the advantages of ultrasonic resolution in combination with EM absorption contrast. PAT is also called optoacoustic tomography (OAT).

[0081] In photoacoustic tomography (PAT), each temporal PA signal, measured at
5 various detection positions, provides one-dimensional radial information about the PA source relative to the detector position; 2D surface scans offer other 2D lateral information about the PA source. Combining the temporal and spatial measurements affords sufficient information for a complete reconstruction of a 3D PA source. Because the PA signal received by each
10 ultrasound detector is the integral of the ultrasound waves over the sensing aperture of the detector, the reconstruction algorithms depend on the detector apertures as well as the scanning geometries. Small-aperture detectors are often used to approximate point detectors, which receive PA signals originating from spherical shells, centered at each point detector, with radii determined by the acoustic times of flight. The three geometries commonly used are planar, cylindrical, and spherical surfaces. Both Fourier- and time-domain reconstruction
15 formulas with point-detector measurements for these geometries have been well established. Besides, algorithms based on other detection methods, such as large-aperture (plane), line, or circle detectors have also been derived.

[0082] Targeting agents may also be used in photothermal ablation therapy. Photothermal ablation (PTA) therapy has gained increasing attention in recent years as a
20 minimally invasive alternative to conventional approaches to cancer treatment such as surgery and chemotherapy (Amin *et al.*, 1993; Nolsoe *et al.*, 1993; Fiedler *et al.*, 2001; Vogeland Venugopalan, 2003). NPs with unique optical properties—primarily gold nanostructures, such as gold nanoshells (Hirsch *et al.*, 2003; Loo *et al.*, 2005), gold nanorods (Dickerson *et al.*, 2008; Park *et al.*, 2010), gold nanocages (Chen *et al.*, 2007; Au *et al.*,
25 2008), and hollow gold nanospheres (Lu *et al.*, 2010; Melancon *et al.*, 2008; (Lu *et al.*, 2009), but also carbon nanotubes (Chakravarty *et al.*, 2008 Burke *et al.*, 2009)—have been investigated as photothermal coupling agents to enhance the efficacy of PTA therapy. These plasmonic nanomaterials exhibit strong absorption in the near-infrared (NIR) region (wavelength 700-1100 nm) and offer an opportunity to convert optical energy to thermal
30 energy, enabling deposition of otherwise benign optical energy into tumors for thermal ablation of tumor cells.

V. Examples

[0083] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 – *In vivo* μ PET/CT Imaging of EphB4 Receptors using ^{64}Cu -Labeled Peptides

[0084] Many solid tumors overexpress EphB4 receptor, a member of the Ephrin receptor tyrosine kinase family. Noninvasive imaging of EphB4 could potentially increase early detection rates, monitor response to therapy directed against EphB4, and improve patient outcomes. Disclosed herein is a series of peptide-based imaging agents with high receptor binding affinity for nuclear imaging of EphB4 receptors. In the first generation imaging probes, the EphB4-binding peptide TNYLFSPNGPIARAW (TNYL-RAW; SEQ ID NO: 1) was conjugated with 1,4,7,10-tetraazadodecane-N,N',N'',N'''-tetraacetic acid (DOTA). DOTA-TNYL-RAW was labeled with ^{64}Cu with high labeling efficiency. *In vitro*, ^{64}Cu -DOTA-TNYL-RAW displayed high binding affinity to EphB4 ($K_d = 2$ nM) and was selectively taken up by CT26 and PC-3M cells, but not by A549 cells. Binding of FITC-TNYL-RAW and ^{64}Cu -DOTA-TNYL-RAW to CT26 and PC-3M cells was blocked by an excess amount of TNYL-RAW. *In vivo*, ^{64}Cu -DOTA-TNYL-RAW showed significantly higher uptake in PC-3M tumors than in A549 tumors, with percentages of injected dose per gram of tumor (%ID/g) values of 0.84 ± 0.09 and 0.44 ± 0.09 at 24 hr after radiotracer injection, respectively. μ PET/CT imaging clearly revealed deposition of ^{64}Cu -DOTA-TNYL-RAW in CT26 and PC-3M tumors but not in A549 tumors. Furthermore, uptake of ^{64}Cu -DOTA-TNYL-RAW in both CT26 and PC-3M tumors could be blocked by cold TNYL-RAW. Thus, the expression of EphB4 receptors can be noninvasively interrogated by μ PET/CT using ^{64}Cu -DOTA-TNYL-RAW.

[0085] Pasquale and colleagues (Koolpe *et al.*, 2005) identified, using phage display technology, several 12-mer peptides that selectively bind to individual Eph receptors. Tyr-Asn-Tyr-Leu-Phe-Ser-Pro-Asn-Gly-Pro-Ile-Ala (TNYLFSPNGPIA; SEQ ID NO: 2), an EphB4 binding peptide from the initial screening, was further modified to include a RAW moiety at the carboxyl terminus. The resulting peptide, Tyr-Asn-Tyr-Leu-Phe-Ser-Pro-Asn-Gly-Pro-Ile-Ala-Arg-Ala-Trp (TNYLFSPNGPIARAW, designated as TNYL-RAW; SEQ ID NO: 1), was shown to be a potent antagonist of EphB4, with a fifty percent inhibition concentration (IC₅₀) of ~15 nM for the binding of ephrin-B2 to murine EphB4 receptors.

[0086] The inventors investigated whether TNYL-RAW can be used as a receptor ligand for the noninvasive imaging of EphB4. The present disclosure shows that ⁶⁴Cu-labeled TNYL-RAW is a promising radiotracer for PET imaging of EphB4 receptor expression in both human prostate and colon cancer xenograft models.

[0087] Materials and Methods

[0088] Materials. ⁶⁴Cu was produced on a CS-15 biomedical cyclotron at Washington University School of Medicine (St. Louis, MO). Recombinant EphB4/Fc chimera, phycoerythrin (PE)-conjugated rat anti-human EphB4 monoclonal antibody, and rabbit anti-EphB4 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit antibody conjugated with near-infrared dye was purchased from LiCOR (Lincoln, NE). The protein assay kit was obtained from Bio-Rad (Hercules, CA). 1,4,7,10-Tetraazadodecane-N,N',N'',N'''-tetraacetic acid (DOTA) was obtained from Macrocyclics (Dallas, TX). The BIAcore sensor chip CM5, amine coupling kit, HBSEP running buffer (0.01 M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.4, 0.15 M NaCl, 3 mM ethylenediaminetetraacetic acid [EDTA], and 0.005% [v/v] surfactant P20 solution), and regeneration buffer were purchased from BIACORE, Inc. (Piscataway, NJ). 4',6-Diamidino-2-phenylindole (DAPI) was obtained from Sigma-Aldrich (St. Louis, MO).

[0089] Radiolabeling of DOTA-TNYL-RAW. ⁶⁴CuCl₂ (74–148 MBq [2–4 mCi]) in 0.1 M sodium acetate (pH 5.2) was added to 10 µg of DOTA-TNYL-RAW in water. The reaction mixture was incubated at 70°C for 1 h. The progress of the reaction was monitored by RP-HPLC with a radiodetector. The reaction was terminated with the addition of EDTA. The ⁶⁴Cu-labeled peptide was further purified, if necessary, by reverse-phase high-

performance liquid chromatography (RP-HPLC) on an Agilent 1100 system (C-18, Vydac, 4.6 × 250 mm, 10 μm) eluted with a linear gradient of 10%–90% acetonitrile in a 0.1% aqueous TFA solution over 35 min at a flow rate of 1.0 mL/min. ⁶⁴Cu-DOTA-TNYL-RAW (retention time, t_R = 12.5 min) was collected in 1- to 2-mL fractions. The solvent was then removed, reconstituted in saline, and passed through a 0.22-μm filter for use in the animal experiments. Natural copper chloride (^{nat}CuCl₂) was used to synthesize ^{nat}Cu-DOTA-TNYL-RAW under identical conditions, and its identity was confirmed by high-resolution electrospray ionization mass spectrometry (HRMS-ESI). ⁶⁴Cu-DOTA-TNYL-RAW was co-injected with ^{nat}Cu-DOTA-TNYL-RAW into the above-mentioned HPLC system equipped with both ultraviolet and radiodetectors to confirm its identity.

[0090] Stability of radiolabeled ⁶⁴Cu-DOTA-TNYL-RAW. ⁶⁴Cu-DOTA-TNYL-RAW was incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) or mouse serum at 37°C. Aliquots were removed at 1, 2, 4, 6, 12, and 24 h and analyzed by RP-HPLC with a radiodetector to assess their stability.

[0091] Immobilization of EphB4 receptor to sensor chip. The stock solution (100 μg/mL) of EphB4/Fc in phosphate-buffered saline (PBS) was diluted to 25, 12.5, and 6.25 μg/mL with 10 mM sodium acetate buffer at pH 4.5 and immobilized to a CM5 sensor chip using the amine coupling reaction following manufacturer-provided procedures (BIAcore). Briefly, the surfaces of the chips in flow cells (FC)-1, -2, -3, and -4 were activated by exposing them to a mixture of 200 mM N-ethyl-N'-dimethylaminopropyl carbodiimide and 50 mM N-hydroxysuccinimide for 7 min. FC-1 was used as a reference surface and was directly deactivated by injecting 1 M ethanolamine at pH 8.5 for 7 min. The other three flow cells were injected with 25 μg/mL, 12.5 μg/mL, and 6.25 μg/mL EphB4/Fc, respectively, followed by injection of 1 M ethanolamine to block the remaining activated ester groups on the surface. The chip was allowed to stabilize for at least 2 h in HBSEP running buffer before injecting test analytes.

[0092] Surface plasmon resonance (SPR) assay of receptor binding affinity. Binding assays were performed at 25°C in HBSEP running buffer. The peptides were diluted in HBSEP buffer, filtered, degassed, and injected at concentrations between 1.6 nM and 800 nM at a flow rate of 30 μL/min. The injection time of peptides into the HBSEP buffer was 4 min, followed by a 4-min dissociation period. The chips were regenerated using a 1-min pulse of 10 mM glycine (pH 2.2) after each binding cycle. Each cycle consisted of a 1-min waiting

period to allow monitoring of the baseline binding stability. For subtraction of bulk effects, caused by changes in the buffer composition or nonspecific binding, double-referencing was performed. Therefore, all analyzed samples were additionally injected onto an uncoated reference surface, including a sample of the running buffer, which was also tested on the EphB4/Fc coated flow cell. Data were evaluated with BIAevaluation software (version 3.0, BIACORE), applying a simple 1:1 binding mass transfer model. The obtained sensorgrams were fitted globally over the whole range of injected concentrations for both the association and dissociation phases. Equilibrium dissociation constants were then calculated from the rate constants ($K_D = k_{\text{off}}/k_{\text{on}}$) (Cooper, 2002).

[0093] Fluorescence microscopy. The CT26 murine colon cancer cell line and A549 human lung adenocarcinoma epithelial cell line were purchased from the American Type Culture Collection (Manassas, VA). The PC-3M human prostate cancer cell line was obtained from Professor Dominic Fan (MD Anderson Cancer Center, Houston, TX). PC-3M, CT26, or A549 cells were seeded (1×10^5 /well) in Lab-Tek II chambered slides (Nalge Nunc International, Naperville, IL) supplemented with RPMI-1640 medium plus 10% FBS one day before the experiment. The cells were incubated with 100 μL of phenol-free RPMI-1640 culture medium containing 10 μM of FITC-TNYL-RAW or scrambled FITC-scTNYL-RAW (AGPFNTYLRLINAWSP; SEQ ID NO: 3) for 20 min at room temperature. For the blocking experiment, 10 μM of FITC-TNYL-RAW and 1 mM of TNYL-RAW were added to the cells. For detection of EphB4 expression, cells were incubated with 10 μL of PE-conjugated rat anti-human anti-EphB4 monoclonal antibody (R&D Systems, Minneapolis, MN) in 100 μL of phenol red-free RPMI-1640 medium. The cells were washed and fixed with 4% paraformaldehyde for 15 min at room temperature. The cell nuclei were counterstained with DAPI. After washing with PBS, the slides were mounted and visualized under a Zeiss Axiovert Z.1 fluorescent microscope (Zeiss, Jena, Germany).

[0094] Cell binding. For the cell binding study, CT26, PC-3M, and A549 cells were grown in 6-cm Petri dishes as described before (Bauer *et al.*, 2005). The culture medium was replaced with 2 mL of RPMI 1640 without FBS containing ^{64}Cu -DOTA-TNYL-RAW ($\sim 40 \mu\text{Ci/mL}$, 100 nM), and cells were incubated at room temperature for 30, 60, or 120 min. For the blocking experiment, ^{64}Cu -DOTA-TNYL-RAW peptide ($\sim 40 \mu\text{Ci/mL}$, 100 nM) was co-incubated with TNYL-RAW (10 μM) under the same conditions. Thereafter, the monolayers were scraped and transferred into 5-mL tubes. The tubes were briefly vortexed and 100 μL of

the cell suspension was transferred into a microcentrifuge tube containing 500 μ L of a 75:25 mixture of silicon oil (density 1.05; Sigma-Aldrich) and mineral oil (density 0.872; Acros, Geel, Belgium). The mixture was centrifuged at 14,000 rpm for 5 min. After the tubes were frozen in liquid nitrogen, the bottom tips containing the cell pellet were cut off. The cell pellets and the supernatants were counted with Packard Cobra Quantum γ -counter (GMI, Ramsey, Minnesota). The protein content in a 100- μ L cell suspension was quantified using the Bio-Rad protein assay kit according to the manufacturer's protocol. The radioactivity in the cell pellets and media was counted, and the data were expressed as activity ratios of the cell pellet to the medium ([cpm/ μ g protein in pellet]/[cpm/ μ g medium]). The experiments were performed in pentaplicate.

[0095] Animal models. All animal studies were performed under the guidelines and approval of the Institutional Animal Care and Use Committee. Athymic nude mice (4-6 weeks old, both sexes) were obtained from Harlan Laboratories (Charles River, Wilmington, Massachusetts). CT26, PC-3M, and A549 cells were grown in DMEM/F12 supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere with 5% CO₂. The cells were harvested by trypsinization. After centrifugation of cell suspension at 5000 rpm for 5 min, the culture medium was aspirated and the cell pellet was re-suspended in culture medium for subcutaneous injection in the right front leg of each mouse. When tumors were about 1 cm in diameter, micro-PET/CT (μ PET/CT) imaging and biodistribution analyses were performed.

[0096] μ PET/CT imaging. Mice bearing PC-3M tumors (n = 4), CT26 tumors (n = 4; EphB4 positive), or A549 tumors (n = 4; EphB4 negative) were imaged with μ PET/CT at various times after intravenous injection of 200 μ Ci ⁶⁴Cu-DOTA-TNYL-RAW (0.5~1 μ g). For the blocking experiment, the tumor-bearing mice (n = 2) were co-injected with 200 μ Ci ⁶⁴Cu-DOTA-TNYL-RAW and cold TNYL-RAW peptide at a dose of 50 μ g/mouse. Images were acquired using an Inveon PET/CT system (Siemens, Knoxville, TN). The spatial resolution of the PET system is approximately 1.4 mm. Tumor-bearing mice were anesthetized with isoflurane (2% in oxygen) and placed in a prone position. The CT imaging parameters were as follows: X-ray voltage, 80 kVp; anode current, 500 mA; exposure time of each of the 360 rotational steps, 300–350 ms. Images were acquired at 1, 4, and 24 h after intravenous administration of ⁶⁴Cu-DOTA-TNYL-RAW. Images were reconstructed using the two-dimensional ordered subsets expectation maximization algorithm. PET and CT image

fusion and image analysis were performed using Inveon Research Workplace (Siemens Preclinical Solutions, Knoxville, TN). For tumor voxel intensity calculation, an irregular 3-dimensional region of interest (VOI) was manually drawn covering the whole tumor on CT and then copied to the corresponding PET images. A circular VOI (approximately 20 mm³) was drawn on the muscle of the legs. VOI was also drawn on a standard (radiotracer solution containing 1% of the injection dose) placed along with the animals. The mean activities within the VOI of the tumor and muscle were calculated in IRW workstation (Siemens).

[0097] Assessment of biodistribution by tissue sampling. In a separate experiment, athymic nude mice bearing PC-3M, CT26, and A549 xenografts (n = 4/group) were injected with 0.74 MBq (20 μ Ci, 50 ng) of ⁶⁴Cu-DOTA-TNYL-RAW peptide to evaluate the biodistribution of the radiotracer. For the blocking experiment, the tumor-bearing mice (n = 2) were co-injected with 20 μ Ci ⁶⁴Cu-DOTA-TNYL-RAW and 200-fold cold TNYL-RAW peptide. The animals were sacrificed at 4 and 24 h after injection. The organs of interest were excised and weighed and their radioactivity counted using an automatic gamma counter. (GMI, Ramsey, Minnesota). The stomach and intestines were not emptied prior to radioactivity measurements. The percentage of injected dose per gram of tissue (%ID/g) was calculated by dividing the %ID/organ by the weight of the organ. Values were expressed as mean $\pm$ standard deviation (SD).

[0098] Immunohistochemical analysis. After the μ PET/CT studies were completed, the mice were sacrificed and their tumors were excised, snap-frozen, and cut into 4- μ m sections. For immunohistochemical analysis, frozen tumor sections were fixed in 4% paraformaldehyde solution for 20 min at room temperature and washed with PBS three times. Tumor sections were blocked with 10% goat serum for 30 min at 37°C, then the slides were then incubated with rabbit anti-EphB4 antibody (U-200, 1:100 dilution) in PBS at 37°C overnight. After incubation with primary antibody, slides were washed with PBS three times and incubated with secondary goat anti-rabbit antibody conjugated with Alexa Flour 488 (1:500 dilution; Invitrogen). Slides were washed again in PBS and counterstained with DAPI. Microphotographs were taken under a Zeiss Axiovert Z.1 fluorescence microscope with the same conditions and displayed at the same scale to make sure that the relative brightness observed in the images reflected the difference in EphB4 expression level.

[0099] Results

[00100] Chemistry, radiochemistry, and stability. The structure of $^{\text{nat}}\text{Cu}/^{64}\text{Cu}$ -DOTA-TNYL-RAW is shown in FIG. 1. The reaction scheme for the synthesis of $^{\text{nat}}\text{Cu}/^{64}\text{Cu}$ -DOTA-TNYL-RAW is shown in FIG. 7A. RAW was eluted as a single peak with a retention time of 13.8 min, $m/z = 1705.7764$ for $[\text{M}+\text{H}]^+$ ($\text{C}_{80}\text{H}_{117}\text{N}_{22}\text{O}_{20}$, calculated $[\text{M}+\text{H}]^+$: 1705.8814). DOTA-TNYL-RAW was eluted as a single peak with a retention time of 12.6 min, $m/z = 2092.0554$ for $[\text{M}+\text{H}]^+$ ($\text{C}_{96}\text{H}_{143}\text{N}_{26}\text{O}_{27}$, calculated $[\text{M}+\text{H}]^+$: 2092.0616). FITC-TNYL-RAW was eluted as a single peak with a retention time of 15.6 min, $m/z = 2094.9003$ for $[\text{M}+\text{H}]^+$ ($\text{C}_{101}\text{H}_{128}\text{N}_{23}\text{O}_{25}\text{S}$, calculated $[\text{M}+\text{H}]^+$: 2094.9172).

[00101] The formation of the $^{\text{nat}}\text{Cu}(\text{II})$ -labeled complex $^{\text{nat}}\text{Cu}$ -DOTA-TNYL-RAW was confirmed by mass spectrometry (FIG. 7B), with $m/z = 1076.9957$ for $[\text{M}+\text{Cu}]^{2+}$ ($\text{C}_{96}\text{H}_{143}\text{CuN}_{26}\text{O}_{27}$, calculated $[\text{M}+\text{Cu}]^{2+}$: 1076.9917). Under the same HPLC conditions, $^{\text{nat}}\text{Cu}$ -DOTA-TNYL-RAW (UV detector) and ^{64}Cu -DOTA-TNYL-RAW (radiodetector) had almost identical retention times (12.5 min and 12.6 min, respectively) (FIG. 7C). The radiochemical purity, defined as the ratio of the main product peak to all peaks, was determined by HPLC to be >95%. The specific activity of ^{64}Cu -DOTA-TNYL-RAW used in the *in vitro* and *in vivo* experiments was typically 7.4-14.8 MBq/nmol (0.2-0.4 Ci/ μmol) at the end of synthesis. ^{64}Cu -DOTA-TNYL-RAW was stable in DMEM culture medium containing 10% FBS for up to 24 h at 37°C. Approximately 30% of the ^{64}Cu -DOTA-TNYL-RAW was degraded after 2 h of incubation in mouse serum at 37°C.

[00102] TNYL-RAW peptides bound to EphB4 receptors with nM affinity. FIG. 2 shows representative sensorgrams obtained from SPR analyses of $^{\text{nat}}\text{Cu}$ -DOTA-TNYL-RAW and a scrambled peptide, with fitting curves obtained using a global 1:1 mass transfer model (gray lines). SPR sensorgrams of TNYL-RAW and DOTA-TNYL-RAW peptides are presented in FIG. 8. The corresponding binding kinetics and affinity data are summarized in Table 1. TNYL had an equilibrium dissociation constant (K_D) of 3.06 nM. Conjugation of DOTA to the N-terminus of the peptide increased the K_D value to 23.3 nM. Chelation of Cu^{2+} to DOTA-TNYL-RAW restored the binding affinity of the resulting metal complex, with a K_D value of 1.98 nM. No binding to EphB4 was detected with a scrambled peptide (AGPFNTYLRINAWSP; SEQ ID NO: 3).

[00103] **Table 1.** Association (k_{on}) and dissociation (k_{off}) rate and equilibrium dissociation (K_D) constants of peptides interacting with immobilized EphB4 receptors obtained from SPR analysis.

Analyte	k_{on} [$M^{-1}s^{-1}$]	k_{off} [s^{-1}]	K_D [M]	χ^2
TNYL-RAW	4.26×10^5	1.3×10^{-3}	3.06^{-9}	0.155
DOTA-TNYL-RAW	1.10×10^5	2.56×10^{-3}	2.33^{-8}	0.281
^{nat} Cu-DOTA-TNYL-RAW	6.89×10^6	1.31×10^{-2}	1.98^{-9}	1.86

5 **[00104]** FITC-TNYL-RAW selectively bound to tumor cells overexpressing EphB4 *in vitro*. Two EphB4-positive cell lines (PC-3M and CT26) and one EphB4-negative cell line (A549) were used for an *in vitro* binding study. Immunohistostaining with PE-conjugated rat anti-human EphB4 monoclonal antibody confirmed the expression of EphB4 on the surface of PC-3M, CT26, and A549 cells. PC-3M, CT26, and A549 cells were treated with FITC-TNYL-RAW (10 μ M) or a scrambled peptide FITC-sc-TNYL-RAW (10 μ M) for 20 min at room temperature. Cells were also stained with PE-conjugated anti-EphB4 antibody for the expression of EphB4 receptors. For the blocking experiment, FITC-TNYL-RAW (10 μ M) was co-incubated with TNYL-RAW (1 mM). The cell nuclei were counterstained with DAPI. Stained cells were imaged by fluorescence microscopy. PC-3M and CT26 cells, but not A549 cells, were readily stained with FITC-TNYL-RAW. A FITC-labeled scrambled TNYL-RAW peptide (FITC-sc-TNYL-RAW) did not show detectable binding to PC-3M and CT26 cells. The binding of FITC-TNYL-RAW to PC-3M and CT26 cells was efficiently blocked by an excess amount of unlabeled TNYL-RAW peptide.

20 **[00105]** ⁶⁴Cu-DOTA-TNYL-RAW peptide selectively binds to EphB4-positive cells. ⁶⁴Cu-DOTA-TNYL-RAW had increased uptake with time in EphB4-positive PC-3M (FIG. 3B) and CT26 (FIG. 3A) cell lines, but not in EphB4-negative A549 cells (FIG. 3C). Co-incubation with cold TNYL-RAW peptide completely abolished the binding of ⁶⁴Cu-DOTA-TNYL-RAW to PC-3M at all time points tested (FIG. 3B) and reduced its binding to CT26 cells by ~10-fold (FIG. 3A).

25 **[00106]** μ PET/CT imaging. FIG. 4 shows μ PET/CT imaging of both coronal and transverse slices that contain tumor. Tumors were clearly visualized at 1 h and 4 h (CT26) and at 4 h and 24 h (PC-3M) after the ⁶⁴Cu-DOTA-TNYL-RAW injection. In

contrast, A549 tumors were barely discernible after radiotracer injection. The uptake of ^{64}Cu -DOTA-TNYL-RAW in CT26 tumors was rapid, reaching 1.3 and 2.6 %ID/g at 1 h and 4 h postinjection, respectively. By 24 h after injection, the level of tumor radioactivity had declined to the body background level (FIG. 4). The uptake values of ^{64}Cu -DOTA-TNYL-RAW in PC-3M tumors were 1.4, 3.2, and 3.6 %ID/g at 1, 4, and 24 h postinjection, respectively. The accumulation of ^{64}Cu -DOTA-TNYL-RAW in A549 tumor was low at all time points examined, reaching a level of 1.7, 1.5, and 1.2 %ID/g at 1, 4, and 24 h after radiotracer administration. These values were only slightly higher than those recorded for muscle tissue in the same animals. *Ex vivo* immunohistochemical staining confirmed the expression of EphB4 throughout the CT26 and PC-3M tumors, whereas A549 tumors did not express EphB4 receptors.

[00107] FIG. 5 compares μPET images obtained in the presence and absence of a large excess of cold TNYL-RAW at 4 h after radiotracer injection in a CT26 tumor-bearing mouse and at 24 h after radiotracer injection in a PC-3M tumor-bearing mouse. In CT26 and PC-3M tumor models, the co-administration of cold TNYL-RAW caused a 77% and 81% reduction in ^{64}Cu -DOTA-TNYL-RAW accumulation in tumors, respectively.

[00108] Biodistribution of ^{64}Cu -DOTA-TNYL-RAW assessed by tissue sampling. Biodistribution of ^{64}Cu -DOTA-TNYL-RAW at 24 h after radiotracer injection in nude mice bearing PC-3M prostate and A549 lung cancer xenografts is summarized in FIG. 6A. There was significantly higher uptake of ^{64}Cu -DOTA-TNYL-RAW in PC-3M than in A549 tumors (0.84 %ID/g vs. 0.44 %ID/g, SD = 0.09, 0.09 respectively, $p = 0.000015$). The liver, spleen, and kidney were the major organs with highest radiotracer retention (FIG. 6A). The tumor-to-muscle ratio was reduced 56.7% in CT26 tumors at 4 h postinjection and 47.6% in PC-3M tumors at 24 h postinjection when ^{64}Cu -DOTA-TNYL-RAW was co-injected with cold TNYL-RAW peptide (FIG. 6B). The results of tissue sampling study corroborated with the pattern of ^{64}Cu -DOTA-TNYL-RAW biodistribution determined by non-invasive *in vivo* PET/CT imaging.

[00109] Discussion

[00110] The ability to identify patients with significant receptor EphB4 receptor expression in tumors and/or to monitor changes in EphB4 expression during treatment using noninvasive imaging techniques would markedly enhance the selection and

evaluation of patients treated with anticancer drugs directed at EphB4. With this aim, the inventors developed ^{64}Cu -DOTA-TNYL-RAW, a novel PET radioligand for EphB4 receptor imaging, based on an EphB4 antagonist peptide with binding to EphB4 receptors in the low nM range (Koolpe *et al.*, 2005).

5 **[00111]** SPR analysis showed that TNYL-RAW peptide had a dissociation constant (K_D) of 3.09 nM, which is comparable to that reported in the literature (K_D 1~2 nM) (Koolpe *et al.*, 2005). TNYL-RAW also had a slow dissociation rate ($\sim 1.3 \times 10^{-3}$ [s $^{-1}$]), which is a better indicator than simple binding affinity for *in vivo* molecular imaging applications (Berezov *et al.*, 2001). Previous studies showed that the N-terminal residues of
10 the peptide could be modified without affecting the stability of the binding complex with EphB4 (Chrencik *et al.*, 2006). However, when the radiometal chelator DOTA was introduced at the N-terminus of TNYL-RAW, an 8-fold reduction in K_D was observed. Introduction of Cu^{2+} to DOTA-TNYL-RAW restored the binding affinity of TNYL-RAW (Table 1). Therefore, subtle structural change at the N-terminus of the peptide can still affect
15 the binding of the peptide to EphB4 receptors. Because no interaction between a scrambled peptide and EphB4 was observed, the binding of TNYL-RAW to EphB4 is sequence-specific.

[00112] Most colon cancer and prostate cancer cell lines overexpress EphB4 receptors (Xia *et al.*, 2005; Liu *et al.*, 2002; Huang *et al.*, 2007). Both the CT26 colon cancer and PC-3M prostate cancer cell lines used in these studies were positive for EphB4
20 expression. Taken together, the results of our binding and uptake studies (FIG. 3) indicate that ^{64}Cu -DOTA-TNYL-RAW exhibits specific, high-affinity binding to EphB4 on the surface of cancer cells, with low nonspecific interaction. This makes ^{64}Cu -DOTA-TNYL-RAW a suitable probe for noninvasive imaging of EphB4 receptors.

[00113] *In vivo* $\mu\text{PET/CT}$ imaging studies revealed that ^{64}Cu -DOTA-TNYL-
25 RAW has very favorable tumor-homing characteristics in CT26 and PC-3M tumor xenografts in mice. In contrast, the radiotracer showed only minimal uptake in A549 tumors at all time points (FIG. 4). *Ex vivo* immunohistostaining of tumor xenografts confirmed that both CT26 and PC-3M tumors expressed high levels of EphB4, while A549 tumors had negligible EphB4 levels. In CT26 tumors, ^{64}Cu -DOTA-TNYL-RAW was washed out of the tumor after
30 4 h, but it was significantly retained by PC-3M tumors for up to 24 h (FIG. 4). Given the fact that the peptide-EphB4 complex has a relatively slow off-rate ($K_{\text{off}} = 1.31 \times 10^{-2}$ [S $^{-1}$]), the level of EphB4 receptor expression should be the predominant factor influencing the

magnitude of ^{64}Cu -DOTA-TNYL-RAW accumulation and retention in tumor tissue. However, the difference in the degradation of ^{64}Cu -DOTA-TNYL-RAW peptide-based radiotracer in different tumor microenvironments may also contribute to the difference in tumor retention of the radiotracer.

5 **[00114]** The stability of radioactive compounds is critical because the molecular integrity of the radiopharmaceutical must be maintained for an adequate time in the blood circulation during biodistribution and imaging studies. ^{64}Cu -DOTA-TNYL-RAW was stable in DMEM with 10% FBS for at least 24 h and was stable in mouse serum for up to 2 h, after which slow degradation was observed. Nevertheless, these $\mu\text{PET/CT}$ studies
10 revealed that ^{64}Cu -DOTA-TNYL-RAW possess sufficient *in vivo* stability for tumor imaging.

[00115] To establish the specificity of tumor uptake of ^{64}Cu -DOTA-TNYL-RAW *in vivo*, blocking studies were performed by co-injection of the radiotracer with a large excess of non-radioactive TNYL-RAW peptide. PET images revealed significant reductions in tumor uptake of ^{64}Cu -DOTA-TNYL-RAW in both CT26 and PC-3M tumors in the
15 presence of cold TNYL-RAW (FIG. 5). Quantitative analysis of radiotracer concentration showed that tumor-to-muscle ratios were reduced 56.7% in CT26 tumors at 4 h postinjection and 47.6% in PC-3M tumors at 24 h postinjection when ^{64}Cu -DOTA-TNYL-RAW was co-
injected with cold TNYL-RAW peptide (FIG. 6B). These results, together with the findings that EphB4-negative A549 tumors were hardly discernible in PET images acquired with
20 ^{64}Cu -DOTA-TNYL-RAW (FIG. 4) and that there was significantly higher uptake of ^{64}Cu -DOTA-TNYL-RAW in EphB4-positive PC-3M tumors than in EphB4-negative A549 tumors (FIG. 6A), support the notion that the uptake of ^{64}Cu -DOTA-TNYL-RAW in colon and prostate cancer xenografts in mice was specific and mediated by EphB4 receptors.

[00116] The ^{64}Cu -DOTA-TNYL-RAW biodistribution data obtained by both
25 non-invasive PET/CT imaging and by tissue sampling indicate that the liver and the kidney were the major organs for physiological uptake and clearance of this radiotracer. In general, hepatobiliary clearance is a major route for hydrophobic peptides (Rusckowski *et al.*, 2001). TNYL-RAW peptide contains several hydrophobic amino acids (Asn, Ile, Leu, Phe, Pro, Ala, Trp) that could contribute to the high liver uptake of ^{64}Cu -DOTA-TNYL-RAW. The high
30 level of accumulation of the radiotracer in the kidneys may be attributed to lysosomal degradation of the peptide within renal cells (Tsai *et al.*, 2001).

Example 2 - Effective Photothermal Chemotherapy Using Doxorubicin-Loaded Gold Nanospheres That Target EphB4 Receptors in Tumors

[00117] Photothermal ablation (PTA) therapy is a recently developed technique that uses near-infrared (NIR) laser light-generated heat to destroy tumor cells. PTA has gained popularity recently because a specific amount of photo-energy is delivered directly into the tumor mass without causing systemic effects, thus promising minimally invasive intervention as an alternative to surgery (Bardhan *et al.*, 2011; Melancon *et al.*, 2009). However, PTA therapy alone is unlikely to kill all tumor cells because the heat distribution is non-uniform, especially in areas peripheral to large blood vessels where heat can be rapidly dissipated by circulating blood. To improve the efficacy and tumor selectivity of laser-induced PTA, light-absorbing photothermal conducting nanoparticles are introduced. In principle, NIR laser-modulated photothermal effects can not only enable PTA of tumor cells but also trigger release of anticancer agents. Such a multimodal approach, which permits simultaneous PTA therapy and chemotherapy, should provide an opportunity for complete eradication of tumor cells.

[00118] Hollow gold nanospheres (HAuNS) are a novel gold nanostructure ideally suited for PTA because of their unique combination of small size (30-50 nm), biocompatibility, and strong and tunable absorption in the entire NIR region (Melancon *et al.*, 2008; Lu *et al.*, 2009). Owing to their hollow interior, exceptionally high doxorubicin (DOX) payload to HAuNS (up to 60% by weight, 4-fold of the payload achievable with solid gold nanoparticles of the similar size) could be achieved with HAuNS (You *et al.*, 2010). To maximize the therapeutic efficacy of DOX-loaded HAuNS (DOX@HAuNS), it is essential that DOX@HAuNS is selectively delivered to the tumor. EphB4 is a particularly promising target for tumor-specific delivery of DOX@HAuNS.

[00119] Using c(TNYL-RAW) as a homing ligand, DOX@HAuNS were selectively targeted to EphB4-positive tumors, and concerted chemo-photothermal therapy mediated by EphB4-targeting DOX@HAuNS induced remarkable antitumor efficacy with reduced systemic toxicity. These results support the concept of integrating multiple functions into a single nanodevice to mediate simultaneous PTA and local drug release.

[00120] Materials and Methods

[00121] Reagents. Indium-111 chloride ($^{111}\text{InCl}_3$, Iso-Tex Diagnostics (Friendswood, TX). Methoxy-polyethylene glycol (PEG)-SH (molecular weight, 5,000) and NH_2 -PEG-COOH (molecular weight, 5,000) were purchased from Nektar (San Francisco, CA). Sodium citrate (>99%), cobalt chloride hexahydrate (99.99%), sodium borohydride (99%), and chloroauric acid trihydrate (American Chemical Society reagent grade) were purchased from Thermo Fisher Scientific (Waltham, MA) and were used as received. All amino acid derivatives and coupling reagents were purchased from EMD Chemicals (Philadelphia, PA), Bachem Americas (Torrance, CA), and Chem-Impex International (Wood Dale, IL). PL-DMA resin was obtained from EMD Chemicals. DOX, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Reagent-grade solvents were used without further purification unless otherwise specified. For the surface plasmon resonance (SPR) assay, the BIAcore sensor chip CM5, amine-coupling kit, HBSEP running buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% [v/v] surfactant P20 solution), and regeneration buffer were purchased from GE Healthcare (Waukesha, WI). ^3H -labeled DOX was purchased from Moravsek Biochemicals Inc. (Brea, CA).

[00122] Synthesis of SH-PEG-c(TNYL-RAW). The heterofunctional PEG linker SATA-PEG-CONHS containing a protected sulfhydryl group (S-acetylthioacetate, SATA) and NHS activated ester on each end of the PEG chain was first synthesized. Briefly, NH_2 -PEG-COOH (0.5 g, 0.25 mmol) and 1.2 equivalent N-succinimidyl-S-acetylthioacetate (SATA) were dissolved in 2 mL anhydrous DCM, and then 3 equivalents of dry DIPEA was added dropwise. The reaction solution was stirred at room temperature overnight. After all organic solvent was removed under a vacuum, the crude product was purified by gel filtration with a PD-10 column. The collected aqueous solution was lyophilized to yield SATA-PEG-COOH, which was further activated with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and NHS to make SATA-PEG-CONHS. c(TNYL-RAW) was synthesized on solid support. The peptide was conjugated to SATA-PEG5000-CONHS in a 1:1 molar ratio in DCM to obtain SATA-PEG-c(TNYL-RAW). The product was purified by PD-10 column and then treated with 0.5 M NH_2OH to obtain SH-PEG-c(TNYL-RAW) before conjugation with HAuNS.

[00123] Synthesis of DOX-loaded, c(TNYL-RAW)-conjugated HAuNS.

HAuNS were synthesized according to a previously reported method (You *et al.*, 2010). Briefly, cobalt nanoparticles were first synthesized by deoxygenating deionized water containing 4.5 mL of 1 M sodium borohydride, 2.8 mL of 0.1 M sodium citrate, and 1.0 mL
5 of 0.4 M cobalt chloride. After chloroauric acid was added into the solution containing cobalt nanoparticles, the cobalt immediately reduced the gold ions onto the surface of the nanoparticles and was simultaneously oxidized to cobalt oxide. Any remaining cobalt was further oxidized by air, resulting in the final product, HAuNS. The size of the HAuNS was determined using dynamic light scattering on a Brookhaven 90Plus particle size analyzer
10 (Holtsville, NY). The UV-visible spectra were recorded on a Beckman Coulter DU-800 UV-visible spectrometer (Brea, CA). The morphology of the HAuNS was examined using a JEM 1010 transmission electron microscope (JEOL USA, Peabody, MA).

[00124] To obtain c(TNYL-RAW)-conjugated HAuNS, SH-PEG-c(TNYL-

RAW) (50 nmol) was mixed with 1.0 mL HAuNS solution (200 optical density [OD], 5.0
15 mg/mL) and stirred overnight. Then, SH-PEG (3 μ mol) was added to the solution. After another overnight stirring, c(TNYL-RAW)-conjugated HAuNS was purified on a PD-10 column.

[00125] For DOX loading, free DOX (5 mg) in water (5 mL) was added to

c(TNYL-RAW)-HAuNS (40 OD) in 5 mL of 2.8 mM citrate solution, and the mixture was
20 stirred at room temperature for 24 h. The resulting DOX-loaded c(TNYL-RAW)-HAuNS (T-DOX@HAuNS) were purified by 3 repeated centrifugation and washing steps.

[00126] Synthesis of cyclic peptide c(TNYL-RAW) (Lys-Thr-Asn-Tyr-Leu-

Phe-Ser-Pro-Asn-Gly-Pro-ILe-Ala-Arg-Ala-Trp-Asp). PL-DMA resin (1 g) was treated with ethylenediamine (30 mL) overnight and washed with dimethylformamide. 4-(4-
25 Hydroxymethyl-3-methoxyphenoxy) acetic acid linker (3 equivalents) was attached to resin in the presence of diisopropylcarbodiimide (DIPCDI) (3 equiv.) and hydroxybenzotriazole (HOBt) (3 equiv.). Fluorenylmethyloxycarbonyl chloride (Fmoc)-Asp-Wang resin)OAll was formed by esterification with DIPCDI (3 equiv.) and 4-dimethylaminopyridine (DMAP) (0.5 equiv.). Fmoc-Lys(Boc)-Thr(tBu)-Asn(Trt)-Tyr(tBu)-Leu-Phe-Ser(tBu)-Pro-Asn(Trt)-Gly-
30 Pro-ILe-Ala-Arg(Pbf)-Ala-Trp(Boc)-Asp(resin)OAll was synthesized using an Fmoc solid-phase strategy. Amino acids (3 equiv.) were coupled stepwise on the solid support in the presence of DIPCDI (3 equiv.) and HOBt (3 equiv.) coupling reagents. After removal of the

allyl-protecting groups using $\text{Pd}^0[\text{P}(\text{C}_6\text{H}_5)_3]_4$ (3 equiv.) in DCM/NMP/AcOH (dichloromethane/*N*-methylpyrrolidone/acetic acid) (37/1/2, v/v/v) and Fmoc-protecting groups using 20% piperidine in dimethylformamide (DMF), head-and-tail cyclization on the solid support was carried out in DMF using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (Py-BOP) (3 equiv.), HOBT (3 equiv.), and *N,N*-diisopropylethylamine (DIPEA) (6 equiv.) as coupling agents. Deprotection and cleavage of the conjugates from the solid support were carried out simultaneously by treatment with trifluoroacetic acid/water/triethylsilane (TFA/ H_2O /TES, 95/1/4, v/v/v) to yield c(TNYL-RAW). The product was purified by high-performance liquid chromatography and validated by electrospray ionization mass spectrometry ($m/z = 1931.0085$ for $[\text{M}+\text{H}]^+[\text{C}_{90}\text{H}_{132}\text{N}_{25}\text{O}_{23}]$, calculated $[\text{M}+\text{H}]^+ = 1930.9928$).

[00127] SPR assay of receptor binding affinity. EphB4/Fc (extracellular domain of human EphB4 fused to the carboxy-terminal Fc region of human IgG1) was immobilized to CM5 sensor chips using the amine coupling reaction according to the manufacturer's instructions (GE Healthcare). Briefly, the surfaces of the chips in flow cells 1, 2, 3, and 4 were activated by exposing the chips to a mixture of 200 mM *N*-ethyl-*N'*-dimethylaminopropyl carbodiimide and 50 mM *N*-hydroxysuccinimide (NHS) for 7 min. Flow cell 1 was used as a reference surface and was directly deactivated by injecting 1 M ethanolamine at pH 8.5 for 7 min. The other three flow cells were injected with 25 $\mu\text{g/mL}$, 12.5 $\mu\text{g/mL}$, and 6.25 $\mu\text{g/mL}$ EphB4/Fc per flow cell, respectively, followed by injection of 1 M ethanolamine to block the remaining activated ester groups on the surface. EphB4/Fc solutions of different concentrations (25, 12.5, and 6.25 $\mu\text{g/mL}$) were obtained by diluting a stock solution of EphB4/Fc (100 $\mu\text{g/mL}$) in phosphate-buffered saline (PBS) with 10 mM sodium acetate buffer (pH 4.5). The chip was allowed to stabilize for at least 2 h in HBSEP running buffer before injecting the test analytes.

[00128] Binding assays were performed at 25°C in HBSEP running buffer. c(TNYL-RAW) was diluted in HBSEP buffer, filtered, degassed, and injected at concentrations between 1.6 nM and 800 nM at a flow rate of 30 $\mu\text{L/min}$. Peptides were injected into the HBSEP buffer over 4 min, and injection was followed by a 4-min dissociation period. The chips were regenerated using a 1-min pulse of 10 mM glycine (pH 2.2) after each binding cycle. Each cycle consisted of a 1-min waiting period to allow monitoring of the baseline binding stability. For subtraction of bulk effects caused by changes

in the buffer composition or nonspecific binding, a double-referencing technique was employed. In this technique, all analyzed samples, including a sample of the running buffer, were additionally injected onto a reference cell capped with ethanolamine but without the EphB4 coating. Data were evaluated with BIAevaluation software (version 3.0, GE Healthcare), by applying a simple 1:1 binding mass transfer model. The resultant sensorgrams were fitted globally over the whole range of injected concentrations for both the association and dissociation phases. Equilibrium dissociation constants were then calculated from the experimental rate constants ($K_D = k_{\text{off}}/k_{\text{on}}$).

[00129] Radiolabeling of HAU NS with ^{111}In . To conjugate a radiometal

chelator to c(TNYL-RAW)-HAuNS, 4-aminobenzyl-diethylenetriaminepentaacetic acid thioctamide (DTPA-TA, 10 mg/mL; 5.0 μL) (Lu *et al.*, 2007) was mixed with 1.0 mL of aqueous solution of HAU NS (200 OD/mL) for 6 h at room temperature. SH-PEG-c(TNYL-RAW) was then added to the DTPA-TA-conjugated HAU NS as described in the previous section. For radiolabeling with ^{111}In , aliquots of DTPA-TA-conjugated c(TNYL-RAW)-HAuNS in 0.1 M sodium acetate solution (pH 5.4) were mixed with an aqueous solution of $^{111}\text{InCl}_3$ (5 mCi) for 1 h. ^{111}In -labeled c(TNYL-RAW)-HAuNS were purified by centrifugation at 10,000 rpm for 10 min and washed three times with PBS. The nanoparticles were then resuspended in sodium citrate solution (3.5 mM). Finally, ^{111}In -labeled c(TNYL-RAW)-HAuNS were loaded with DOX to create ^{111}In -labeled T-DOX@HAuNS. The radiolabeling efficiency and the stability of ^{111}In -labeled T-DOX@HAuNS were analyzed using instant thin-layer chromatography. The paper strips were developed with PBS (pH 7.4) containing 4.0 mM EDTA, and the radioactivity was quantified using a Bioscan IAR-2000 thin-layer chromatography imaging scanner (Washington, DC). Free $^{111}\text{In}^{3+}$ moved to the solvent front ($R_f = 0.9\text{--}1.0$), and the nanoparticles remained at the original spot ($R_f = 0.0$). The labeling efficiency was >95%.

[00130] Cell culture. MDA-MB-231 (human breast carcinoma), Hey (human

ovarian carcinoma), and A549 (human lung adenocarcinoma) cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, Inc., Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO_2 . A2780 human ovarian carcinoma cells were kindly provided by Dr. Stephen J. Williams (Fox Chase Cancer

Center, Philadelphia, PA). These cells were maintained at 37°C in RPMI-1640 medium containing 10% fetal bovine serum and insulin (0.25 units/mL).

[00131] Cell uptake of HAU NS. Cells were transferred and cultured onto 20-mm glass cover slips in a 24-well plate and allowed to grow for 2 days. The medium was replaced with 1 mL of fresh culture medium containing T-DOX@HAuNS. After incubation for 2 h, cell nuclei were stained with DAPI. The cell monolayer on the cover slip was removed, repeatedly rinsed with PBS, and then mounted for microscopic examination. The cellular fluorescence and dark-field light scattering images were examined under a Zeiss Axio Observer Z1 fluorescence microscope equipped with a dark-field condenser.

[00132] Quantitative cellular uptake was determined using ¹¹¹In-labeled nanoparticles. Hey and A549 cells (~1 × 10⁶/dish) were cultured in 60 mm × 15 mm dishes. Cells were incubated with ¹¹¹In-labeled T-DOX@HAuNS or ¹¹¹In-labeled DOX@HAuNS (1.5 μCi/dish) for 3 h at room temperature in culture medium lacking fetal bovine serum and phenol red. For the blocking experiments, cells were incubated with free c(TNYL-RAW) (1.0 μM) for 0.5 h and then with ¹¹¹In-labeled T-DOX@HAuNS or ¹¹¹In-labeled DOX@HAuNS under the same conditions. Cells were rinsed five times with PBS (pH 7.4). After the PBS was removed, the cells were scraped off the dish, suspended in PBS, and the radioactivity of the cell suspension was then measured with a gamma counter. Protein concentration in cell suspension was quantified using the Bio-Rad protein assay kit (Richmond, CA). The data are expressed as radioactivity (dpm per μg protein).

[00133] Analysis of EphB4 expression. For western blot analysis, cells were harvested at 70% confluence by adding CellLytic M cell lysis buffer (Sigma-Aldrich) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), followed by centrifugation at 13,500 rpm. The supernatants were collected, and protein concentrations were measured with Coomassie protein assay reagent (Thermo Fisher Scientific). Equal amounts of protein from each experimental group were run on a 4-12% NuPAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane. The EphB4 expression was probed with mouse anti-EphB4 antibody and Alexa Fluor 680-conjugated goat anti-mouse IgG (Invitrogen). β-Actin was used as a control to indicate the loading and transfer efficiency. Protein bands were visualized with a LI-COR Odyssey system (Lincoln, NE).

[00134] A separate experiment was carried out to optically observe the binding between the antibody against EphB4 and the studied cell lines. Cells were cultured on 20-mm glass cover slips and incubated with primary antibody (mouse anti-EphB4 antibody) for 1 h at 4 °C. The cells were then washed with PBS three times and incubated with secondary antibody (Alexa Fluor 680-conjugated goat anti-mouse IgG). The cell-coated cover slip was then repeatedly rinsed with PBS and mounted for microscopic examination using an Axio Observer Z1 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Germany).

[00135] Cytotoxicity. Cytotoxicity was measured using an MTT assay according to the manufacturer's suggested procedures. EphB4-positive A2780 cells and EphB4-negative A549 cells were exposed to free DOX, DOX@HAuNS, or T-DOX@-HAuNS for 72 h. The data are expressed as the percentage of surviving cells and are reported as the mean values of three measurements.

[00136] Pharmacokinetics and biodistribution. All animal studies were carried out under Institutional Animal Care and Use Committee-approved protocols. For the pharmacokinetic analysis, 8 healthy female Swiss mice (22-25 g; Charles River Laboratories, Wilmington, MA) for each group were each injected intravenously with a dose of either ¹¹¹In-labeled T-DOX@-HAuNS or ¹¹¹In-labeled DOX@HAuNS (both 20 µCi, 1.0 mg/mL [40 OD] in 0.2 mL citrate buffered solution). At predetermined intervals up to 48 h, blood samples (10 µL) were taken from the tail vein, and the radioactivity of each sample was measured with a gamma counter. The percentage of the injected dose per gram of blood (%ID/g) was calculated. The blood pharmacokinetic parameters for the radiotracer were analyzed using a noncompartmental model with WinNonlin 5.0.1 software (Pharsight, Sunnyvale, CA).

[00137] To investigate the *in vivo* distribution of T-DOX@-HAuNS, female nude mice (Harlan, Indianapolis, IN) bearing 6-8 mm subcutaneous A2780, MDA-MB-231, or Hey tumors were intravenously injected with ¹¹¹In-labeled T-DOX@-HAuNS or ¹¹¹In-labeled DOX@HAuNS (20 µCi/mouse in 0.2 mL). For the blocking studies, the mice were intravenously injected with a mixture of ¹¹¹In-labeled T-DOX@-HAuNS (20 µCi/mouse in 0.2 mL) and an excess of free c(TNYL-RAW) (0.3 µmol). Mice were killed at 24 h after injection. Various tissues, including tumors, were collected and weighted. The radioactivity for each sample was measured with a gamma counter. Uptake of nanoparticles in various tissues was calculated as %ID/g.

[00138] Single photon emission computed tomography (SPECT) imaging.

Mice bearing subcutaneous Hey tumors were intravenously injected with ^{111}In -labeled T-DOX@HAuNS or DOX@HAuNS (8.0 mCi/kg, 0.525 mg/mL [25 OD]). For the blocking studies, mice were injected intravenously with a mixture of ^{111}In -labeled DOX@c(TNYL-RAW)-HAuNS and free c(TNYL-RAW) (0.3 μmol). The mice were placed in a prone position and anesthetized with 0.5%-2.0% isoflurane gas (Iso-Thesia, Rockville, NY) in oxygen. SPECT images were generated at 24 h after injection. After imaging, the mice were killed, and their tumors were removed. The tumors were snap-frozen and cut into 5- μm slices that were then used for autoradiography analysis on a Fujifilm FLA-5100 imaging system (Stamford, CT).

[00139] NIR laser-triggered DOX release *in vivo*.

For measuring the photothermal effect of T-DOX@HAuNS, mice bearing Hey tumors were injected intravenously with saline (5.0 mL/kg) or T-DOX@-HAuNS (5.0 mL/kg of 50 OD HAuNS). At 24 h after injection, the tumor was irradiated with an NIR laser (3 W/cm²) for 5 min; Diomed 15 Plus, UK) through the skin surface. Temperature was measured with two thermocouples inserted into the tumor. Care was taken to ensure the thermocouple was not directly exposed to the laser beam.

[00140] The DOX release mediated by the photothermal effect in the Hey

tumors was studied using ^{111}In - and ^3H -labeled T-DOX@-HAuNS, in which the HAuNS were labeled with ^{111}In and the DOX with ^3H . Tumors were irradiated by NIR laser light (3 W/cm² for 5 min) at 1 h after intratumoral injection of the dual-labeled nanoparticles (^3H : 10 μCi ; ^{111}In : 20 μCi) into the center of the tumor. The mice were killed 5 min after laser irradiation, and the tumors were removed, snap-frozen, and sliced into 10- μm sections. Injected tumors that did not receive NIR laser treatment were similarly prepared and used as controls.

[00141] The radioactive signals from ^{111}In and ^3H were detected using a

Fujifilm FLA-5100 imaging system. Briefly, the sections of the tumors were exposed to phosphorous screen film (an SR imaging plate) for 15 min at -10°C, and the ^{111}In autoradiograph was obtained by scanning the film. After the ^{111}In was completely decayed (stored at -80°C for 5 weeks), the same sections were exposed to phosphorous screen film (a TR imaging plate) for 3 days at -10°C, and the ^3H autoradiograph was obtained by scanning the film. Concurrently, control specimens from tumors that were not laser-treated were

subjected to the same procedures. The autoradiographic distribution of ^{111}In -HAuNS and ^3H -DOX was compared by overlaying the two autoradiograms.

[00142] Antitumor activity *in vivo*. Hey tumors were generated by subcutaneous injection of Hey cells (5.0×10^6 cells/mouse). When the mean tumor volume reached $\sim 200 \text{ mm}^3$, mice were divided into four groups consisting of 6-8 mice each. Mice in groups 1 through 4 were injected intravenously with saline ($n = 6$, 5.0 mL/kg), HAuNS ($n = 6$, 5.0 mL/kg of 1.25 mg HAuNS/mL solution), DOX@HAuNS ($n = 7$, 10 mg equivalent DOX/kg, 5.0 mL/kg of 1.25 mg HAuNS/mL solution), and T-DOX@HAuNS ($n = 8$, 10 mg equivalent DOX/kg, 5.0 mL/kg of 1.25 mg HAuNS/mL solution), respectively. All mice received NIR laser irradiation through the skin surface at 24 h postinjection (2.0 W/cm^2 for 3 min). The tumor dimensions were measured with a caliper, and the tumor volume was calculated according to the equation: volume = (tumor length) $\times$ (tumor width) $^2/2$. At the end of the experiment (when the tumor size reached $>1500 \text{ mm}^3$ or 22 days after initial injection, whichever came first), mice were killed by CO_2 asphyxiation, and the tumors were collected and weighed. Parts of the tumors were fixed in formalin and cut into 5- μm slices for hematoxylin and eosin staining. Body weight was measured weekly to assess systemic toxicity.

[00143] Statistics. Pharmacokinetic data were analyzed using non-compartmental WinNonlin method. Statistical analysis was performed using the SYSTAT program. P values were obtained using the two-sample *t*-test. For cellular uptake, biodistribution, and antitumor activity data, statistic analyses were performed by ANOVA, with $P < 0.05$ considered to be statistically significant.

[00144] Results

[00145] Synthesis and characterization of EphB4-targeted T-DOX@HAuNS.
Prior to carrying out efficacy studies, the inventors performed a number of experiments to fully characterize the targeted DOX@HAuNS system for *in vivo* delivery. The average diameter of the HAuNS was $36.8 \pm 1.6 \text{ nm}$, as determined by dynamic light scattering. Transmission electron microscopy (TEM) confirmed that the HAuNS consisted of a thin gold shell ($\sim 4 \text{ nm}$ thickness) with a hollow interior. The extinction spectrum showed that the plasma resonance peak for HAuNS was $\sim 800 \text{ nm}$ (FIG. 14).

[00146] The targeting ligand cyclic peptide c(TNYL-RAW) is a second-generation EphB4-binding antagonist. The peptide had an equilibrium dissociation constant (K_d) of 4.4 nM as determined by surface plasmon resonance sensorgram (FIG. 15A). No degradation of ^{64}Cu -labeled c(TNYL-RAW) was observed by high-performance liquid chromatography after incubation of the peptide in mouse plasma over a period of 24 h, whereas ^{64}Cu -labeled linear TNYL-RAW was degraded as soon as 2 h after incubation (FIG. 15B). c(TNYL-RAW) was linked to SATA-PEG-NHS through an activated ester. After deprotection of the SH group, SH-PEG-c(TNYL-RAW) was conjugated to HAU NS in an aqueous solution via S-Au bonding (FIG. 9). The amount of c(TNYL-RAW) conjugated to the HAU NS was determined by quantitative amino acid analysis after complete dissolution of c(TNYL-RAW)-conjugated HAU NS. The conjugation efficiency was 13.7% and there were about 880 molecules of c(TNYL-RAW) on each HAU NS nanoparticle. DOX was readily loaded into c(TNYL-RAW)-conjugated HAU NS using a previously reported method to give T-DOX@HAU NS (You *et al.*, 2010). DOX loading efficiency was over 90%, and DOX content was 30% (w/w).

[00147] In vitro uptake in cancer cells. Next, the inventors evaluated the selectivity of uptake of c(TNYL-RAW)-conjugated DOX@HAU NS in EphB4-positive tumor cells. Western blotting analysis indicated high expression levels of EphB4 receptor in MBA-MD-231, A2780, and Hey cells, but only low expression level in A549 cells (FIG. 16A). Immunostaining using anti-EphB4 antibody confirmed strong EphB4 signals from A2780, MDA-MB-231, and Hey cells but weak signal from A549 cells. On the basis of these findings, Hey cells were selected for subsequent efficacy studies.

[00148] FIG. 10A shows representative photomicrographs of fluorescence and dark-field images of Hey cells incubated with T-DOX@HAU NS. The nanoparticles were readily taken up by the tumor cells. The fluorescence signal from the DOX was colocalized with the signal from the HAU NS, indicating that the DOX remained associated with the HAU NS after T-DOX@HAU NS were internalized. Under the same conditions, significantly more T-DOX@HAU NS was internalized in the cells with high EphB4 receptor expression (Hey) than in the cells with low EphB4 receptor expression (A549) ($P < 0.05$, FIG. 10B). The addition of free c(TNYL-RAW) peptide into the culture medium (Hey cells) induced a significant decrease in the cellular uptake of the nanoparticles ($P < 0.005$) but did not cause a change in uptake in the A549 cells that had low EphB4 receptor expression (FIG. 10B).

These data indicate that T-DOX@HAuNS was taken up by EphB4-positive cells via receptor-mediated endocytosis.

[00149] Cytotoxicity. For cells with low EphB4 expression (A549), the difference in cytotoxicity between T-DOX@HAuNS and non-targeted DOX@HAuNS was minimal, demonstrating the importance of EphB4-mediated uptake (FIG. 17A). Free DOX exhibited higher toxicity in A549 cells than both T-DOX@HAuNS and DOX@HAuNS. The lower cytotoxic potency of T-DOX@HAuNS and DOX@HAuNS can be attributed to relatively stable complexes formed between DOX and HAuNS and delayed DOX release inside cells. However, significantly higher toxicity was shown for T-DOX@HAuNS in A2780 cells than either free DOX or DOX@HAuNS (FIG. 17B). The higher cellular cytotoxicity with targeted nanoparticles can be attributed to the increased uptake of T-DOX@HAuNS in the target cells.

[00150] Pharmacokinetics, biodistribution, and SPECT imaging. FIG. 11A shows the mean blood activity time profile of ^{111}In -labeled T-DOX@HAuNS and DOX@HAuNS. The pharmacokinetic parameters are summarized in Table 2. DOX@HAuNS had moderately higher area under the blood drug concentration-time curve extrapolated to infinite time ($\text{AUC}_{0-\infty}$, $322.3 \pm 72 \% \text{ID h/mL}$) than T-DOX@HAuNS ($242.8 \pm 46 \% \text{ID h/mL}$, $P = 0.046$). However, there was no significant difference between the two HAuNS formulations in any other pharmacokinetic parameters ($P \geq 0.05$), suggesting that the conjugation of c(TNYL-RAW) to HAuNS did not significantly change the pharmacokinetic properties of the nanoparticles. Both types of nanoparticles (T-DOX@HAuNS and DOX@HAuNS) were almost completely eliminated from the blood at 48 h after injection. FIG. 11B shows the biodistribution of T-DOX@HAuNS, T-DOX@HAuNS with blocking by free c(TNYL-RAW), and DOX@HAuNS in nude mice at 24 h after injection. Most nanoparticles were taken up by the liver, spleen, and kidney. Interestingly, significantly less T-DOX@HAuNS than DOX@HAuNS accumulated in the liver ($P = 0.0009$) and the spleen ($P = 0.0006$). Conversely, T-DOX@HAuNS had significantly higher uptake in the kidney than DOX@HAuNS did ($P = 0.004$). With the exception of kidney uptake, co-injection with an excess of c(TNYL-RAW) did not affect the biodistribution pattern of T-DOX@HAuNS. c(TNYL-RAW) blocking reduced the kidney uptake of T-DOX@HAuNS from $17.2 \% \text{ID/g}$ to $12.9 \% \text{ID/g}$ ($P = 0.02$). This finding may be reflective of EphB4 expression in venous endothelium of the kidney (Ozgur *et al.*, 2011).

Table 2. Pharmacokinetic parameters of DOX@HAuNS and T-DOX@HAuNS after intravenous injection in female Swiss mice.

PK Parameters	DOX@HAuNS	T-DOX@HAuNS	P Value
Elimination half-life (h)	11.9 ± 3.8	9.75 ± 1.7	0.229
C _{max} (%ID/mL)	28.9 ± 4.9	30.8 ± 3.7	0.459
AUC _{0-∞} (%ID h/mL)	322.3 ± 72	242.8 ± 46	0.046
CL (mL/h)	0.323 ± 0.068	0.426 ± 0.092	0.050
Vd (mL)	5.30 ± 0.81	5.85 ± 0.69	0.239
Vss (mL)	4.94 ± 0.70	5.25 ± 0.61	0.439
MRT (h)	16.0 ± 5.0	12.6 ± 1.8	0.137

Analysis of variance showed no differences between the two HAuNS formulations. C_{max}, maximum blood concentration; AUC_{0-∞}, area under the blood drug concentration-time curve extrapolated to infinite time; CL, systemic clearance; Vd, volume of distribution; Vss, volume of distribution at steady-state; MRT, mean residence time.

[00151] To demonstrate efficiency of tumor targeting, the inventors carried out biodistribution studies in tumor-bearing mice. T-DOX@HAuNS displayed significantly higher accumulation than nontargeted DOX@HAuNS in tumors with high EphB4 receptor expression, with 3.0-, 2.1-, and 1.6-fold mean increases in Hey (P = 0.0016), A2780 (P = 0.0005), and MDA-MB-231 tumors (P = 0.019), respectively (FIG. 11C). Furthermore, the addition of free c(TNYL-RAW) peptide significantly reduced the uptake of T-DOX@HAuNS in these tumors, with 2.3-, 1.5-, and 2.0-fold decreases, respectively (FIG. 11C). These results indicated that T-DOX@HAuNS was actively targeted to tumors expressing EphB4.

[00152] microSPECT/CT images showed significant blood activity in the liver and spleen for both ¹¹¹In-labeled T-DOX@HAuNS and DOX@HAuNS in nude mice bearing Hey tumors after intravenous injection of ¹¹¹In-labeled T-DOX@HAuNS, ¹¹¹In-labeled T-DOX@HAuNS plus free c(TNYL-RAW), and ¹¹¹In-labeled DOX@HAuNS. By 24 h after injection, accumulation of T-DOX@HAuNS in the tumor was clearly visualized. The uptake of T-DOX@HAuNS in the Hey tumors was blocked by an excess of free c(TNYL-RAW). Similarly, tumor uptake of then non-targeted DOX@HAuNS was barely visible. Autoradiographs of slices sectioned from all 3 tumors (Hey, A2780, and MDA-MB-231) 24 h

after injection showed stronger ^{111}In radioactivity signals with T-DOX@HAuNS than with T-DOX@HAuNS plus free c(TNYL-RAW) or with DOX@HAuNS.

[00153] *In vivo* antitumor activity. The temperature measured by the thermocouple within the tumor reached $\sim 53^\circ\text{C}$ after 5 min of NIR laser exposure on the tumor surface at an output power of 3 W/cm^2 in mice injected with T-DOX@HAuNS (FIG. 12). No change in temperature was noted under the same conditions in the tumors of control mice. Dual-tracer autoradiography showed that immediately after NIR laser irradiation, ^3H -DOX was released and dispersed into the area surrounding the site where T-DOX@HAuNS was introduced. Conversely, ^3H -DOX was mostly colocalized with ^{111}In -HAuNS in mice that did not undergo NIR laser treatment.

[00154] FIG. 13A shows the Hey tumor growth curves after intravenous injections of saline, HAuNS (5.0 mL/kg of 1.25 mg HAuNS/mL saline [50 OD], no DOX, no targeting), DOX@HAuNS (10 mg equivalent DOX/kg, 5.0 mL/kg of 1.25 mg HAuNS/mL), and T-DOX@HAuNS (10 mg equivalent DOX/kg, 5.0 mL/kg of 1.25 mg HAuNS/mL). Mice in each group received NIR laser treatment (2.0 W/cm^2 for 3 min) 24 h after injection. The mice in the saline-plus-laser group were killed on day 9 after injection because most of the tumors in this group were $\sim 1500\text{ mm}^3$ at that time. Mice in the other three groups were killed on day 22. Treatment with T-DOX@HAuNS-plus-laser showed significantly enhanced antitumor activity compared with saline-plus-laser, HAuNS-plus-laser, and DOX@HAuNS-plus-laser. The mean tumor weight in T-DOX@HAuNS-plus-laser group on day 22 after treatment was $0.0038 \pm 0.0007\text{ g}$ ($n = 8$), which was significantly smaller than that of the saline-plus-laser ($1.2 \pm 0.51\text{ g}$ on day 9, $n = 6$; $P < 0.0001$), HAuNS-plus-laser ($1.5 \pm 0.34\text{ g}$ on day 22, $n = 6$; $P < 0.0001$), and DOX@HAuNS-plus-laser ($0.36 \pm 0.24\text{ g}$ on day 22, $n = 7$; $P < 0.001$) groups (FIG. 13B). The tumors in this group of mice became whitish immediately after treatment, suggesting disruption of blood perfusion. Remarkably, the tumors in six of the eight mice treated with T-DOX@HAuNS-plus-laser regressed completely and became scar tissue by 22 days after the injection. Histological analysis showed the presence of scar tissue and a lack of residual tumor cells in mice treated with T-DOX@HAuNS-plus-laser (FIG. 13C). Tumors in the nontargeted DOX@HAuNS-plus-laser group became loose and discrete, suggesting damage to tumor cells. However, tumors could not be completely eradicated without targeting ligand. Tumors in the saline-plus-laser group appeared to be intact. In the saline-plus-laser and DOX@HAuNS-plus-laser treatment groups, viable tumor

cells were found throughout the tumor volumes (FIG. 13C). No apparent signs of toxicity were observed throughout the course of the study. The body weight of mice in all treatment groups steady increased during the study period (FIG. 18).

[00155] Discussion

5 **[00156]** In this study, the inventors showed that T-DOX@HAuNS had significantly greater cytotoxic effects in tumor cells with high EphB4 receptor expression than DOX@HAuNS and free DOX did *in vitro*. Moreover, T-DOX@HAuNS demonstrated enhanced antitumor activity when combined with NIR laser irradiation than DOX@HAuNS plus laser treatment *in vivo*. These findings indicate that c(TNYL-RAW) peptide is highly effective for selective delivery of T-DOX@HAuNS to tumors with high expression of EphB4. The significantly enhanced antitumor activity of T-DOX@HAuNS could be attributed to 1) increased accumulation of the nanoparticles in tumors, 2) controlled release of DOX mediated by NIR laser irradiation, and 3) synergistic interaction between chemotherapy and PTA therapy, both of which were activated concurrently by NIR laser.

15 **[00157]** In our nanoconstruct, c(TNYL-RAW) peptide with high EphB4 binding affinity and high plasma stability was conjugated to HAuNS through a PEG linker, which ensured availability of the peptide to the target receptor (FIG. 9). Additional monofunctional SH-PEG chains were introduced together with SH-PEG-c(TNYL-RAW) to ensure that all available gold surface was covered by PEG. This process was used to create DOX@HAuNS and ligand conjugated T-DOX@HAuNS, both of which had high colloidal stability.

25 **[00158]** Both *in vitro* and *in vivo* data showed that tumor uptake of T-DOX@HAuNS could be partially blocked by free c(TNYL-RAW) peptide, confirming that cell uptake of T-DOX@HAuNS was mediated by EphB4 (FIGS. 10 and 11). *In vivo*, T-DOX@HAuNS displayed significantly higher accumulation than nontargeted DOX@HAuNS in all three tumor models evaluated. Because DOX@HAuNS and T-DOX@HAuNS exhibited similar pharmacokinetic behaviors (FIG. 11A, Table 2), the difference in tumor uptake between targeted and nontargeted HAuNS is unlikely a result of enhanced permeability and retention effect. Hence, these data support successful EphB4 receptor-mediated targeted delivery of T-DOX@HAuNS after intravenous injection.

[00159] The temperature in tumors of mice that received an intravenous injection of T-DOX@HAuNS reached $\sim 53^{\circ}\text{C}$ after 5 min of continuous-wave NIR laser exposure at 3 W/cm^2 (FIG. 12). This temperature is sufficient for causing irreversible damage to cancer cells (Melancon *et al.*, 2011). As expected, there was no temperature change in the tumors of mice that did not receive the nanoparticle injection followed by NIR irradiation. Therefore, T-DOX@HAuNS mediated efficient photothermal effect. In aqueous solution, the release of DOX from DOX@HAuNS could be activated by NIR laser irradiation (You *et al.*, 2010). To demonstrate DOX could be released *in vivo* upon NIR irradiation, we used an autoradiographic technique with dual-radiotracer labeling. ^3H undergoes beta decay with a 12.3 year half-life, releasing 18.6 keV of energy in the process. On the other hand, ^{111}In emits gamma radiation with a half-life of 2.81 days at much higher energy (171 KeV and 245 KeV). Taking advantage of these differences in the decay properties between ^3H and ^{111}In , the inventors were able to separate signals from HAuNS and DOX in the tumors. Thus, immediately after NIR laser irradiation at 3 W/cm^2 , ^3H -DOX was dissociated from ^{111}In -HAuNS, whereas in the control tumor not exposed to NIR laser irradiation, signals from ^3H -DOX colocalized perfectly with ^{111}In -HAuNS (FIG. 13B). These data confirm that free DOX was released from T-DOX@HAuNS and then diffused away from the nanoparticles when tumors were exposed to the NIR laser.

[00160] The antitumor efficacy of free DOX, DOX@HAuNS, DOX@HAuNS-plus-NIR laser was investigated against MDA-MB-231 tumors in the inventors previous work (You *et al.*, 2012). There was no significant difference in tumor size between groups treated with DOX (15 mg/kg, one injection) and DOX@HAuNS (15 mg equivalent DOX/kg per injection, 2 injections). However, DOX@HAuNS-plus-NIR laser (15 mg equivalent DOX/kg per injection, 2 injections) reduced tumor volume significantly more than the other 2 treatments. In these earlier studies, free DOX at a single dose of 15 mg/kg caused serious systemic toxicity and cardiotoxicity. Therefore, free DOX was not used as a control in the current study. Instead, non-targeted DOX@HAuNS was used as a control and compared the antitumor studies of T-DOX@HAuNS against ovarian Hey tumors with that of DOX@HAuNS. Combined T-DOX@HAuNS-plus-laser treatments demonstrated significantly better antitumor activity than combined HAuNS-plus-laser (PTA only) and nontargeted DOX@HAuNS-plus-laser treatments (FIG. 13).

[00161] Significantly, this higher antitumor activity was achieved without causing significant systemic toxicity, as indicated by continued increase in body weight in mice in all treatment groups (FIG. 18). The inventors previously compared the toxicity of free DOX, liposomal DOX, and DOX@HAuNS (You *et al.*, 2012). DOX@HAuNS after a single
 5 dose at 60 mg equivalent DOX/kg had no cardiotoxicity compared to liposomal DOX (two doses at a total dose of 30 mg DOX/kg) and free DOX (single dose of 15 mg/kg). In the heart, 100% of both liposomal DOX- and free DOX-treated mice had a vacuolar cardiomyopathy. However, for mice treated with DOX@HAuNS, the histopathologic features in the heart were similar to those observed in the saline-treated control mice and no
 10 abnormal features were observed.

Example 3 – Heterodimeric Peptides for Bispecific Targeting of EphB4 and EphA2 Receptors

[00162] Both EphA2 and EphB4 receptors are over-expressed in a variety of solid tumors, including ovarian, breast, colorectal, brain, and prostate. These receptors are
 15 also expressed in angiogenic blood vessels. Therefore, members of the Ephrin receptor family are attractive targets for cancer imaging and therapy (Pasquale, 2010). Herein is disclosed a series of peptidyl heterodimers with high receptor binding affinity to EphA2 and EphB4 suitable for molecular imaging of Ephrin receptors. The heterodimers exemplified by YSA-TNYL-DOTA-⁶⁴Cu had significantly higher target-to-background ratio than its corresponding
 20 monomeric imaging probes ⁶⁴Cu-DOTA-TNYL-RAW and ⁶⁴Cu-DOTA-YSA targeting EphB4 and EphA2, respectively. Thus, imaging probes that simultaneously bind to both EphA2 and EphB4 can potentially increase early detection rates and be used to monitor response to therapy directed against EphA2 and EphB4.

[00163] A peptidyl heterodimer, YSAYPDSVPMMS(SEQ ID NO: 4)-PEG-
 25 TNYLFSPNGPIARAW(SEQ ID NO: 1) (YSA-TNYL), was synthesized by linking the two peptides YSAYPDSVPMMS (YSA; SEQ ID NO: 4) targeting EphA2 and TNYLFSPNGPIARAW (TNYL; SEQ ID NO: 1) targeting EphB4 together with a polyethylene glycol (PEG) linker. YSA-TNYL was then labeled with the positron emitter ⁶⁴Cu through 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) chelator
 30 (FIG. 19). The receptor-binding characteristics and tumor-targeting efficacy of heterodimer YSA-TNYL were evaluated *in vitro* with Surface Plasmon Resonance (SPR) sensor chip technology (Tables 3-5) and *in vivo* using μ PET imaging. YSA-TNYL-DOTA peptide had

comparable binding affinity to EphB4 compared with TNYL peptide and higher binding affinity to EphA2 than YSA peptide. The K_D decreased from about 10^{-9} to about 10^{-11} when both EphB4 and EphA2 receptors were coated on the sensor chip, indicating that the binding affinity of the heterodimer increased when both EphB4 and EphA2 receptors were coated on the sensor chip.

[00164] Binding of the YSA-TNYL heterodimer to EphB4 and EphA2 was blocked by their natural ligands. Surface competition assays were performed with YSA-TNYL-RAW dimer and ephrinB2 with the surface coated with EphB4 using an increasing concentration of heterodimer and a constant concentration of ephrinB2, the natural ligand of EphB4 (FIG. 20A, highest concentration is the bottom line on the sensorgram; lowest is the top). Surface competition assays were also performed with YSA-TNYL-RAW dimer and EphA2 with the surface coated with ephrinA1, the natural ligand of EphA2, using an increasing concentration of heterodimer and a constant concentration of EphA2 (FIG. 20B, highest concentration is the bottom line on the sensorgram; lowest is the top).

[00165] In human ovarian tumor HEY cells *in vitro*, YSA-TNYL-DOTA- ^{64}Cu dimer showed high uptake in tumor cells over a period of 2-hr incubation (FIG. 21). The tumor uptake of YSA-TNYL-DOTA- ^{64}Cu could be partially blocked with an excess amount of cold TNYL, YSA, or mixture of TNYL-RAW and YSA peptides.

[00166] The inventors also performed μPET imaging of EphA2 and EphB4 receptors in ovarian cancer xenografts using ^{64}Cu -labeled bispecific YSA-TNYL-RAW heterodimer. The dimeric radiotracer exhibited higher tumor-to-target ratio (FIG. 22) and improved imaging properties as compared to its corresponding monomeric radiotracers.

[00167] Compared with ^{64}Cu -DOTA-TNYL and ^{64}Cu -DOTA-YSA monomeric tracers, the heterodimer YSA-TNYL-DOTA- ^{64}Cu also showed improved pharmacokinetics, resulting in a significantly higher target-to-background ratio. Thus, this class of radiotracers directed at both EphA2 and EphB4 should be useful as imaging probes for early tumor detection, monitoring treatment response to therapies directed at Ephrin receptors, and noninvasive characterization of Ephrin receptors. Unexpectedly, K_D 2, which represents binding between the bispecific peptide with EphB4, was two orders of magnitude lower when both EphB4 and EphA2 were coated onto the sensor chip than when only EphB4 was coated

onto the sensor chip, suggesting that binding of EphA2 enhanced the binding of the specific heterodimer to EphB4.

Table 3. Peptide binding with EphB4 coated to the sensor chip.

Analyte	k_{on} [$M^{-1}S^{-1}$]	k_{off} [s^{-1}]	K_D [M]	χ^2
TNYL-RAW	4.26×10^5	1.3×10^{-3}	3.06^{-9}	0.155
YSA-TNYL Dimer	1.09×10^6	4.82×10^{-3}	4.41^{-9}	4.37

5 **Table 4.** Peptide binding with EphA2 coated to the sensor chip.

Analyte	k_{on} [$M^{-1}S^{-1}$]	k_{off} [s^{-1}]	K_D [M]	χ^2
YSA	4.26×10^4	1.3×10^{-2}	3.06^{-7}	1.11
YSA-TNYL Dimer	46	1.3×10^{-5}	2.76^{-7}	12.1

Table 5. Heterodimer binding to coated mixture of EphB4 and EphA2 (1:1).

k_{on1} [$M^{-1}S^{-1}$]	k_{off1} [s^{-1}]	k_{on2} [$M^{-1}S^{-1}$]	k_{off2} [s^{-1}]	K_{D1} [M]	K_{D2} [M]	χ^2
1.31×10^3	1.17×10^{-3}	2.66×10^7	1.98×10^{-3}	8.93×10^{-7}	7.44×10^{-11}	6.46

Example 4 – Dual Labeled Peptides for Targeting of EphB4 Receptors

10 **[00168]** Herein is disclosed a dual labeling approach by introducing both a radionuclide and a near-infrared dye to an EphB4-targeting peptide that allows for dual modal imaging of the receptors. Such imaging probes can provide increased information content and are useful for both diagnostic imaging and guiding surgery intraoperatively.

15 **[00169]** ^{64}Cu -DOTA and Cy5.5 dye were introduced to TNYL peptide to synthesize a dual-tracer imaging probe. *In vitro*, dual labeled TNYL displayed significantly higher binding to U251 glioma cells over-expressing EphB4 (FIG. 23) than to U87 cells that express low levels of EphB4. The binding of dual labeled TNYL-RAW to U251 cells could be blocked by a large excess of unlabeled peptide. *In vivo*, μ PET/CT and near-infrared fluorescence optical imaging clearly showed the uptake of the dual labeled TNYL-RAW peptide in both U251 and U87 tumors in the brains of nude mice 1 h and 24 h after
20 intravenous injection. The specific uptake of dual labeled peptide in both tumors was

confirmed by blocking experiments. In U87 tumors, Cy5.5-labeled peptide was found co-localized only with CD31- and EphB4-expressing tumor blood vessels (FIG. 24). In U251 tumors, ^{64}Cu -DOTA-TNYL-Cy5 5 was found to bind to both brain tumor cells and angiogenic blood vessels expressing EphB4 receptors.

5 **Example 5 - μ PET/CT Imaging of EphB4 Receptors in Melanoma Xenografts with ^{68}Ga Labeled Cyclic TNYL-RAW Peptides**

10 **[00170]** Herein is disclosed a cyclic peptide with high receptor binding affinity for EphB4 with high *in vivo* stability suitable for molecular imaging of Ephrin receptors and for targeted drug delivery. The purpose of this study was to design, synthesis, and evaluate peptidomimetics with enhanced *in vivo* stability and low background through conformation constrain. The lactam formation of the side chain to side chain peptide cyclization (FIG. 25) was utilized to improve stability of peptides in the presence of peptidases and proteinases. Surface plasma resonance (SPR) sensor chip technology was used to screen the resulting peptides with regard to their receptor binding affinity. The lead cyclic peptide was conjugated with 1,4,7-triazacyclononanetriacetic acid (NOTA) and labeled with ^{68}Ga . Biodistribution, and small-animal PET/CT studies were performed in nude mice bearing EphB4-positive human melanoma. The inventors identified candidate peptides with binding affinity (K_d) in the lower nanomolar range (4.4 nM; FIG. 26) with significantly improved stability in mouse plasma (FIG. 27) and reduced retention in the liver and the spleen (FIG. 28B). μ PET/CT studies demonstrated clear visualization of EphB4 expressing tumors (FIGS. 28A). ^{68}Ga labeled cyclic(TNYL-RAW) peptide can be used as a PET/CT tracer to image tumor expression of EphB4, with a high tumor-to-background ratio. The improved metabolic stability, along with favorable pharmacokinetic profile and high receptor binding affinity, suggest that these peptides are promising candidates as novel therapeutic and imaging agents targeted to EphB4 receptors.

25 **[00171]** Using doxorubicin (DOX)-loaded hollow gold nanospheres (DOX@HAuNS) as an example, the inventors demonstrated targeted delivery of nanoparticles conjugated to the cyclic(TNYL-RAW) peptide that targets EphB4, T-DOX@HAuNS. Increased uptake of targeted nanoparticles T-DOX@HAuNS was observed in three EphB4-positive tumors both *in vitro* and *in vivo*.

[00172] *In vivo* release of DOX from DOX@HAuNS, triggered by near-infrared laser, was confirmed by dual radiotracer technique. Treatment with T-DOX@HAuNS followed by near-infrared laser irradiation resulted in significantly decreased tumor growth when compared to treatments with non-targeted DOX@HAuNS plus laser or
5 HAuNS plus laser. The tumors in six of the eight mice treated with T-DOX@HAuNS plus laser regressed completely with only residual scar tissue by 22 days following injection, and none of the treatment groups experienced a loss in body weight.

[00173] Together, our findings demonstrate that concerted chemo-photothermal therapy with a single nanodevice capable of mediating simultaneous PTA and local drug
10 release may have promise as a new anticancer therapy.

Example 6 - μ PET/CT Imaging of EphB4 Receptors in Melanoma Xenografts with Co-injection of ^{68}Ga -Labeled Cyclic TNYL-RAW Peptides and Metformin

[0001] Herein is disclosed a method of imaging EphB4 receptors by co-injection of ^{68}Ga -labeled cyclic TNYL-RAW with metformin, an anti-diabetic drug in the biguanide
15 class. Most peptide-based radionuclide therapy and imaging agents are cleared through the kidneys and reabsorbed and partially retained in the proximal tubules, causing dose-limiting nephrotoxicity. Various attempts have been made to reduce renal toxicity of radiolabeled compounds. Competitive inhibition of proximal tubular reabsorption can cause reduction in the renal uptake of the radiolabeled compounds. A maximum reduction of the kidney
20 radiation dose of approximately 50% has been achieved by co-infusion with basic (positively charged amino acids).

[0002] The inventors have found a more than 3-fold reduction in renal uptake of ^{68}Ga -labeled cyclic(TNYL-RAW) ^{68}Ga -NOTA-c(TNYL-RAW) when the radiotracer was co-injected with metformin (FIG. 29). These data indicate that metformin can be potentially used
25 with radiolabeled compounds in radiotherapy and imaging to reduce renal toxicity.

* * *

[0003] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be
30 apparent to those of skill in the art that variations may be applied to the methods and in the

steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar
5 substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS

1. An Ephrin receptor targeting agent comprising a first peptide comprising the amino acid sequence of SEQ ID NO: 1 or a cyclic peptide comprising the sequence of SEQ ID NO: 1 wherein the first peptide is conjugated to a therapeutic agent or an imaging agent.
- 5 2. The method of claim 1, wherein the first peptide is a cyclic peptide.
3. The targeting agent of claim 1, wherein the first peptide is conjugated to a radioisotope, a nanoparticle, a toxin, a chemotherapeutic agent, a fluorescent dye or a combination thereof.
4. The targeting agent of claim 1, wherein the first peptide is conjugated to a therapeutic
10 agent.
5. The targeting agent of claim 1, wherein the first peptide is conjugated to an imaging agent.
6. The targeting agent of claim 5, wherein the imaging agent is a SPECT imaging agent, PET imaging agent or an MRI contrast agent.
- 15 7. The targeting agent of claim 3, wherein the radioisotope is a gamma emitter, a positron emitter or a beta-emitter.
8. The targeting agent of claim 3, wherein the radioisotope is conjugated to the first peptide through a chelating moiety.
9. The targeting agent of claim 8, wherein the chelating moiety is DOTA or NOTA.
- 20 10. The targeting agent of claim 3, wherein the radioisotope is Cu-64, Cu-67, In-111, Tc-99m, Ga-67, Ga-68, Y-90 or Lu-177.
11. The targeting agent of claim 3, wherein the chemotherapeutic agent is doxorubicin.
12. The targeting agent of claim 3, wherein the nanoparticle is a hollow gold nanosphere (HAuNS).
- 25 13. The targeting agent of claim 12, wherein the HAuNS is a doxorubicin-loaded nanosphere.

14. The targeting agent of claim 1, comprising a second peptide targeting agent.
15. The targeting agent of claim 14, wherein the second peptide targeting agent comprises an amino acid sequence of SEQ ID NO: 4.
16. The targeting agent of claim 14, wherein the first and second peptides are comprised
5 as a fusion protein.
17. The targeting agent of claim 14, wherein the first and second peptides are connected by a linker.
18. The targeting agent of claim 17, wherein the linker polyethylene glycol (PEG).
19. An imaging probe for noninvasive imaging of EphB4 receptors comprising a
10 heterodimer peptide, wherein said peptide has motifs which target both EphA2 and EphB4.
20. The imaging probe of claim 19, wherein the heterodimer polypeptide comprises the amino acid sequence of SEQ ID NO: 1; a cyclic peptide comprising the sequence of SEQ ID NO: 1; or the sequence of SEQ ID NO: 4.
21. The imaging probe of claim 20, wherein the heterodimer polypeptide comprises the
15 amino acid sequence of SEQ ID NO: 1 and 4.
22. The imaging probe of claim 20, wherein the heterodimer polypeptide comprises a cyclic peptide comprising the sequence of SEQ ID NO: 1 and a peptide comprising the sequence of SEQ ID NO: 4.
23. The imaging probe of claim 19, wherein the heterodimer polypeptide comprises an
20 EphA2-targeting motif and an EphB4-targeting motif separated by a linker.
24. The imaging probe of claim 23, wherein the linker is a PEG linker.
25. The imaging probe of claim 21, wherein the heterodimer polypeptide comprises from amino- to carboxy-terminus the amino acid sequence of SEQ ID NO: 1; a PEG linker; and the amino acid sequence of SEQ ID NO: 4.
- 25 26. A method of imaging a subject comprising:
(a) administering an effective amount of a targeting agent of claim 5 to the subject;
and

(b) imaging the subject to detect the presence of the targeting agent.

27. The method of claim 26, wherein imaging the agent comprises performing the imaging comprises positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), fluorescence imaging or
5 photoacoustic imaging.

28. The method of claim 26, wherein the subject has a cancer.

29. The method of claim 28, wherein the cancer is an EphB4- or EphA2-overexpressing cancer.

30. The method of claim 28, wherein the targeting agent is conjugated to a therapeutic
10 and imaging agent, further defined as a method of dual imaging and therapy.

31. A method of treating a subject comprising administering an effective amount of a targeting agent of claim 4 to the subject.

32. The method of claim 31, wherein the subject has a cancer.

33. The method of claim 31, wherein the cancer is an EphB4- or EphA2-overexpressing
15 cancer.

34. The method of claim 31, wherein the targeting agent is conjugated to a therapeutic and imaging agent, further defined as a method of dual imaging and therapy.

35. The method of claim 31, wherein the targeting agent comprises a hollow gold nanosphere (HAuNS) conjugate.

20 36. The method of claim 36, wherein the HAuNS is a DOX-loaded HAuNS.

37. The method of claim 35, wherein the method further comprises administering a photothermal ablation therapy to the subject.

38. The method of claim 38, wherein the photothermal ablation therapy is administered by application of a photothermal ablation therapy.

25 39. A composition for use in the treatment or imaging of a subject comprising a targeting agent in accordance with claim 1.

40. A method of monitoring a cancer patient comprising the steps of:
injecting the patient with the peptide of claim 19;
imaging the patient using a PET/CT scan imaging device wherein the device produces a PET image;
- 5 acquiring the PET image of a tumor in the patient;
determining tumor-to-muscle ratio in the patient; and
administering a cancer treatment to the patient based on the tumor-to-muscle ratio.
41. A method of identifying EphB4 receptor expression in a subject in need thereof comprising administering to the subject a therapeutic amount of the heterodimer peptide of claim 19, and determining uptake or binding of the peptide in said subject.
- 10 42. A method of reducing renal uptake of a radio-labeled agent in a subject comprising administering the radio-labeled agent to the subject in conjunction with metformin.
43. The method of claim 42, wherein the radio-labeled agent is a therapeutic or imaging agent.
- 15 44. The method of claim 42, wherein the radio-labeled agent is a radio-labeled targeting agent in accordance with claim 1.
45. The method of claim 42, wherein the radio-labeled agent is administered before or essentially simultaneously with the metformin.
- 20 46. The method of claim 42, wherein the radio-labeled agent is administered after the metformin.
47. A pharmaceutical composition comprising a radio-labeled therapeutic or imaging agent and metformin formulated together in a pharmaceutically acceptable carrier.
48. The composition of claim 47, wherein the radio-labeled therapeutic or imaging agent is a radio-labeled targeting agent in accordance with claim 1.
- 25 49. A composition for use in reducing renal uptake of a radio-labeled agent, the composition comprising a radio-labeled therapeutic or imaging agent and metformin formulated together in a pharmaceutically acceptable carrier.

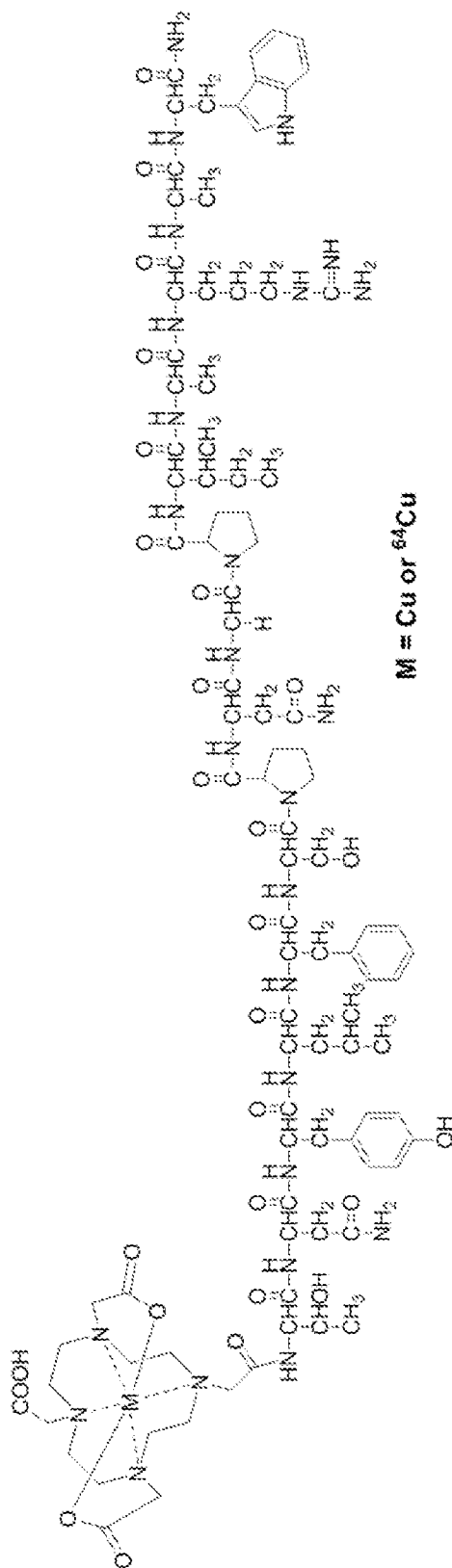


FIG. 1

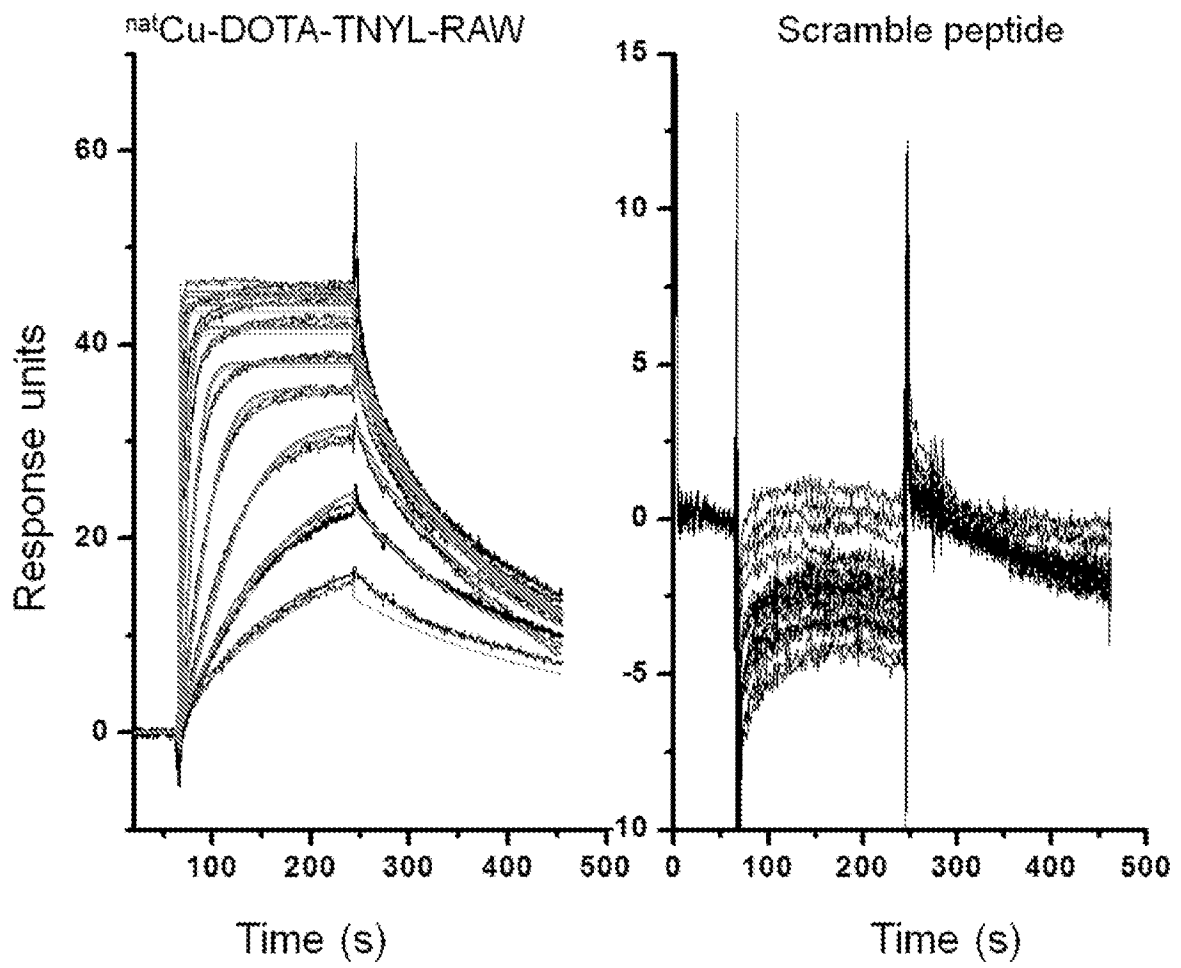


FIG. 2

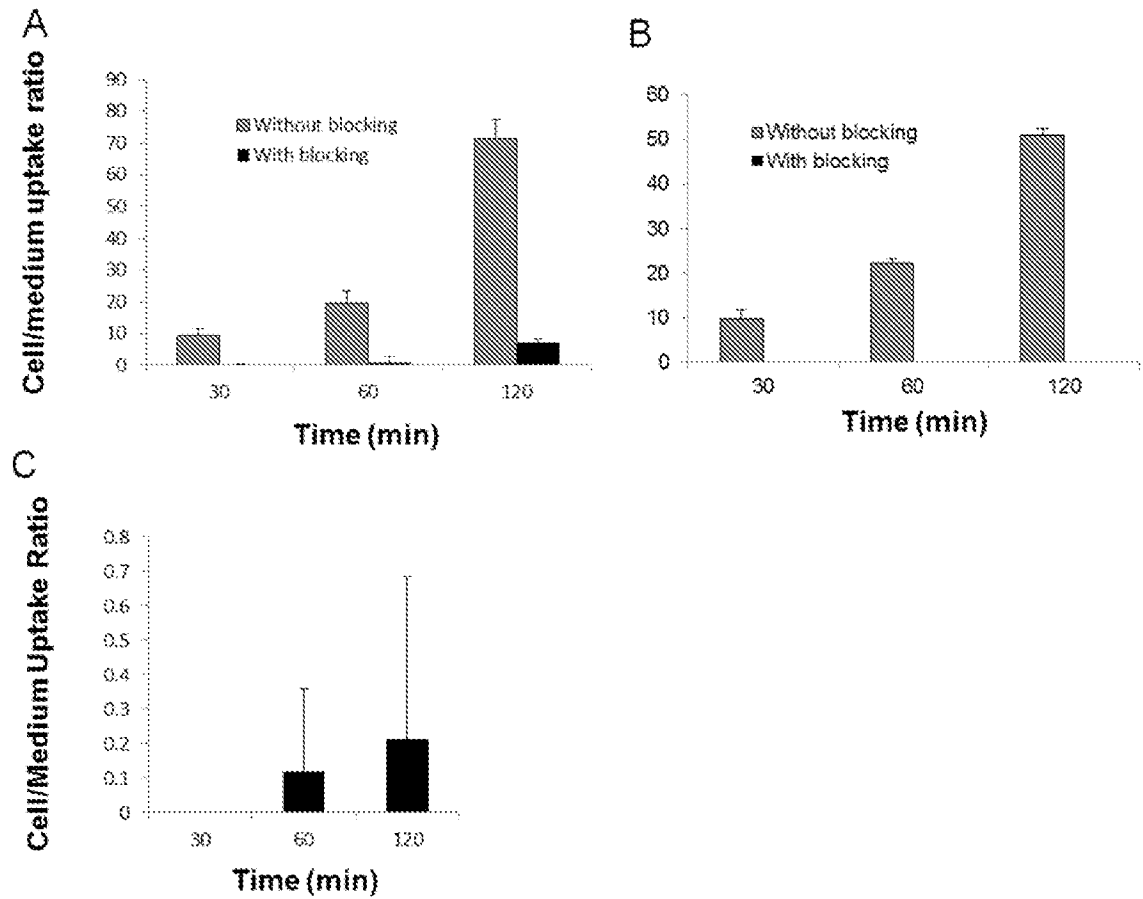


FIG. 3

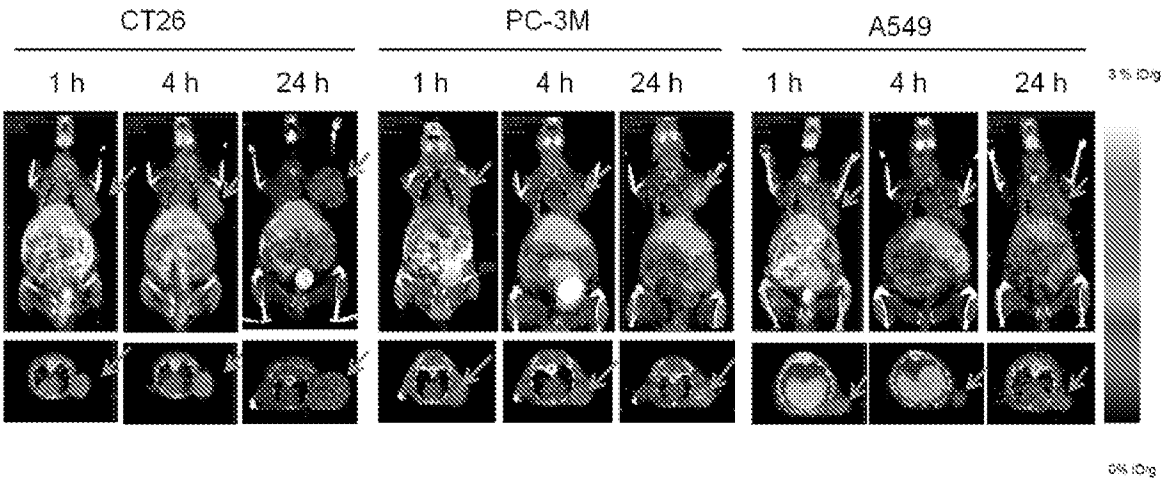


FIG. 4

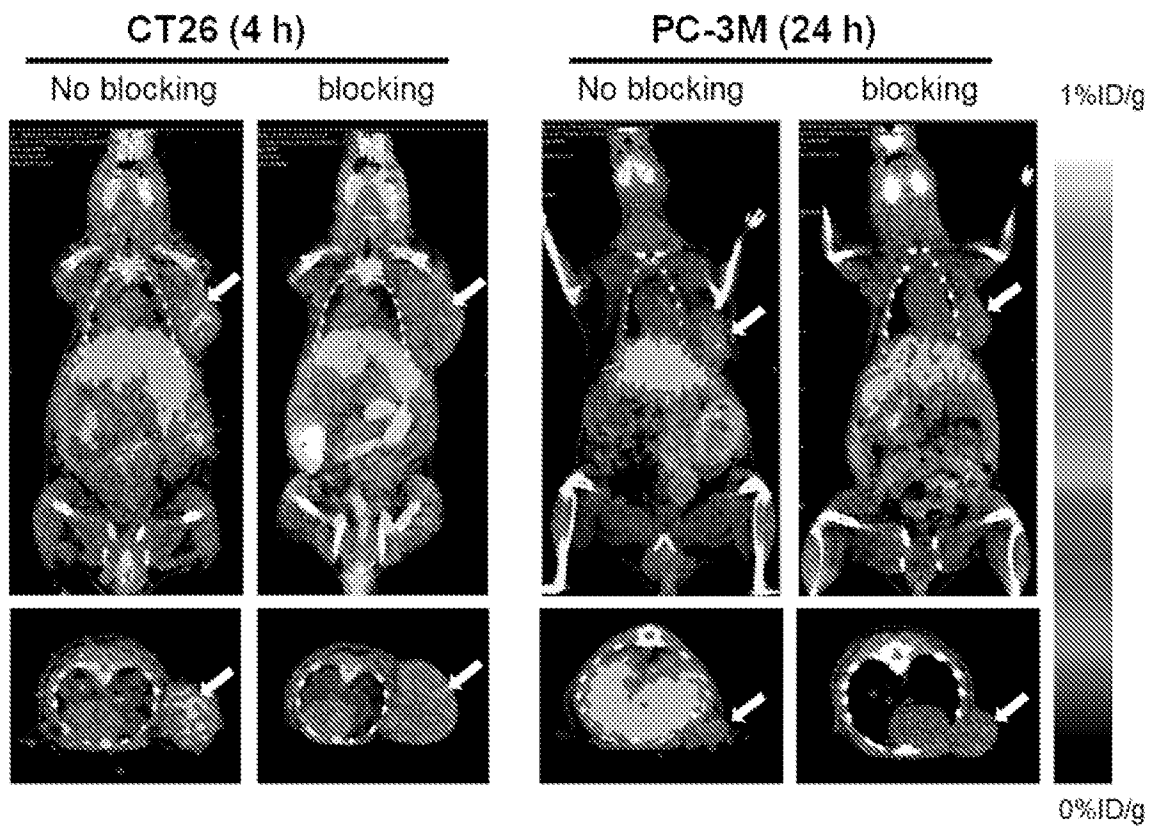


FIG. 5

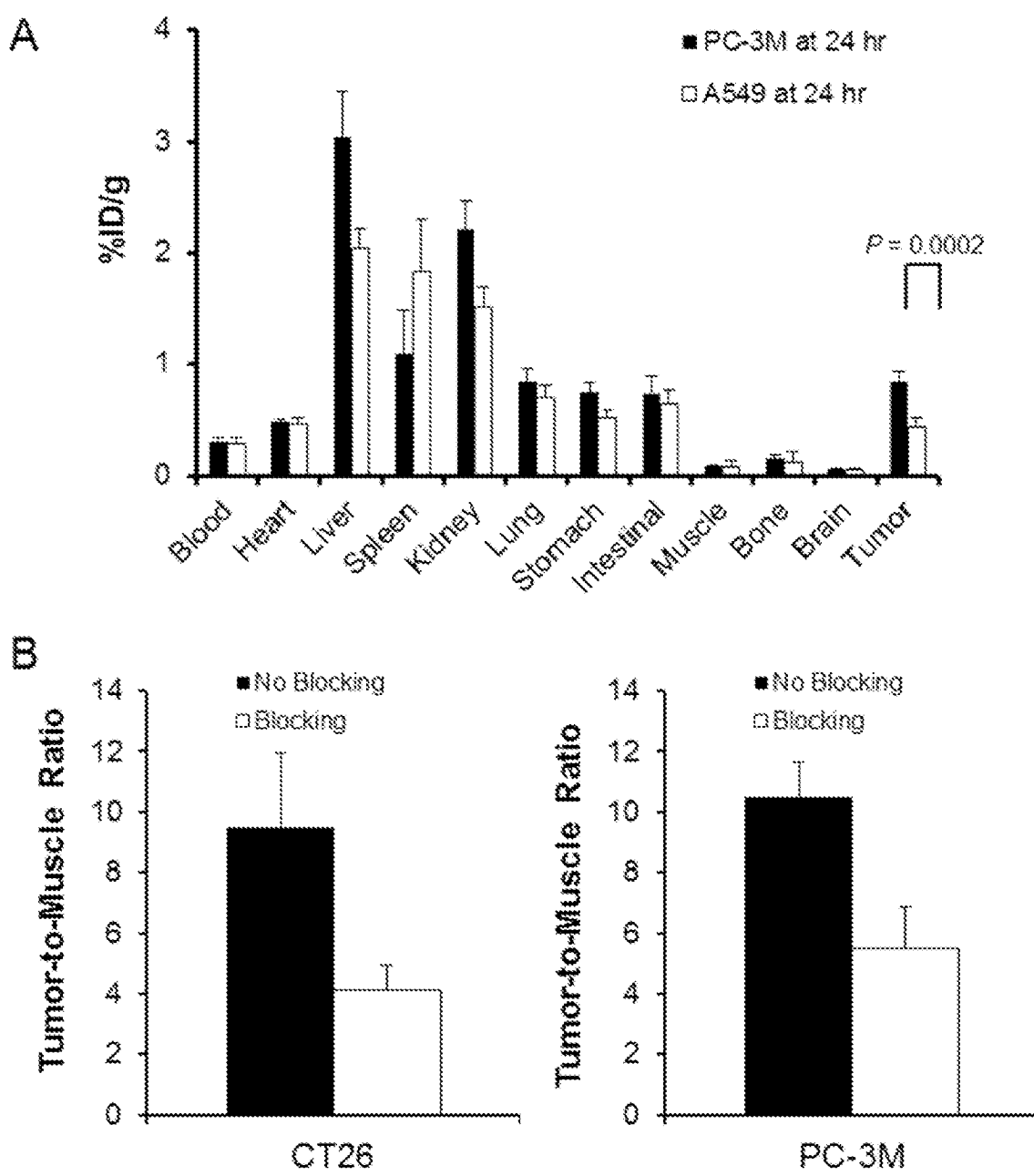


FIG. 6

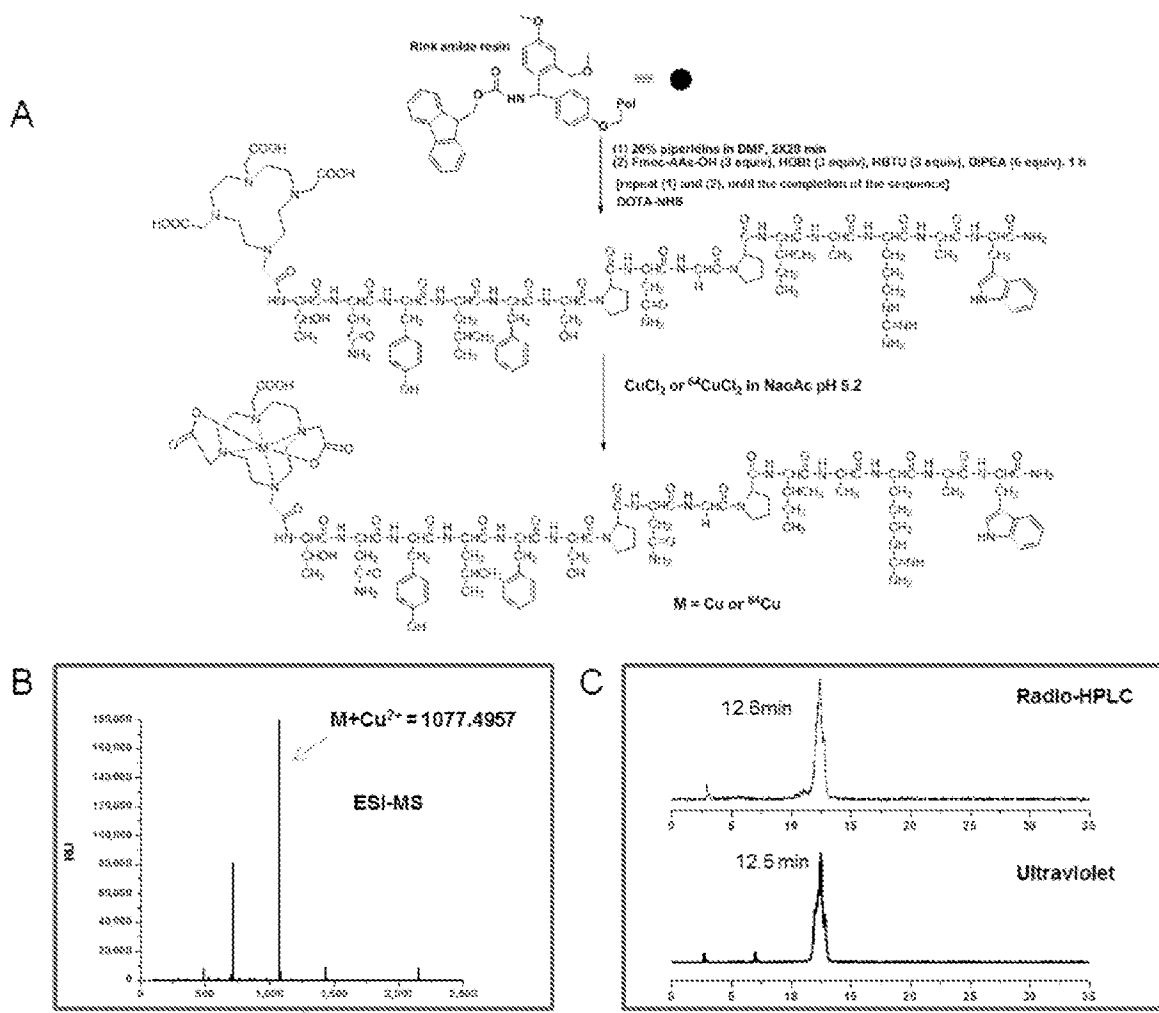


FIG. 7

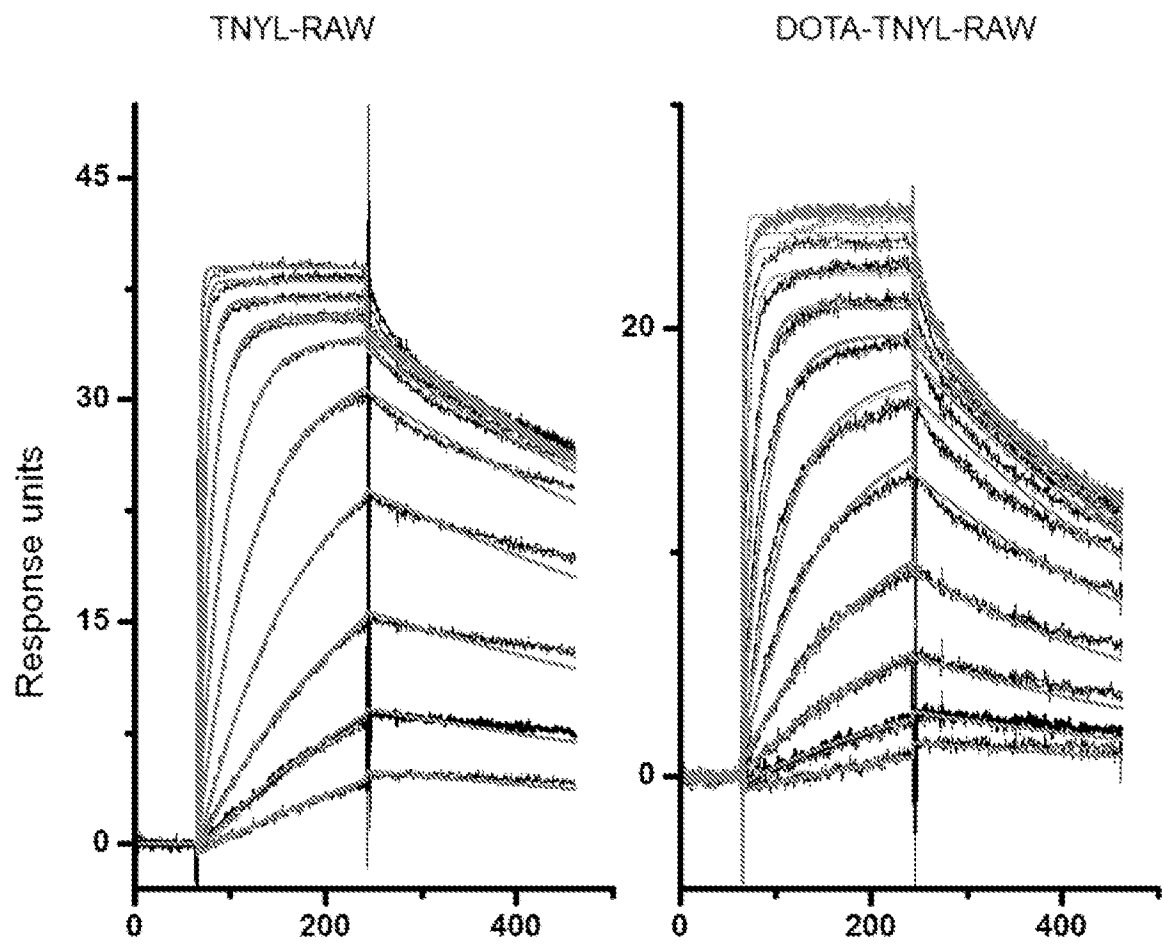


FIG. 8

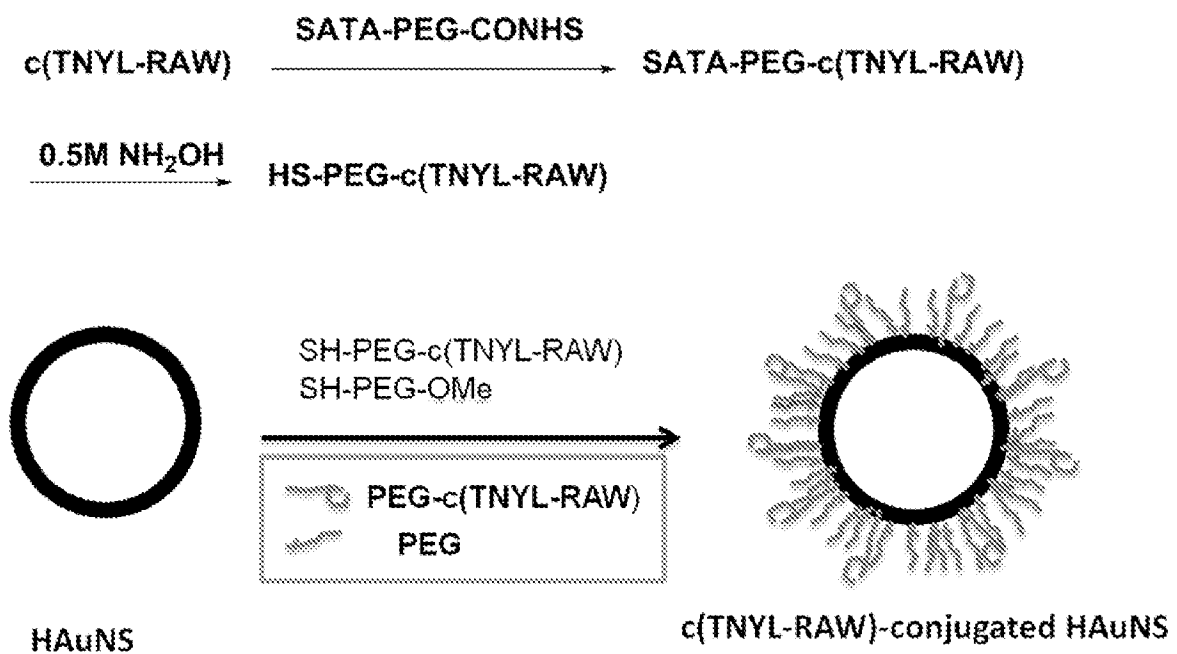


FIG. 9

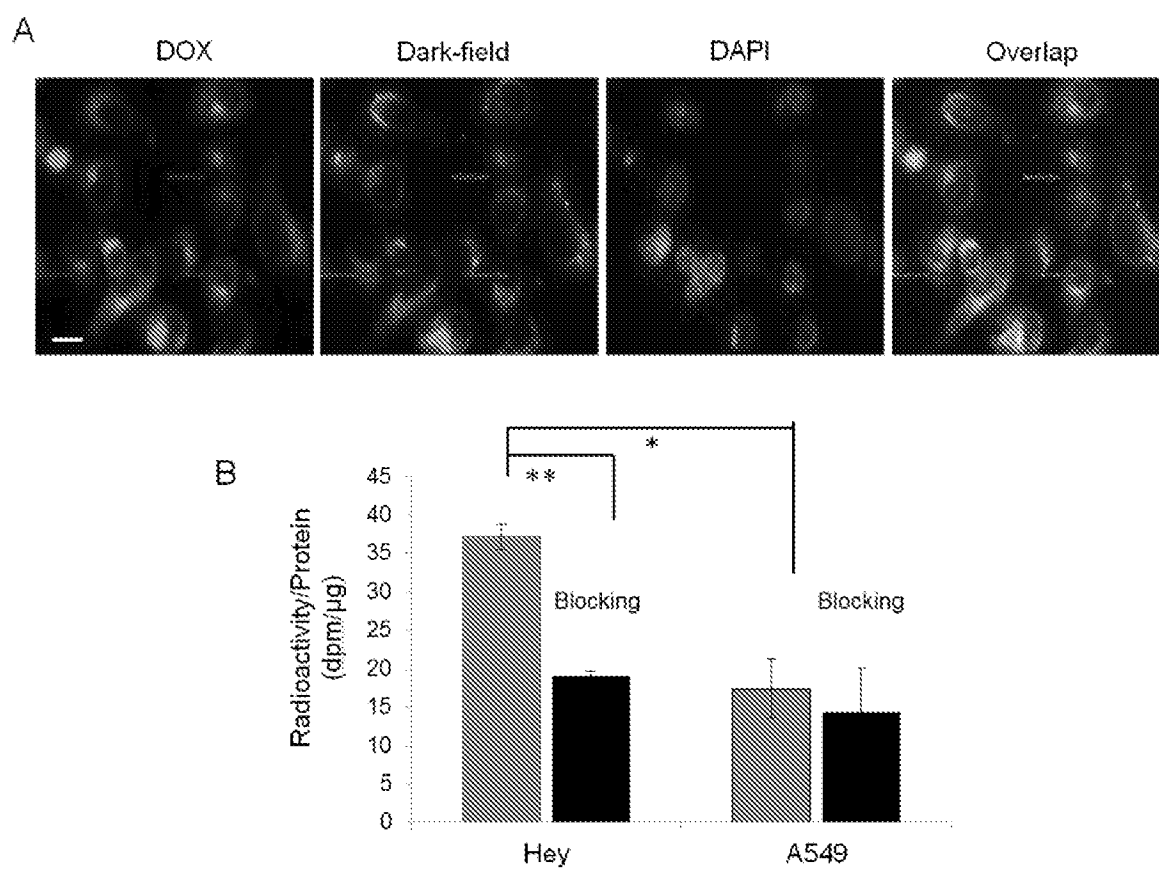


FIG. 10

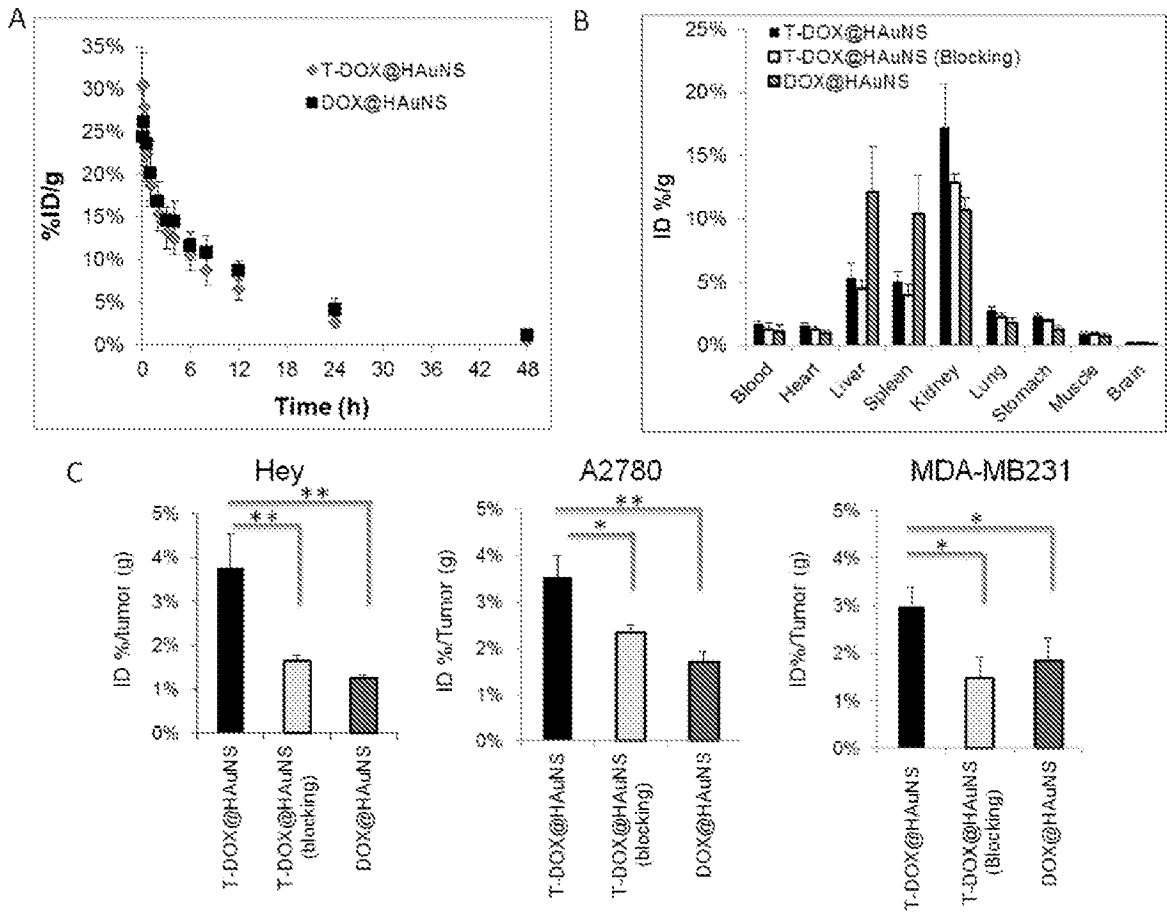


FIG. 11

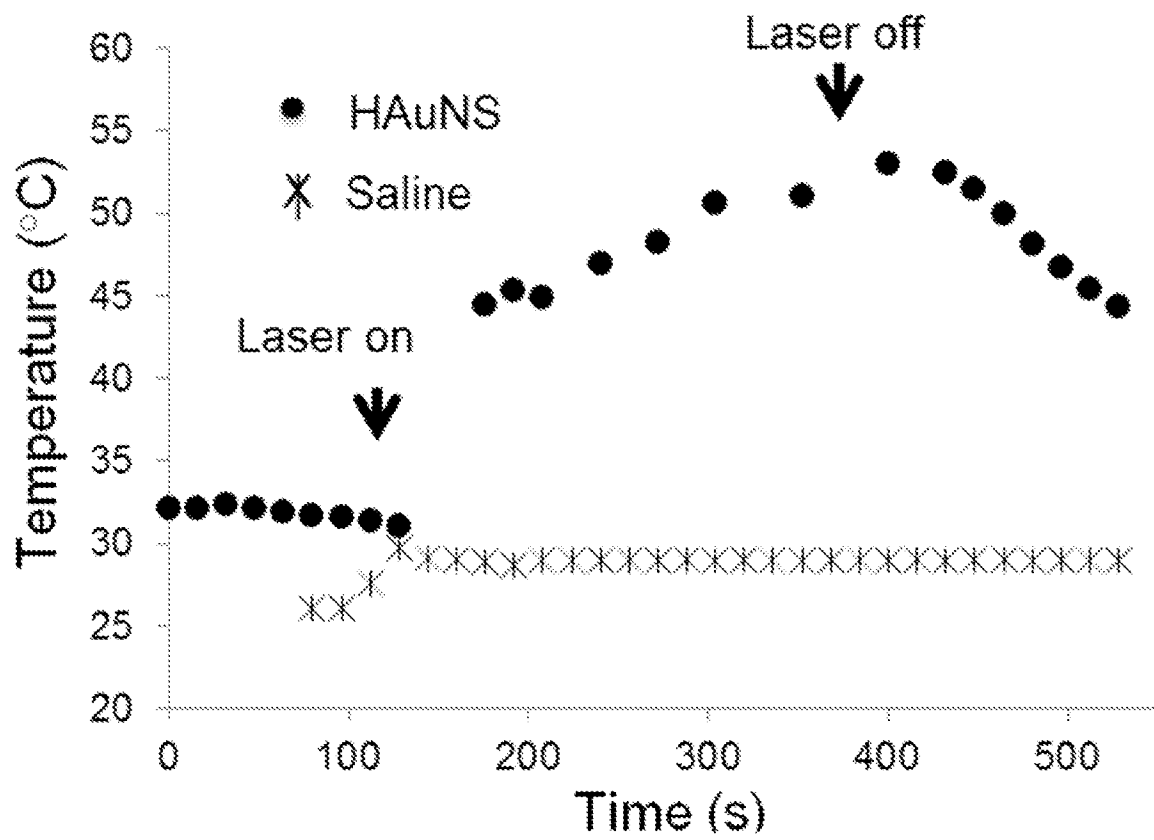


FIG. 12

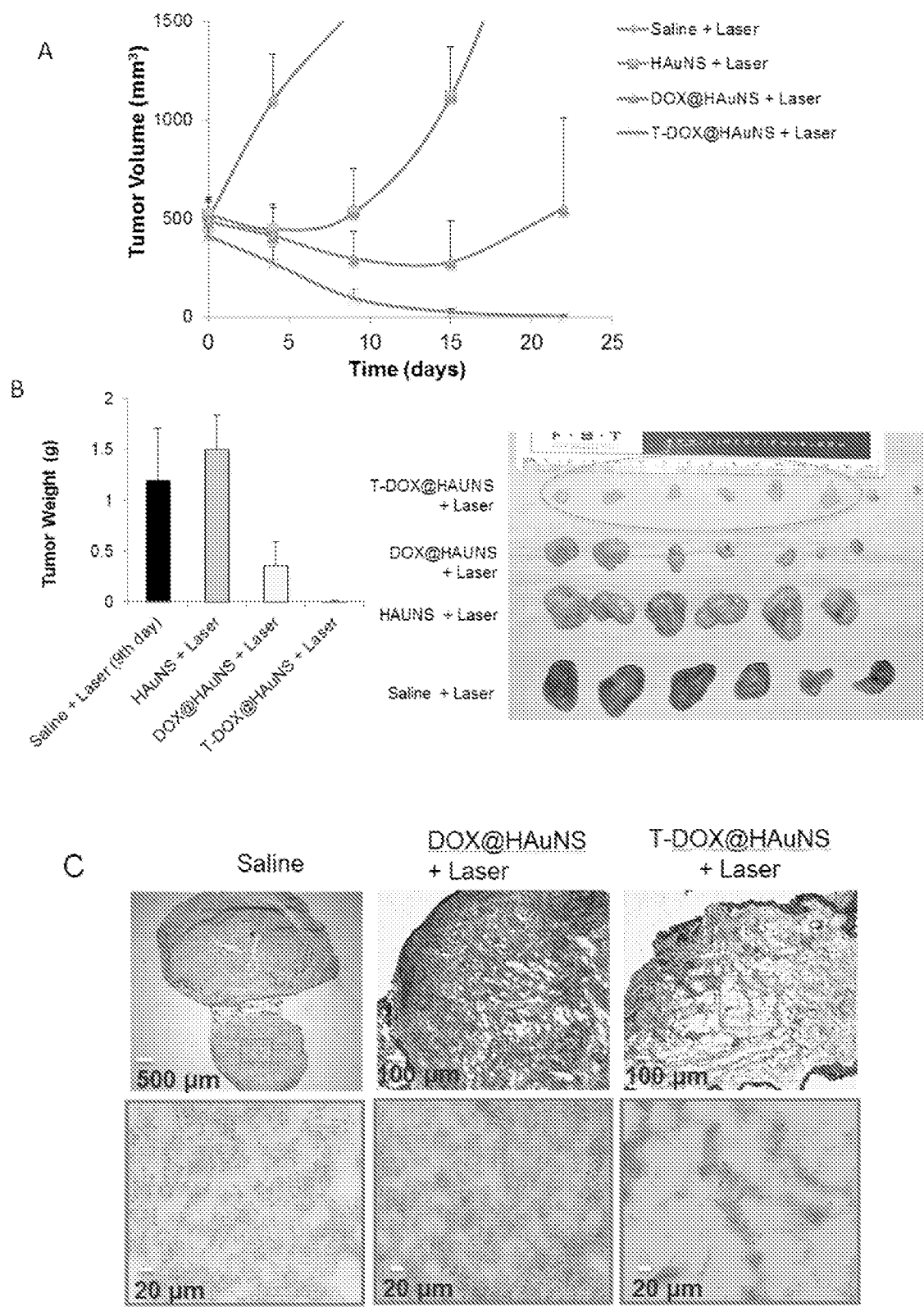


FIG. 13

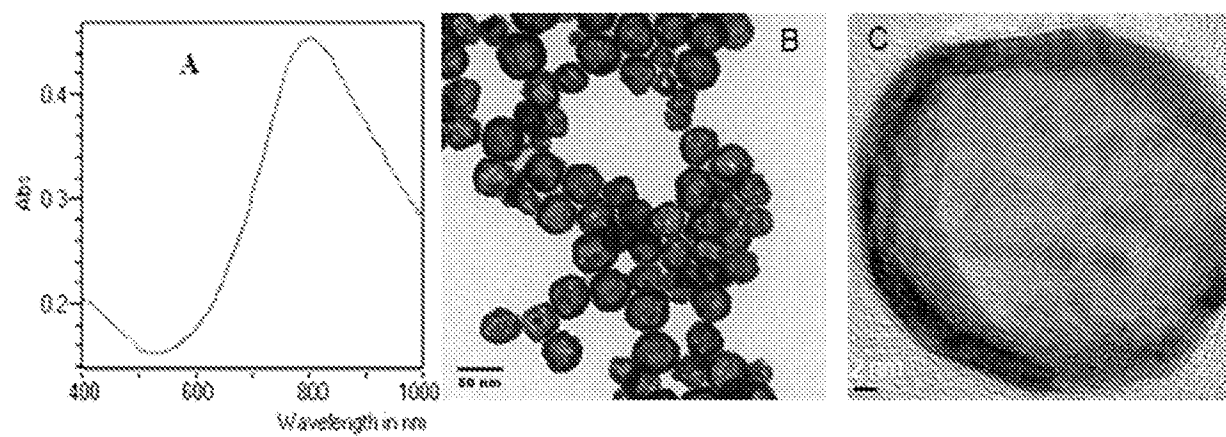
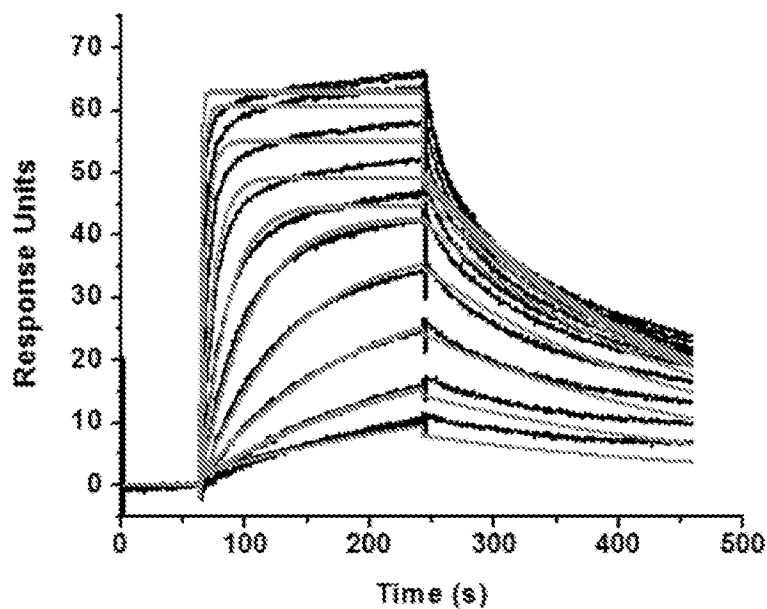


FIG. 14

A



B

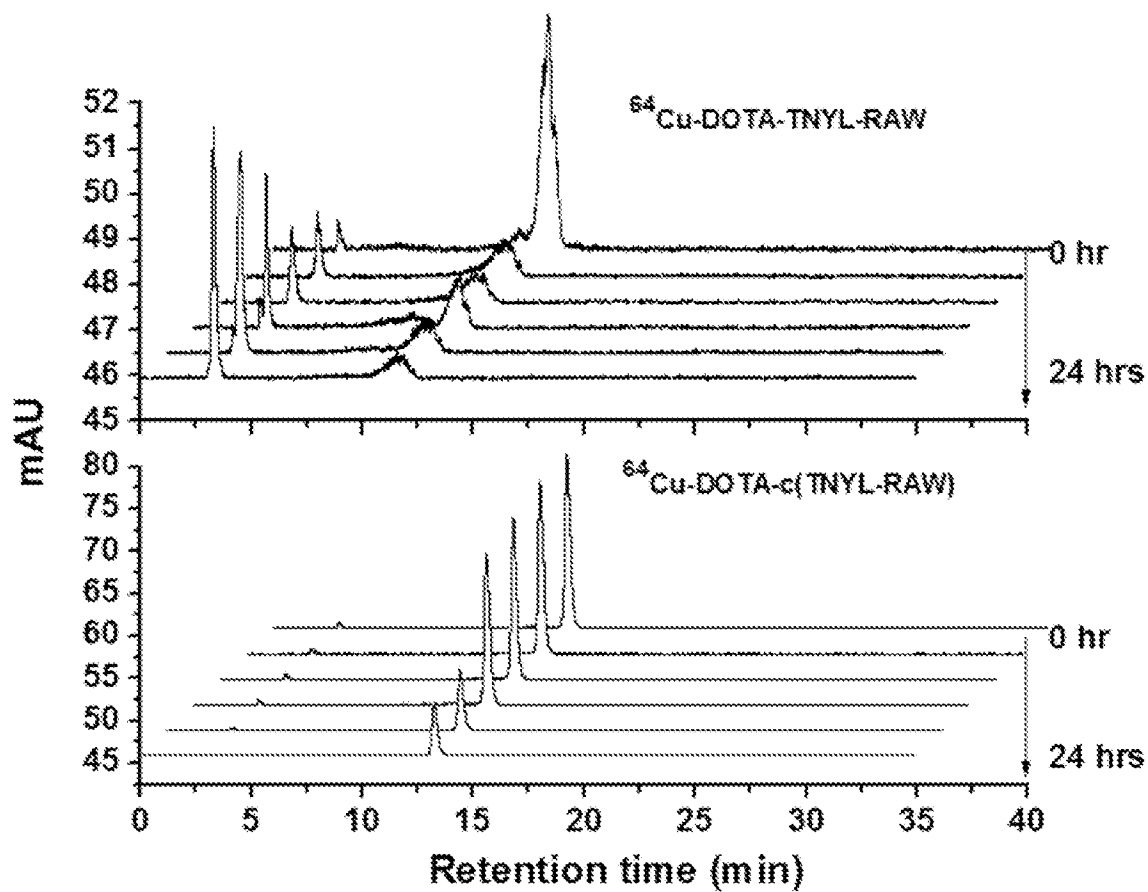


FIG. 15

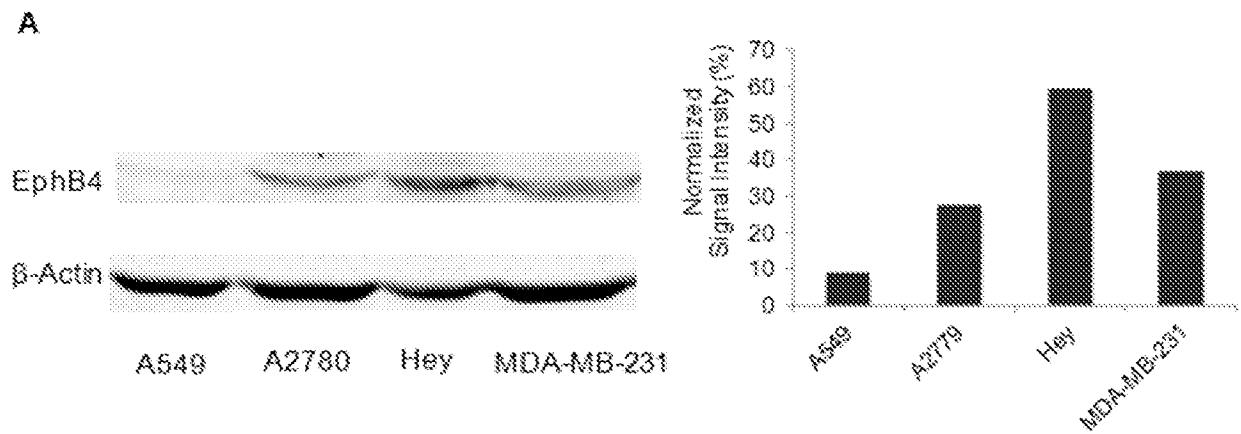


FIG. 16

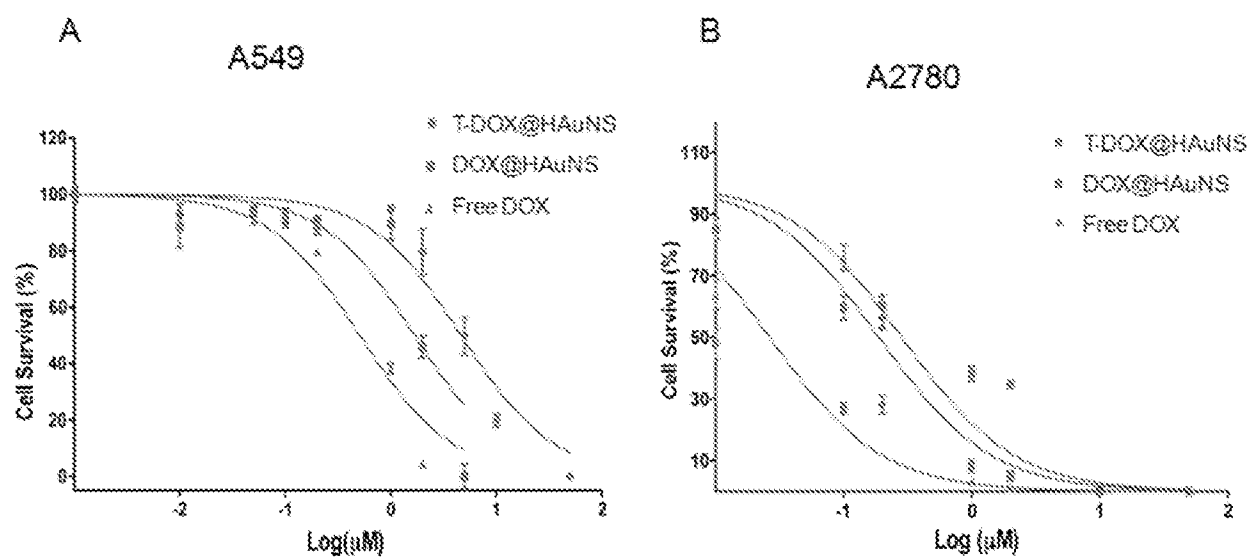


FIG. 17

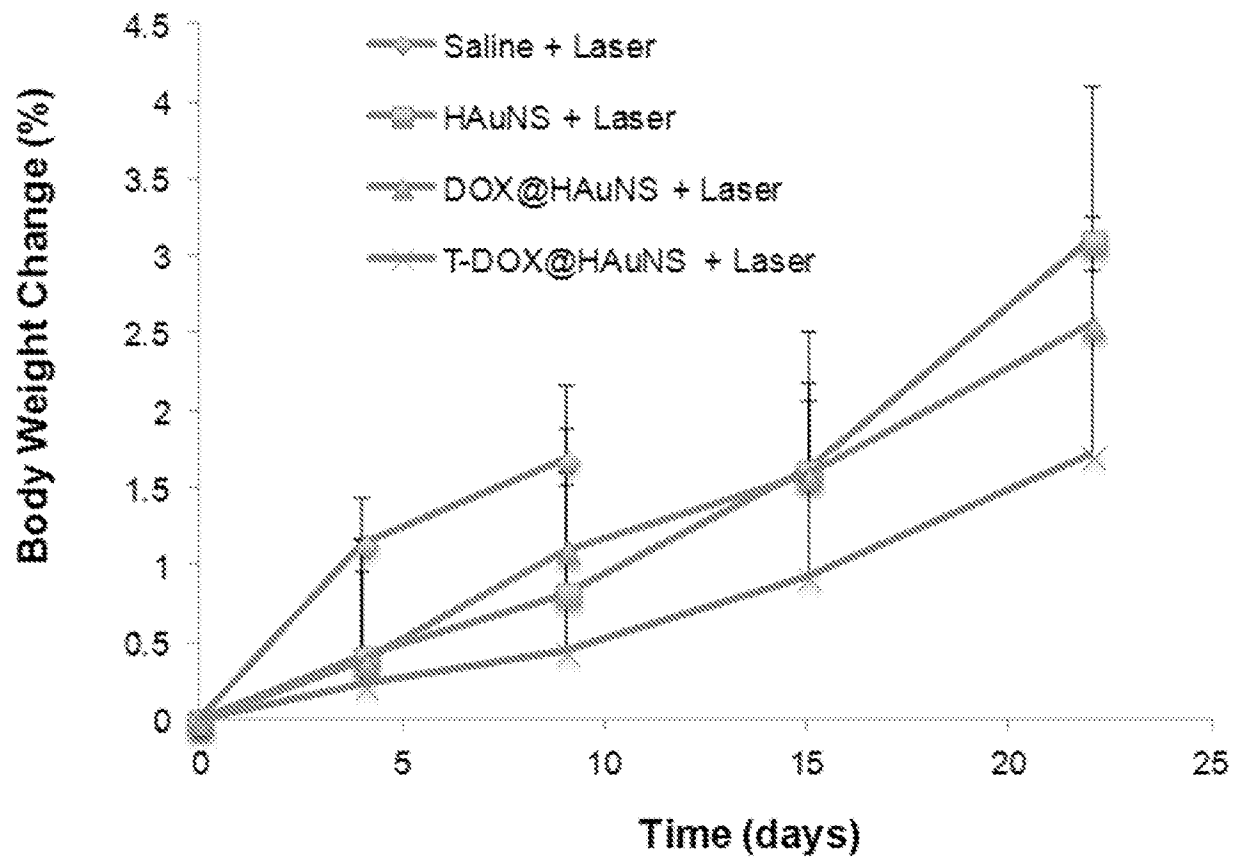


FIG. 18

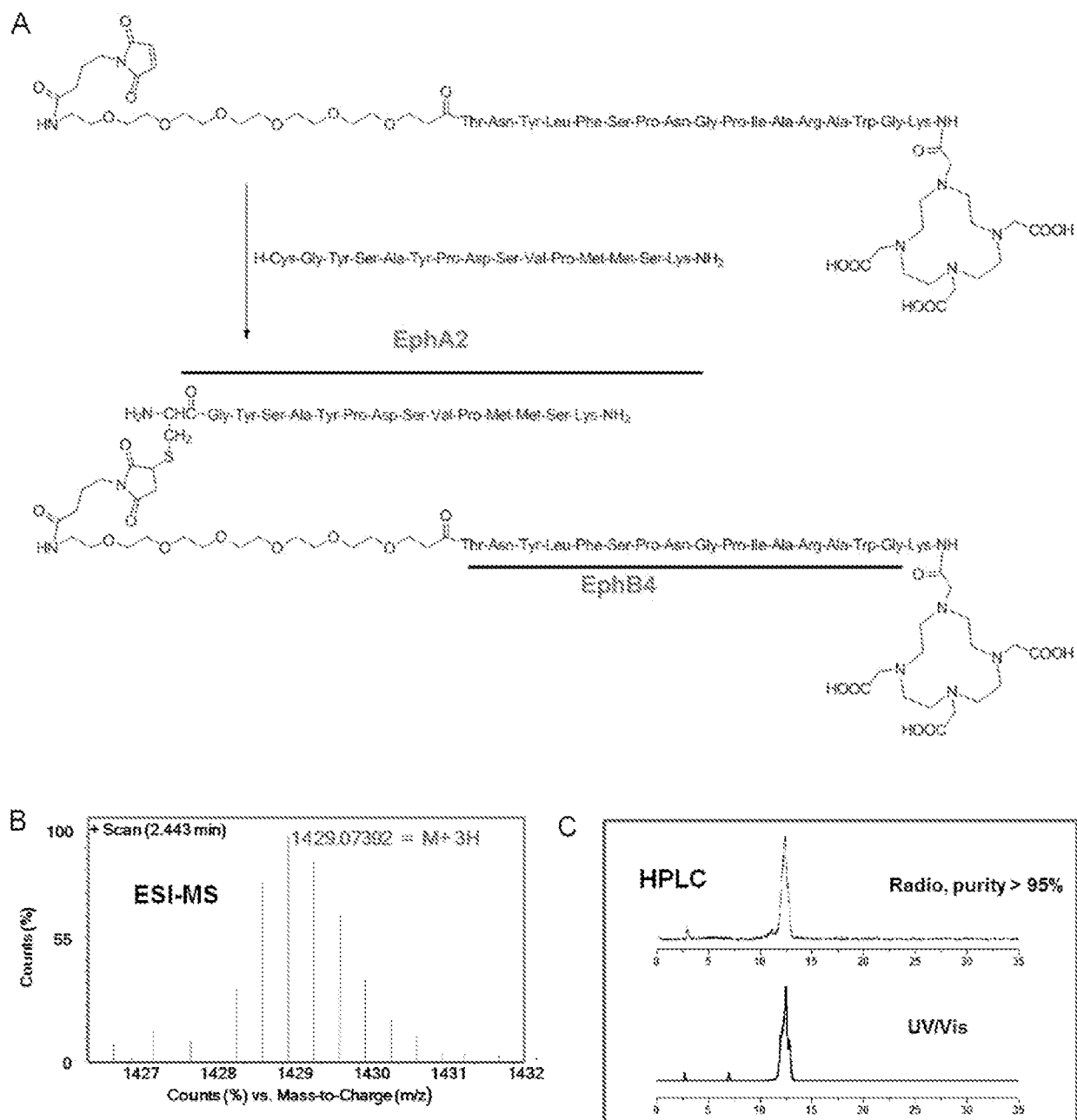


FIG. 19

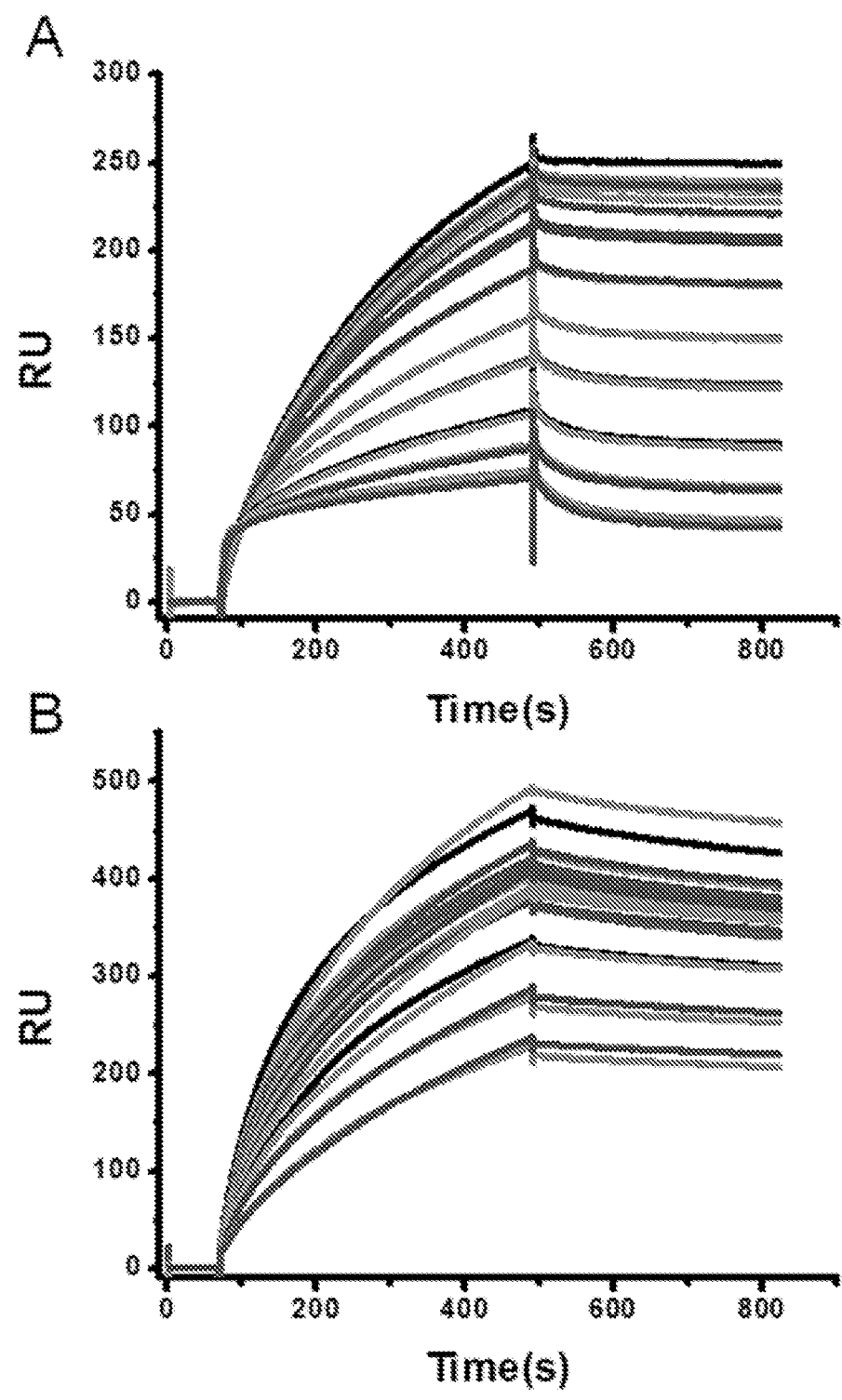


FIG. 20

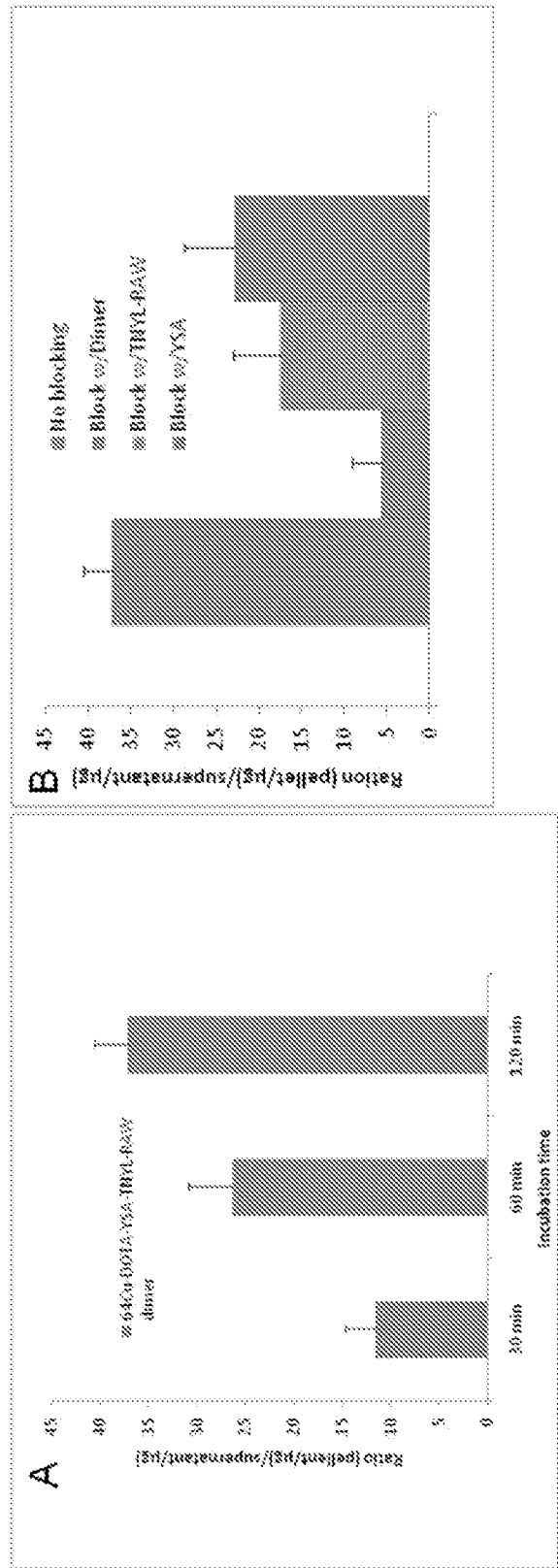


FIG. 21

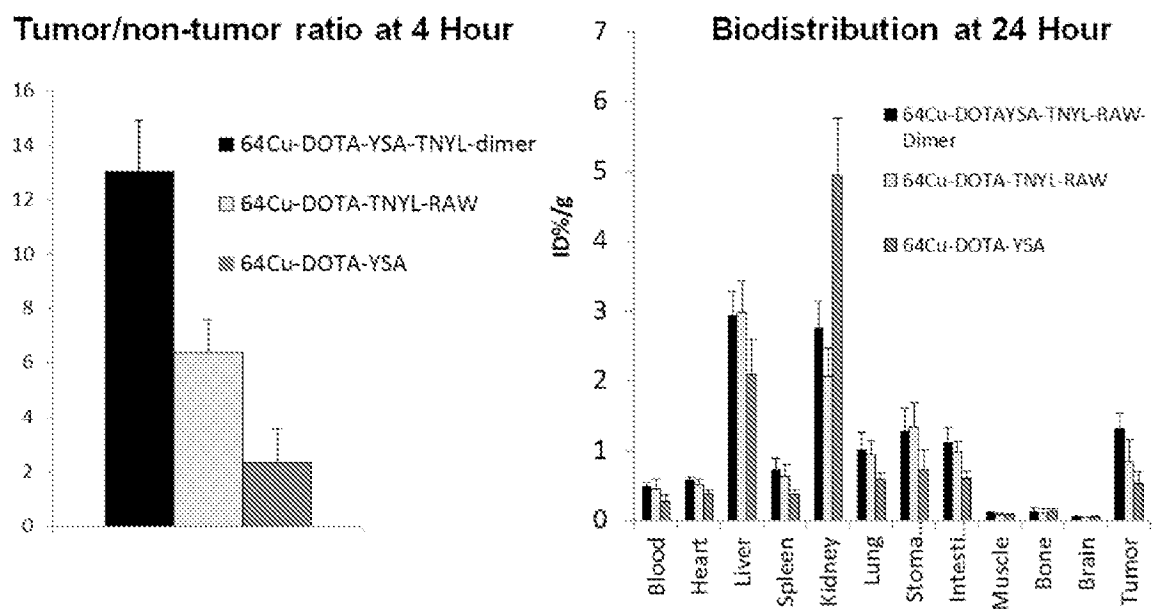


FIG. 22

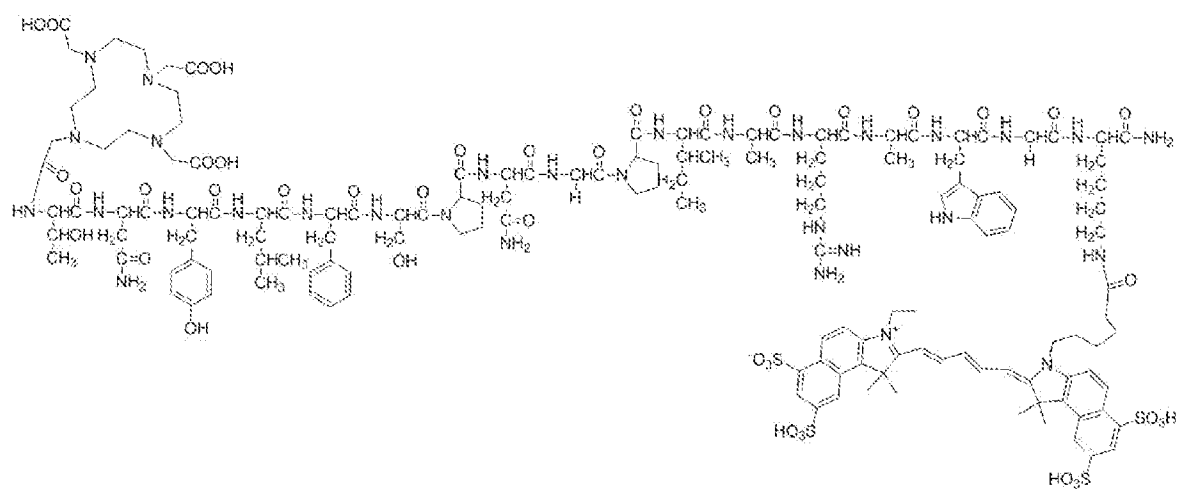


FIG. 23

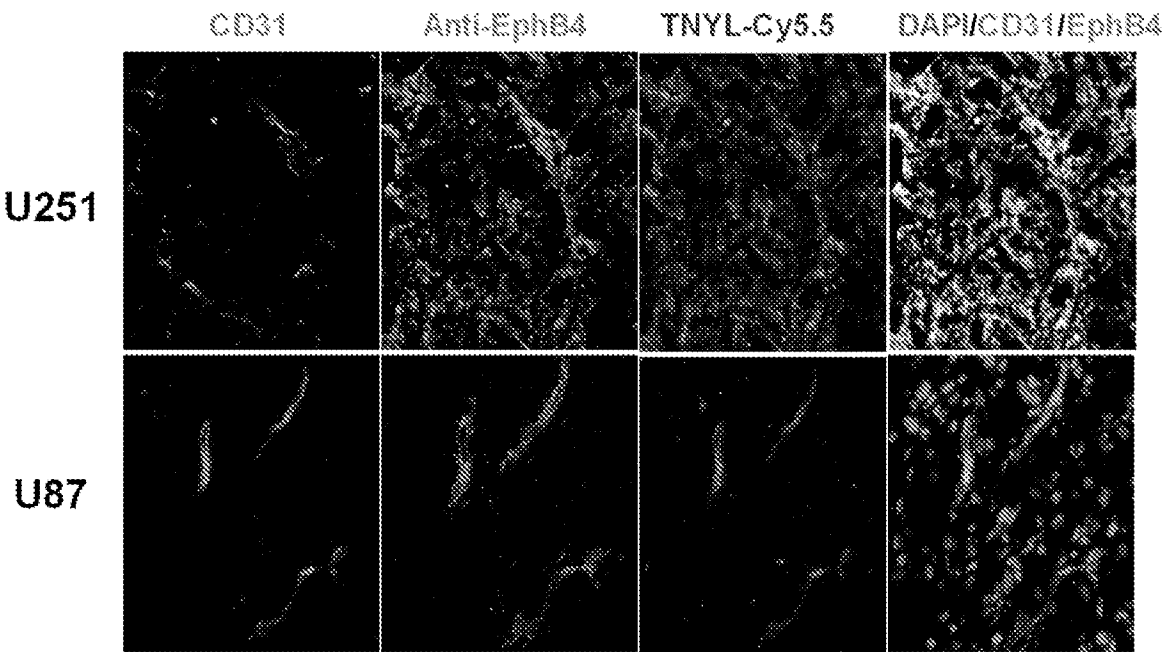


FIG. 24

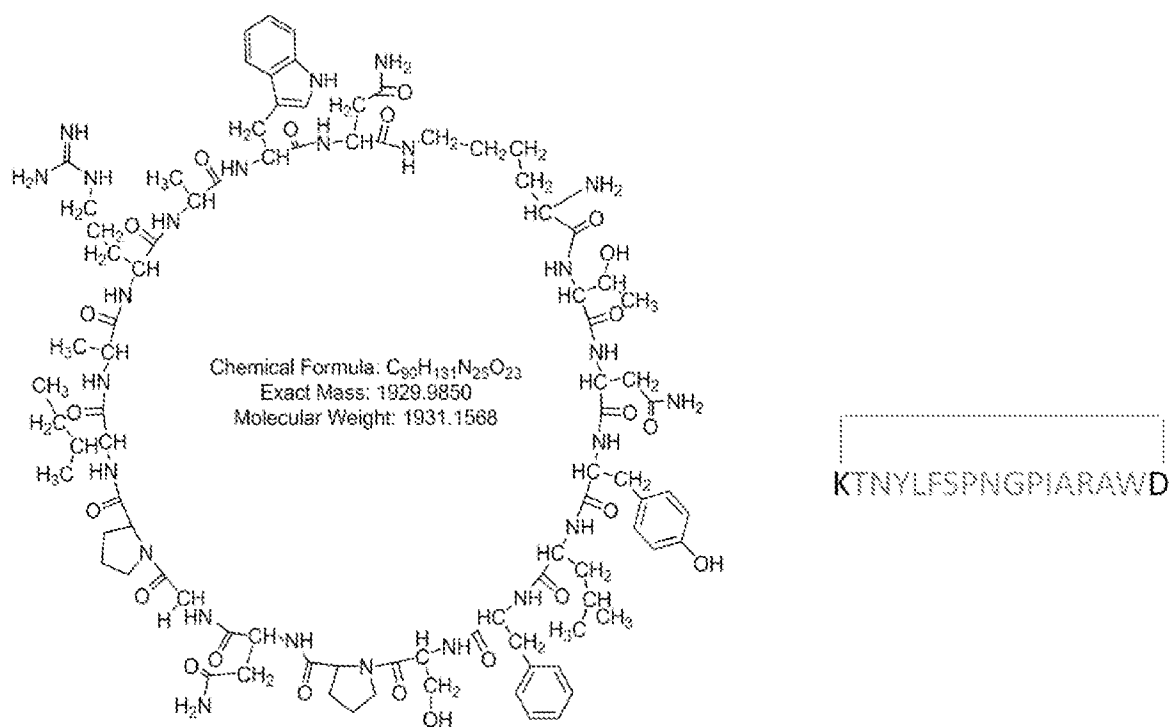


FIG. 25

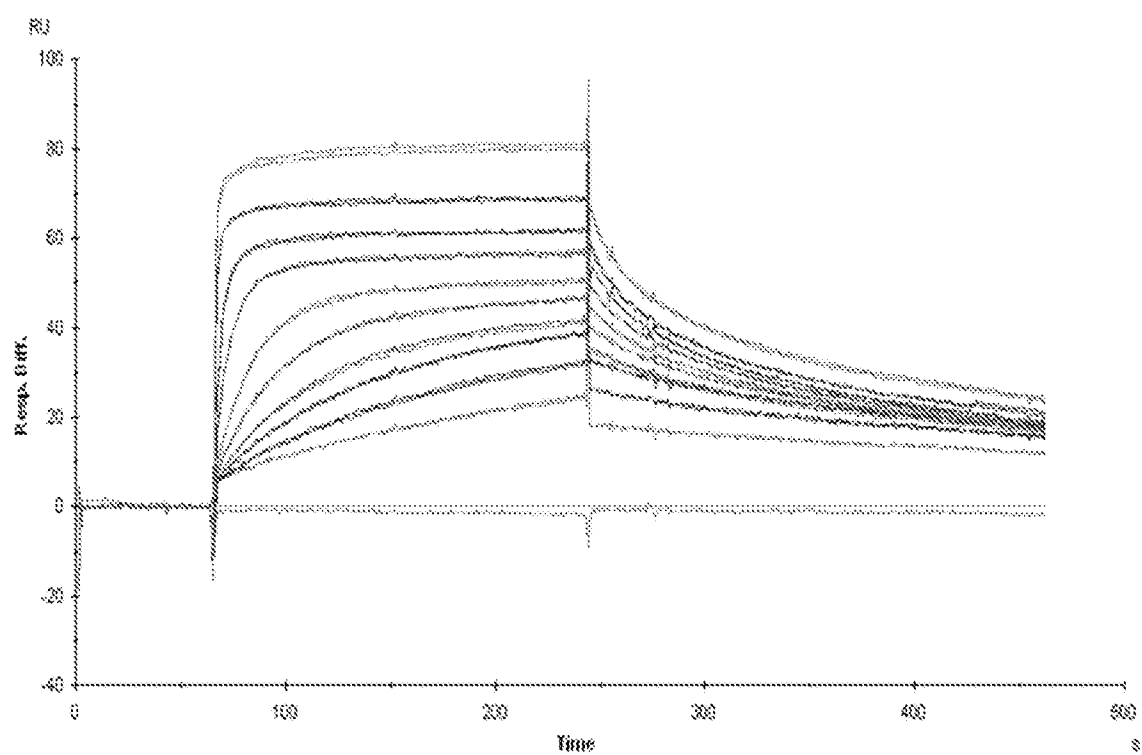


FIG. 26

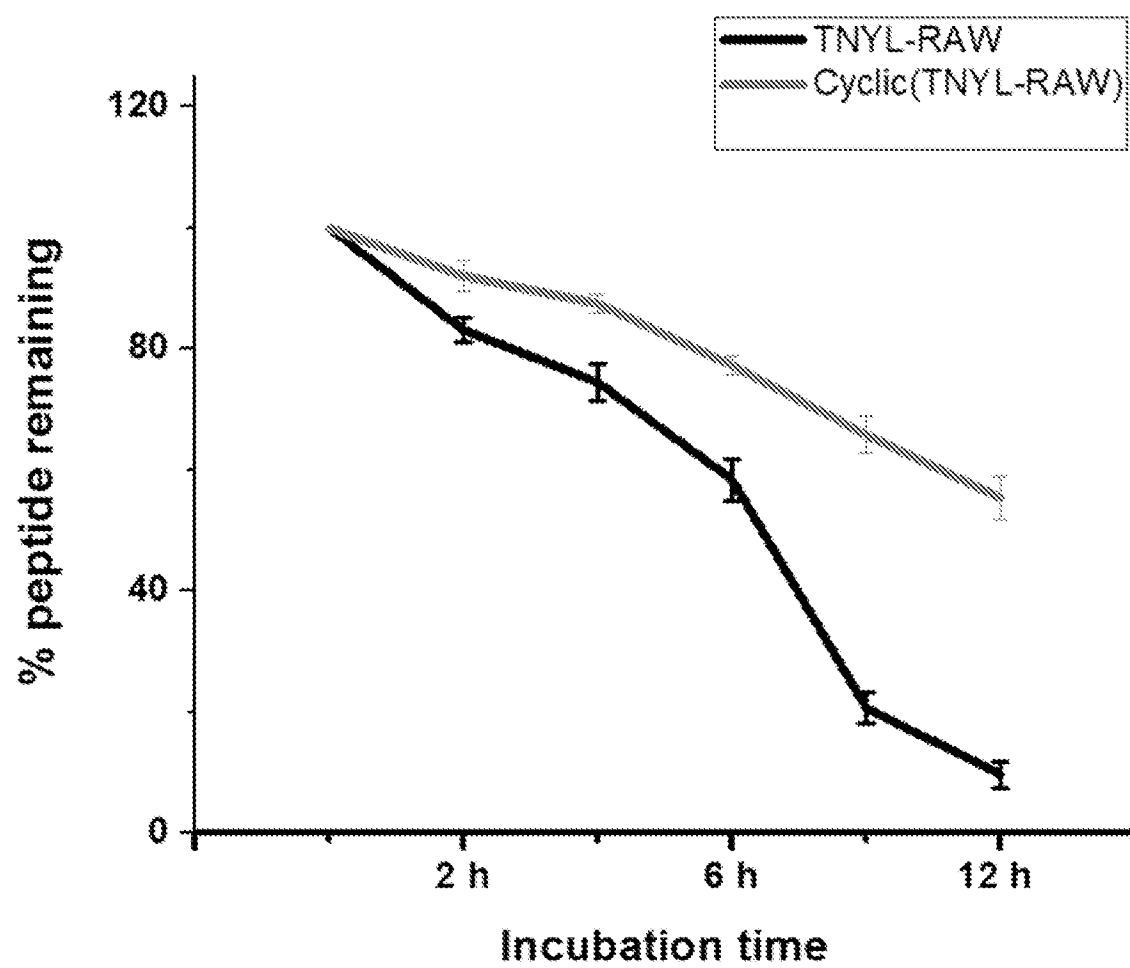
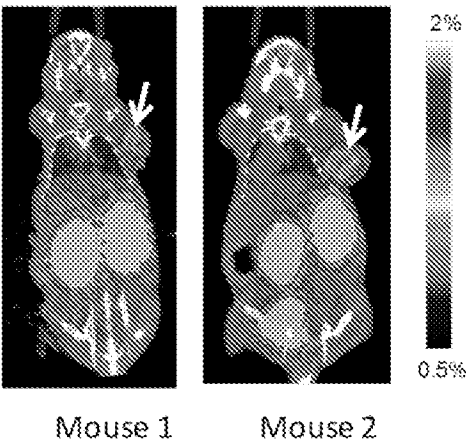


FIG. 27

A



B

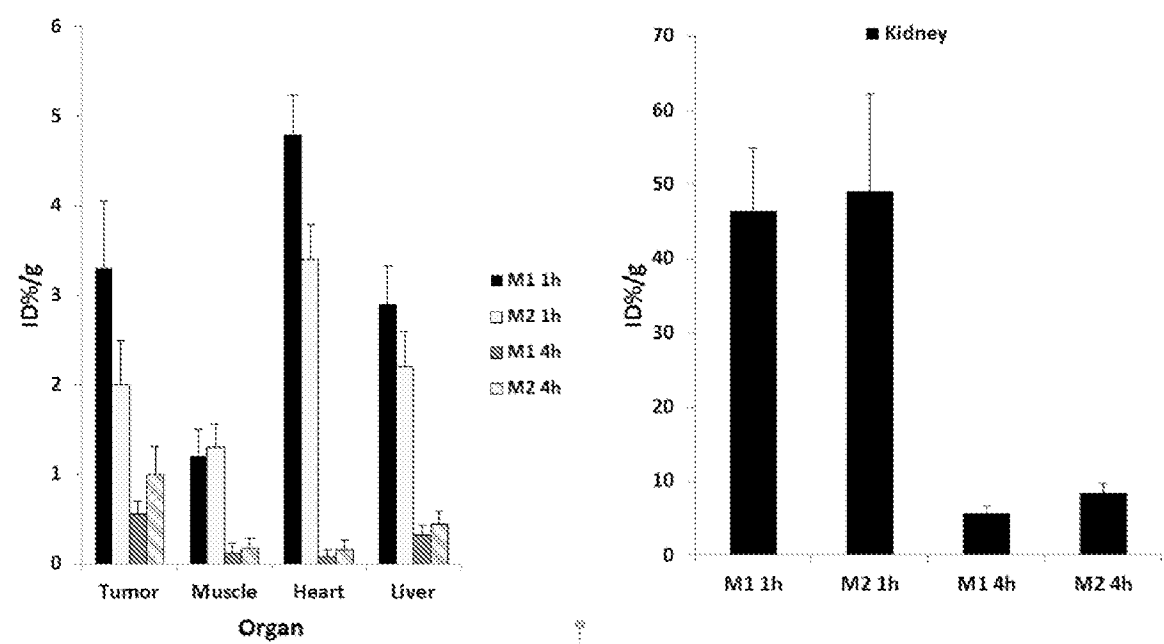


FIG. 28

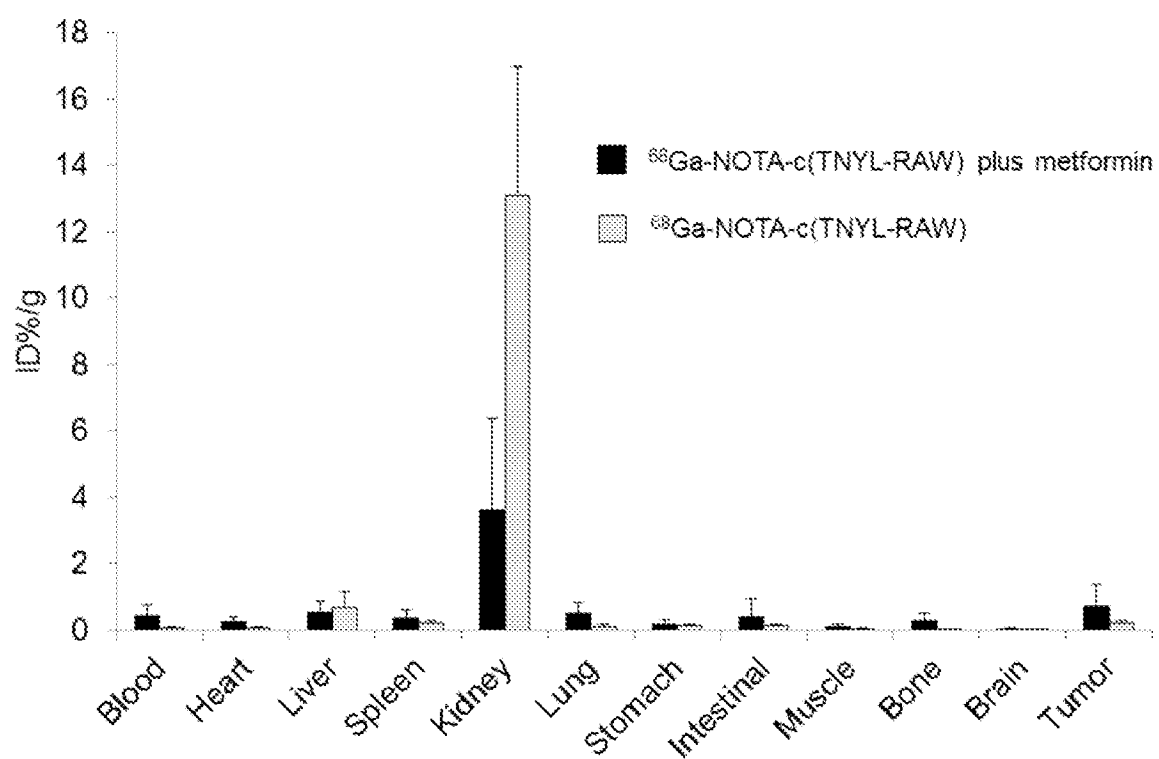


FIG. 29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/021434**A. CLASSIFICATION OF SUBJECT MATTER*****A61K 49/14(2006.01)i, A61K 31/7042(2006.01)i, A61K 47/48(2006.01)i, A61K 9/16(2006.01)i, A61P 35/00(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)

A61K 49/14; A61K 9/14; A61K 039/395; C07H 021/04; A61K 9/50; C07K 1/00; A61K 38/00; A61K 38/04; A61K 9/16; G01N 33/53; A61K 31/713

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords: ephrin receptor targeting, imaging agent, EphB4, EphA2, metformin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	US 7582438 B2 (PASQUALE, E. B. et al.) 01 September 2009 See abstract; column 10, lines 53-61, column 25, lines 7-10 and claims 1, 6-14.	1,3-13,39 2,14-25,47-49
A	WO 2008-122993 A1 (PANACEA BIOTEC LIMITED) 16 October 2008 See claims 1, 4, 5.	1-25,39,47-49
A	US 2005-0147612 A1 (YAYON, A. et al.) 07 July 2005 See abstract; paragraph [0033] and claims 1, 4.	1-25,39,47-49
A	US 2011-0091562 A1 (LYON, L. A. et al.) 21 April 2011 See abstract; paragraphs [0053]-[0055]; and claims 27-46.	1-25,39,47-49
A	US 2002-0111300 A1 (BEHR, T. M. et al.) 15 August 2002 See abstract and claims 1-4.	1-25,39,47-49

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

30 April 2013 (30.04.2013)

Date of mailing of the international search report

30 April 2013 (30.04.2013)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan
City, 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

CHOI, Sung Hee

Telephone No. 82-42-481-8740



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/021434

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. a sequence listing filed or furnished

- ☐ on paper
☒ in electronic form

b. time of filing or furnishing

- ☐ contained in the international application as filed
☒ filed together with the international application in electronic form
☐ furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/021434**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.: 26-38, 40-46
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 26-38, 40-46 pertain to a method for treatment of the human by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.
2. ☒ Claims Nos.: 38
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:

Claim 38 is not clear because it is worded in reference to claim 38, itself (PCT Article 6).
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 an additional fee, this Authority did not invite payment of any additional fee.
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.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2013/021434

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2013/021434

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