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(54) Title: MULTI-MODAL BIOPROBE FOR BLADDER CANCER IMAGING AND PHOTODYNAMIC THERAPY

(57) Abstract: Provided herein is a new generation of PDT agents based on porphyrin-lanthanide complexes with specific functional groups which can specifically localize on particular tumors, and their PDT processes can be monitored via NIR emission from erbium. In particular, provided herein is a multi-modal lanthanide-porphyrin PDT agent (Er-R₃) that are capable of killing the bladder tumor cells selectivity via ¹O₂ from porphyrin moiety and affording the fluorescence imaging simultaneously upon Er-R₃ binding with the integrin $\alpha_v\beta_3$ isoform in bladder cancer cells.



WO 2018/064961 A1

MULTI-MODAL BIOPROBE FOR BLADDER CANCER IMAGING AND PHOTODYNAMIC THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 The present application claims benefit from U.S. Patent Application No. 15/352,561, filed on November 15, 2016 and U.S. Provisional Application No. 62/404,222 filed on October 5, 2016, both of which are hereby incorporated by reference in their entirety.

TECHNICAL FIELD

- 10 [0001] The present invention relates to a new generation of photodynamic therapy agents based on porphyrin-lanthanide complexes, and the photodynamic therapy processes that can be monitored via NIR emission from erbium. In particular, the present invention provides a multi-modal lanthanide-porphyrin photodynamic therapy agent that are capable of killing bladder tumor cells.

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BACKGROUND

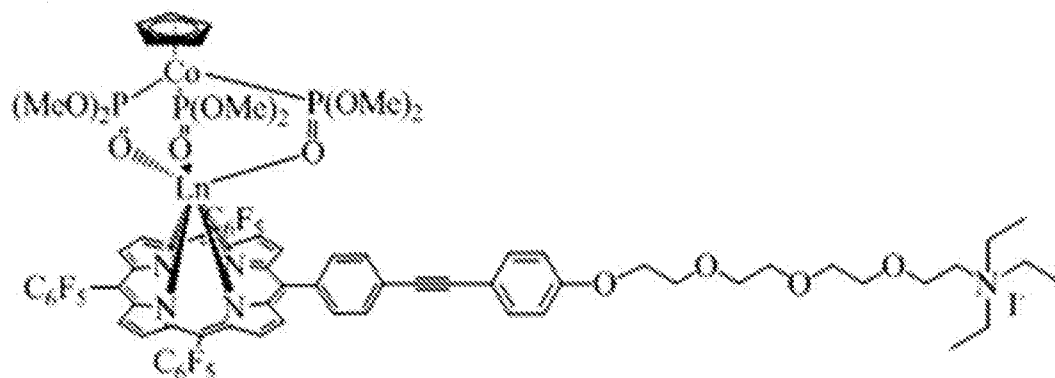
- [0002] Photodynamic Therapy (PDT) is emerging as a novel cancer treatment modality to help patients to live longer and to improve their quality of life without causing long-term side-effects. PDT is still failing to obtain deserved popularity in society due to current technological
- 20 constraints and absence of practical advances as it is only offered in some treatment center and studied with little clinical trials. Recently, a renaissance of PDT has commenced with its wide recognition as a valuable treatment option for localized cancers (i.e. non-metastatic cancers), as well as pre-cancers of the skin and in the mouth, after three PDT photosensitizing agents have been approved by the United States Food and Drug Administration (FDA), e.g. Aminolevulinic
- 25 acid (ALA). Nevertheless, conventional PDT still has from several limitations and drawbacks: (i) it is only able to treat diseased areas where light can be reached, that is, on or just under the skin; (ii) currently-used PDT drugs could leave people very sensitive to light, and therefore special precautions must be taken after the drugs are put in or on the body; (iii) adverse in-vitro/in-vivo reactions occur due to the variation in physiological conditions and notched distribution of
- 30 cytotoxic singlet oxygen; and (iv) non-specific therapeutic nature may jeopardize normal cells during PDT treatment.

[0003] In this regard, porphyrin-based moieties, another novel class of promising PDT agents, have been investigated extensively by scientists worldwide to develop photodynamic therapy can be available and effective to other types of cancers and diseases particularly in the skin, bladder, mouth, and brain. As far as the light penetration depth for the singlet oxygen ($^1\text{O}_2$) generation is concerned, several porphyrin moieties have successfully showed the possibility to achieve near-infrared (NIR) excitation (via multi-photon/Second harmonic generation). NIR photons can penetrate deep and emit expeditiously from tissues without causing cell damage with their strong two-photon absorption properties being at $\sim 860\text{nm}$. In the literature and referenced patent filing, a porphyrinato metal complex is disclosed which can serve as an in-vivo anti-cancer torpedo equipped with visible-to-NIR emission for imaging and discriminating radar for tumor cell selectivity, and $^1\text{O}_2$ explosive ammunition. However, the cancer selectivity of these PDT agents are still not yet solved and there exists a need to provide PDT agents with better cancer selectivity.

[0004] It is an objective of the present invention to provide for PDT probes that can specifically localize on particular tumors, and their PDT processes can be monitored via NIR emission.

SUMMARY OF THE INVENTION

[0005] In a first aspect of the present invention there is provided a composition for photodynamic therapy and imaging of cancer cells comprising gadolinium porphyrinate complex (**Gd-N**) of Formula (I):



Formula (I)

wherein Ln = **Gd**, or a pharmaceutically acceptable salt thereof.

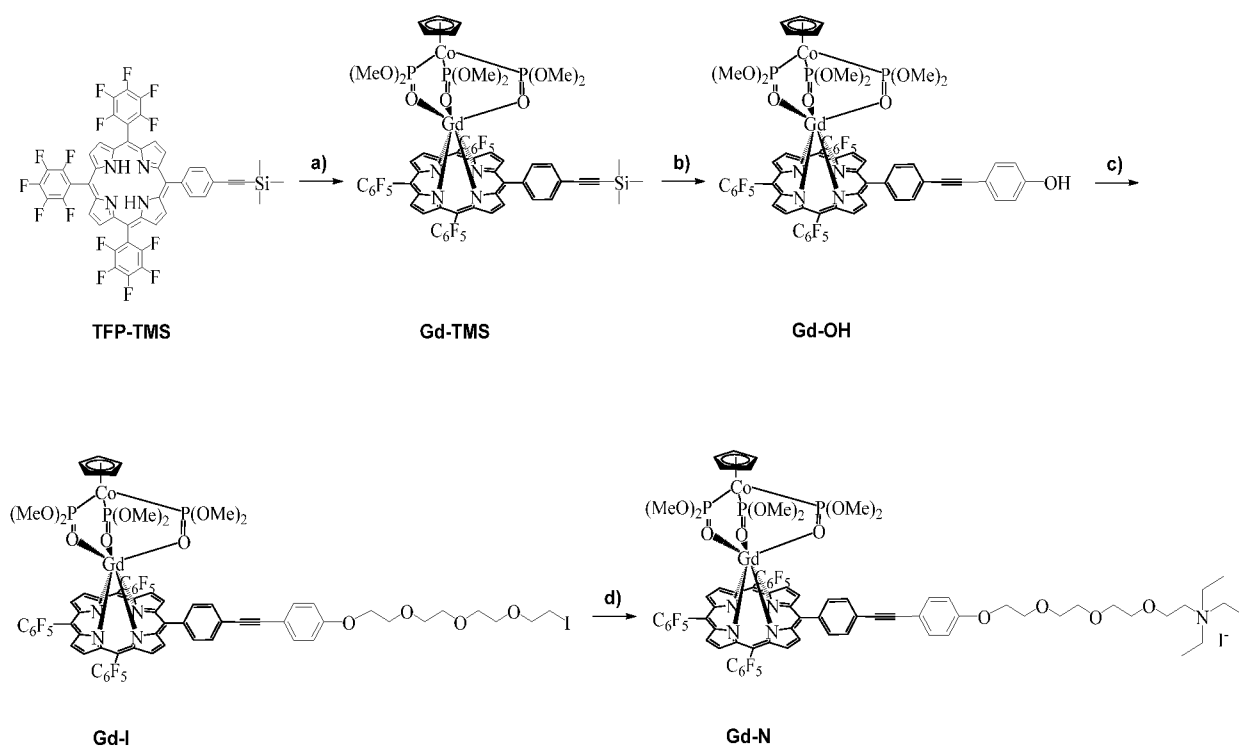
[0006] In a first embodiment of the first aspect of the present invention there is provided a composition for photodynamic therapy and imaging of cancer cells wherein the cancer cells have anionic cell membranes.

5 [0007] In a second aspect of the present invention there is provided a method of photodynamic therapy and imaging of cancer cells comprising administering to a subject in need thereof the composition according to the first aspect of the present invention and irradiating a radiation source to the cancer cells in the subject in need thereof.

10 [0008] In a first embodiment of the second aspect of the present invention, the administration of said composition is performed intravenously or by injection to site of said cancer cells.

15 [0009] In a second embodiment of the second aspect of the present invention there is provided a method of photodynamic therapy and imaging of cancer cells comprising administering to a subject in need thereof a composition according to the first aspect of the present invention and irradiating the cancer cells in the subject in need thereof with a radiation source, wherein said radiation source is a light source of about 860 nm in wavelength.

20 [0010] In a third aspect of the present invention there is provided a method of synthesizing the compound of Formula (I) comprising steps according to scheme 1:



a) (i) $\text{Gd}[\text{N}(\text{SiMe}_3)_2]_3 \cdot [\text{LiCl}(\text{THF})_3]_x$, toluene, reflux, 12h; (ii) $\text{Na}\{(\eta^5\text{-C}_5\text{H}_5)\text{Co}[\text{P}(=\text{O})(\text{OMe})_2]_3\}$, toluene, rt, 1h; b) (i) TBAF (THF, 1M), CH_2Cl_2 , rt, 30min; (ii) a) 4-iodophenol, $\text{Pd}(\text{PPh}_3)_4$, CuI, THF, NEt_3 , 40°C , 12h; c) Tetraethyleneglycol diiodide, DMF, K_2CO_3 , 80°C , 8h; d) Triethylamine, DMF, 85°C , 24h.

Scheme 1

wherein

Step a): Removing solvent from a solution of $\text{Gd}[\text{N}(\text{SiMe}_3)_2]_3 \cdot [\text{Li}(\text{THF})_3\text{Cl}]_x$ to form a precipitation of LiCl; adding dichloromethane (CH_2Cl_2) to the precipitation of LiCl to form a first mixture wherein the first mixture is centrifuged to separate a clear layer from said first mixture; transferring the clear layer to a porphyrin free base trifluoropropyl-trimethoxysilane(TFP-TMS) dissolved in toluene solution to form a second mixture; refluxing the second mixture until the porphyrin free base is coordinated with a metal ion to form a refluxed second mixture; cooling the refluxed second mixture to room temperature to form a cooled refluxed second mixture; adding dry $\text{Na}\{(\eta^5\text{-C}_5\text{H}_5)\text{Co}[\text{P}(=\text{O})(\text{OMe})_2]_3\}$ to the cooled refluxed second mixture to form a third mixture; stirring the third mixture; removing solvent from the third mixture to form a first residue; dissolving the first residue in CH_2Cl_2 to form a fourth mixture; filtering and column chromatographing the fourth mixture using CH_2Cl_2 /Hexane as eluent to produce **Gd-TMS**;

Step b): Adding Tetrabutylammonium fluoride to a solution of the **Gd-TMS** in CH_2Cl_2 , and stirring the **Gd-TMS** solution to create a chemical reaction; after completion of the chemical reaction, the solution is passed through column chromatography to form a fifth mixture; removing solvent from the fifth mixture to obtain an intermediate; dissolving the intermediate

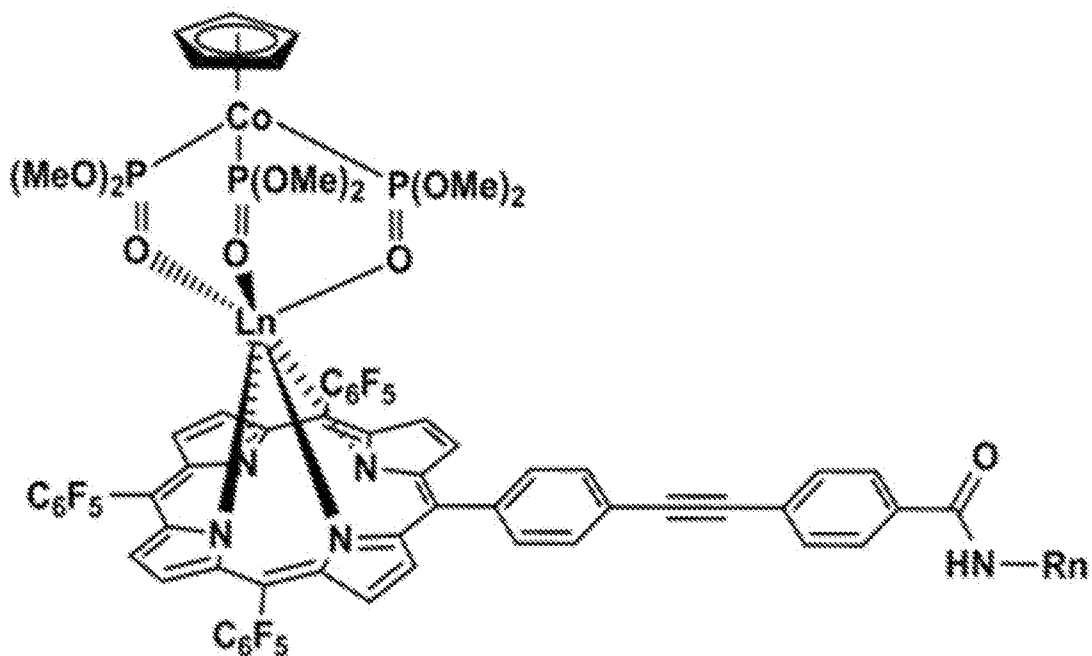
and 4-iodophenol in dry tetrahydrofuran and triethylamine to form a sixth mixture; mixing the sixth mixture with nitrogen to form a nitrogenized sixth mixture; adding $\text{Pd(PPh}_3)_4$ and CuI to said nitrogenized sixth mixture to form a seventh mixture; stirring the seventh mixture at least 35°C for at least 10 hours under a nitrogen atmosphere to produce a stirred seventh mixture; removing solvent from the stirred seventh mixture to produce a second residue; purifying the second residue using column chromatography with $\text{CH}_2\text{Cl}_2/\text{Methanol}$ as eluent to produce **Gd-OH**;

Step c): Adding anhydrous K_2CO_3 to a solution of **Gd-OH** and tetraethyleneglycol diiodide in dry N,N-Dimethylmethanamide to form an eighth mixture; heating said eighth mixture to at least 80°C for at least 8 hours under a nitrogen atmosphere to form a heated eighth mixture; removing solvent from the heated eighth mixture to form a first crude product; purifying the first crude product using column chromatography eluted by $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ to produce **Gd-I**, and

Step d): Adding anhydrous Net_3 to a solution of **Gd-I** in dry DMF to form a ninth mixture; heating the ninth mixture to at least 85°C for at least 24 hours under nitrogen atmosphere to form a heated ninth mixture; removing the solvent from the heated ninth mixture to obtain a second crude product; purifying the second crude product using column chromatography with $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ as the eluent to remove unreacted **Gd-I** and other impurities, and further purifying with $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ as the eluent to obtain **Gd-N**.

[0011] In a fourth aspect of the present invention there is provided a multi-modal lanthanide-porphyrin PDT agent (**Er-R₃**) that are capable of killing bladder tumor cells selectivity via $^1\text{O}_2$ from porphyrin moiety and affording fluorescence imaging simultaneously upon **Er-R₃** binding with integrin $\alpha_v\beta_3$ isoform in bladder cancer cells.

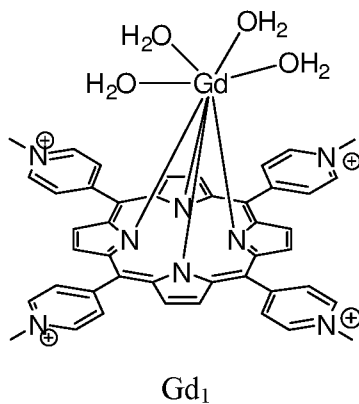
[0012] In a fifth aspect of the present invention there is provided a composition for photodynamic therapy and imaging of cancer cells comprising Erbium porphyrin based complexes or Ytterbium porphyrin based complexes or Gadolinium porphyrin based complexes represented by the molecular formula:

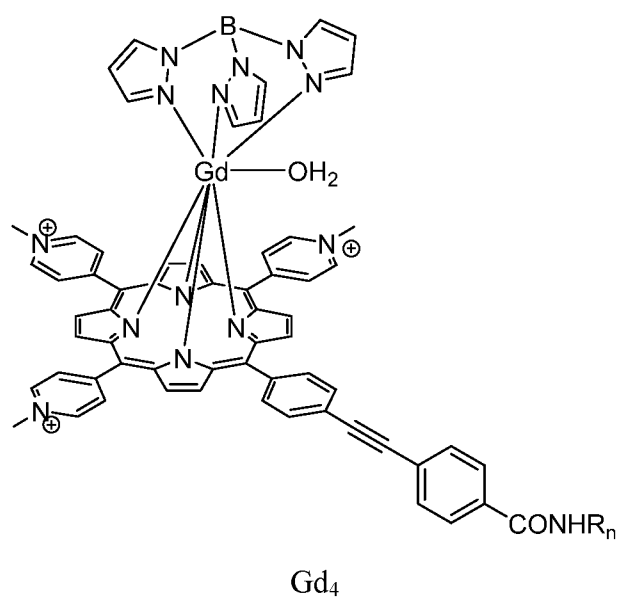
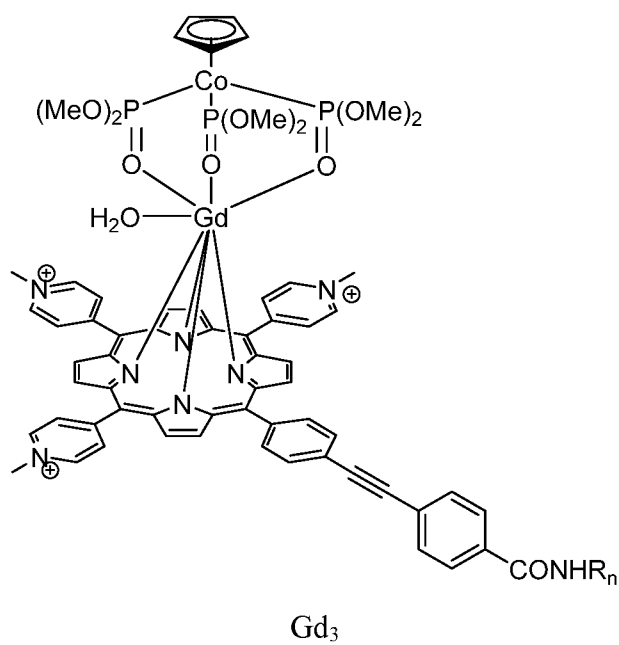
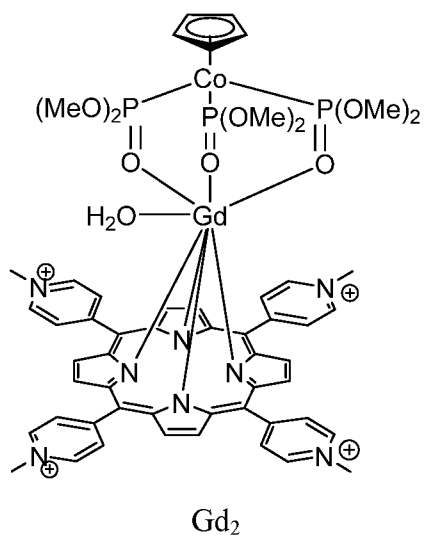


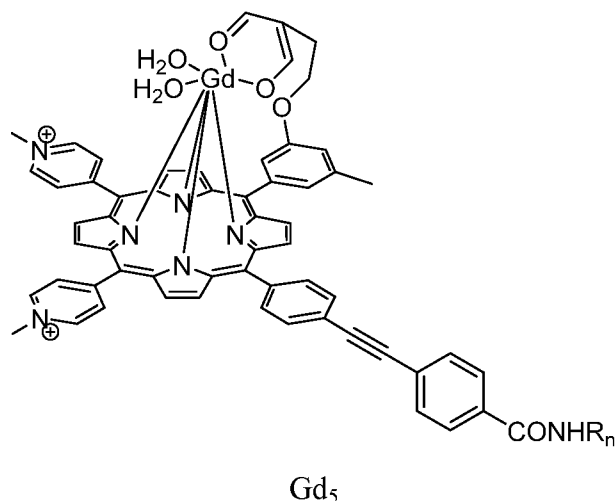
wherein Ln is Er, Yb, or Gd; and

R_n is a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 1; SEQ ID NO: 2; and SEQ ID NO: 3; or

- 5 a water-soluble porphyrin-based Gadolinium complex represented by a molecular formula selected from the group consisting of Gd₁, Gd₂, Gd₃, Gd₄, and Gd₅:

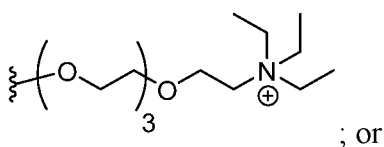






wherein

R_n is



a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 1; SEQ ID NO: 2; and SEQ ID NO: 3

or a pharmaceutically acceptable salt thereof.

[0013] In a first embodiment of the fifth aspect of the present invention there is provided a composition wherein the Erbium porphyrin based complexes are conjugated with integrin $\alpha_v\beta_3$ isoform-specific peptides.

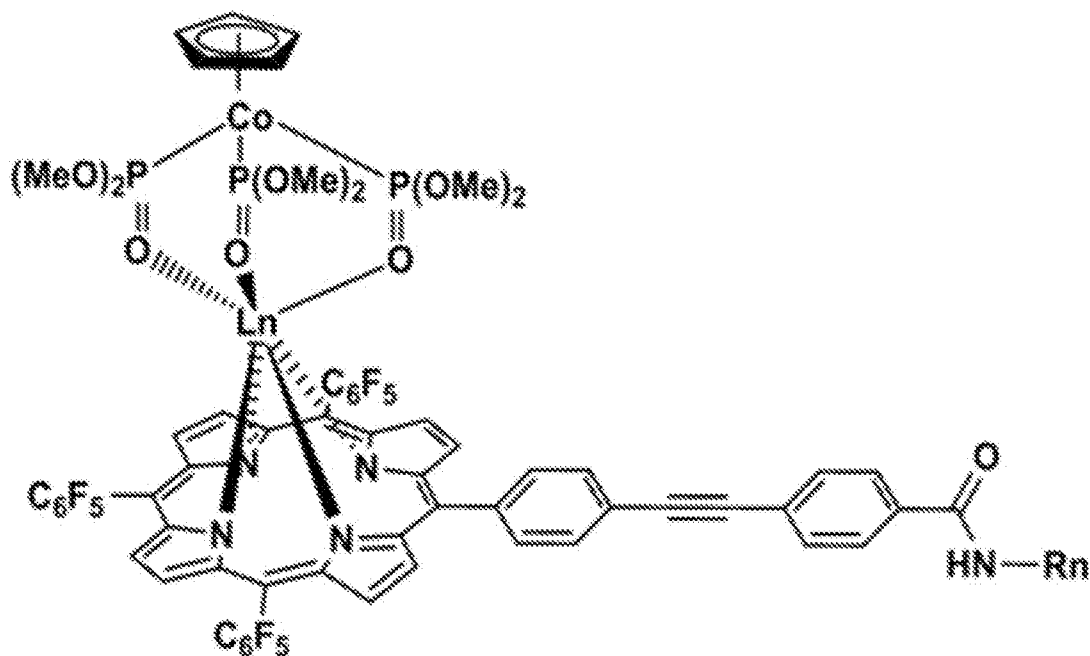
[0014] In a second embodiment of the fifth aspect of the present invention there is provided a composition wherein the Erbium porphyrin based complexes are conjugated with peptide RrRk (SEQ ID NO: 4).

[0015] In a third embodiment of the fifth aspect of the present invention there is provided a composition wherein the Erbium porphyrin based complexes are conjugated with integrin $\alpha_v\beta_3$ isoform specific peptide sequence (-cGRLKEKKc-) (SEQ ID NO: 5).

[0016] In a fourth embodiment of the fifth aspect of the present invention there is provided a composition wherein the Erbium porphyrin based complexes are conjugated with both

peptide RrRk (SEQ ID NO: 4) and integrin $\alpha_v\beta_3$ isoform specific peptide sequence (-cGRLKEKKc-) (SEQ ID NO: 5).

[0017] In a fifth embodiment of the fifth aspect of the present invention there is provided a composition comprising the Erbium porphyrin based complex represented by the molecular formula:



wherein Ln is Er and R_n is a polypeptide having an amino acid sequence of SEQ ID NO: 3.

[0018] In a sixth embodiment of the fifth aspect of the present invention there is provided a composition wherein the cancer cells comprising of bladder cancer cells, cervical cancer cells and lung cancer.

[0019] In a sixth aspect of the present invention there is provided a method of photodynamic therapy and imaging of cancer cells comprising administering to a subject in need thereof the composition and irradiating the cancer cells in the subject in need thereof with a radiation source.

[0020] In a first embodiment of the sixth aspect of the present invention there is provided a method wherein the administration of said composition is performed intravenously or by injection to site of said cancer cells.

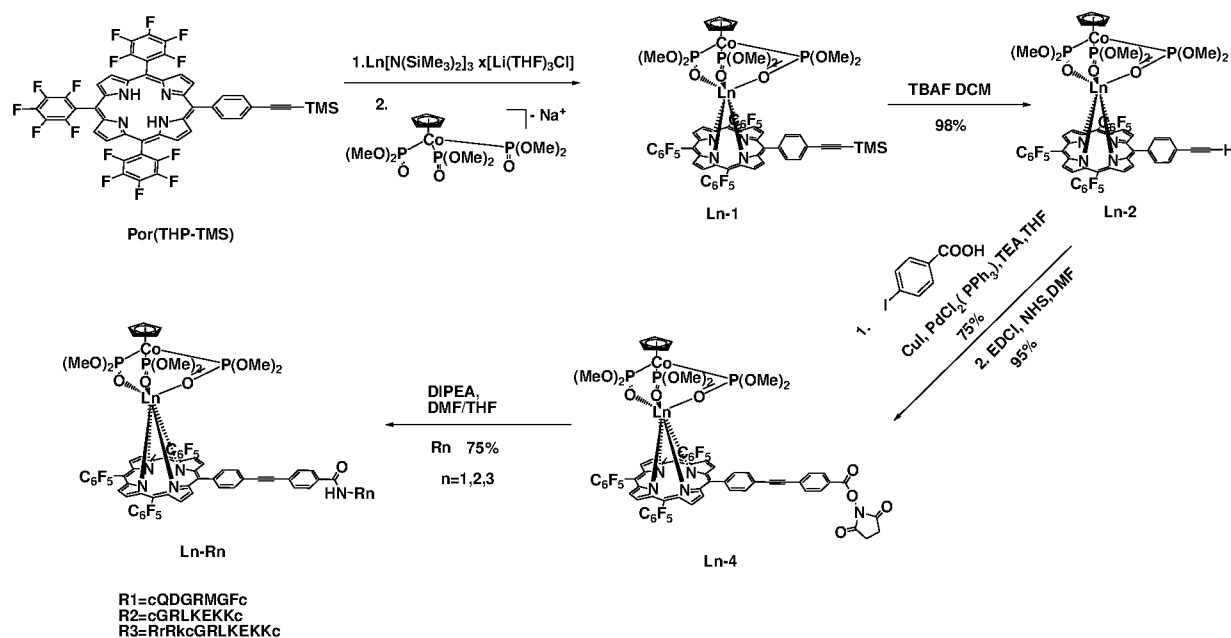
5 [0021] In a second embodiment of the sixth aspect of the present invention there is provided a method wherein said radiation source is a light source with a wavelength in the Q band of porphyrin.

[0022] In a third embodiment of the sixth aspect of the present invention there is provided a method wherein said radiation source is a light source with a wavelength beyond 550 nm or
10 is at 860nm.

[0023] In a fourth embodiment of the sixth aspect of the present invention there is provided a method wherein the imaging is performed using fluorescent imaging, NIR imaging or MRI imaging

15 [0024] In a fifth embodiment of the sixth aspect of the present invention there is provided a method wherein the imaging is performed using MRI imaging when Ln is Gd, or Ln is Gd₁, Gd₂, Gd₃, Gd₄, or Gd₅.

[0025] In a seventh aspect of the present invention there is provided a method of synthesizing
20 the composition according to claim 1 wherein Ln = Er or Ln = Yb comprising steps according to the following scheme:



wherein

- 5 said compound Por(THP-TMS) is synthesized via steps comprising:
- dissolving Pyrrole, pentafluorobenzaldehyde and 4-[2-(trimethylsilyl)ethynyl]benzaldehyde 6 in CH_2Cl_2 under an argon atmosphere to produce a first solution;
- leaving the first solution for at least 10 minutes;
- 10 adding $\text{BF}_3 \cdot \text{O}(\text{Et})_2$ to the first solution;
- stirring the first solution for at least 1 hour at room temperature;
- adding DDQ(2,3-Dichloro-5,6-dicyano-1,4-benzoquinone) to the first solution;
- stirring the first solution for at least another 1 hour at room temperature;
- removing the solvent from the first solution in vacuo to produce a first mixture;
- 15 passing the first mixture through a silica column (hexanes- CH_2Cl_2) concentrated under reduced pressure to produce 5,10,15-Tris(pentafluorophenyl)-20-[4-{2-(trimethylsilyl)ethynyl}phenyl]porphyrin or Por(THP-TMS);
- said compound Ln-1 is synthesized via steps comprising:
- dissolving $\text{Ln}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{LiCl}(\text{THF})_3] : \text{HN}(\text{SiMe}_3)_2$ in THF at about 0
- 20 degrees Celcius to produce a second solution;
- adding n-BuLi slowly over at least 30-minutes period to the second solution;
- stirring the second solution for at least 12 hours;

transferring the second solution to a Schlenk flask with LnCl_3 suspended in THF to produce a second mixture;

stirring the second mixture for at least 24 hours until all of the solid LnCl_3 disappeared to produce $\text{Ln}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$ ($x = 3 \sim 5$) wherein

$\text{Ln}=\text{Er}$ or $\text{Ln}=\text{Yb}$;

said compound Yb-1 is further synthesized via steps comprising:

transferring $\text{Yb}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$ ($x = 3 \sim 5$) to a Schlenk flask;

removing the solvent from $\text{Yb}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$ ($x = 3 \sim 5$) under vacuum to produce a first residue;

adding CH_2Cl_2 to the first residue for the precipitation of LiCl to produce a third mixture;

centrifuging the third mixture until a clear layer is produced;

transferring the clear layer to another Schlenk flask with dry $\text{Por}(\text{THP-TMS})$ free base dissolved in toluene to produce a third solution;

refluxing the third solution until most of the free base coordinated with the metal ion;

adding dry NaLOMe [LOMe^- ((cyclopentadienyl)tris(dimethylphosphito)-cobaltate or an anionic tripodalligand) to the third solution to produce a fourth mixture;

stirring the fourth mixture for at least another 12 hours;

cooling down the fourth mixture to room temperature;

removing the solvent from the fourth mixture in vacuum to produce a second residue;

dissolving the second residue in CHCl_3 ;

filtering and chromatographing the dissolved second residue on silica gel using CHCl_3 /petroleum ether as eluent;

further dissolving the output from chromatography in CH_2Cl_2 , and filtering the solution to produce compound Yb-1.

Said compound Er-1 is further synthesized via steps comprising:

the same steps as for Yb-1, replacing $\text{Yb}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$ ($x = 3 \sim 5$)

with $\text{Er}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$ ($x = 3 \sim 5$);

said compound Ln-2 wherein $\text{Ln} = \text{Yr}$ is synthesized via steps comprising:

- adding TBAF to a solution of Yb-1 in CH_2Cl_2 to produce a fifth solution;
stirring the fifth solution for at least 30 minutes;
monitoring the progress of the reaction of the fifth solution by TLC;
after completion of the reaction, passing the fifth solution through a short of silica
5 gel column;
removing the solvent from the fifth solution to produce Yr-2;
said compound Er-2 is further synthesized via steps comprising:
the same steps as for Yb-2, replacing Yb-1 with Er-1;
said compound Ln-4 wherein Ln = Yr is synthesized via steps comprising:
10 mixing $\text{Pd}(\text{PPh}_3)_4$, CuI, Yb-2 and 4-iodobenzoic acid in a dried flask under
nitrogen to produce a fifth mixture;
adding THF and Net_3 to the fifth mixture and degassing said fifth mixture with
nitrogen;
stirring said fifth mixture at least 40 °C for at least 12 hours;
15 removing the solvent from said fifth mixture under reduced pressure to produce a
third residue;
purifying the third residue by chromatography;
eluting the purified third residue with CH_2Cl_2 /Methanol to produce an eluted
compound;
20 mixing the eluted compound, EDCI, NHS in a dried flask and under nitrogen to
produce a sixth mixture;
adding dry DMF to the sixth mixture;
stirring the sixth mixture at room temperature for at least 48 hours;
removing the solvent from the stirred sixth mixture to produce a fourth residue;
25 recrystallizing the fourth residue by diethyl ether and drying the crystals to
produce Yb-4;
said compound Er-4 is further synthesized via steps comprising:
the same steps as for Yb-4, replacing Yb-2 with Er-2;
said compound Yb- R_1 is synthesized via steps comprising:
30 mixing a stirred solution of Yb-4 in anhydrous DMF with N,N'-
diisopropylethylamine (DIPEA) to produce a seventh mixture;
adding peptide R_1 to the seventh mixture;
leaving the seventh mixture to react at room temperature for at least 24 hours;

removing the solvent from the seventh mixture under vacuum to produce a dry fifth residue;

recrystallizing the dry fifth residue by diethyl ether for at least three times;

drying the recrystallized dry fifth residue to produce Yb-R₁;

5 said compound Yb-R₂ is further synthesized via steps comprising:

the same steps as for Yb-R₁, replacing R₁ with R₂;

said compound Yb-R₃ is further synthesized via steps comprising:

the same steps as for Yb-R₁, replacing R₁ with R₃;

said compound Er-R₁ is further synthesized via steps comprising:

10 the same steps as for Yb-R₁, replacing Yb-4 with Er-4;

said compound Er-R₂ is further synthesized via steps comprising:

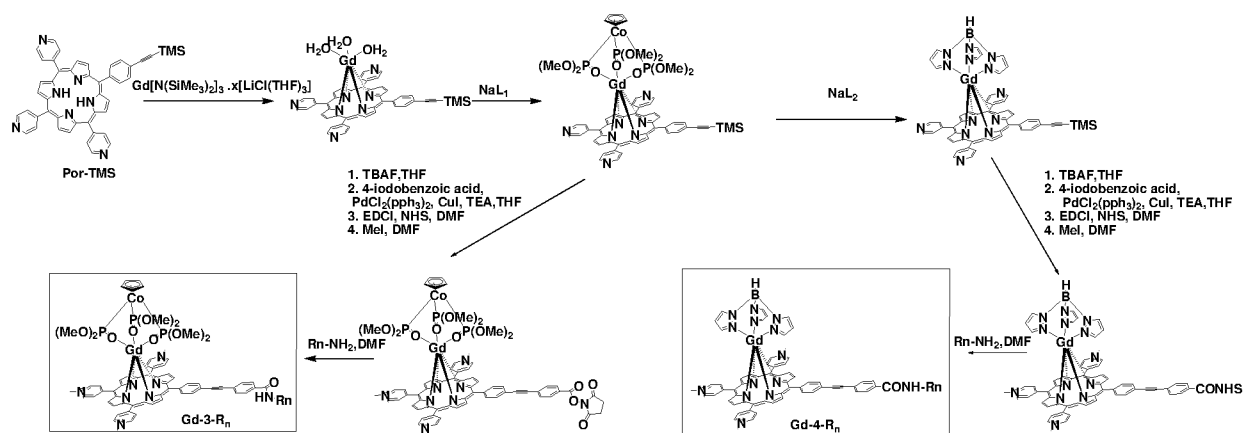
the same steps as for Yb-R₂, replacing Yb-4 with Er-4;

said compound Er-R₃ is further synthesized via steps comprising:

the same steps as for Yb-R₃, replacing Yb-4 with Er-4.

15

[0026] In a eighth aspect of the present invention there is provided a method of synthesizing the composition according to claim 1 comprising steps according to the following scheme:



20

wherein

said compound Por-TMS is synthesized via steps comprising:

mixing 4-((trimethylsilyl)ethynyl)benzaldehyde with Pyridine-4-carboxaldehyde in propionic acid to produce an eighth mixture;

25 stirring the eighth mixture for at least half an hour in at least 130°C;

adding pyrrole dropwise into the eighth mixture with the temperature increased to at least 140 °C;

stirring the eighth mixture for at least 30 minutes in open air;

cooling down the eighth mixture to room temperature;

5 removing the solvent from the eighth mixture under reduce pressure to produce a crude product;

dissolving the crude product in CH₂Cl₂ to produce a sixth solution;

purifying the sixth solution by column chromatography on silica gel column CH₂Cl₂/Methanol to produce Por –TMS;

10 said compound Gd[N(SiMe₃)₂]₃·x[LiCl(THF)₃] is synthesized via steps comprising:

dissolving HN(SiMe₃)₂ in THF at about 0 degrees Celcius to produce a seventh solution;

adding n-BuLi to the seventh solution over at least a 30-minutes period;

15 stirring the seventh solution at least for 12 hours until a clear pale yellow solution was obtained;

transferring the seventh solution a Schlenk flask with GdCl₃ suspended in THF to produce a ninth mixture;

stirring the ninth mixture for at least 24 hours until all of the solid GdCl₃ disappeared to produce the resultant solution Gd[N(SiMe₃)₂]₃·x[LiCl(THF)₃] (x = 3 ~ 5);

20 said compound Gd-1-L1 is synthesized via steps comprising:

transferring Gd[N(SiMe₃)₂]₃·x[LiCl(THF)₃] (x = 3 ~ 5) to a Schlenk flask and

removing the solvent therein under vacuum to produce a sixth residue;

adding CH₂Cl₂ to the sixth residue for the precipitation of LiCl to produce a tenth mixture;

25 centrifuging the tenth mixture until a clear layer is produced;

transferring the clear layer to another Schlenk flask with dry Por –TMS free base dissolved in toluene to produce an eighth solution;

refluxing the eighth solution until most of the free base coordinated with the metal ion;

30 adding dry NaL₁ (0.1 g, 0.22mmol) [L₁⁻ ((cyclopentadienyl)tris(dimethylphosphito)-cobaltate, an anionic tripodal ligand) to the eighth solution to produce an eleventh mixture;

stirring the eleventh mixture for at least another 12 hours;

cooling down the eleventh mixture to room temperature;
removing the solvent from the eleventh mixture in vacuum to produce a seventh residue;
dissolving the seventh residue in CHCl_3 ;
5 filtering and chromatographing the dissolved second residue on silica gel using $\text{CHCl}_3/\text{CH}_3\text{OH}$ ether as eluent;
further dissolving the output from chromatography in CH_2Cl_2 , and filtering the dissolved output to produce compound Gd-1-L1.

Said compound Gd-1-L2 is synthesized via steps comprising:

10 the same steps as for Gd-1-L1, replacing NaL_1 with KL_2 (potassium tris(1-pyrazolyl) borohydride);

said compound Gd-3 is synthesized via steps comprising:

adding TBAF to a solution of Gd-1-L1 in DCM to produce a ninth solution;
stirring the ninth solution for at least 30 minutes;
15 monitoring the reaction of the ninth solution by TLC;
passing the ninth solution through a short of silica gel column using DCM to remove the solvent therein to produce a pure product;
placing the pure product and $\text{Pd}(\text{PPh}_3)_4$, CuI, 4-iodobenzoic acid in a dried flask and under nitrogen to produce a twelfth mixture;
20 adding THF and Net_3 to the twelfth mixture;
degassing the twelfth mixture with nitrogen;
stirring the twelfth mixture at a temperature at least 40°C for at least 12 hours;
removing the solvent from the twelfth mixture under reduced pressure to produce an eighth residue;

25 purifying the eighth residue by chromatography;
eluting the purified eighth residue with $\text{CH}_2\text{Cl}_2/\text{Methanol}$;
placing the eluted purified eighth residue, EDCI, NHS in a dried flask and under nitrogen to produce a thirteenth mixture;
30 adding dry DMF to the thirteenth mixture;
stirring the thirteenth mixture at room temperature for at least 48 hours;
removing the solvent from the thirteenth mixture to produce a ninth residue;

recrystallizing the ninth residue by diethyl ether and dried said crystals to produce Gd-3;

dissolving the Gd-3 in DMF;

adding CH₃I to the dissolved Gd-3;

5 stirring the dissolved Gd-3 for at least 5 hours;

removing the solvent from the stirred dissolved Gd-3 to produce a tenth residue;

washing the tenth residue with ether .DCM. to produce pure Gd-3;

said compound Gd-4 is synthesized via steps comprising:

the same steps as for Gd-3, replacing Gd-1-L1 with Gd-1-L2;

10 said compound Gd-3-Rn is synthesized via steps comprising:

mixing a stirred solution of Gd-3 in anhydrous DMF with N,N'-

diisopropylethylamine (DIPEA) to produce a fourteenth mixture;

adding peptide Rn to the fourteenth mixture;

reacting the fourteenth mixture at room temperature for at least 24 hours;

15 removing the solvent from the fourteenth mixture under vacuum to produce a dry eleventh residue;

recrystallizing the dry eleventh residue by diethyl ether for at least three times and further dry the result to produce Gd-3-Rn;

said compound Gd-4-Rn is synthesized via steps comprising:

20 the same steps as for Gd-3-Rn, replacing Gd-3 with Gd-4.

[0027] Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the present invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

25 [0028] Those skilled in the art will appreciate that the present invention described herein is susceptible to variations and modifications other than those specifically described.

[0029] The present invention includes all such variation and modifications. The present
30 invention also includes all of the steps and features referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

[0030] Throughout this specification, unless the context requires otherwise, the word “comprise” or variations such as “comprises” or “comprising”, will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. It is also noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as “comprises”, “comprised”, “comprising” and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean “includes”, “included”, “including”, and the like; and that terms such as “consisting essentially of” and “consists essentially of” have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the present invention.

[0031] Furthermore, throughout the specification and claims, unless the context requires otherwise, the word “include” or variations such as “includes” or “including”, will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0032] Other definitions for selected terms used herein may be found within the detailed description of the present invention and apply throughout. Unless otherwise defined, all other technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the present invention belongs.

[0033] Other aspects and advantages of the present invention will be apparent to those skilled in the art from a review of the ensuing description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034] The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, in which:

[0035] **Figure 1A** shows molecular structure of the cancer cells specific photodynamic therapy agent (**Gd-N**) and their control analogues **Yb-N**, **Gd-RhB** and **Yb-RhB**.

[0036] **Figure 1B** is 3D in vitro imaging of **Gd-N** after 15-hour incubation in HeLa cells.

[0037] **Figure 1C** and **Figure 1D** show difference in subcellular localization of **Gd-N** in cancer cells (HeLa) and normal cells. (WPMY-1), respectively.

5 [0038] **Figure 2** shows emission spectra of **Gd-N** (HEPES buffer solution, 10 μ M, λ_{ex} = 430 nm, pH = 7.4) and $^1O_2$ quantum yield measurement (Near-IR phosphorescence spectra of $^1O_2$ CHCl₃, 10 μ M, λ_{ex} = 430nm, abs(λ_{ex}) = 0.03). **Yb-N** and H₂TPP are measured similarly as control.

10 [0039] **Figure 3** shows in vitro imaging of **Gd-N** and **Gd-RhB** in tumor cells – HeLa and normal cells – MRC5 (as the controls) after 2-hour incubation. PDT effect is triggered upon 860 nm excitation. A) **Gd-RhB** in HeLa; b) **Gd-RhB** in MRC-5; c) **Gd-N** in HeLa; d) **Gd-N** in MRC-5 (1 μ M).

15 [0040] **Figure 4** shows photocytotoxicities of **Gd-N**, **Gd-RhB** (control) and **Yb-N** (control) towards (A) cancer cell (HeLa) and (B) normal cell (QSG 7701). **Gd-N** ($^1O_2$ available, tumor specific, strong photocytotoxicity in cancer cells, but no photocytotoxicity in normal cells), **Gd-RhB** (control – $^1O_2$ available, non tumor specific, strong cancer and normal cell photocytotoxicity) and **Yb-N** (control, $^1O_2$ not available, no photocytotoxicity in both cancer and normal cells). Photocytotoxicity curves are obtained using 1 μ M of conjugates and various light
20 doses from 0 to 1 J/cm²; MTT assays are carried out after incubation for 24 hours. (37°C, 5% CO₂).

[0041] **Figure 5** shows the in vitro photocytotoxicity (λ_{ex} = 430 nm) of the tumor-specific **Gd-N** in four tumor cell lines (HeLa, SK-N-SH, HK-1 and A549) and three normal cell lines (QSG
25 7701, MRC-5, WPMY-1), as well as the two controls – **Yb-N** and **Gd-RhB**.

[0042] **Figure 6** shows in vivo studies of **Gd-N** as the cancer cell-specific PDT agent. A) the representative gross images of tumors after PDT using 860nm laser for excitation, and candidates are divided into four groups (Group 1: **Yb-N**; Group 2: **Gd-N**; Group 3: **Yb-RhB**;
30 Group 4: **Gd-RhB**); b) the measurement of tumor volume in a); c) In vivo biodistribution of **Gd-N** via ICP-MS studies; d) Two-photon microscopic images of tumor samples in c); e) In vivo tumor inhibition assays of **Gd-N**; f) In vivo tumor inhibition via **Gd-N** induced $^1O_2$ through caudal vein injection.

[0043] **Figure 7** shows **Gd-N** and **Gd-RhB** induced $^1\text{O}_2$ activated the inhibitor of apoptosis protein family and mTOR pathway. (A) western blot of HeLa cells dosed with $1\mu\text{M}$ **Gd-N** or **Gd-RhB** and irradiated with 0.5 J/cm^2 . Untreated or free of chemicals samples are served as the controls. (B) Cellular protein changes are semi-quantitatively measured using Gel-Pro Analyzer software of western blotting bands in (A) and showed as the ratio to β -actin (loading control of total proteins). P values are calculated between untreated and **Gd-N** or **Gd-RhB** plus laser groups by One-way Analysis of Variance.

[0044] **Figure 8** shows A) High-resolution MALDI-TOF mass spectrum of **Gd-N**; B) Isotopic patterns for the molecular ion **Gd-N**; C) Calculated MS patterns of the molecular ion **Gd-N** (using the software: IsoPro 3.0).

[0045] **Figure 9** shows the absorption spectra of **Gd-N** and **Gd-RhB**.

[0046] **Figure 10** shows the schematic representation of energy absorption, migration and emission (indicated by $\cdots\rightarrow$) processes in the (A) gadolinium porphyrinate complex (**Gd-N**) and (B) ytterbium porphyrinate complex (**Yb-N**).

[0047] **Figure 11** shows the open-aperture Z-scan trace of **Gd-N** (351 GM) and **Gd-RhB** (418 GM) excited at 800 nm in DMSO ($5\mu\text{M}$). The average power of the laser beam is 0.271 mW.

[0048] **Figure 12** shows (a) the molecular structures of **Ln-R_n**, (b) the visible spectra of **Ln-R_n** in aqueous solution with 430 nm excitation (conc. = 1 M, Ln = Er or Yb, n = 1, 2 and 3) and (c) near-infrared emission spectra of **Ln-R_n** in aqueous solution with 430 nm excitation (conc. = 1M, Ln = Er or Yb, n = 1, 2 and 3).

[0049] **Figure 13** shows subcellular localization of **Er-R_n** and **Yb-R_n** porphyrin complexes in human bladder carcinoma (T24 and 5637) cells, normal lung fibroblast (MRC-5) cells, and Human cervical carcinoma (HeLa) cells.

[0050] **Figure 14** shows the cellular uptake analyzed by flow cytometry of **Er-R_n** and **Yb-R_n** porphyrin complexes in 5637, T24, HeLa and MRC-5 cells incubated for 0, 3, 6, and 24 hours as

indicated by arrows. The y-axis and x-axis are corresponding to cell counts and fluorescence intensity in FL3 channel (wavelength >650 nm).

[0051] **Figure 15** shows the comparison of in vitro photo-cytotoxicity of **Er-R_n** and **Yb-R_n** porphyrin complexes with ALA in (A) T24, (B) HeLa and (C) MRC-5 cells irradiated at 10 J cm⁻² with 550 nm long-pass filter, D) Summary of IC₅₀ value of **Er-R_n** and **Yb-R_n** porphyrin complexes and ALA in the presence and absence of irradiation in T24, HeLa and MRC-5 cells.

[0052] **Figure 16** shows HPLC chromatogram of the complexes. Elution conditions: column, Agilent ZORBAXSB-C18 (4.6 X 150 mm, particle size 5; flow rate, 1.0 mL/min; gradient elution; detection wavelength, 430 nm. Retention time: (A) **Yb-4**, 7.24min ; (B) **Er-4**, 7.23 min; (C) **Yb-R₁**, 10.00 min; (D) **Yb-R₂**, 10.21 min; (E) **Yb-R₃**, 10.01 min; (F) **Er-R₁**, 9.66min; (G) **Er-R₂**, 10.09min; and (H) **Er-R₃**, 9.80 min.

[0053] **Figure 17** shows the 400 MHz-¹H-NMR (CDCl₃) spectrum of **Por(THP-TMS)**.

[0054] **Figure 18** shows the MALDI-TOF spectrum of **Por(THP-TMS)**.

[0055] **Figure 19** shows the 400 MHz-¹H-NMR (CDCl₃) spectrum of **Yb-1**.

[0056] **Figure 20** shows the MALDI-TOF spectrum of **Yb-1**.

[0057] **Figure 21** shows the 400 MHz-¹H-NMR (CDCl₃) spectrum of **Er-1**.

[0058] **Figure 22** shows the MALDI-TOF spectrum of **Er-1**.

[0059] **Figure 23** shows the 400 MHz-¹H-NMR (CDCl₃) spectrum of **Yb-2**.

[0060] **Figure 24** shows the MALDI-TOF spectrum of **Yb-2**.

[0061] **Figure 25** shows the 400 MHz-¹H-NMR (CDCl₃) spectrum of **Er-2**.

[0062] **Figure 26** shows the MALDI-TOF spectrum of **Er-2**.

[0063] **Figure 27** shows the 400 MHz-¹H-NMR (CDCl₃) spectrum of **Yb-4**.

[0064] **Figure 28** shows the MALDI-TOF spectrum of **Yb-4**.

[0065] **Figure 29** shows the 400 MHz-¹H-NMR (CDCl₃) spectrum of **Er-4**.

[0066] **Figure 30** shows the MALDI-TOF spectrum of **Er-4**.

[0067] **Figure 31** shows the MALDI-TOF spectrum of **Yb-R₁**.

[0068] **Figure 32** shows the MALDI-TOF spectrum of **Yb-R₂**.

[0069] **Figure 33** shows the MALDI-TOF spectrum of **Yb-R₃**.

[0070] **Figure 34** shows the MALDI-TOF spectrum of **Er-R₁**.

[0071] **Figure 35** shows the MALDI-TOF spectrum of **Er-R₂**.

[0072] **Figure 36** shows the MALDI-TOF spectrum of **Er-R₃**.

[0073] **Figure 37** shows the subcellular localization of **Er-R_n** and **Yb-R_n** porphyrin complexes by staining with Lyso Tracker green in (A) 5637 cells, (B) T24 cells, (C) HeLa cells and (D) MRC-5 cells.

[0074] **Figure 38A** shows NIR emission of existing organelle/DNA specific lanthanide complex. *Meso*-pyridinium-substituted porphyrin-based ytterbium complexes shows a responsive NIR emission upon the addition of DNA; **Figure 38B** shows NIR emission of water-soluble and mitochondria specific porphyrin-based Yb(III) complex (**Yb-2**).

[0075] **Figure 39A** shows affinity of Yb-N to phosphatidylserine and cancer cells; a strong binding to phosphatidylserine and the capability to differentiate of cancer cells via targeting the

anionic phospholipid membrane; **Figure 39B** shows the development of organometallic complexes as in vitro and vivo tumor specific PDT agents as PDT agents.

[0076] **Figure 40** shows the molecular structures of multi-modal porphyrin based metal complexes A) Gd-1, B) Gd-2, C) Gd-3-R₁ and D) Gd-3-N which are used as PDT, optical and MRI agents.

[0077] **Figure 41** shows flow cytometry analysis of cellular uptake of **Gd-3-R₁**. **Gd-3-R₁** has the fastest uptake rate among the four porphyrin complexes in the cancer cells and also with the better selectivity towards (B) bladder cancer T24 cells rather than (A) normal MRC-5 cells.

[0078] **Figure 42** shows emission intensities of Gd-3-R₁ complex and H₂TPP irradiated at 430nm excitation; similar ¹O₂ and emission quantum yield upon protonation of the Gd-3-R₁ complex compared with H₂TPP reported in PNAS, 2014, E5492–E5497, which are around ~70% ¹O₂ quantum yield and 46% emission quantum yield with the excitation at 430 nm respectively.

[0079] **Figure 43** shows the t₁ relaxivity of **Gd-3-R₁** and Gd-DOTA of various concentrations; t₁ relaxivity of Gd-3-R₁ is three times greater than Gd-DOTA.

[0080] **Figure 44** shows concentration of Gd-1, Gd-2, Gd-3-R₁ and Gd-3-N in ppm level per gram of different tissues.

[0081] **Figure 45** shows the structures (A) Gd-3-R_n, (B) Gd-4-R_n and (C) Gd-5-R_n with the change of different substituent groups (improve water solubility), and organometallic/molecular caps (for stability/relaxivity).

[0082] **Figure 46** shows the reaction scheme of the complexes **Gd-3-R_n** and **Gd-4-R_n** intermediates for Figure 40 and 45.

[0083] **Figure 47** shows structures of (A)Gd-5-R_n, (B)Gd-3-R_n, (C)Gd-4-R_n, (D)Gd-6-R_n that achieve better MRI and PDT effects. (With better cellular permeability, t₁ relaxivity and NIR emission for optical imaging).

[0084] **Figure 48** shows the development of multi-modal water-soluble, lanthanide-based PDT agents for optical imaging and inhibition of bladder cancer.

[0085] **Figure 49A** shows the selectivity towards (T24 bladder cancer, xenograft tumor) by comparing the *in vivo* magnetic resonance images of Gd-DOTA and Gd-3-R₁ (**Figure 49B**).

DETAILED DESCRIPTION OF THE INVENTION

[0086] The present invention is not to be limited in scope by any of the specific embodiments described herein. The following embodiments are presented for exemplification only.

[0087] Without wishing to be bound by theory, the inventors of the present invention have developed gadolinium porphyrinate (**Gd-N**; Figure 1A), a PDT agent which is synthesized on the basis of **Yb-N** and shown 51% singlet oxygen quantum yield with characteristic NIR emission of porphyrin upon photoexcitation. (Figure 1B-D) Comprehensive studies have revealed that **Gd-N** can recognize tumor cells by their anionic phosphatidylserine membrane in the first six hours after administration. Upon administration of Gd-N, laser-irradiation at certain wavelengths, Gd-N enters the tumor cells and produce ¹O₂ in addition to exhibiting TP-induced NIR emission. Results of the *in vivo* mouse models and biodistribution assays further illustrates that **Gd-N** is found to be located in the tumor after simple injection of **Gd-N** into the blood vessel. Upon ¹O₂ releasing from the porphyrin, the solid tumor is found to be reduced after 24-hour treatment. To the best of current knowledge in the art, there is a lack of *in vivo* lanthanide-based PDT agents. The present invention provide a novel PDT agent, Gd-N, and the use thereof for practical cancer tracking, imaging and treatment.

[0088] Results and Discussion

[0089] The detailed synthesis and characterization of **Gd-N**, which is the motif structure of the ytterbium complex (**Yb-N**) reported in the inventors' previous work, are shown in Scheme 1 and **Figure 8**. **Gd-N** and **Yb-N** are structurally different in the lanthanide ion present in the complex (the vector ligated to **Gd-N** is also the same as **Yb-N**). It is self-evident that porphyrin's coordination with different lanthanides can cause changes not only in the NIR emission, but also the ¹O₂ generation. (**Figure 2 and Figure 9**) Such phenomena, in principle, arise from the fact that better orbital overlapping between the metal center and the ligand results in better energy transfer (i.e. the bonding orbitals of Yb which consists of a smaller atomic radius than Gd is thus

overlap more preferably and compatibly with the porphyrin's orbitals). The heavy atom effect exerted by the lanthanide can also augment the triplet-state decay rate and lead to higher triplet-state quantum yields of the porphyrin system. According to spectroscopic studies, the singlet oxygen quantum yield of **Yb-N** is measured to be 0% and **Gd-N** is determined to be 51%. The calculations are based on (i) the NIR phosphorescence intensity of the $^1\text{O}_2$ (at 1270 nm) produced from the two complexes and (ii) the lowest excited states of ytterbium $^2\text{F}_{5/2}$ ($\sim 10200\text{ cm}^{-1}$) and gadolinium $^6\text{P}_{7/2}$ ($\sim 32000\text{ cm}^{-1}$) respectively. It should be noted that the latter energy level of $^6\text{P}_{7/2}$ is much higher than the singlet/triplet levels of the porphyrin unit (singlet states = ~ 23200 and 15300 cm^{-1} ; triplet state = 12500 cm^{-1}). A large energy gap between porphyrin and Gd, there is no energy transfer from porphyrin to Gd; the energy gained can therefore purely be either dissipated in the form of light or employed to form singlet oxygen, making direct determination of $^1\text{O}_2$ quantum yield feasible. (**Figure 10(A)**) This is entirely not the same case for Yb. As the energy gap between porphyrin and Yb is small, most of the energy absorbed by the porphyrin unit would just be simply transferred to the ytterbium efficiently (via heavy atom effect) and afford the characteristic *f-f* emission exclusively. (**Figure 10(B)**) The two percentages have clearly showcased that nearly half of the energy absorbed by the porphyrin of **Gd-N** would be involved in the $^1\text{O}_2$ generation, while the rest will be normally used for the porphyrin's NIR emissions; in contrast, for **Yb-N**, ytterbium's *f-f* luminescence at $1.08\mu\text{m}$ is the dominant process of energy consumption under the same photoexcitation. (Linear and two-photon excitation at 430 nm and 860 nm respectively; the two-photon absorption cross section of **Gd-N** and **Yb-N** are similar to be $\sim 351\text{GM}$ (**Figure 11**).

[0090] Investigations with relation to real PDT-applications of **Gd-N** *in vitro* and, particularly, *in vivo* had been accomplished in terms of tumor selectivity, cytotoxicity and photocytotoxicity, imaging, PDT efficiency, as well as biodistribution. The selectivity of **Gd-N** against tumor and normal cells are superiorly distinct. As shown in **Figure 1B-1D**, in the HeLa cancer cell, strong red emission from the porphyrin of **Gd-N** can be observed on the periphery, that is, the membrane surface, after 2-hour incubation. Upon incubation with more than 15 hours, several red emission enter and scatter internally to the cytoplasm. In the normal cell MRC-5, however, no emission can be detected on the surface of or inside the cell even after the 12 hours of incubation. In order to have a fair comparison, **Gd-RhB** had been synthesized for the control experiments. Rhodamine B (**RhB**) is a well-known mitochondria vector common for conjugation. Under the same experimental condition (incubation time, concentration, cell lines and laser

power), the inventors find **Gd-RhB**'s emission in both normal and cancer cells' mitochondria, and this very observation becomes the clear, cognizant, and convincing evidence of the tumor-specific property of the **Gd-N**. (**Figure 3**) Through the MTT assays, the cytotoxicity of the three complexes, **Gd-N**, **Yb-N** and **Gd-RhB** in dark can be subsequently determined against the two kinds of cell lines. The IC₅₀ values of them are 0.78, 0.80, and 0.65 mM in cancer cells (HeLa) and 0.70, 0.70, and 0.45 mM in normal cells (MRC-5) respectively. The underlying reason of the vast difference in the dark cytotoxicity of **Gd-RhB** towards cancer/normal cells compared with **Gd-N** and **Yb-N** can be largely due to **Gd-RhB**'s non-selectivity. Again, **Gd-N** of the present invention exhibits crucial tumor selectivity. The *in vitro* PDT effect of the three complexes is evaluated using *in vitro* confocal microscopy and photocytotoxicity assays. **Gd-N**, **Yb-N** and **Gd-RhB** complexes are dosed in HeLa cells and MRC-5 cells for 6 hours, and then subjected to excitation at 860 nm for triggering any PDT effect. (Three complexes are all available for TP-induced *in vitro* imaging with TP cross-section ~351 GM; given the limitation of the confocal spectroscopy, the emission from porphyrin had only been monitored from 600 nm to 750 nm only) In **Figure 3**, the emission of **Gd-RhB** can be noticed in the mitochondria. Upon suitable laser-induction, only small quantities of ¹O₂ are produced but the cancer cells are killed within a few minutes; in effect, the normal cells are also killed rapidly under the same conditions. The PDT effect of **Gd-RhB** is therefore efficient enough but obviously non-selective and undesirable; it accumulates inside the mitochondria of cancer and normal cells, annihilating them unselectively. Although **Yb-N** is cancer-specific, its incapability to produce any ¹O₂ imposes a restriction on any PDT practice. The red emissive **Gd-N**, not only it recognizes and localizes on the anionic membrane of tumor cells, but also access to certain parts of cytoplasm and induce cancer apoptosis via ¹O₂ upon 9-minutes light dose flashing 5 seconds per minute. More time is required to trigger cancer cell death by **Gd-N** after definite laser irradiation; however, there is no significant cell death in the normal cells, far outweighing its slow-response drawback.

[0091] The concentration-dependent photocytotoxicity of **Gd-N**, **Yb-N** and **Gd-RhB**, ranging from 0.2 to 1 μM dosage, are measured under varying light doses from 0.25 to 1 J/cm² in cancer cells and normal cells. The light dose-response curves obtained are displayed in **Figure 4**. In HeLa cancer cells, **Gd-RhB** and **Gd-N** exhibit strong photocytotoxicity, whereas **Yb-N** (without singlet oxygen) has no photocytotoxicity (**Figure 4 (A)**). From **Figure 4(B)**, in normal cell QSG 7701, no photocytotoxicity is found from **Gd-N**, while **Gd-RhB** gives very similar results as it behaved in the cancer cells. Such trend correlates with the selective cellular uptake of **Gd-N** by

cancer and normal cells. The inventors had extended the studies with the use of more cancer cell and normal cell lines, and the results are shown in **Figure 5** — **Gd-N** maintains its good tumor selectivity towards total of 7 cell lines (four cancer cells and three normal cells), thereby acting as an outstanding and specific PDT agent.

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[0092] To demonstrate *in vivo* uptakes of complexes of the present invention, studies of biodistribution on these complexes' specialty towards infections of particular organs are carried out via xenograft mouse models and ICP-MS. Four complexes are classified into four groups. HeLa cells pre-incubated separately with **Gd-N**, **Yb-N**, **Gd-RhB** and **Yb-RhB**, and are subcutaneously injected into BALB/c nude mice and then irradiated the injected sites with 860 nm laser. Two weeks later, mice are pictured and the tumor volumes are measured (picture of the mice and measurement of tumor volumes are shown in **Figure 6 a)** and **6b)**, respectively). The tumors are found effectively inhibited in the groups of **Gd-N** and **Gd-RhB**, compared with their counterparts **Yb-N** and **Yb-RhB**; **Gd-N**, among the four complexes, is the best *in vivo* PDT agent that devastate the tumor with 100% efficiency. In biodistribution study, BALB/c nude mice with tumor xenograft attaining a size of approximately 0.1 cm³ are caudal vein injected with **Gd-N** (1.0 mg/kg). Two days after administration, concentrations of Gd-N and Gd-RhB in different tissues or circulating blood are examined using ICP-MS. As seen in **Figure 6 c)**, tumors have the largest enrichment of **Gd-N** (4.84 ppm/g), demonstrating the specific recognition of the **Gd-N** towards tumor cells.

[0093] This result is also confirmed by two-photon microscopic imaging of the tumor tissues extracted from **Gd-N** administrated BALB/c nude mice. There are obvious two-photon microscopic signal from Gd-N (image of Gd-N, circled points), while the control image (showed as background, imaged by bright field shows no specific signal. The merge image is the overlap photon of Background and Gd-N which is shown **Figure 6 d)**. Further verification of the inhibiting effect of **Gd-N** and **Gd-RhB** towards tumor growth in tumor-bearing mice is done by intratumorally injecting BALB/c nude mice of HeLa xenograft tumor of approximately 0.3 cm³ with **Gd-N** (2.0 mg/kg), **Gd-RhB** (2.0 mg/kg) and **ALA** (60 mg/kg) (5-aminolevulinic acid, which can produce protoporphyrin in living cells and herein serve as the control PDT chemical), and irradiating with 860 nm light for three hours after injection. The total light dosage to tumor is 50 J/cm². Tumors are then allowed for growth for another 7 days and subjected for final

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extraction and picturing. As shown in **Figure 6 e**), **Gd-N** is capable of tremendously inhibiting and even reducing the size of solid tumor by half from 2 cm to 1 cm within a short period of time.

[0094] Alternatively, mice with xenograft tumor are caudal vein injected with **Gd-N** and **Gd-RhB** (2.0 mg/kg body weight) and allowed for full circulation for 6 hours. Then tumors are irradiated with 860 nm light similarly as above. The tumor with light untreated serves as a control. The treatments are repeated for three times in the following days in a one-time-per-day manner. Consistently, it is found that **Gd-N** plus light treated tumors are inhibited compared to their opposite flank controls of tumor or **Gd-RhB** groups. Pharmacokinetics analyses also show that **Gd-N** persisted in animals for longer time with a larger MRT (mean residence time) value (12.50 hours), while **Gd-RhB** is fast cleared (with MRT of 5.04 hours) (results are illustrated in **Figure 6 f**) and **Table 1**).

Parameters	Gd-N	Gd-RhB
Equation	$C_{(t)}=138.61 e^{-0.08t}$	$C_{(t)}=176.08 e^{-0.1986t}$
AUC _(0-t) (μg/mL×h)	1732.63	886.61
MRT _(0-t) (h)	12.50	5.04
t _{1/2} (h)	8.66	3.49
V _d (mL)	0.269	0.251

Table 1. Pharmacokinetic parameters of **Gd-N** and **Gd-RhB** in plasma after caudal vein injections of 20 nmol of **Gd-N** (37.34 μg) or **Gd-RhB** (44.28 μg) to BALB/c nude mice (n=3), respectively. AUC, area under the concentration–time curve; MRT, mean residence time; t_{1/2}, statistical half life; V_d, volume of distribution.

[0095] Molecular mechanism of PDT, protein levels of cellular surviving and inhibitors of apoptosis protein family (IAP) in protein lysates of PDT-treated HeLa cells are also investigated. Survivin and the IAP family members, c-IAP1, c-IAP2 and XIAP, are all remarkably expressed in **Gd-N** plus laser-treated samples. mTOR pathway is found to be involved in response to the PDT treatment of the cancer cells. The levels of two critical members, mTOR and GβL, are obviously elevated upon **Gd-N** or **Gd-RhB** induced ¹O₂ stress stimulus. These results demonstrate the successful cell killing effect of **Gd-N** promoted photodynamic therapy at the molecular level and may also shed new light on the design and improvement of current PDT agents (**Figure 7**).

[0096] **Conclusion**

[0097] The present invention provides theranostic gadolinium complex **Gd-N** for use as an anti-cancer agent which is equipped with visible-to-NIR emission for imaging, tumor cell selectivity, and $^1\text{O}_2$ generation. Through a string of *in vitro* and *in vivo* studies, the effectiveness and advantages of **Gd-N** of the present invention for use as smart dual-functional PDT agent is shown. The present invention also provides a method of tracking and imaging long-term live cancer cell, using **Gd-N**, as well as selective photodynamic therapy.

[0098] **Experimentation Methods**[0099] *Linear induced photophysical properties*

[00100] UV-Visible absorption spectra (ranging from 200 to 1100 nm) and single-photon luminescence spectra are recorded with an HP UV-8453 spectrophotometer and an Edinburgh Instrument FLS920 Combined Fluorescence Lifetime and Steady state spectrophotometer equipped with a UV-to-NIR-sensitive photomultiplier inside a nitrogen flow cooled housing. The Inventors had corrected all the spectra from the detector response and stray background light phosphorescence, measuring the quantum yields of the lanthanide complexes by a demountable 142 mm (inner) diameter barium sulphide-coated integrating sphere supplied with the two access ports in Edinburgh Instrument FLS920.

[00101] *Singlet oxygen quantum yield*

[00102] With phosphorescence at 1270 nm, the singlet oxygen with an InGaAs detector on the PTI QM4 luminescence spectrometer are detected, and the quantum yields (Φ_Δ) of all compounds in CHCl_3 through comparing the $^1\text{O}_2$ emission intensity of the sample solution to that of a reference material ^[4] (H_2TPP , $\Phi_\Delta = 0.55$ in CHCl_3) are determined and as illustrated in the following equation:

$$\Phi_\Delta^S = \Phi_\Delta^{\text{REF}} \times \left(\frac{n_S}{n_{\text{REF}}} \right)^2 \frac{G_\Delta^S}{G_\Delta^{\text{REF}}} \times \frac{A_{\text{REF}}}{A_S}$$

where Φ_Δ denotes the singlet oxygen quantum yield, G_Δ indicates the integrated emission intensity, A represents the absorbance at the operation excitation wavelength, n reflects the solvent's refractive index, given that the Superscripts REF and S stand for the reference and sample respectively. In all cases, the inventors had measured the $^1\text{O}_2$ emission spectra upon due

excitation. To reduce the impacts of re-absorption of the emitted light, all absorbance were set at 0.05 as well.

[00103] ***Cell culture***

5 [00104] Human HeLa (cervical carcinoma) and WPMY-1 (normal prostate stroma immortalized cell) cells are grown in DMEM medium; A549 (lung adenoma) are maintained in a mixture of Ham's F12K medium and L-glutamine (N3520, Sigma, St. Louis, MO, USA); QSG 7701 (normal liver cell), HK-1, HONE1 (nasopharyngeal carcinoma) are grown in RMPI-1640 medium; MRC-5 (normal lung fibroblasts) and SK-N-SH (neuroblastoma) cells are grown in
10 MEM medium. (i) 10% (v/v) fetal bovine serum (FBS), (ii) 100 µg/ml streptomycin, and (iii) 100 units/ml penicillin are also added in the all the medium.

[00105] ***In vitro imaging***

[00106] To test the suitability of the water-soluble complexes of the present invention as
15 bioprobes, a commercial confocal laser scanning microscope, Leica TCS SP5, equipped with a Ti: Sapphire laser (Libra II, Coherent) as well as a 980 nm wavelength laser for excitation are used to in vitro image HeLa/WPMY-1/MRC-5 cells incubated with the five complexes of the present invention.

20 [00107] ***MTT cell viability assay***

[00108] After 24 hours, the water-soluble complexes and the treated cells are incubated further with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/ml) for 4 hours, so that formazan is formed along with the cell's metabolic pathways. Then, the formazan are extracted and dissolved by dimethyl sulfoxide (DMSO), with the absorbance of the
25 subsequent solutions being measured in a Bio-Rad iMark microplate reader (490 nm). Quadruplicates are performed and the data are interpreted and analyzed by plottings using the GraphPad Prism 5 software.

[00109] ***Photodynamic treatment (PDT) assay***

30 [00110] On a 96-well plate, cancer cells (2×10^4 /well) are first incubated overnight and then treated with complexes of the present invention and control analogues for 6 hours in dark. After the old medium being replaced with the fresh one, the cells are accordingly exposed to yellow light ($1-8 \text{ J/cm}^2$) generated from a 400 W tungsten lamp fitted with a heat-isolation filter

and a 500 nm long-pass filter under the fluency rate of mW/cm². After 24 hours, post-PDT cell viability is examined by MTT assay. Cell monolayers are rinsed with PBS prior to incubation with 250 µg/mL MTT solution at 37 °C for 3 hours. The formazan crystals formed and dissolved in DMSO then undergo absorbance measurement at 540 and 690 nm by a 96-well plate reader (Elx800 Absorbance Microplate Reader).

[00111] **Animals:** All the experiments entailing animal models are performed on athymic nude mice (BALB/c-nu/nu) which are all obtained from Guangdong Medical Lab Animal Center (license number: SCXK-2008-0002). Mice are raised and operated according to the strict protocol the National Standard of Animal Care and Use Procedures (20080820).

[00112] **Pharmacokinetics analysis: Gd-N and Gd-RhB** (1.0 µmol/kg body weight each) are caudal vein injected into the mice. Then sera are collected at different time points from 0-20 hours as indicated. The concentrations of **Gd-N** and **Gd-RhB** are measured by PerkinElmer EnVision Multilabel Reader 2104 at 570 nm, and calculated using standard absorptions via concentration curve. Pharmacokinetic parameters ($t_{1/2}$, Vd, MRT, AUC) are calculated by fitting with one compartment model.

[00113] ***In vivo bio distribution via ICP-MS***

[00114] Biodistribution studies of In vivo uptakes of complexes of the present invention in particular organs/bacterial infections are carried out via ICP-MS. **Gd-N** and **Gd-PhB** are administered to mice at a dosage of 1.0 µmol/kg body weight when tumor xenograft attains a size of approximately 0.1 cm³. 2 days later, around 0.02-0.04 gram of sample tissues are collected in tumor, liver, lung, kidney, spleen, brain, prostate, skin and blood (80-90 µL). All samples are incubated with 500 µL nitric acid at 37 °C for releasing the metal ions for further ICP-MS examinations, in addition to dissolving the interfering organic molecules.

[00115] ***In vivo photodynamic therapy studies***

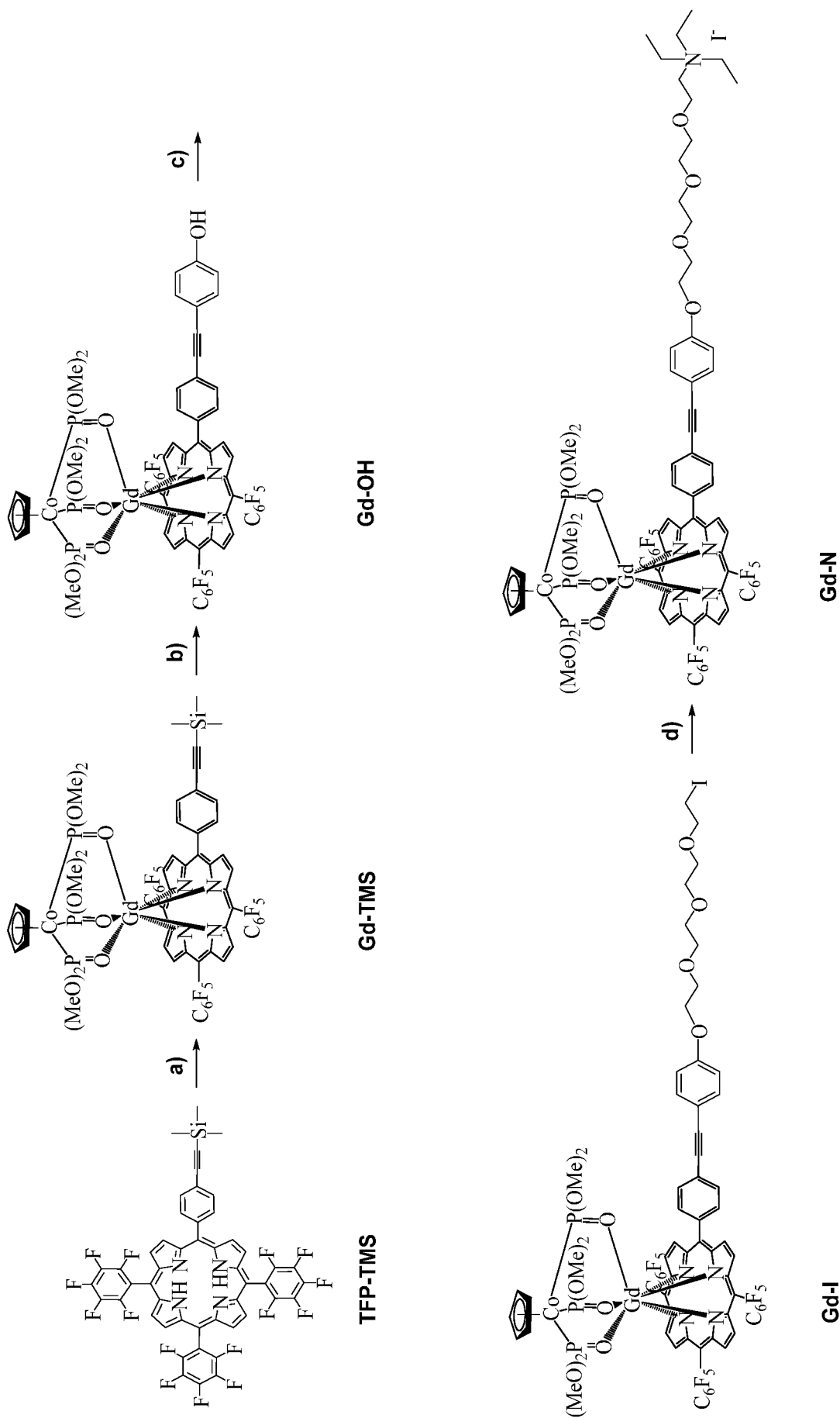
[00116] For the establishment of mouse tumor xenograft mode, cells are trypsinized, harvested and suspended in the culture medium. 1×10^6 cells in 100 µL volume are s.c. injected into the flanks of female athymic nude mice (with 5-week old) and waited for 10–15 days. When the tumor volume reaches to size of 100–150 mm³, the animals are randomly divided into different groups for further experiments. Tumor volume is measured by calipers (accuracy of

0.02 mm) and then calculated independently on the basis of the equation $V = (L \times W^2)/2$, where L and W correspond to the larger and smaller dimensions, respectively. One-way analysis of variance towards statistical significances between groups is assessed by the GraphPad Prism 5.0 software.

5

[00117] **Materials and Methods**

[00118] All chemicals used are of reagent-grade and are purchased from Sigma–Aldrich and used without further purification. Preparations of intermediates $\text{Yb}[\text{N}(\text{SiMe}_3)_2]_3 \cdot [\text{LiCl}(\text{THF})_3]^1$ and starting porphyrin free base TFP-TMS² are performed according to the literature procedures. Preparations of the control compounds **Gd-RhB**, **Yb-RhB**⁴ and **Yb-N**⁵ are accomplished according to the inventors' previous procedures. All analytical-grade solvents are dried by standard procedures, distilled and deaerated before use. High-resolution mass spectra, reported as m/z, are obtained on a Bruker Autoflex MALDI-TOF mass spectrometer. Elemental analyses carried out at the School of Chemical Engineering,
10
15 Northwest University, P. R. China. The synthetic route of intermediates and **Gd-N** is shown in Scheme 1:



a) (i) $\text{Gd}[\text{N}(\text{SiMe}_3)_2]_3 \cdot [\text{LiCl}(\text{THF})_3]_3$, toluene, reflux, 12h; (ii) $\text{Na}\{(\eta^5\text{-C}_5\text{H}_5)\text{Co}[\text{P}(=\text{O})(\text{OMe})_2]_3\}$, toluene, rt, 1h; b) (i) TBAF (THF, 1M), CH_2Cl_2 , rt, 30min; (ii) a) 4-iodophenol, $\text{Pd}(\text{PPh}_3)_4$, CuI , THF, NEt_3 , 40°C , 12h; b) Tetraethyleneglycol diiodide, DMF, K_2CO_3 , 80°C , 8h; c) Triethylamine, DMF, 85°C , 24h.

[00119] **Scheme 1.** The synthetic routes of **Gd-NGd-TMS**: A solution of $\text{Gd}[\text{N}(\text{SiMe}_3)_2]_3 \cdot [\text{Li}(\text{THF})_3\text{Cl}]_x$ (5.0 ml, 0.6 mmol Gd) is transferred to a Schlenk flask and the solvent is removed under vacuum. Then 10 ml dichloromethane (CH_2Cl_2) is added for the precipitation of LiCl. The mixture is centrifuged and the clear layer is transferred to another Schlenk flask with the porphyrin free base TFP-TMS (196 mg, 0.2 mmol) dissolved in 20 ml toluene. The resulting solution is refluxed for 12 hours until most of the free base are coordinated with the metal ion. The reaction solution is cooled to room temperature. Then dry $\text{Na}\{(\eta^5\text{-C}_5\text{H}_5)\text{Co}[\text{P}(=\text{O})(\text{Ome})_2]_3\}$ (104 mg 0.22 mmol) is added to the mixture which is magnetically stirred for another 1 hour. After the reaction was complete, the solvent is removed under vacuum and the residue dissolved in CH_2Cl_2 , filtered and chromatographed on silica gel using CH_2Cl_2 /Hexane as eluent to afford the pure product as a red solid. Yield: 86%; MALDI-TOF MS: calcd. For $[\text{M}^+]$: M. p. > 300 °C; 1587.1965, found: 1587.2154; Anal. Calc. For $[\text{C}_{60}\text{H}_{44}\text{CoF}_{15}\text{N}_4\text{O}_9\text{P}_3\text{SiGd}]$: C, 45.40; H, 2.79; N, 3.53%, Found: C, 45.46; H, 2.83; N, 3.51%; UV/Vis (DMSO, 25°C): λ_{max} (log ϵ) = 427 (5.68), 558 (4.34), 597 (3.29 $\text{dm}^3 \text{mol}^{-1}\text{cm}^{-1}$).

[00120] **Gd-OH**: Tetrabutylammonium fluoride (TBAF, 1.0 M in THF, 200 μL , 0.2 mmol) is added to a solution of **Gd-TMS** (182 mg, 0.1 mmol) in 10 ml CH_2Cl_2 , and the solution is stirred for 30 minutes. The progress of the reaction is monitored by TLC. After completion of the reaction, the mixture is passed through a short column of silica gel. After removal of the solvent, the intermediate is obtained and used for the next step without further purification. Then the obtained intermediate and 4-iodophenol (33 mg, 0.15 mmol) are dissolved in dry tetrahydrofuran (THF, 15 ml) and triethylamine (Net_3 , 5 mL), and the mixture is bubbled with nitrogen for 30 minutes. After that, $\text{Pd}(\text{PPh}_3)_4$ (12 mg 0.01 mmol) and CuI (3.8 mg, 0.02 mmol) are added to the above solution. The reaction mixture is stirred at least 35°C for at least 10 hours under a nitrogen atmosphere. Then the solvent is removed under reduced pressure. The residue is purified by column chromatography on silica gel using CH_2Cl_2 /Methanol (50:1) as eluent to afford the pure product as a red solid. Yield: 73% (**Table 2**); M. p. > 300 °C; MALDI-TOF MS: calcd. For $[\text{M}^+]$: 1607.0291, found: 1608.0308; Anal. Calc. For $[\text{C}_{63}\text{H}_{40}\text{CoF}_{15}\text{N}_4\text{O}_{10}\text{P}_3\text{Gd}]$: C, 47.08; H, 2.51; N, 3.49%, Found: C, 47.10; H, 2.49; N, 3.51%; UV/Vis (DMSO, 25°C): λ_{max} (log ϵ) = 426 (5.70), 555 nm (4.48 $\text{dm}^3 \text{mol}^{-1}\text{cm}^{-1}$).

Time (hours) \ Temperature (°C)	10	12	15
35	60	64	69
40	68	73	74
60	59	62	66

Table 2 Yield in different cross-coupling reaction condition(%). Considering both time and temperature, 40 °C and 12 hours is selected as the major reaction condition.

[00121] **Gd-I:** To a solution of **Gd-OH** (161 mg, 0.1 mmol) and tetraethyleneglycol diiodide (207 mg, 0.5 mmol) in dry N,N-Dimethylmethanamide (DMF, 10 ml) is added anhydrous K₂CO₃ (69 mg, 0.5 mmol), and the mixture is heated to 80 °C for 8 hours under a nitrogen atmosphere. Then the solvent is removed under reduced pressure. The crude product is purified by column chromatography on silica gel eluted by CH₂Cl₂/CH₃OH (v/v, 100:1) to afford the pure product as a red solid. Yield: 82%; M. p. > 300 °C; MALDI-TOF MS: calcd. For [M⁺]: 1893.2210, found 1893.1038; Anal. Calc. for [C₇₁H₅₅CoF₁₅IN₄O₁₃P₃Gd]: C, 45.04; H, 2.94; N, 3.11%, Found: C, 45.21; H, 2.99; N, 3.06%; UV/Vis (DMSO, 25°C): $\lambda_{\max}$ (log ϵ) = 425 (5.71), 555 nm (4.50 dm³ mol⁻¹cm⁻¹).

[00122] **Gd-N:** To a solution of **Gd-I** (95 mg, 0.05 mmol) in dry (DMF, 10 ml), anhydrous Net₃ (1ml, excess) is added, and the mixture is heated to 85 °C for 24h under the nitrogen atmosphere. Then the solvent is removed under reduced pressure. The obtained crude product is purified by silica gel column chromatography using CH₂Cl₂/CH₃OH (v/v, 80:1) as the eluent to remove unreacted **Gd-I** and other impurities, then using CH₂Cl₂/CH₃OH (v/v, 10:1) to obtain the pure product as a red solid. Yield: 80%; M. p. > 300 °C; MALDI-TOF MS: calcd. For [M⁺]: 1867.5095, found 1867.2538; Anal. Calc. For [C₉₉H₈₅CoF₁₅N₆O₁₆P₃Gd]: C, 46.37; H, 3.54; N, 3.51%, Found: C, 46.40; H, 3.59; N, 3.48%; UV/Vis (DMSO, 25°C): $\lambda_{\max}$ (log ϵ) = 426 (5.74), 555 nm (4.53 dm³ mol⁻¹cm⁻¹).

[00123] Two-photon-absorption measurements

[00124] The two-photon-absorption spectra (i.e., Z-scan traces) are measured at 800 nm by the open-aperture Z-scan method using 100 fs laser pulses with a peak power of 276 GWcm⁻² from an optical parametric amplifier operating at a repetition rate of 1 kHz generated from a

Ti:sapphire regenerative amplifier system. The laser beam is split into two parts by a beam splitter. One is monitored by a photodiode (D1) as the incident intensity reference, I_0 , and the other is detected as the transmitted intensity by another photodiode (D2). After passing through a lens with $f = 20$ cm, the laser beam is focused and passed through a quartz cell. The position of the sample cell, z , is moved along the direction of the laser beam (z axis) by a computer-controlled translatable table so that the local power density within the sample cell could be changed under the constant incident intensity laser power level. Finally, the transmitted intensity from the sample cell is detected by the photodiode D2. The photodiode D2 is interfaced to a computer for signal acquisition and averaging. Each transmitted intensity datum represents the average of over 100 measurements. Assuming a Gaussian beam profile, the non-linear absorption coefficient, β , can be obtained by curve-fitting to the observed open-aperture traces, $T(z)$, with Equation (1)⁶, where α_0 is the linear absorption coefficient, l is the sample length (the 1 mm quartz cell) and z_0 is the diffraction length of the incident beam. After obtaining the nonlinear absorption coefficient, β , the 2PA cross-section, $\sigma^{(2)}$, of the sample molecule (in units of $1 \text{ GM} = 10^{-50} \text{ cm}^4 \text{ photon}^{-1}$) can be determined by using Equation (2), where N_A is Avogadro's constant, d is the concentration of the sample compound in solution, h is Planck's constant and ν is the frequency of the incident laser beam.

$$(1) \quad T(z) = 1 - \frac{\beta I_0 (1 - e^{-\alpha_0 l})}{2\alpha_0 (1 + (z/z_0))^2}$$

$$(2) \quad \sigma_2 = \frac{1000 \beta h \nu}{N_A d}$$

[00125] **Further embodiments of the present inventions**

[00126] In a further embodiment of the present invention, new generation of PDT agents based on porphyrin-lanthanide complexes with specific functional groups are provided which can specifically localize on particular tumors, and their PDT processes can be monitored via NIR emission from erbium (Er). The newly developed erbium porphyrin complexes are conjugated with integrin $\alpha_v\beta_3$ isoform-specific peptides. The porphyrin and erbium emission from **Er-R₃** show that **Er-R₃** are able to significantly interrupt bladder cancer tumor growth that specific binds to "integrin $\alpha_v\beta_3$ isoform" with responsive emission for imaging.

[00127] The water solubility of Er porphyrin complexes are improved compared with previously reported analogues with conjugation of hydrophilic peptide RrRk (SEQ ID NO: 4).

The integrin $\alpha_v\beta_3$ isoform specific peptide sequence (-cGRLKEKKc-) (SEQ ID NO: 5) is chosen to conjugate with RrRk (SEQ ID NO: 4) in different positions for the estimation of binding selectivity to integrin $\alpha_v\beta_3$ isoform in bladder cancer cells (Scheme 3). The amphiphilic character of the peptides is synthesized with the combination of hydrophilic RrRk (SEQ ID NO: 4) and hydrophobic cGRLKEKKc (SEQ ID NO: 5) to improve the cell permeability. The absorption coefficient (Porphyrin: Soret Band at 430 nm, $199,526 \text{ cm}^{-1}$) and emission quantum yield (Porphyrin: Soret Band and Er: $^2F_{5/2} \rightarrow ^2F_{7/2}$) of **Er-R₁**, **Er-R₂** and **Er-R₃** are similar. The details of photophysical measurement of **Ln-R_n** are shown in the **Table 3**. The Er moiety demonstrates stronger singlet oxygen quantum efficiency than Yb moiety due to the energy transfer from porphyrin Yb for f-f emission which much better than from porphyrin to Er f-f emission. All of the **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes are characterized by ^1H NMR and mass spectrometry (**Figure 17-36**).

Table 3 . Photophysical properties Summary of **Ln-R_n** (Ln=Yb, Er, n=1,2,3)

Compound	Absorption (λ_{max})[nm] Log($\epsilon[\text{dm}^3 \text{mol}^{-1} \text{cm}^{-1}]$) ^a	Excitation (λ_{exc})[nm]	Emission(λ_{em}) [nm] ^a (Φ_{em}, τ) ^{b,c}	Φ_{Δ} ^d
Yb-R ₁	425 (5.37), 554 (4.09)	430	656,712 (0.012) 975 (29.86 s)	Not Found
Yb-R ₂	425 (5.34), 554 (4.16)	430	56,712 (0.013) 975 (30.08 s)	Not Found
Yb-R ₃	425 (5.27), 554 (4.04)	430	656,712(0.013) 975 (29.97 s)	Not Found
Er-R ₁	426 (5.32), 554 (4.05)	430	654 ,715 (0.014), 1531	0.11
Er-R ₂	426 (5.50), 554 (4.53)	430	654 ,715 (0.014), 1531	0.12
Er-R ₃	426 (5.36), 554 (4.24)	430	654 ,715 (0.015), 1531	0.12

[a] Absorption and Emission are measured in water (3% DMSO) at room temperature. [b] The emission quantum yield standard used in this study is tetraphenylporphyrin (H₂TPP) in anhydrous DCM ($\Phi_{\text{em}} = 0.120$ at 298 K). [c] lifetime were measured in water (3% DMSO) at room temperature [d] The Singlet oxygen quantum yield standard used in this study was tetraphenylporphyrin (H₂TPP) in anhydrous DCM ($\Phi_{\Delta} = 0.62$ at 298 K).

In Figure 12, the photophysical properties of Er or Yb porphyrin based complexes are similar. However, the in vitro subcellular localization, uptake and toxicity (light and dark) are different due to the conjugation of the peptides. First of all, the subcellular localization of Er-R_n

porphyrin complexes and Yb-Rn porphyrin complexes (Ln= Er or Yb; n = 1, 2 and 3; R1= cQDGRMGFc={ Ahx }-(D-Cys)-Gln-Asp-Gly-Arg-Met-Gly-Phe-(D-Cys) (SEQ ID NO: 1); R2= cGRLKEKKc = { Ahx }-(D-Cys)-Gly-Arg-Leu-Lys-Glu-Lys-Lys-(D-Cys) (SEQ ID NO: 2); R3= RrRkcGRLKEKKc= { Ahx }-Arg-Arg-(D-Arg)-Lys-{ Ahx }-(D-Cys)-Gly-Arg-Leu-Lys-Glu-Lys-Lys-(D-Cys) (SEQ ID NO: 3) in bladder cancer cells-T24 and -5637, cervical cancer cells – HeLa and normal lung normal cells-MRC5 are different (Figure 13, dosed concentration = 5 μ M, incubation time = 6 hours). The in vitro fluorescent intensity of three erbium porphyrin complexes is higher than its ytterbium motif analogue due to efficient energy transfer from the porphyrin molecules to the Yb³⁺ ion and emits Yb near-infrared fluorescence. In bladder cancer cells T24 and 5637, red porphyrin emission from Er-R1 is found only on the cell membrane, however, the red emission of Er-R2 and Er-R3 are found inside the cells. Ytterbium analogues also show the same subcellular localization; emission of porphyrin Yb-R1 is found in the cell membrane. The co-localization experiments have been done with green Lyso tracker, the red emission from Er-R2, Er-R3, Yb-R2 and Yb-R3 overlap well with the green fluorescence from Lyso Tracker in T24 and 5637 cells in Figure 37, but Er-R1 and Yb-R1 are not, indicating the Er-R2 porphyrin complexes, Er-R3 porphyrin complexes, Yb-R2 porphyrin complexes and Yb-R3 porphyrin complexes are mostly localized in the lysosome of T24 cells and 5637 cells and Er-R1 and Yb-R1 are localized in T24 and 5637 cell membrane. To further confirm, the peptides sequence in SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3 can recognize the $\alpha_v\beta_3$ integrin. The in vitro imaging of Er-Rn porphyrin complexes and Yb-Rn porphyrin complexes (n = 1, 2 and 3) have been done in the non-bladder cancer cells, HeLa and MRC-5 under the same experimental condition. Red emission signal is detected in neither HeLa cells nor MRC-5. The lack of $\alpha_v\beta_3$ integrin in HeLa and MRC-5 cells should limit the uptake of Er-Rn and Yb-Rn. The porphyrin complexes Er-Rn and Yb-Rn (n = 1, 2 and 3) will not bind to HeLa and MRC-5 cells, so only green emission signal from Lyso tracker is shown in fluorescent staining experiment (Figure 37).

[00128] To further prove that the selective uptake of Er-Rn and Yb-Rn (n = 1, 2 and 3) porphyrin complexes in bladder cancer cells was induced by the recognition of the $\alpha_v\beta_3$ integrin on the surface of the T24 through the tailor-made cyclic peptide R1, R2 and R3, the flow cytometry analysis of erbium (Er) and ytterbium (Yb) complexes in three cell lines is performed and the results are shown in the Figure 14.

[00129] Molecular docking simulates the present porphyrin complexes and provides great steric hindrance to the peptides and facilitates interaction with the $\alpha_v\beta_3$ integrin. Zhang et al. (Urologic Oncol. 2012, 30, 635-645) have tested the peptides **R₁** and **R₂** of the complexes of the subject invention in different cell lines and screened through the OBOC combinatorial library to demonstrate the binding specificity towards bladder cancer. The amphiphilic peptide **R₃** is the modification of **R₂** by adding RrRk (SEQ ID NO: 4) to improve water solubility and cellular uptake. Therefore, as shown in Figure 14, the T24 cells display significant fluorescence within a 6 hours incubation of **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes in FL3 channel (Emission filter: 670 long-pass filter), whereas the HeLa and MRC-5 (cell surface $\alpha_v\beta_3$ integrin receptor-negative) incubated with **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes show little fluorescence signal under similar experimental condition. In addition, the cellular uptake increases along with the incubation time in T24 cells which is quantified as median fluorescence intensity after 24-hour (Table 4).

Median fluorescence intensity	T24	HeLa	MRC-5
Er-R ₁	11.80	4.86	4.07
Yb-R ₁	6.68	4.31	3.56
Er-R ₂	21.21	4.11	3.09
Yb-R ₂	14.07	4.21	3.02
Er-R ₃	40.52	4.13	3.59
Yb-R ₃	26.07	3.36	3.33

Table 4. Summary of **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes median fluorescence intensity in T24, HeLa and MRC-5 cells incubated for 24 hours.

[00130] After verification of specific uptake of **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes into T24 cells, *in vitro* PDT in various cell lines are carried out. Low-dark and high-light cytotoxicity are the necessary properties of a photosensitizer for the application in PDT. The cytotoxicity of **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes to T24, HeLa and MRC-5 cells are examined in both presence (550 nm long-pass filter, 6mW cm⁻², 28 minutes) and absence of irradiation using MTT assay. **Er-R_n** and **Yb-R_n** exhibit high photo-cytotoxicity under irradiation of 10 Jcm⁻². Furthermore, the photo-cytotoxicity increased along with the concentration of **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes with a half lethal dose (IC₅₀) calculated in Figure 15 after incubation for 24 hours. The IC₅₀ of **Er-R_n**

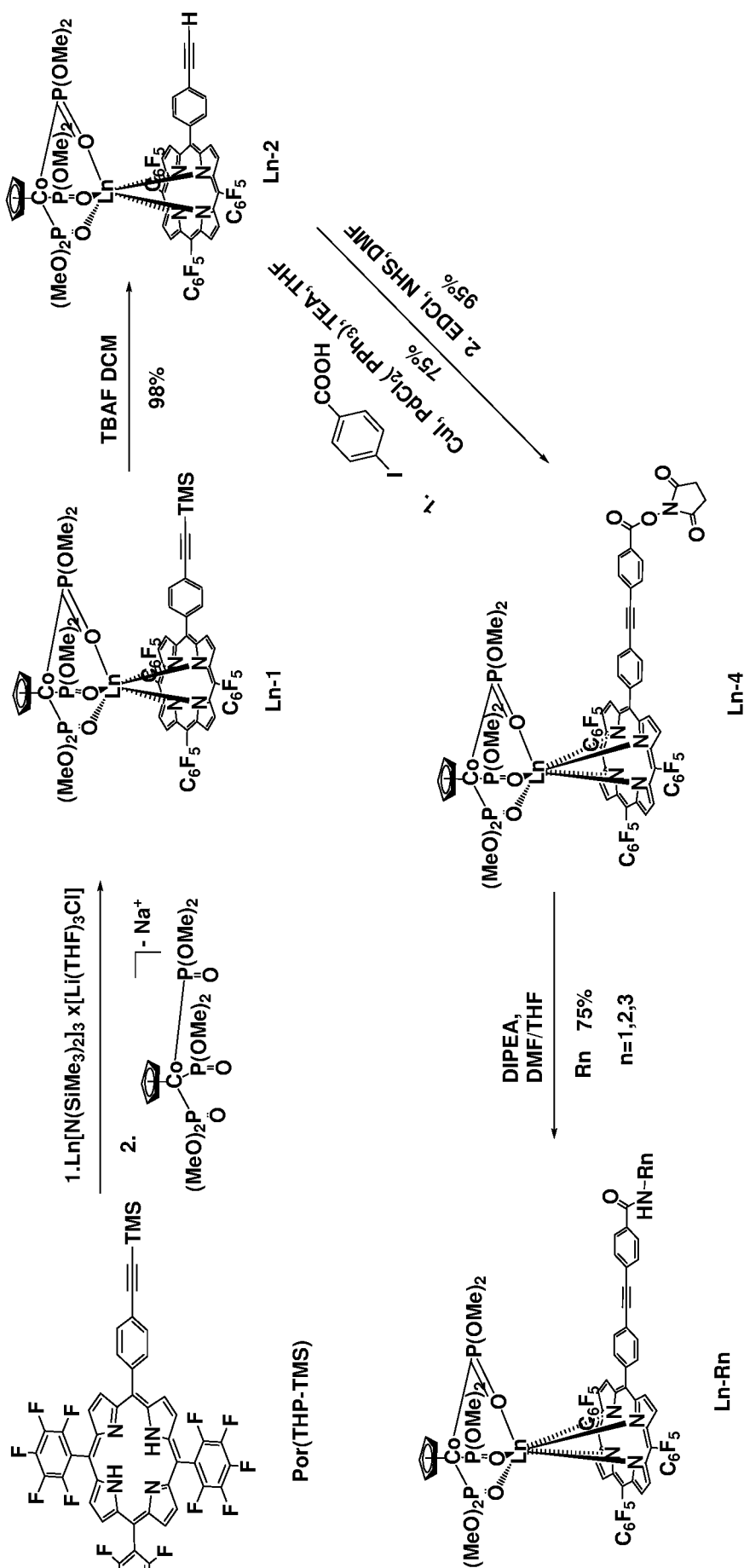
porphyrin complexes and **Yb-R_n** porphyrin complexes to T24 is 8 to 10 times lower than HeLa and MRC-5 which demonstrates **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes selectively kill bladder cancer. Attributed from the RrRK (SEQ ID NO:4) peptide sequence in **Er-R₃** porphyrin complexes and **Yb-R₃** porphyrin complexes, the cellular uptake of them is higher than **Er-R₁** porphyrin complexes, **Er-R₂** porphyrin complexes, **Yb-R₁** porphyrin complexes and **Yb-R₂** porphyrin complexes which lead to higher photo-cytotoxicity. Moreover, the excitation wavelength 550nm is located in the Q band of porphyrin which would provide better tissue penetration in practice. However, it cannot trigger efficient PDT effect comparable to the FDA approved Aminolevulinic acid, (ALA). ALA is excited at 400-450 nm. In some embodiments of the present invention, **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes cause more intense photo-cytotoxic effect than ALA when excited beyond 550 nm. Among all of the **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes, **Er-R₃** porphyrin complexes kill bladder cancer cells mostly effectively (IC₅₀ as low as 31 μM can be reached) due to brightest in vitro fluorescence and highest cellular uptake. However, in the absence of light, all of the **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes are basically non-cytotoxic. (IC₅₀ over 1000μM) Based on the results above, **Er-R₃** porphyrin complexes is of the preferred embodiment for PDT agent to selectively kill bladder cancer.

[00131] In summary, the present invention provides a multi-modal lanthanide-porphyrin PDT agent that is capable of killing the tumor cells via ¹O₂ from porphyrin moiety and affording the fluorescence imaging simultaneously. **Er-R₃** porphyrin complexes are synthesized and shown to be highly selective to bladder cancer cells by specific targeting integrin α_vβ₃ isoform in bladder cancer cells with strong NIR and ¹O₂ emission. The cancer cells selectivity uptake property of the porphyrin complexes of the present invention is confirmed by flow cytometry and in-vitro imaging and is able to significantly interrupt the bladder cancer cells growth with specific binding to “integrin α_vβ₃ isoform” of bladder cancer cells.

[00132] **General Information about the compound synthesis.**

[00133] All chemicals used are of reagent – grade and are purchased from Sigma – Aldrich and used without further purification. All analytical – grade solvents are dried by standard procedures, distilled and deaerated before use. NMR spectra are recorded on a Bruker Ultra shield 400 Plus NMR spectrometer. The ¹H NMR chemical shifts are referenced to tetramethylsilane, TMS (δ = 0.00). High – resolution mass spectra, reported as m/z, are obtained

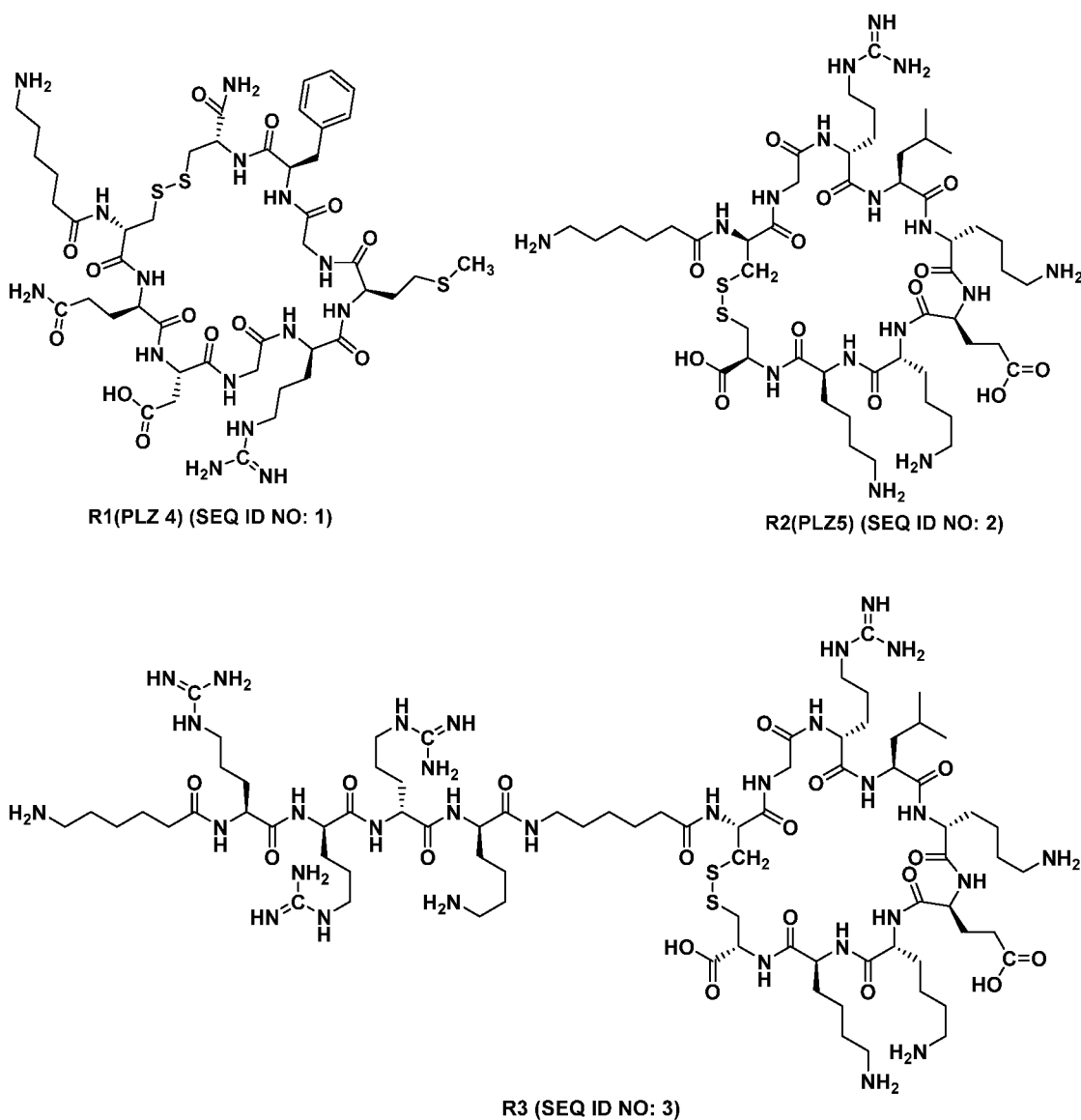
on a Bruker Autoflex MALDI – TOF mass spectrometer. The synthetic Route of intermediates and Ln-Rn (Ln=Yb,Er, n=1,2,3) are shown in Scheme 2. All the Ln-Rn (Ln=Yb or Er, n=1,2,3) complexes are purified by High Performance Liquid Chromatography. The solvent system was shown in Table 5.

Scheme 2. The synthetic scheme of Ln-Rn. (Ln=Yb or Er, n=1,2,3)

R1=cQDGRMGFc (SEQ ID NO: 1)

R2=cGRLKEKKc (SEQ ID NO: 2)

R3=RrRkcGRLKEKKc (SEQ ID NO: 3)



Scheme 3. The molecular structures of bladder cancer peptides.

[00134] **Synthesis of the intermediates and Ln-R_n** (Ln=Yb or Er, n=1,2,3)

[00135] **Preparation of compound Por(THP-TMS)**

[00136] Pyrrole (280uL, 4.0mmol), pentafluorobenzaldehyde (588mg, 3.0 mmol) and 4-[2-(trimethylsilyl)ethynyl]benzaldehyde 6 (202 mg, 1.0 mmol) are dissolved in 410mL CH₂Cl₂ under an argon atmosphere. After 10 minutes BF₃O(Et)₂ (0.60mL of 2.65M stock solution, 1.32 mmol) is added *via* syringe with vigorous stirring. After addition is complete, the reaction is left to stir for 1 hour at room temperature. DDQ (2,3-

Dichloro-5,6-dicyano-1,4-benzoquinone) (0.68g, 3.0mmol) is added and after 1 hour stirring at room temperature the solvent is removed in vacuo. The crude reaction mixture is passed through a short silica column (hexanes-CH₂Cl₂ (9:1)) concentrated under reduced pressure, to give product 5,10,15-Tris(pentafluorophenyl)-20-[4-{2-(trimethylsilyl)ethynyl}phenylporphyrin], a pink/purple solid (238 mg, 22.8%); ¹HNMR (CDCl₃): -2.87 (2 H, s, NH), 7.91 (2 H, d, *J* 8.1 Hz, Ar-H), 8.17 (2 H, d, *J* 8.1 Hz, Ar-H), 8.89 (2 H, d, *J* 4.7 Hz, P-pyrrole), 8.932 (4 H, s, P-pyrrole), 8.94 (2 H, d, *J* 4.7 Hz, P-pyrrole); 0.387 (9H, s) MS (MALDI) for [M]⁺, calcd. for C₄₉H₂₃F₁₅N₄Si 980.1513, found 981.1519.

[00137] **Preparation of compound Ln-1**

[00138] Ln[N(SiMe₃)₂]₃ · x[LiCl(THF)₃]: HN(SiMe₃)₂ (Ln = Yb or Er, 10.8 ml 0.050 mol) is dissolved in 20ml of THF in ice bath, then n-BuLi (1.6 M in hexane) is added slowly over 30-minutes period. The resulting solution is magnetically stirred for 12 hours until a clear pale yellow solution is obtained. Then the solution is transferred slowly to a Schlenk flask with LnCl₃ (Ln = Yb or Er, 4.74 g, 0.017mol) suspended in 20 ml THF. The resulting mixture is magnetically stirred for 24 hours until all of the solid LnCl₃ (Ln = Yb or Er,) disappear. The resultant solution Ln[N(SiMe₃)₂]₃ · x[Li(THF)₃Cl] (Ln = Yb or Er, x = 3 ~ 5) is used for next synthetic step.

[00139] **Yb-1:** Yb[N(SiMe₃)₂]₃ · x[Li(THF)₃Cl] (2.5 ml, 0.52 mmol of Yb) as prepared above is transferred to a Schlenk flask and the solvent is removed under vacuum. Then 10ml CH₂Cl₂ is added, for the precipitation of LiCl. The mixture is centrifuged and the clear layer is transferred to another Schlenk flask with dry Por(THP-TMS) (0.1g, 0.16mmol) dissolved in 15ml toluene. The resulting solution is refluxed until most of the free base coordinated with the metal ion. Then, dry NaLOMe (0.1 g, 0.22 mmol)[LOMe-((cyclopentadienyl)tris(dimethylphosphito)-cobaltate, an anionic tripodalligand) is added and magnetically stirred for another 12 hours before the reaction solution is cooled down to room temperature. Upon completion of the reaction, the solvent is removed in vacuum and the residue dissolved in CHCl₃, filtered and chromatographed on silica gel using

CHCl_3 /petroleum ether (V/V 1:1) as eluent. The product is dissolved in CH_2Cl_2 (5 ml) and the solution is filtered.

[00140] **Yb-1:** Yield: 81%; ^1H NMR (CDCl_3): δ -5.02(s, 5H), 0.93 (s, 9H), 6.37 (s, 18H), 8.70 (s, 1H), 8.97 (d, $J = 4.96$ Hz, 1H), 10.88 (s, 1H), 14.65 (s, 2H), 14.89(s, 2H), 15.18 (s, 2H), 15.58 (s, 2H), 17.40 (s, 1H); MALDI-TOF MS: calcd. For $\text{C}_{60}\text{H}_{44}\text{CoF}_{15}\text{N}_4\text{O}_9\text{P}_3\text{SiYb}$ $[\text{M}]^+$ 1603.0571, found: 1603.0556.

[00141] **Er-1:** The same procedure with Yb-1, replace $\text{Yb}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$ with $\text{Er}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$; Yield: 80%. ^1H NMR (CDCl_3): δ -35.54 (s, 5H), 3.48 (s, 9H), 14.09(s, 1H), 13.50 (s, 1H), 21.73 (s, 18H), 21.16 (s, 1H), 31.22 (s, 2H), 32.93 (s, 2H), 36.37 (s, 2H), 37.76 (s, 2H), 46.77 (s, 1H); MALDI-TOF MS: calcd. For $\text{C}_{60}\text{H}_{44}\text{CoErF}_{15}\text{N}_4\text{O}_9\text{P}_3\text{Si}$ $[\text{M}]^+$ 1597.1878, found 1597.2927.

[00142] **General procedure for the preparation of Ln-2**

[00143] **Yb-2:** TBAF (1.0 M in THF, 0.2 mL, 0.2 mmol) is added to a solution of Yb-1 (0.05 mmol 76.55mg,) in 10 ml CH_2Cl_2 , and the solution is stirred for 30min. The progress of the reaction is monitored by Thin-layer Chromatography (TLC). After completion of the reaction, the mixture is passed through a short of silica gel column. After removal of solvent, pure product is obtained.

[00144] **Yb-2:** Yield: 92%; ^1H NMR (CDCl_3): δ -4.82 (s, 5H), 4.13 (s, 1H), 6.30 (s, 18H), 8.63(s, 1H), 8.95 (d, $J = 4.44$ Hz, 1H), 10.83 (s, 1H), 14.51 (s, 2H), 14.90(s, 2H), 15.08 (s, 2H), 15.44 (s, 2H), 17.21 (s, 1H); MALDI-TOF MS: calcd. For $\text{C}_{57}\text{H}_{36}\text{CoF}_{15}\text{N}_4\text{O}_9\text{P}_3\text{Yb}$ $[\text{M}+\text{Na}+\text{Cl}]^+$ 1587.0176, found 1587.0514.

[00145] **Er-2:** The same procedure with Yb-2, replace Yb-1 with Er-1; Yield: 92%; ^1H NMR (CDCl_3): δ -35.05 (s, 5H), 13.94(s, 1H), 13.19 (s, 1H), 20.56 (s, 18H), 21.02 (s, 1H), 30.97 (s, 2H), 32.77 (s, 2H), 36.44 (s, 2H), 37.36 (s, 2H), 46.20 (s, 1H); MALDI-TOF MS: calcd. For $\text{C}_{57}\text{H}_{36}\text{CoErF}_{15}\text{N}_4\text{O}_9\text{P}_3$ $[\text{M}+\text{H}]^+$: 1525.0067, found: 1525.0143.

[00146] **General procedure for the preparation of Ln-4**

[00147] **Yb-4** :Pd(PPh₃)₄ (22.16 mg 0.08 mmol), CuI (7.65 mg, 0.04 mmol), Yb-2 (30.62. mg, 0.02mmol)and 4-iodobenzoic acid 5.087mg are placed in a dried flask and under nitrogen. THF (15 mL) and Net₃ (5 mL) are added and the reaction mixture degassed with nitrogen. The reaction mixture is stirred at 40 °C for 12 hours. After that, the solvent is removed under reduced pressure. The residue is purified by chromatography. Elution with CH₂Cl₂/Methanol (12:1). The eluted compound (26mg, 0.0157mmol), EDCI (6.04mg, 0.031mmol), NHS (3.57mg, 0.031mmol) are placed in a dried flask and under nitrogen,10mL dry DMF is added. Stirred at room temperature for 48 hours, then remove the solvent. The residue is recrystallized by diethyl ether and dried to give **Yb-4**.

[00148] **Yb-4**: Yield: 72%; ¹HNMR (CDCl₃): δ -4.82 (s, 5H), 4.16 (s, 1H), 6.39 (s,18H), 8.68 (s, 1H), 8.98 (d, *J* = 4.44 Hz, 1H), 8.47 (s, *J* = 4.44 Hz, 2H), 8.45 (s, *J* = 4.44 Hz, 2H) 10.91 (s, 1H), 14.63 (s, 2H), 14.92 (s, 2H), 15.24 (s, 2H), 15.61 (s, 2H), 17.39 (s, 1H); MALDI-TOF MS: calcd. For C₆₈H₄₃CoF₁₅N₅O₁₃P₃Yb [M]⁺1748.0176,found 1748.0460. HPLC characterization: retention time = 7.24min (Figure 16 (A)).

[00149] **Er-4**: The same procedure with Yb-4, only replace Yb-2 with Er-2; Yield: 80%; ¹HNMR (CDCl₃): δ -35.94 (s, 5H), 6.04 (s, 1H), 8.64 (d, *J* = 7.96 Hz, 2H),10.80 (d, *J* = 5.12 Hz, 2H), 13.12 (s, 1H), 13. 76 (s, 1H), 20.67 (s, 18 H), 20.90 (s,1H), 31.06 (s, 2H), 32.94 (s, 2H), 36.39 (s, 2H), 37.62 (s, 2H), 46.54 (s, 1H);MALDI-TOF MS: calcd. For C₆₈H₄₃CoErF₁₅N₅O₁₃P₃ [M+Cl]⁺ : 1777.2035, found: 1777.4591. HPLC characterization: retention time = 7.23 min(Figure 16 (B)).

[00150] **General procedure for the preparation of Ln-Rn**

[00151] **Yb-R₁** : A stirred solution of Yb-4(16mg, 1equiv.) in anhydrous DMF is mixed with N,N'-diisopropylethylamine (DIPEA) (1 equiv.). The mixture solution is added into a vial which contains peptide R₁ (1.3 equiv.) It was then reacted at room temperature overnight, after that, the solvent is removed under vacuum to get the dry

compound. The residue is recrystallized by diethyl ether three times and dried to give **Yb-R₁**.

[00152] **Yb-R₁**: Yield: 69%. MALDI-TOF MS: calculated for C₁₀₉H₁₀₉CoF₁₅N₁₉O₂₃P₃S₃Yb [M+H]⁺: 2760.4878 found: 2760.6458. HPLC characterization: retention time = 10.00 min (Figure 16 (C)).

[00153] **Yb-R₂**: The same procedure with Yb-R₁, peptide R₂ is used; Yield: 69% MALDI-TOF MS: calculated for C₁₁₃H₁₂₉CoF₁₅N₂₁O₂₂P₃S₂Yb [M+H]⁺ 2808.6835 found: 2808.6715. HPLC characterization: retention time = 10.21 min (Figure 16 (D)).

[00154] **Yb-R₃**: The same procedure with Yb-R₁, peptide R₃ is used; Yield: 65% MALDI-TOF MS: calculated for C₁₄₃H₁₈₇CoF₁₅N₃₅O₂₈P₃S₂Yb [M+H]⁺ 3520.2985 found: 3520.2543. HPLC characterization: retention time = 10.01 min (Figure 16 (E)).

[00155] **Er-R₁**: The same procedure with Yb-R₁, replace Yb-4 with Er-4; Yield: 75% MALDI-TOF MS: calculated for C₁₀₉H₁₀₉CoErF₁₅N₁₉O₂₃P₃S₃ [M+K]⁺: 2791.4826. found: 2791.3747. HPLC characterization: retention time = 9.66min (Figure 16 (F)).

[00156] **Er-R₂**: The same procedure with Yb-R₂, replace Yb-4 with Er-4; Yield: 72% MALDI-TOF MS: calculated for C₁₁₃H₁₂₉CoErF₁₅N₂₁O₂₂P₃S₂ [M+K]⁺: 2839.6015 found: 2839.2967. HPLC characterization: retention time = 10.09 min (Figure 16 (G)).

[00157] **Er-R₃**: The same procedure with Yb-R₃, replace Yb-4 with Er-4; Yield: 70% MALDI-TOF MS: calculated for C₁₄₃H₁₈₇CoErF₁₅N₃₅O₂₈P₃S₂ [M]⁺: 3511.4955 found: 3511.5162. HPLC characterization: retention time = 9.80 min (Figure 16 (H)).

[00158] **Cell culture**

[00159] Human bladder carcinoma (T24) and (5637) cells are cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotics (penicillin, 50gmL⁻¹; streptomycin, 50gmL⁻¹). Human cervical carcinoma

(HeLa) cells are cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco) and antibiotics (penicillin, 50gmL⁻¹; streptomycin, 50gmL⁻¹). Human normal lung fibroblast (MRC-5) cells are maintained in minimum essential medium (MEM) supplemented with 10% FBS and 1% 50gmL⁻¹ penicillin; 50gmL⁻¹ streptomycin. All cells are incubated at 37°C in a humidified environment with 5% CO₂.

[00160] Dark cytotoxicity

[00161] T24, HeLa and MRC-5 cells (1×10^5) are treated with **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes for 24 hours at six concentrations (1, 5, 10, 50, 100, 500M). The cell monolayers are rinsed once with phosphate-buffered saline (PBS) and incubated with 500gmL⁻¹ 3-(4, 5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide (MTT) solution. The cellular inhibitory potency of the complexes is examined by treating the cells with MTT for 3 hours to allow formazan production during cell metabolism. After that, the formazan crystals are fully dissolved in DMSO with oscillation. Finally, the absorbance of solution is measured with Biotek PowerWave XS microplate reader at the wavelengths of 570 and 690 nm.

[00162] Photo-cytotoxicity

[00163] T24, HeLa and MRC-5 cells (1×10^5) are treated with **Er-R_n** porphyrin complexes and **Yb-R_n** porphyrin complexes for 24 hours at four concentrations (1, 5, 10, 50M). Then, the cells are irradiated at 6mWcm⁻² (equipped with 550 nm long pass filter) for about 27 minutes and further incubated for 24 hours. The cells are then treated according to the same protocol as the previous MTT assay.

[00164] In vitro confocal microscopy

[00165] To investigate the suitability of the obtained complexes as bioprobes, T24, 5637, HeLa and MRC-5 cells (1×10^5) are imaged. After incubation with the complexes at 5M for 24 hours, the cells are washed with PBS for three times before imaging. LysoTracker Green DND-26 was used as costaining dye. Images were acquired on a Leica TCS SPE confocal laser-scanning microscope. The samples and LysoTracker were excited at wavelength of 561 and 488 nm respectively.

[00166] Flow cytometry measurements of cellular uptake

[00167] 5637, T24, HeLa and MRC-5 cells (1×10^5 per sample) are seeded onto 35 mm Petri dishes and incubated overnight. Then the cells are incubated with the **Er-R_n** and **Yb-R_n** porphyrin complexes (5M) for 3, 6 and 24 hours. Cells are harvested with trypsin and washed twice with PBS. The uptake of the complexes by the 5637, T24, HeLa and MRC-5 cells is analyzed by flow cytometry. The cells are excited with a 488 nm argon laser and emission is collected in the FL-3 channel (with a 650 nm long-pass filter); 10000 events are analyzed.

[00168] **HPLC characterization of the complexes.**

[00169] **Table 5.** Solvent gradient for HPLC

Time /min	0.05% TFA in water /%	MeOH /%
0	50	50
5	20	80
20	0	100

[00170] **Another further embodiment of the present invention.**

[00171] **i. Development and comprehensive studies of various organometallic/lanthanide complexes for biological studies**

[00172] Another series of organometallic complexes are provided in the present invention and their structure-photophysical property relationship in multi-photon and non-linear processes are studied. These complexes are applicable for biological imaging. Water-soluble lanthanide(III) porphyrinate complexes and *meso*-pyridinium-substituted porphyrin are obtained by methylation of the corresponding pyridyl complexes with methyl iodide and unambiguously characterized. The binding interactions and photocleavage activities of these water-soluble lanthanide(III) porphyrinate complexes towards DNA are investigated (Figure 38A). Additionally, the inventors reported a new water-soluble, mitochondria-specific porphyrinato Yb(III) complex (Figure 38B) capped with a tripodal $[(\eta^5\text{-C}_5\text{H}_5)\text{Co}\{(\text{MeO})_2\text{P=O}\}_3]^-$ anion which showed a remarkable NIR emission quantum yield in water.

[00173] **ii. Development of organometallic complexes as *in vivo* tumor specific PDT agents**

[00174] The present invention provides another set of novel organelle specific markers (for lysosome, mitochondria, Golgi apparatus). These complexes *in-vitro* simultaneously trigger the generation of $^1\text{O}_2$ *in-vitro* and give luminescent images of the organelles upon irradiation by visible/NIR excitation. Such behavior affords spatial control using dual laser excitations to damage selected cell compartments/components. Previously, the inventors reported a porphyrinato ytterbium complex which shows a strong binding to phosphatidylserine and the capability to differentiate of cancer cells via targeting the anionic phospholipid membrane (Figure 39A) and recently, its motif structure (Gd-N) has demonstrated the availability as *in vivo* tumor specific PDT agents. (Figure 39B).

[00175] In the present invention, another set of multi-modal porphyrinato lanthanide-based complexes for biological imaging are provided — NIR optical and t_1 magnetic resonance imaging — that bind strongly to the targets, phospholysation anionic membrane/ integrin $\alpha_v\beta_3$ isoform, and generates $^1\text{O}_2$ as anticancer agents. The present invention provides (1) complexes for use as dual probe for optical and MRI **imaging** and **cancer specific PDT effect**, via the bio-conjugation with the known functional groups and peptides, and $^1\text{O}_2$ from porphyrin moieties; (2) *in vitro* anticancer effects via optical imaging and other typical protocols; (3) *in vivo* pharmacokinetics and bio-distribution of these complexes (with anticancer effects) by MRI imaging/ICPMS.

[00176] Herein, the inventors have designed and synthesized 4 water-soluble, cell-permeable porphyrin-based gadolinium complexes, Gd-1, Gd-2, Gd-3-R₁, Gd-3-N (Figure 40), which has the same general chemical formula as Figure 12 a). These complexes for use as multi-modal PDT agents are examined (1. Bio-stability – P_M; 2. PDT and in-situ imaging – $^1\text{O}_2$ and emission quantum yield; 3. MR imaging – t_1 relaxivity; and 4. Cancer or bladder cells specific –*ex vivo* toxicity)

[00177] (a) **The stability, solubility and uptake profile of gadolinium complexes, Gd-1, Gd-2, Gd-3-R₁, Gd-3-N**

[00178] The protonation of the substituent groups on the porphyrin of Gd-1, Gd-2, Gd-3-R₁, Gd-3-N demonstrates an improvement of the water solubility. All the complexes have been purified by HPLC. The organometallic system **Gd-2, Gd-3-R₁** and **Gd-3-N** show better stability than **Gd-1**, with the P_m values of **Gd-2, Gd-3-R₁** and **Gd-3-N** being around 8.15. P_m value is the negative log of the concentration of free metal ion left uncomplexed by a given chelator, $pM = -\log[M]_{\text{free}}$. The cellular uptake profile from flow cytometry also shows that **Gd-3-R₁** has the fastest uptake rate among the four complexes in the cancer cells and also with the better selectivity towards bladder cancer T24 cells rather than normal MRC-5 cells (Figure 41).

[00179] (b) **The $^1O_2$ and NIR emission quantum yield**

[00180] Upon protonation of the complexes, similar $^1O_2$ and emission quantum yield are shown and compared with the existing complex, H₂TPP, which are around ~70% $^1O_2$ quantum yield (Figure 42) and 46% emission quantum yield with the excitation at 430 nm respectively

[00181] (c) **The t_1 relaxivity and *ex vivo* toxicity /distribution of Gd-1, Gd-2, Gd-3-R₁, Gd-3-N**

[00182] With an aim to develop them as MR contrast agents, the water exchange rate of the complexes is one of key preliminary studied. The t_1 relaxivity of **Gd-1, Gd-2, Gd-3-R₁, Gd-3-N** are evaluated and **Gd-3-R₁** has three times t_1 relaxivity greater than Gd-DOTA (Figure 43). The effectiveness *ex vivo* tumor toxicity of **Gd-3-R₁** has been compared with the control (**Gd-2**) (Figure 44).

[00183] (d) **The *in vivo* magnetic resonance imaging (MRI)**

[00184] After tail vein injection of **Gd-3-R₁** and Gd-DOTA into the mice, MRI experiment is conducted immediately. As shown in Figure 49A and 49B, the contrast effect of **Gd-3-R₁** on the xenograft tumor (T24 bladder cancer cells) is enhanced

significantly and specifically, while the Gd-DOTA enhances the whole body signal without selectivity toward the tumor.

[00185] Multi-modal lanthanide-based bio-probes with integrin $\alpha_v\beta_3$ isoform, specific peptide coatings as a simultaneous imaging (optical and MR) and anti-cancer agent *in vitro* and *in vivo*.

[00186] **Task 1- Synthesis of “smart” organometallic complexes that can selectively differentiate tumor cells for effective PDT and available for MR and optical imaging**

[00187] There are two major problems associated with commercially or literature available photosensitizers in photodynamic treatment: (i) the recognition of cancer cells and (ii) the monitoring of their effectiveness. Studies show that newly developed lanthanide complex, **Gd-3-R₁**, is able to identify cancer cells through its anionic PS membrane, generate $^1\text{O}_2$ with certain laser wavelength, and display two-photon induced NIR emissions and MR availability. The inventors extend their previous findings and run a comprehensive program to obtain new lanthanide complexes (on top of cancer cell selectivity with better $^1\text{O}_2$ yield) as a new generation of PDT agents specifically for cancer disease, especially for bladder cancer. The new development agents are able to treat tumors that are deeper under the skin or in body tissues, more selective for cancer cells as opposed to normal cells and removed from the body more quickly, reducing the time people need to worry about photosensitivity reactions. **Selection criteria for the best photosensitizers.** There are a number of criteria for the selection of photosensitizers. First of all, it must be water soluble. The photosensitizer(s) should be able to be excited in the near infrared region, especially between 800 nm to 900 nm. Also, the $^1\text{O}_2$ quantum yield of the best photosensitizers should be >20% and with specific mitochondria subcellular localization. The photocytotoxicity of valid bladder cancer specific photosensitizer (i.e. LC₅₀ in 1J laser dosed is 1M) must be 100 times lower than dark cytotoxicity. (IC₅₀ have to be > 0.1mM).

[00188] **Absorption and fluorescence details of porphyrin-based compounds**

[00189] Porphyrin is a highly conjugated molecule with 11 delocalized double bonds. The electronic absorption bands of metalloporphyrins are found at $\sim 410\text{-}430\text{ nm}$ (the Soret band or B band) and $550\text{-}650\text{ nm}$ (Q bands) with strong one photon absorption coefficients ($> 100\text{K M}^{-1}\text{cm}^{-1}$). The emission band of porphyrin is always located at $\sim 650\text{ to }700\text{ nm}$. As a result, the emission and excitation bands of metalloporphyrins are always located within biological windows. In addition, the strong two photon absorption cross section of metalloporphyrins are recorded with $>100\text{ GM}$, which indicates that the porphyrin can be excited at 860 nm and give two photon induced emission at $650\text{ nm to }700\text{ nm}$ for molecular imaging, as well as generation of $^1\text{O}_2$ and PDT.

[00190] **1.1 Synthesis of water-soluble, highly emissive, MR and $^1\text{O}_2$ available porphyrin-based gadolinium (III) complexes which target anionic phospholipid membrane**

[00191] Water-soluble porphyrin-based gadolinium complex (**Gd-N**) exhibits strong selectivity toward the cancer cell membrane, emits strong visible-to-NIR emission, and undergoes high metabolism that can be removed from the body quickly. To improve the relaxivity and stability of the complexes as multi-modal agents, the present invention provides two more types of organometallic complexes in which the Gd ions are stabilized by other organometallic compounds (**Gd-4-R_n**) or with carboxylic pendant arm (**Gd-5-R_n**) (Figure 45 and 46).

[00192] **1.2 Synthesis of water-soluble, highly emissive, MR and $^1\text{O}_2$ available porphyrin-based gadolinium (III) complexes which target bladder cancer cell/tumor**

[00193] For bladder cancer diagnosis, the physical examination on the detection of bladder cancer has high risk to damage the bladder function. When it comes to specific treatment, PDT is a newer treatment method that is now being studied to see if it is useful in treating especially early stage bladder cancers. The limitations of using the traditional PDT are the poor penetration power of light and the difficulty to monitor the performance in real time. The present invention provides multi-modal PDT agents for comprehensive diagnosis and treatment on bladder cancer – MR for diagnosis, NIR induced $^1\text{O}_2$ for PDT and NIR induced NIR emission for real time monitoring the effectiveness of PDT. In the

inventors' publication (PNSA, 2014, E5492–E5497) and the above examples, the stability, relaxivity, NIR-induced emission, $^1\text{O}_2$ ability and selectivity of the present porphyrin based complexes in cancer cells are shown. The design of the theranostic complexes targeting bladder cancer is shown in the Figure 45 (**Gd-3-R₁/Gd-4-R₁/Gd-5-R₁**) and the several bladder cancer specific peptide(s) as vector(s) (**Gd-3-R_n/ Gd-4-R_n/ Gd-5-R_n/ Gd-6-R_n**) in Figure 47 for the $\alpha_v\beta_3$ isoform of integrin protein will be conjugated to the multi-modal complexes in the section 1.1.

[00194] **Preparation of compound Por-TMS**

[00195] 4-((trimethylsilyl)ethynyl)benzaldehyde(2.02g,10mmol) was mixed with Pyridine-4-carboxaldehyde(3.21g,30mmol) in propionic acid (700 mL) and the mixture was stirred for half an hour in 130°C. Then pyrrole(2.64g,40mmol) was added dropwise into the reaction mixture with the temperature increased to 140 °C. Then the mixture continued to be stirred for 30 minutes in open air. After cooled down to room temperature, the solvent was removed under reduce pressure to afford black solid. The crude product was dissolved in minimum amount of CH_2Cl_2 and purified by column chromatography on silica gel column CH_2Cl_2 /Methanol(12:1) to give purple color solid. Yield 8%.

[00196] **Gd[N(SiMe₃)₂]₃·x[LiCl(THF)₃]**: HN(SiMe₃)₂ (10.8ml, 0.050 mol) was dissolved 20ml of THF in ice bath, then n-BuLi (1.6M in hexane) was added slowly over 30-min period. The resulting solution was magnetically stirred for 12 hours until a clear pale yellow solution was obtained. Then the solution was transferred slowly to a Schlenk flask with GdCl₃ (4.47 g, 0.017 mol) suspended in 20 ml THF. The resulting mixture was magnetically stirred for 24 h until all of the solid GdCl₃ was disappeared. The resultant solution Gd[N(SiMe₃)₂]₃·x[LiCl(THF)₃] (x = 3 ~ 5) was referred to as solution C

[00197] **Gd-1-L1**: Solution C (2.5 ml, 0.52 mmol of Gd) prepared above was transferred to a Schlenk flask and the solvent was removed under vacuum. Then 10 ml CH_2Cl_2 was added for the precipitation of LiCl. The mixture was centrifuged and the clear layer was transferred to another Schlenk flask with dry Por-TMS (0.099 g, 0.14 mmol) dissolved in 15 ml toluene. The resulting solution was refluxed until most of the

free base coordinated with the metal ion. Dry NaL_1 (0.1 g, 0.22mmol) [L_1^- ((cyclopentadienyl)tris(dimethylphosphito)-cobaltate, an anionic tripodal ligand) was then added and magnetically stirred for another 12 hours before the reaction solution was cooled down to room temperature. Upon completion of the reaction, the solvent was removed in vacuum and the residue dissolved in CHCl_3 , filtered and chromatographed on silica gel using $\text{CHCl}_3/\text{CH}_3\text{OH}$ ether (V/V 200:1) as eluent. The product was dissolved in CH_2Cl_2 (5 ml) and the solution was filtered. Yield: 61%

[00198] **Gd-1-L2:** The similar procedure with **Gd-1-L1**, replace **NaL_1** with **KL_2** (potassium tris(1-pyrazolyl) borohydride, 0.055g, 0.22mmol). Yield: 50%.

[00199] **Gd-3:** TBAF (1.0M in THF, 0.2mL, 0.2mmol) was added to a solution of Gd-1-L1 (0.133mg, 0.1mmol) in 10ml DCM, and the solution was stirred for 30 min. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was passed through a short of silica gel column using DCM. After removal of solvent, pure product was obtained, the pure product (33.2. mg, 0.02mmol) and $\text{Pd}(\text{PPh}_3)_4$ (2.2 mg 0.008 mmol), CuI (0.77 mg, 0.004 mmol), 4-iodobenzoic acid 5.1mg were placed in a dried flask and under nitrogen. THF (15 mL) and Net_3 (5 mL) were added and the reaction mixture degassed with nitrogen. The reaction mixture was stirred at 40 °C for 12 h. After that, the solvent was removed under reduced pressure. The residue was purified by chromatography. Elution with CH_2Cl_2 /Methanol(10:1) afforded the pure product, the pure product (30mg, 23.75mmol), EDCI (9.02g, 0.048mmol), NHS (5.52mg, 0.048mmol) were placed in a dried flask and under nitrogen, 10mL dry DMF was added. Stirred at room temperature for 48h. then remove the solvent, The residue was recrystallized by diethyl ether and dried to give the title product, the product (33.37mg, 0.025mol) was dissolved in DMF (10 ml), Then CH_3I (0.25mmol) was added and stirred for 5h, After completion of the reaction, solvent was removed. The residue was washed with ether. DCM. afforded the pure product. Yield 52%.

[00200] **Gd-4:** The similar procedure with Gd-3, replace Gd-1-L1 with Gd-1-L2. Yield 50%.

[00201] **Gd-3-Rn** : A stirred solution of Gd-3(20mg, 1equiv) in anhydrous DMF was mixed with N,N'-diisopropylethylamine (DIPEA) (1 equiv.). the mixture solution was added into a vial which containing peptide (1.3 equiv.) (R_n). It was then reacted at RT overnight, after that, the solvent was removed under vacuum to get the dry compound. The residue was recrystallized by diethyl ether three times and dried to give the titled product. Yield 70%.

[00202] **Gd-4-Rn**: The same procedure with Gd-3-Rn, replace Gd-3 with Gd-4. Yield 68%.

[00203] **Task 2: Examination of the selectivity and relaxivity of Gd-3-R₁ with bladder cancer**

[00204] The general photophysical properties, such as emission quantum efficiency and emission lifetime are examined. The magnetic properties of these gadolinium porphyrin complexes are measured. The binding affinity between the $\alpha_v\beta_3$ isoform and the present invented porphyrin complexes **Gd-3-R₁**, is the prime factor for developing the multi-modal probe for monitoring $\alpha_v\beta_3$ isoform bladder cancer. The binding affinities of the present invented complexes for $\alpha_v\beta_3$ isoform bladder cancer are determined by enthalpy changes and electrostatic interactions. The cell-permeable and water-soluble porphyrin-based gadolinium porphyrin complexes are to be bioconjugated with a cancer-specific vector (peptide). The tailored peptide can be used to trace the integrin $\alpha_v\beta_3$ isoform in bladder cancer cell membrane. Bladder cancer (T24) model are under studied and other cancer cell models such as HeLa, SK-N-SH, A549, C666-1 and normal cells: MRC-5 are served as control (Figure 48).

[00205] **2.1 Photophysical properties and stability of the newly synthesized gadolinium (III) complex, Gd-3-R₁**

[00206] The linear, multi-photon photophysical properties (i.e. emission spectra, emission lifetime, quantum yield and two-photon absorption cross section) and $^1O_2$ yield and also photo-bleaching quantum yield (compared with standard, such as uroporphyrin)

of **Gd-3-R₁** is measured following the literature protocols. Titration experiments are conducted to investigate the stabilities of the synthesized porphyrin complexes toward several common biological anions and human serum albumin (HSA); P_M and P_{Ka} are determined. Liquid-concentrated stock solutions of each anion, as well as HSA, are added gradually to a solution of the complexes concerned separately. Absorption, fluorescence as well as ^{31}P NMR spectroscopy are used to monitor the stability of the complexes in aqueous solution upon the addition of various biological small molecules such as HSA, citrate etc.

[00207] **2.2 Binding affinity via electrophoretic mobility shift assay**

[00208] Electrophoretic Mobility Shift Assay is a powerful method for determining the binding affinity of the inventors' gadolinium porphyrin complexes to $\alpha_v\beta_3$ isoform cancer cell which is specific for bladder cancer. The $\alpha_v\beta_3$ isoform cell are expressed in an E.coli system and further purified by glutathione affinity chromatography before carrying out agarose gel electrophoresis. Experiments are to confirm physical structure of the $\alpha_v\beta_3$ isoform would not be altered by the binding of the lanthanide bio-probes.

[00209] **2.3 Evaluation the binding affinity via emission and isothermal titration calorimetric**

[00210] The binding affinity of the complexes and $\alpha_v\beta_3$ isoform is studied by isothermal titration calorimetry (ITC), a solution state method that measures the interactions between molecules, e.g. macro-proteins and ligands. The binding affinity (K_a), binding stoichiometry (N) and the enthalpy changes (H) of the interaction could all be determined by ITC experiments directly. From the enthalpy change, the Gibbs energy and entropy change are determined by established equations. Advantages of ITC include a real-time observation of inter-molecule interactions without limitation on molecular weight in, most importantly, a nondestructive manner.

[00211] **2.4 Relaxivity determination of gadolinium porphyrin complex, Gd-3-R₁**

[00212] The relaxivity of the inventors' synthesized complexes will be calculated from the relaxation time obtained by a Bruker DPX300 NMR spectrometer in D₂O solutions. An inversion-recovery pulse sequence is used and a ten × T₁ delay is maintained between successive pulses. The relaxivity (r₁) is obtained by a plot of the inverse of longitudinal time (1/T₁) versus Gd concentrations:

$$[00213] \quad \frac{1}{T_{1obs}} = r_1 \times [Gd] + \frac{1}{T_{1b}}$$

where T_{1obs} and T_{1b} are the longitudinal relaxation times of the sample and the solvent background respectively.

[00214] **2.5 Tumour models or cell lines will be used, and details of biological studies**

[00215] Bladder cancer (T24) model is under studied and other cancer cell models such as HeLa, SK-N-SH, A549, C666-1 and normal cells: MRC-5 are served as control. Cancer/normal cells (Cancer cells: T24-bladder cancer, HeLa, SK-N-SH, A549, C666-1 and normal cells: MRC-5, (2 × 10⁴/well) are incubated in 96-well plates overnight. ***In vitro* imaging for selective binding-** the cells are treated with **Gd-3-R₁** (Task 1) for 6, 12 and 24 hours in the dark. The culture medium is replaced by fresh medium and the cells are exposed to light (1-8 J/cm²) produced from a laser (linear and multi-photon femtosecond Ti:sapphire laser) in the multi-photon confocal microscope. The time-lapse confocal images of **Gd-3-R₁** in cells are carried out and their *in vitro* subcellular localization are compared. The subcellular localization of **Gd-3-R₁** are different in bladder cell T24 and other non-bladder cancer cell lines, such as HeLa, C666-1 and SK-N-SH.

[00216] ***In vitro* photo-cytotoxicity** – Bladder cancer T24 cells are treated with several concentrations of complex and incubated for 12 hours. The free complex in the medium will be removed by changing the medium several times. The cells will be irradiated by laser to initiate the release of ¹O₂ from the complex and MTT assay is performed to measure the cell viability after a number of incubation time points. Control

experiment is performed with the same experimental condition, such as light dosed amount, incubation time and concentration of proposed complexes in non-bladder cancer cell lines.

[00217] The *in vitro* dark toxicity of the invented complexes are tested. After 24 hours, the water-soluble complexes and the targeted cells are incubated further with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/ml), otherwise known as MTT, for 4 hours, so that formazan can be formed along with the cell's metabolic pathways. Then, the formazan is extracted and dissolved by dimethyl sulfoxide (DMSO), with the absorbance of the subsequent solutions measured in a Bio-Rad iMark microplate reader (490 nm). Quadruplicates are performed and the data are interpreted and analyzed using the GraphPad Prism 5 software.

[00218] The photocytotoxicity of valid bladder cancer specific photosensitizer (i.e. LC₅₀ in 1J laser dosed is 1M) must be 100 times lower than its dark cytotoxicity. (IC₅₀ have to be > 0.1mM).

[00219] **Task 3 — Structure and biological activity (In vitro/-vivo imaging and specific PDT effect)**

[00220] In this section, effectiveness of the **Gd-3-R₁** *in vivo* are evaluated. Comprehensive *in vitro* and *in vivo* examinations are employed, such as multi-confocal *in vivo* real time studies, MR imaging and metabolism studies (Figure 49B).

[00221] **3.1 Determine the stability of lanthanide complexes for biological assays**

[00222] There is great challenge of the coordination stability lanthanide complexes for cellular studies. Therefore, the aqueous/tissue culture medium stability must be carried out. The aqueous stability of the present complexes in the presence of various biomolecules, including citrate and human serum albumin (HSA), and in varies pH are examined as well by simple UV-vis absorption /fluorescence titration via the aforementioned procedures. Liquid concentrated stock solutions of each anion, as well as

HSA, are added individually and gradually to a solution of the complex concerned. Addition is ceased either when the volume of added anion totaled 5% of the complex solution or the influence on complex absorption/luminescence was saturated.

[00223] **3.2 *In vitro* cytotoxicity studies and rate of cellular uptake**

[00224] For the establishment of the mouse tumor xenograft mode, bladder cancer cells (T24) or non-bladder cancer cells (HeLa) are trypsinized, harvested and suspended in serum-free culture medium. 5×10^6 cells in a 100 μL volume is injected subcutaneously into the flanks of female athymic nude mice (5-week old) When the tumor volume reaches the size of around 100 mm^3 , animals are divided randomly into four experimental group with SEVEN mice in each group, as follows: group 1, vehicle control group; group 2, cisplatin treatment group; group 3, Gd-N low dose treatment group; group 4, Gd-N high dose treatment group. Treatments is administered via intratumoral injection once every 5 days, for 21-28 days. The experiment is repeated three times. The tumor volume is measured every 2 days with electronic calipers (accuracy of 0.02 mm) and then calculated independently on the basis of the equation $V = (L \times W^2)/2$, where L and W correspond to the larger and smaller dimensions respectively. All animal experiments is carried out in accordance with the guidelines of the Committee on Use of Human and Animal Subjects in Teaching and Research, Hong Kong Baptist University. One-way analysis of variance towards statistical significances between groups was assessed by the GraphPad Prism 5.0 software.

[00225] **3.3 Real time analysis of $\alpha_v\beta_3$ isoform and evaluation of effectiveness (tumorigenically assays) of tumor inhibition-days/week tracing of tumor development of xenograft mouse via multi-photon confocal and magnetic resonance imaging for pharmacokinetic studies**

[00226] The development of xenograft mice is achieved by transplanting human bladder tumor cells (T24) to mice which are allowed to grow. The complexes is injected at the tail vein, the peritoneum or buccally delivered and after 24 to 48 hours, the xenograft are surgically extracted for two-photon confocal microscopy and MRI experiments, with the peritumor cells extracted being the control (no lanthanide

complexes signals should be obtained). *In vivo* MRI experiments are carried out on the xenograft in a Co-I institute with a Bruker Biospec 4.7 T/30 cm scanner (Bruker Inc., MA). Moreover, the tumor sizes will be measured weekly.

[00227] **3.4 *In vivo* bio-distribution evaluation of proposed gadolinium complexes**

[00228] **Gd-3-R₁** is injected intravenously to BALB/c athymic mice bearing xenografted cancer tumors. After 24 hours of incubation, the mice are sacrificed and its main organs including the tumor is removed and fixed in 10% PBS buffered formalin. Control models are athymic mice with only the buffered formalin injected. The tissue samples are frozen and lyophilized for 24 h before being digested by conc. HNO₃ at 70 °C for 4 hours. The gadolinium content, reflective of the quantity of the complexes, are determined by ICP-MS. The gadolinium content in urine of the mouse are evaluated to confirm the metabolism of these complexes *in vivo*. The results in 3.3 and 3.4 should be correlated (Figure 44).

INDUSTRIAL APPLICABILITY

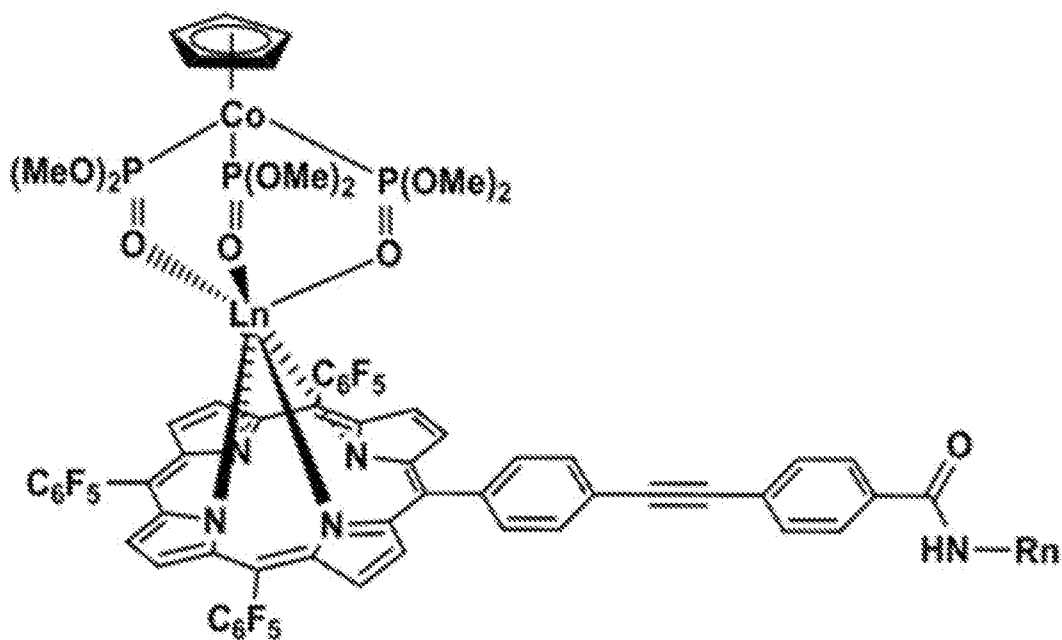
[00229] The present invention relates to a new generation of PDT agents based on porphyrin-lanthanide complexes with specific functional groups which can specifically localize on particular tumors, and their PDT processes can be monitored via NIR emission from erbium. In particular, the present invention provides a multi-modal lanthanide-porphyrin PDT agent (**Er-R₃**) that are capable of killing the bladder tumor cells selectivity via ¹O₂ from porphyrin moiety and affording the fluorescence imaging simultaneously upon **Er-R₃** binding with the integrin $\alpha_v\beta_3$ isoform in bladder cancer cells.

[00230] If desired, the different functions discussed herein may be performed in a different order and/or concurrently with each other. Furthermore, if desired, one or more of the above-described functions may be optional or may be combined.

[00231] While the foregoing invention has been described with respect to various embodiments and examples, it is understood that other embodiments are within the scope of the present invention as expressed in the following claims and their equivalents. Moreover, the above specific examples are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present invention to its fullest extent. All publications recited herein are hereby incorporated by reference in their entirety.

CLAIMS

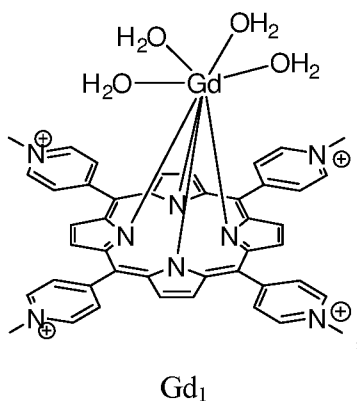
1. A composition for photodynamic therapy and imaging of cancer cells comprising an Erbium porphyrin based complex, Ytterbium porphyrin based complex, or Gadolinium porphyrin based complex represented by the molecular formula:

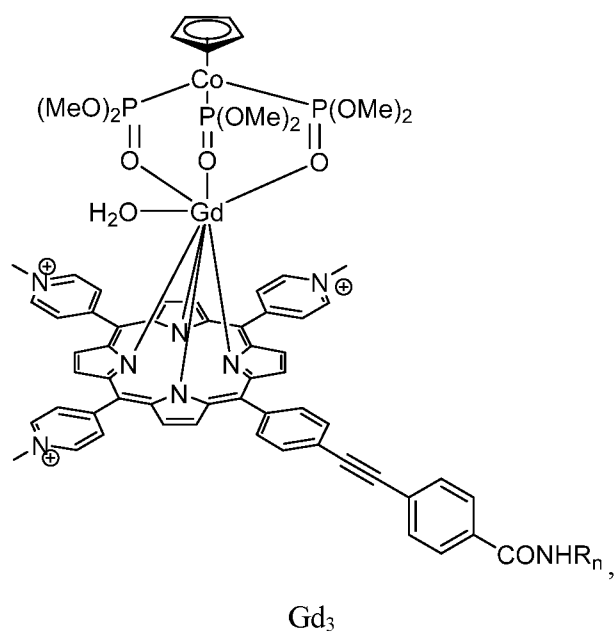
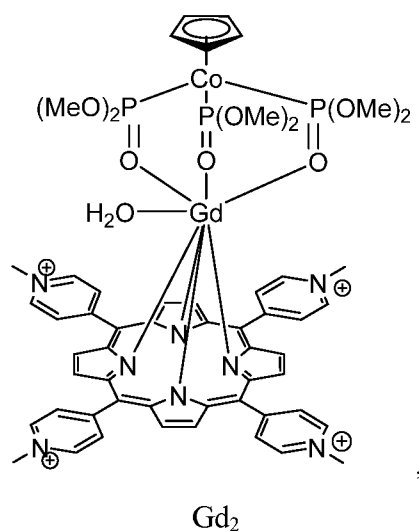


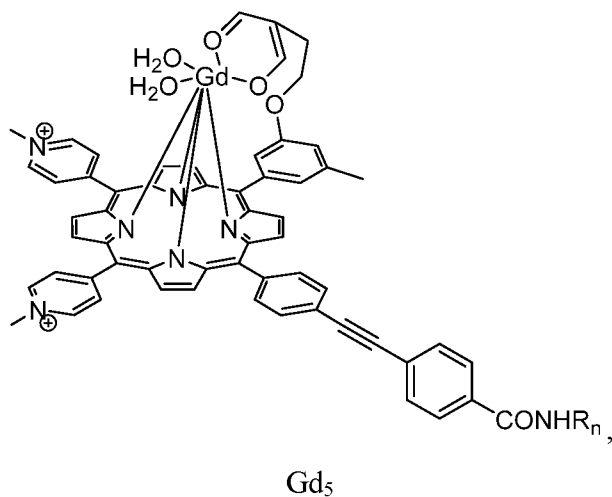
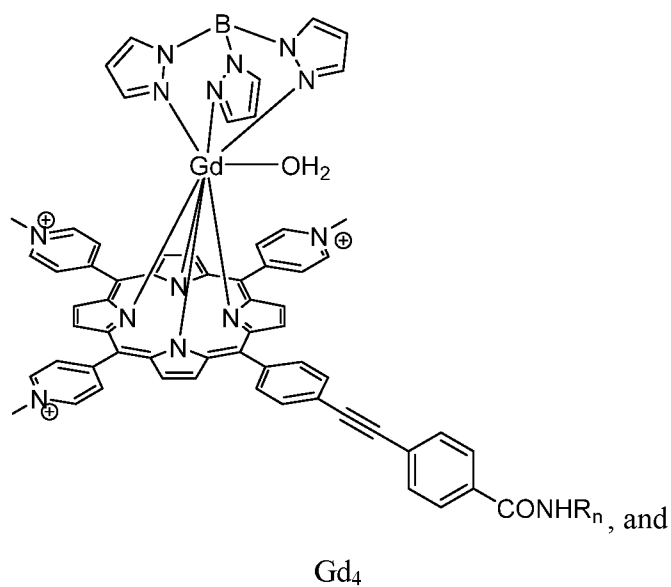
wherein Ln is Er, Yb, or Gd; and

R_n is a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 1; SEQ ID NO: 2; and SEQ ID NO: 3; or

a water-soluble porphyrin-based Gadolinium complex represented by a molecular formula selected from the group consisting of Gd₁, Gd₂, Gd₃, Gd₄, and Gd₅:

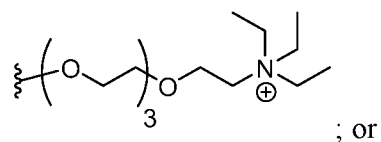






wherein

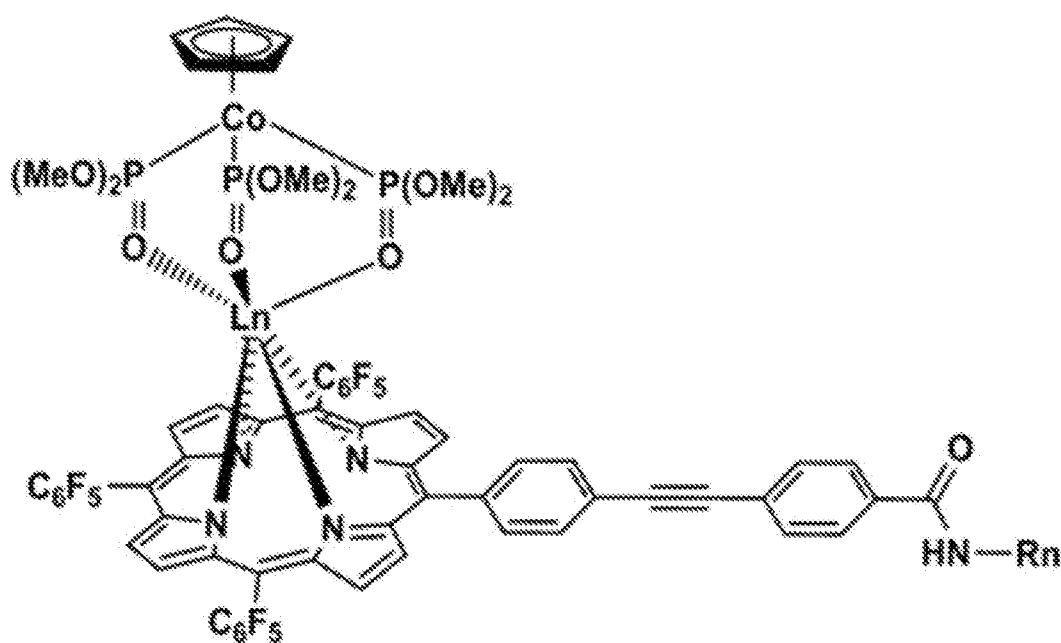
R_n is



a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 1; SEQ ID NO: 2; SEQ ID NO: 3; SEQ ID NO: 4; and SEQ ID NO: 5 or a pharmaceutically acceptable salt thereof.

2. The composition according to claim 1 wherein the Erbium porphyrin based complexes are conjugated with integrin $\alpha_v\beta_3$ isoform-specific peptides.

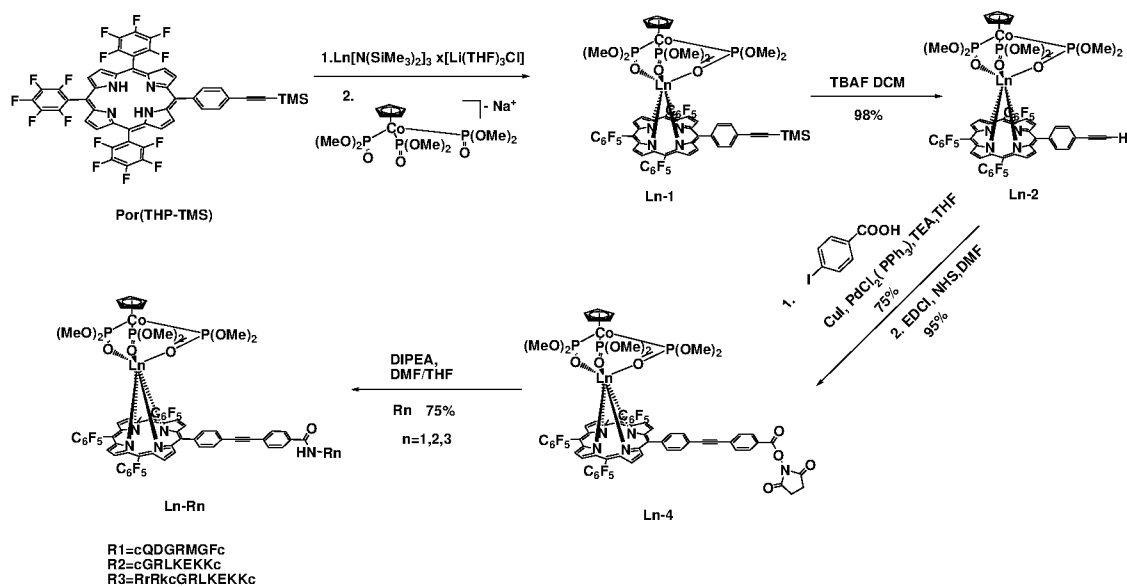
3. The composition according to claim 1, wherein the composition comprises the Erbium porphyrin based complex and R_n is SEQ ID NO: 4.
4. The composition according to claim 1, wherein the composition comprises the Erbium porphyrin based complex and R_n is SEQ ID NO: 5.
5. The composition according to claim 1, wherein the composition comprises the Erbium porphyrin based complex and R_n is SEQ ID NO: 3.
6. The composition according to claim 1, wherein the composition comprises the Erbium porphyrin based complex represented by the molecular formula of:



wherein Ln is Er and R_n is a polypeptide having an amino acid sequence of SEQ ID NO:

- 3.
7. The composition according to claim 1 wherein the cancer cells comprising of bladder cancer cells, cervical cancer cells and lung cancer.
8. A method of photodynamic therapy and imaging of cancer cells comprising administering to a subject in need thereof the composition according to claim 1 and irradiating the cancer cells in the subject in need thereof with a radiation source.
9. The method according to claim 8 wherein the administration of said composition is performed intravenously or by injection to site of said cancer cells.

10. The method according to claim 8, wherein said radiation source is a light source with a wavelength in the Q band of porphyrin.
11. The method according to claim 8, wherein said radiation source is a light source with a wavelength beyond 550 nm.
12. The method according to claim 8, wherein said radiation source is a light source with a wavelength is at 860 nm.
13. The method according to claim 8 wherein the imaging is performed using fluorescent imaging.
14. The method according to claim 8 wherein the imaging is performed using NIR imaging.
15. The method according to claim 8 wherein the imaging is performed using MRI imaging.
16. The method according to claim 8 wherein the composition comprises the Gadolinium porphyrin based complex and Ln is Gd; or the composition comprises a compound selected from the group consisting of Gd₁, Gd₂, Gd₃, Gd₄, and Gd₅.
17. A method of synthesizing the composition according to claim 1 wherein Ln is Er or Yb comprising steps according to the following scheme:



wherein

said compound Por(THP-TMS) is synthesized via steps comprising:

dissolving Pyrrole, pentafluorobenzaldehyde and 4-[2-(trimethylsilyl)ethynyl]benzaldehyde 6 in CH_2Cl_2 under an argon atmosphere to produce a first solution;

leaving the first solution for at least 10 minutes;

adding $\text{BF}_3 \cdot \text{O}(\text{Et})_2$ to the first solution;

stirring the first solution for at least 1 hour at room temperature;

adding DDQ(2,3-Dichloro-5,6-dicyano-1,4-benzoquinone) to the first solution;

stirring the first solution for at least another 1 hour at room temperature;

removing the solvent from the first solution in vacuo to produce a first mixture;

passing the first mixture through a silica column (hexanes- CH_2Cl_2) concentrated under reduced pressure to produce 5,10,15-Tris(pentafluorophenyl)-20-[4-{2-(trimethylsilyl)ethynyl}phenyl]porphyrin or Por(THP-TMS);

said compound Ln-1 is synthesized via steps comprising:

dissolving $\text{Ln}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{LiCl}(\text{THF})_3] : \text{HN}(\text{SiMe}_3)_2$ in THF at about 0 degrees Celcius to produce a second solution;

adding n-BuLi slowly over at least 30-minutes period to the second solution;

stirring the second solution for at least 12 hours;

transferring the second solution to a Schlenk flask with LnCl_3 suspended in THF to produce a second mixture;

stirring the second mixture for at least 24 hours until all of the solid LnCl_3 disappeared to produce $\text{Ln}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$ ($x = 3 \sim 5$) wherein $\text{Ln} = \text{Er}$ or $\text{Ln} = \text{Yb}$;

said compound Yb-1 is further synthesized via steps comprising:

transferring $\text{Yb}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$ ($x = 3 \sim 5$) to a Schlenk flask;

removing the solvent from $\text{Yb}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$ ($x = 3 \sim 5$) under vacuum to produce a first residue;

adding CH_2Cl_2 to the first residue for the precipitation of LiCl to produce a third mixture;

centrifuging the third mixture until a clear layer is produced;

transferring the clear layer to another Schlenk flask with dry Por(THP-TMS) free base dissolved in toluene to produce a third solution;

refluxing the third solution until most of the free base coordinated with the metal ion;

adding dry NaLOMe [LOMe-((cyclopentadienyl)tris(dimethylphosphito)-cobaltate or an anionic tripodal ligand) to the third solution to produce a fourth mixture;
stirring the fourth mixture for at least another 12 hours;
cooling down the fourth mixture to room temperature;
removing the solvent from the fourth mixture in vacuum to produce a second residue;
dissolving the second residue in CHCl_3 ;
filtering and chromatographing the dissolved second residue on silica gel using CHCl_3 /petroleum ether as eluent;
further dissolving the output from chromatography in CH_2Cl_2 , and filtering the solution to produce compound Yb-1.

Said compound Er-1 is further synthesized via steps comprising:

the same steps as for Yb-1, replacing $\text{Yb}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$ ($x = 3 \sim 5$) with $\text{Er}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{Li}(\text{THF})_3\text{Cl}]$ ($x = 3 \sim 5$);

said compound Ln-2 wherein $\text{Ln} = \text{Yr}$ is synthesized via steps comprising:

adding TBAF to a solution of Yb-1 in CH_2Cl_2 to produce a fifth solution;
stirring the fifth solution for at least 30 minutes;
monitoring the progress of the reaction of the fifth solution by TLC;
after completion of the reaction, passing the fifth solution through a short of silica gel column;

removing the solvent from the fifth solution to produce Yr-2;

said compound Er-2 is further synthesized via steps comprising:

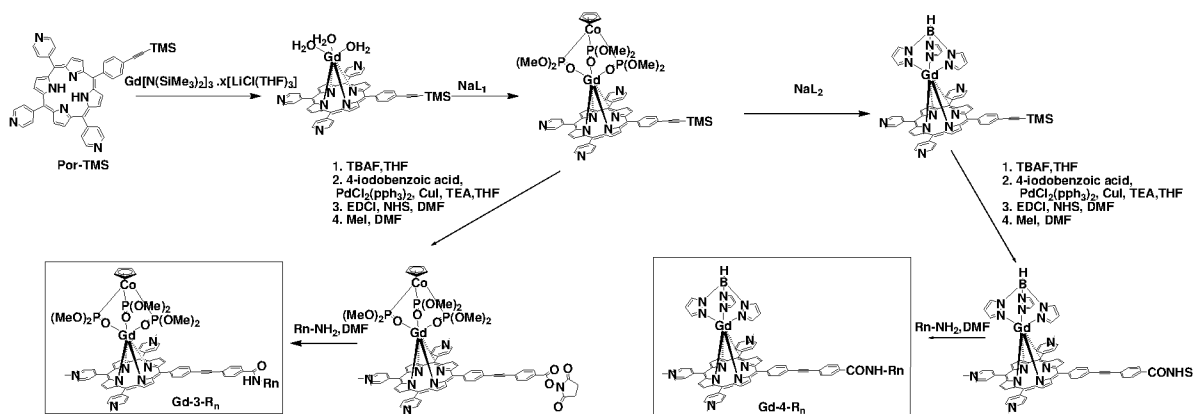
the same steps as for Yb-2, replacing Yb-1 with Er-1;

said compound Ln-4 wherein $\text{Ln} = \text{Yr}$ is synthesized via steps comprising:

mixing $\text{Pd}(\text{PPh}_3)_4$, CuI , Yb-2 and 4-iodobenzoic acid in a dried flask under nitrogen to produce a fifth mixture;
adding THF and Net_3 to the fifth mixture and degassing said fifth mixture with nitrogen;
stirring said fifth mixture at least 40°C for at least 12 hours;
removing the solvent from said fifth mixture under reduced pressure to produce a third residue;
purifying the third residue by chromatography;
eluting the purified third residue with CH_2Cl_2 /Methanol to produce an eluted compound;

mixing the eluted compound, EDCI, NHS in a dried flask and under nitrogen to produce a sixth mixture;
adding dry DMF to the sixth mixture;
stirring the sixth mixture at room temperature for at least 48 hours;
removing the solvent from the stirred sixth mixture to produce a fourth residue;
recrystallizing the fourth residue by diethyl ether and drying the crystals to produce Yb-4;
said compound Er-4 is further synthesized via steps comprising:
the same steps as for Yb-4, replacing Yb-2 with Er-2;
said compound Yb-R₁ is synthesized via steps comprising:
mixing a stirred solution of Yb-4 in anhydrous DMF with N,N'-diisopropylethylamine (DIPEA) to produce a seventh mixture;
adding peptide R₁ to the seventh mixture;
leaving the seventh mixture to react at room temperature for at least 24 hours;
removing the solvent from the seventh mixture under vacuum to produce a dry fifth residue;
recrystallizing the dry fifth residue by diethyl ether for at least three times;
drying the recrystallized dry fifth residue to produce Yb-R₁;
said compound Yb-R₂ is further synthesized via steps comprising:
the same steps as for Yb-R₁, replacing R₁ with R₂;
said compound Yb-R₃ is further synthesized via steps comprising:
the same steps as for Yb-R₁, replacing R₁ with R₃;
said compound Er-R₁ is further synthesized via steps comprising:
the same steps as for Yb-R₁, replacing Yb-4 with Er-4;
said compound Er-R₂ is further synthesized via steps comprising:
the same steps as for Yb-R₂, replacing Yb-4 with Er-4;
said compound Er-R₃ is further synthesized via steps comprising:
the same steps as for Yb-R₃, replacing Yb-4 with Er-4.

18. A method of synthesizing the composition according to claim 1 comprising steps according to the following scheme:



wherein

said compound Por-TMS is synthesized via steps comprising:

mixing 4-((trimethylsilyl)ethynyl)benzaldehyde with Pyridine-4-carboxaldehyde in

propionic acid to produce an eighth mixture;

stirring the eighth mixture for at least half an hour in at least 130°C;

adding pyrrole dropwise into the eighth mixture with the temperature increased to at least 140 °C;

stirring the eighth mixture for at least 30 minutes in open air;

cooling down the eighth mixture to room temperature;

removing the solvent from the eighth mixture under reduce pressure to produce a crude product;

dissolving the crude product in CH₂Cl₂ to produce a sixth solution;

purifying the sixth solution by column chromatography on silica gel column CH₂Cl₂/Methanol to produce Por -TMS;

said compound Gd[N(SiMe₃)₂]₃ · x[LiCl(THF)₃] is synthesized via steps comprising:

dissolving HN(SiMe₃)₂ in THF at about 0 degrees Celcius to produce a seventh solution;

adding n-BuLi to the seventh solution over at least a 30-minutes period;

stirring the seventh solution at least for 12 hours until a clear pale yellow solution was obtained;

transferring the seventh solution a Schlenk flask with GdCl₃ suspended in THF to produce a ninth mixture;

stirring the ninth mixture for at least 24 hours until all of the solid GdCl₃ disappeared to produce the resultant solution Gd[N(SiMe₃)₂]₃ · x[LiCl(THF)₃] (x = 3 ~ 5);

said compound Gd-1-L1 is synthesized via steps comprising:

transferring $\text{Gd}[\text{N}(\text{SiMe}_3)_2]_3 \cdot x[\text{LiCl}(\text{THF})_3]$ ($x = 3 \sim 5$) to a Schlenk flask and removing the solvent therein under vacuum to produce a sixth residue;

adding CH_2Cl_2 to the sixth residue for the precipitation of LiCl to produce a tenth mixture;

centrifuging the tenth mixture until a clear layer is produced;

transferring the clear layer to another Schlenk flask with dry Por -TMS free base dissolved in toluene to produce an eighth solution;

refluxing the eighth solution until most of the free base coordinated with the metal ion;

adding dry NaL_1 (0.1 g, 0.22mmol) [L_1^- ((cyclopentadienyl)tris(dimethylphosphito)-cobaltate, an anionic tripodal ligand) to the eighth solution to produce an eleventh mixture;

stirring the eleventh mixture for at least another 12 hours;

cooling down the eleventh mixture to room temperature;

removing the solvent from the eleventh mixture in vacuum to produce a seventh residue;

dissolving the seventh residue in CHCl_3 ;

filtering and chromatographing the dissolved second residue on silica gel using $\text{CHCl}_3/\text{CH}_3\text{OH}$ ether as eluent;

further dissolving the output from chromatography in CH_2Cl_2 , and filtering the dissolved output to produce compound Gd-1-L1.

Said compound Gd-1-L2 is synthesized via steps comprising:

the same steps as for Gd-1-L1, replacing NaL_1 with KL_2 (potassium tris(1-pyrazolyl)borohydride);

said compound Gd-3 is synthesized via steps comprising:

adding TBAF to a solution of Gd-1-L1 in DCM to produce a ninth solution;

stirring the ninth solution for at least 30 minutes;

monitoring the reaction of the ninth solution by TLC;

passing the ninth solution through a short of silica gel column using DCM to remove the solvent therein to produce a pure product;

placing the pure product and $\text{Pd}(\text{PPh}_3)_4$, CuI, 4-iodobenzoic acid in a dried flask and under nitrogen to produce a twelfth mixture;

adding THF and Net_3 to the twelfth mixture;
degassing the twelfth mixture with nitrogen;
stirring the twelfth mixture at a temperature at least $40\text{ }^{\circ}\text{C}$ for at least 12 hours;
removing the solvent from the twelfth mixture under reduced pressure to produce an eighth residue;
purifying the eighth residue by chromatography;
eluting the purified eighth residue with CH_2Cl_2 /Methanol;
placing the eluted purified eighth residue, EDCI, NHS in a dried flask and under nitrogen to produce a thirteenth mixture;
adding dry DMF to the thirteenth mixture;
stirring the thirteenth mixture at room temperature for at least 48 hours;
removing the solvent from the thirteenth mixture to produce a ninth residue;
recrystallizing the ninth residue by diethyl ether and dried said crystals to produce Gd-3;
dissolving the Gd-3 in DMF;
adding CH_3I to the dissolved Gd-3;
stirring the dissolved Gd-3 for at least 5 hours;
removing the solvent from the stirred dissolved Gd-3 to produce a tenth residue;
washing the tenth residue with ether .DCM. to produce pure Gd-3;
said compound Gd-4 is synthesized via steps comprising:
the same steps as for Gd-3, replacing Gd-1-L1 with Gd-1-L2;
said compound Gd-3-Rn is synthesized via steps comprising:
mixing a stirred solution of Gd-3 in anhydrous DMF with $\text{N,N}'$ -diisopropylethylamine (DIPEA) to produce a fourteenth mixture;
adding peptide Rn to the fourteenth mixture;
reacting the fourteenth mixture at room temperature for at least 24 hours;
removing the solvent from the fourteenth mixture under vacuum to produce a dry eleventh residue;
recrystallizing the dry eleventh residue by diethyl ether for at least three times and further dry the result to produce Gd-3-Rn;
said compound Gd-4-Rn is synthesized via steps comprising:
the same steps as for Gd-3-Rn, replacing Gd-3 with Gd-4.

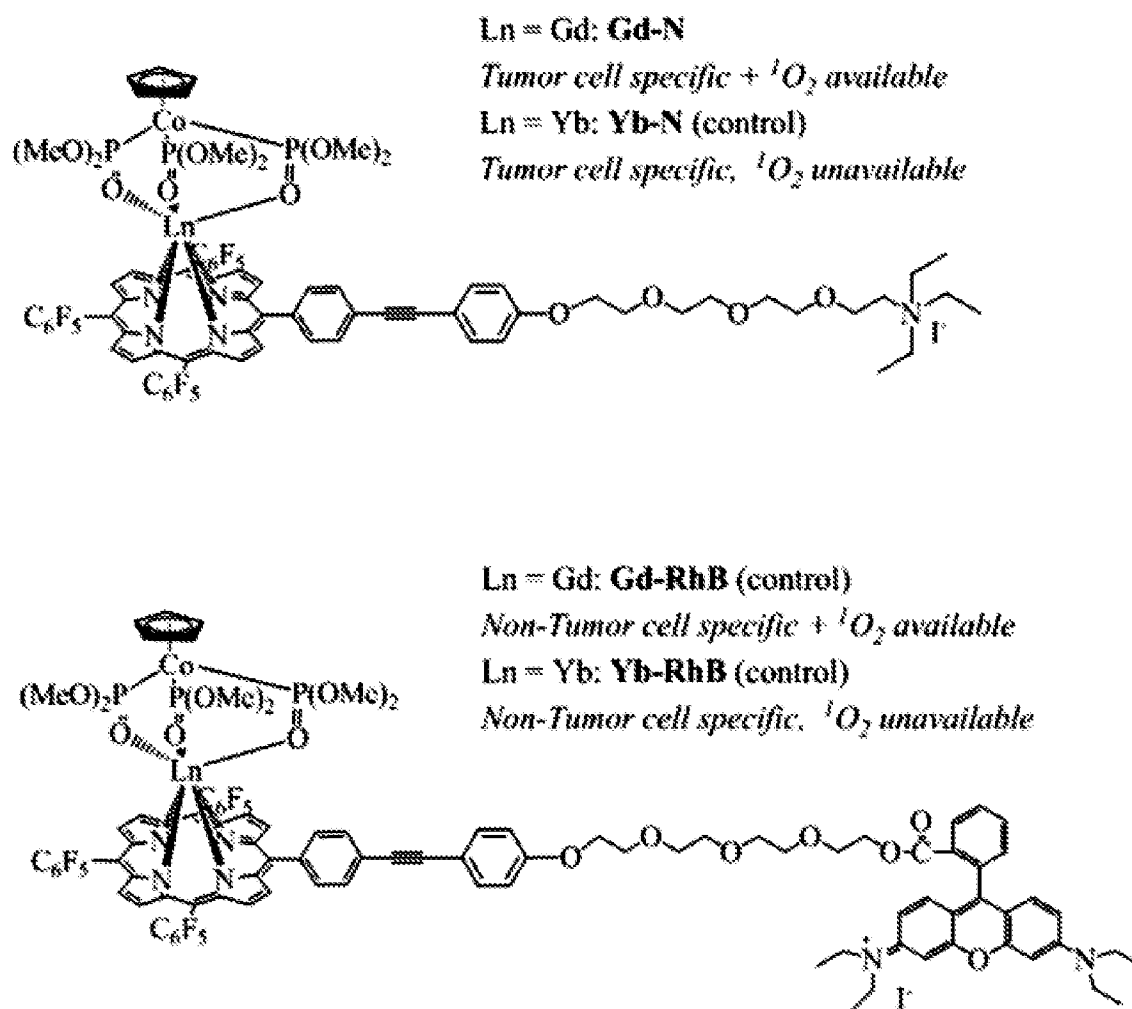


Figure 1A

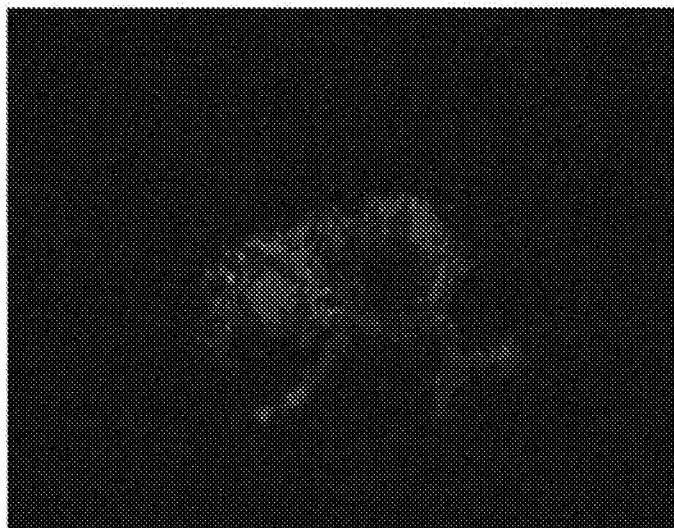


Figure 1B

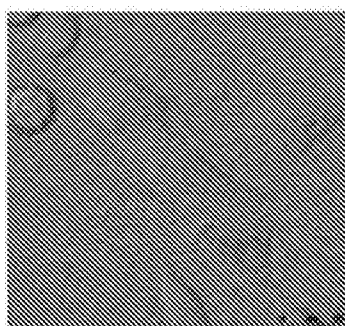


Figure 1C

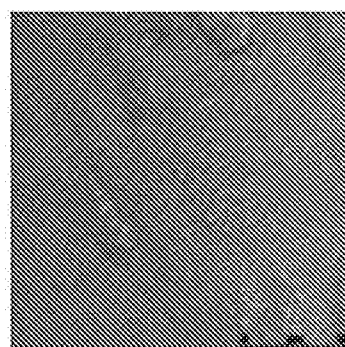


Figure 1D

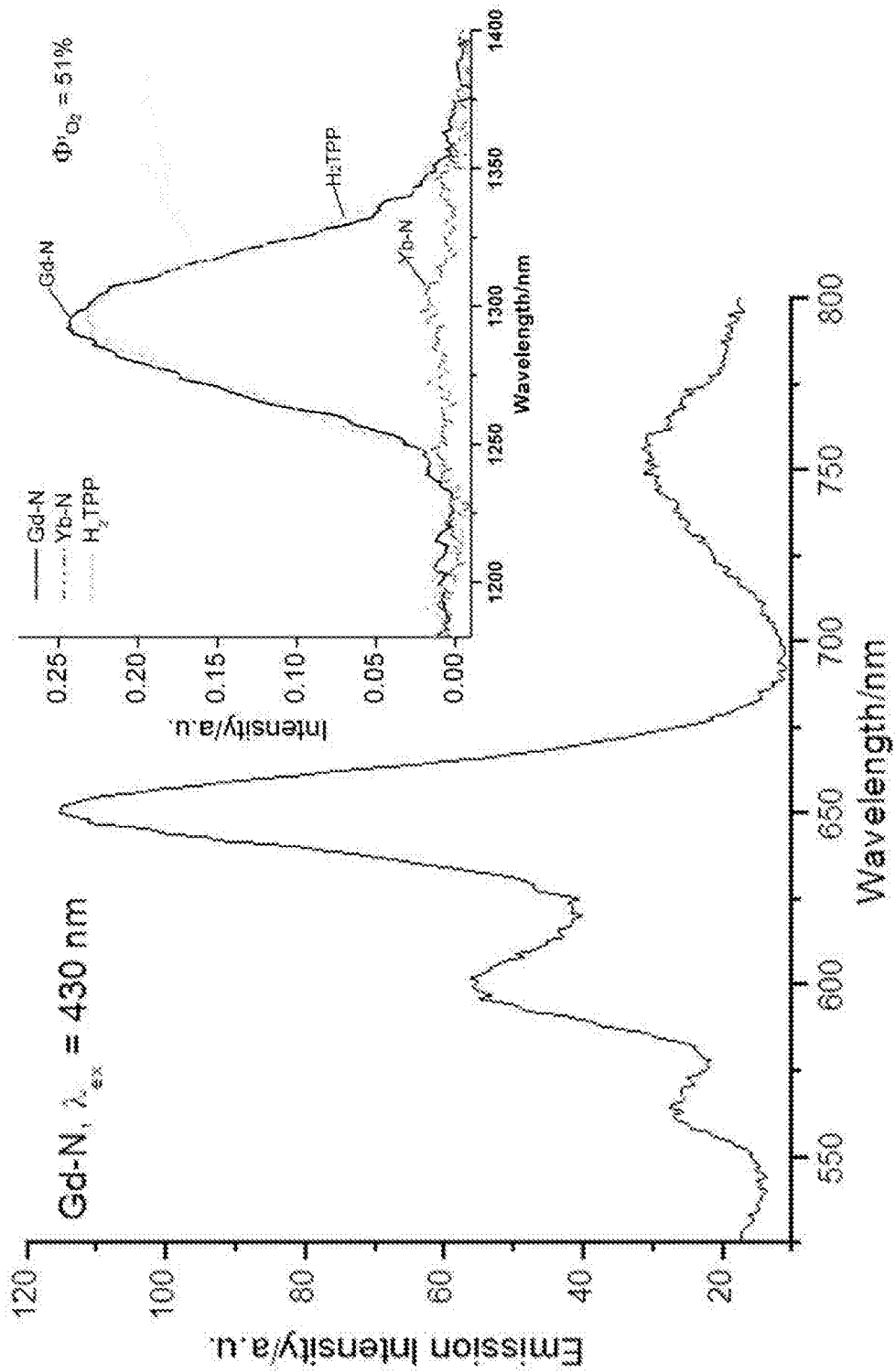


Figure 2

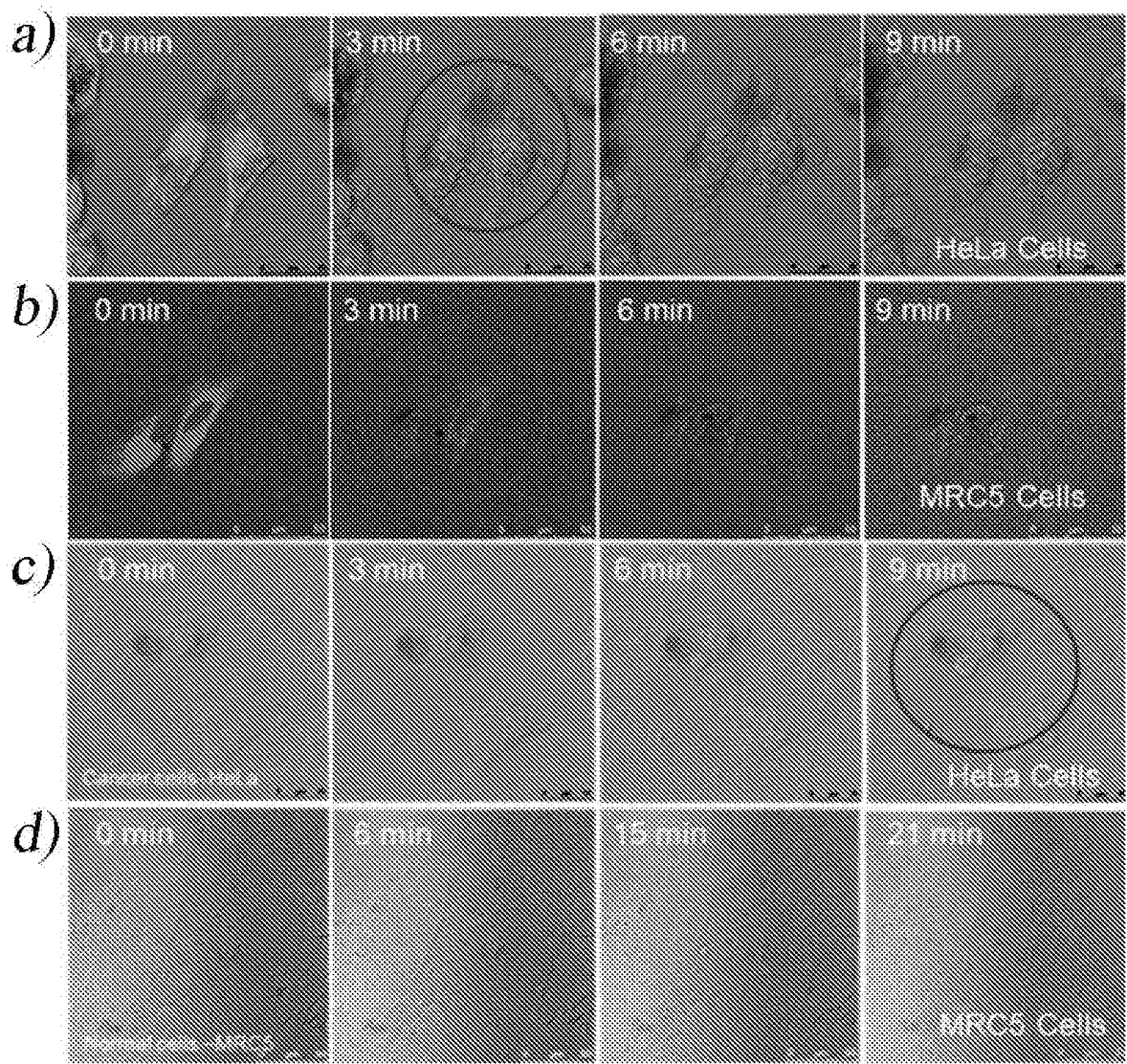


Figure 3

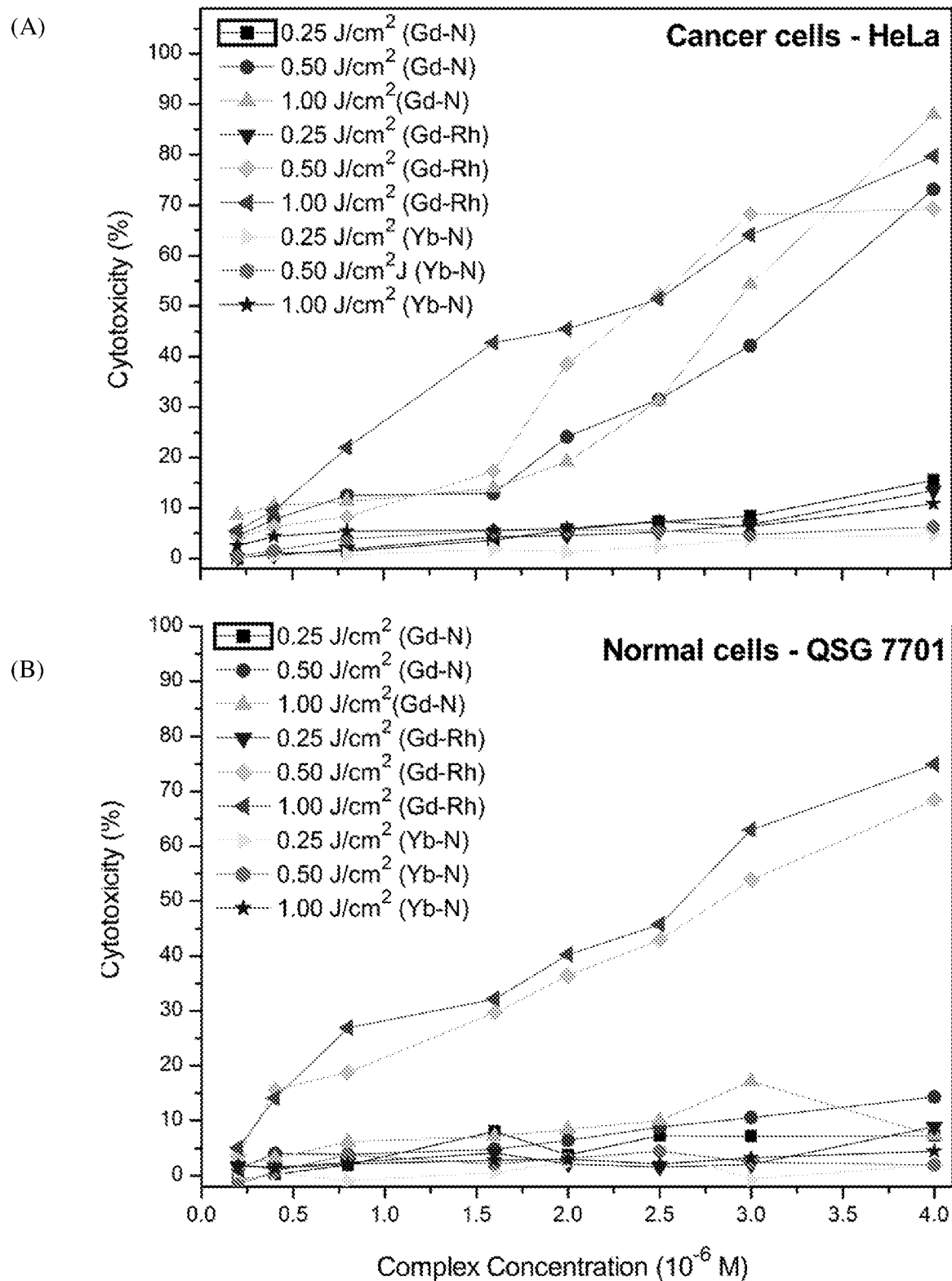


Figure 4

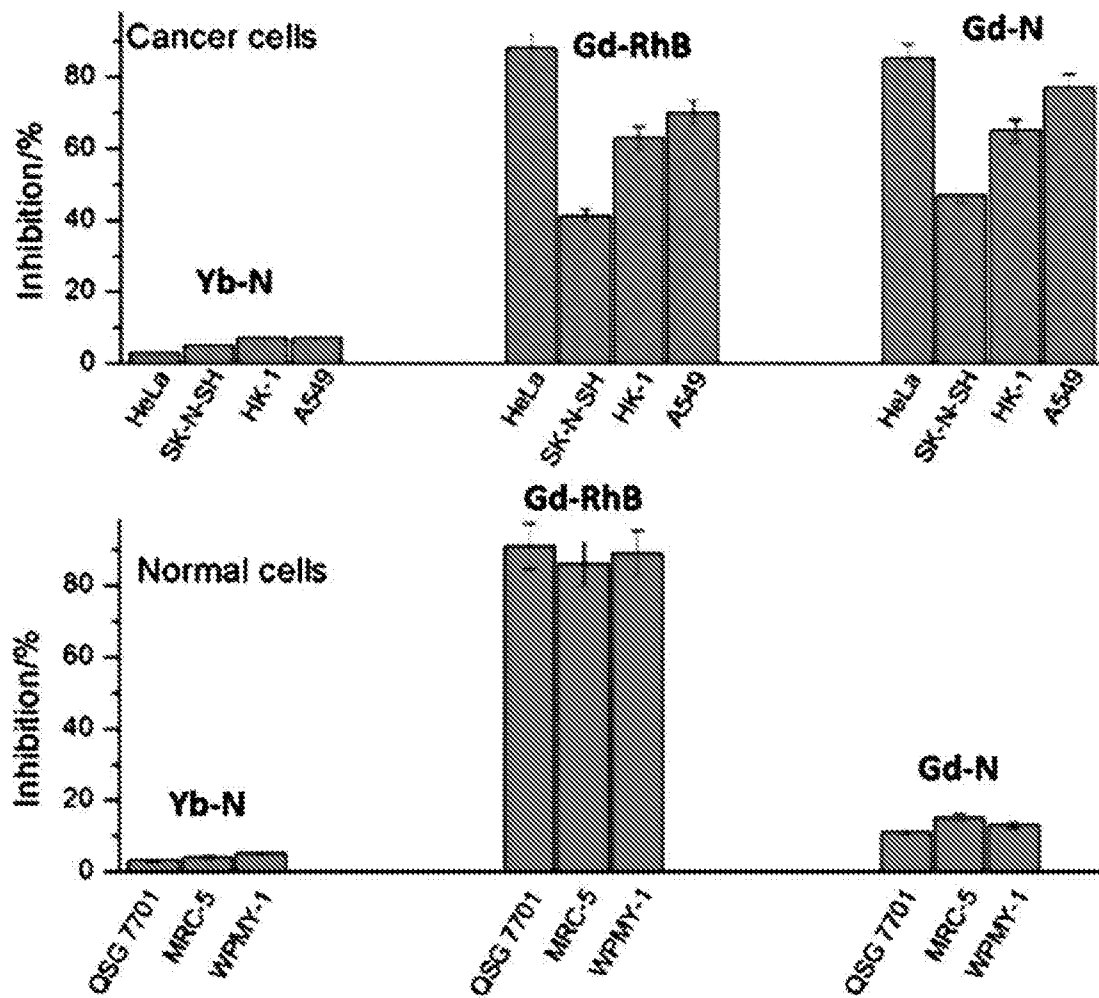
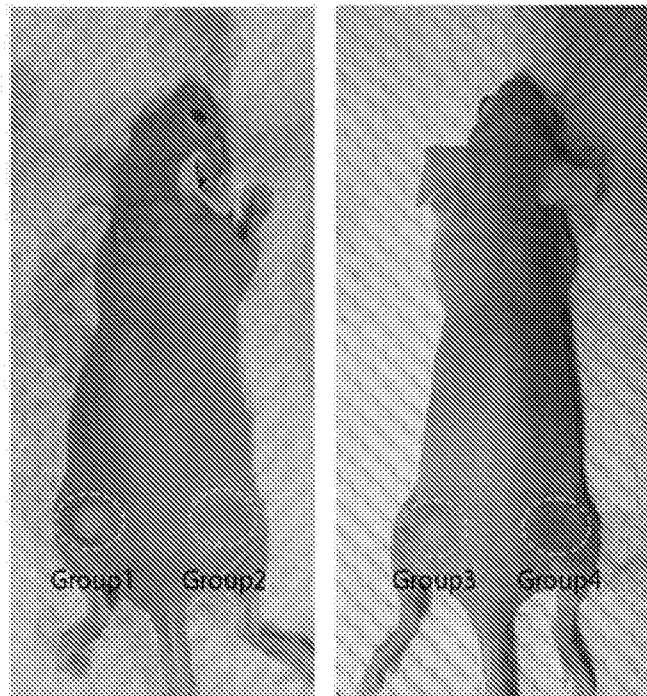


Figure 5

a)



Group 1: Yb-N+laser
Group 2: Gd-N+laser

Group 3: Yb-RhB+laser
Group 4: Gd-Rhb+laser

b)

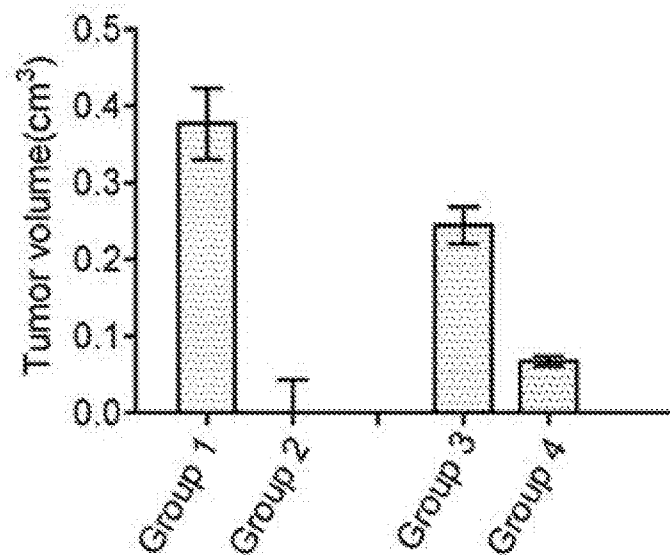


Figure 6

C)

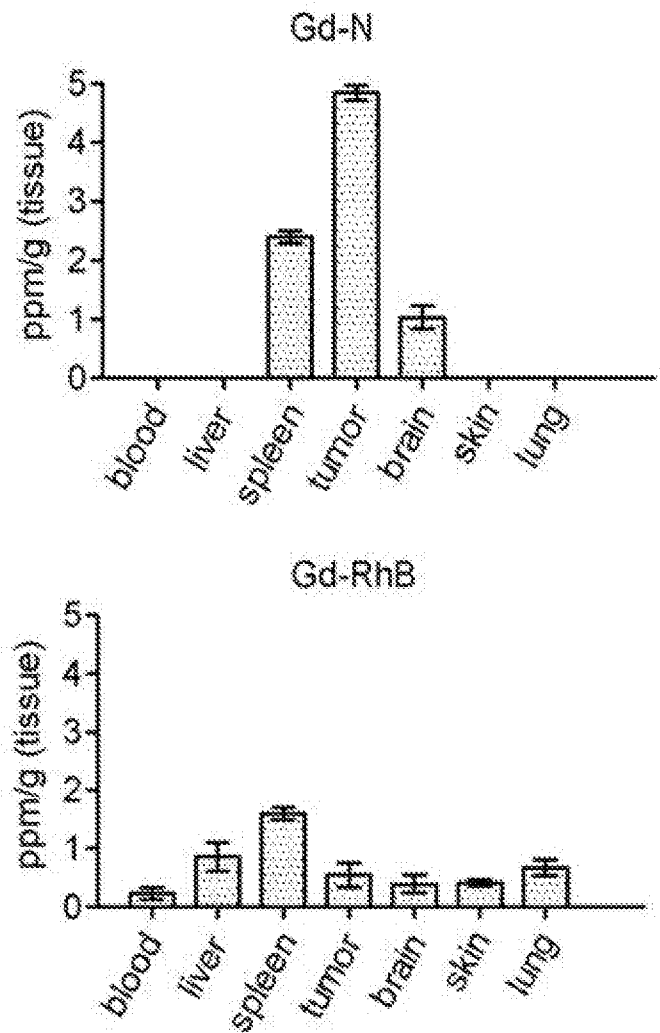
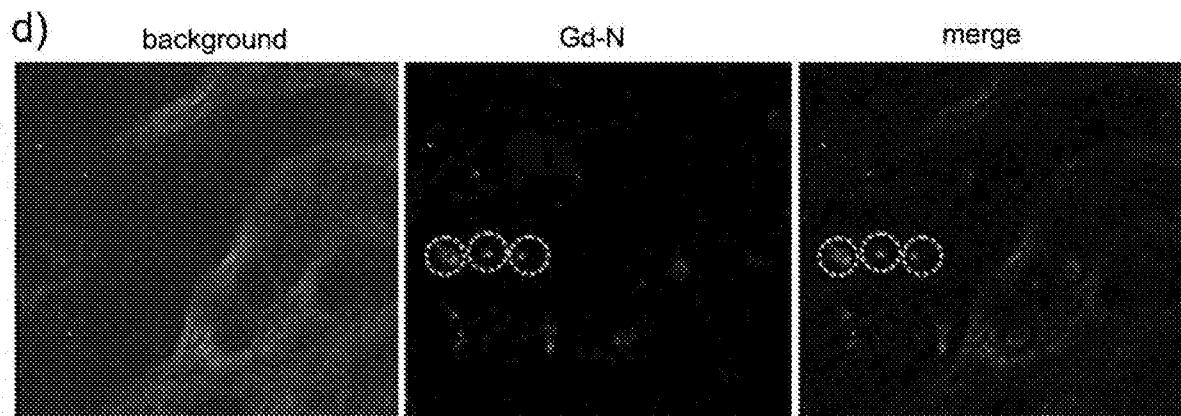


Figure 6 (continued)



e)

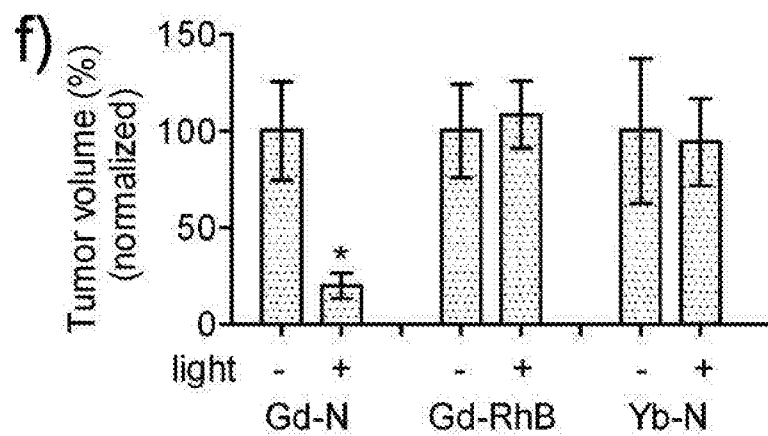
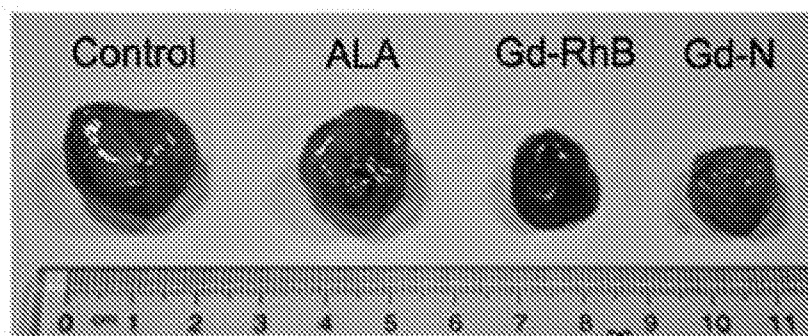


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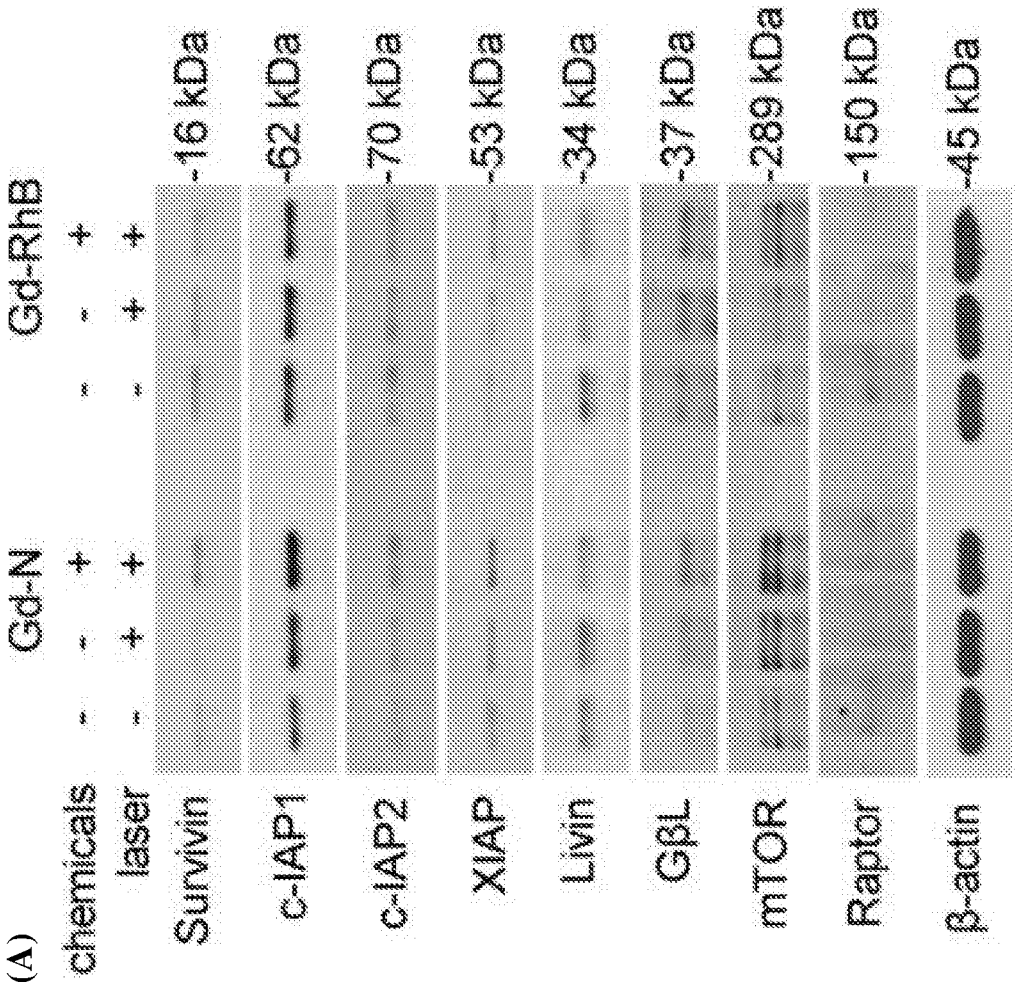


Figure 7

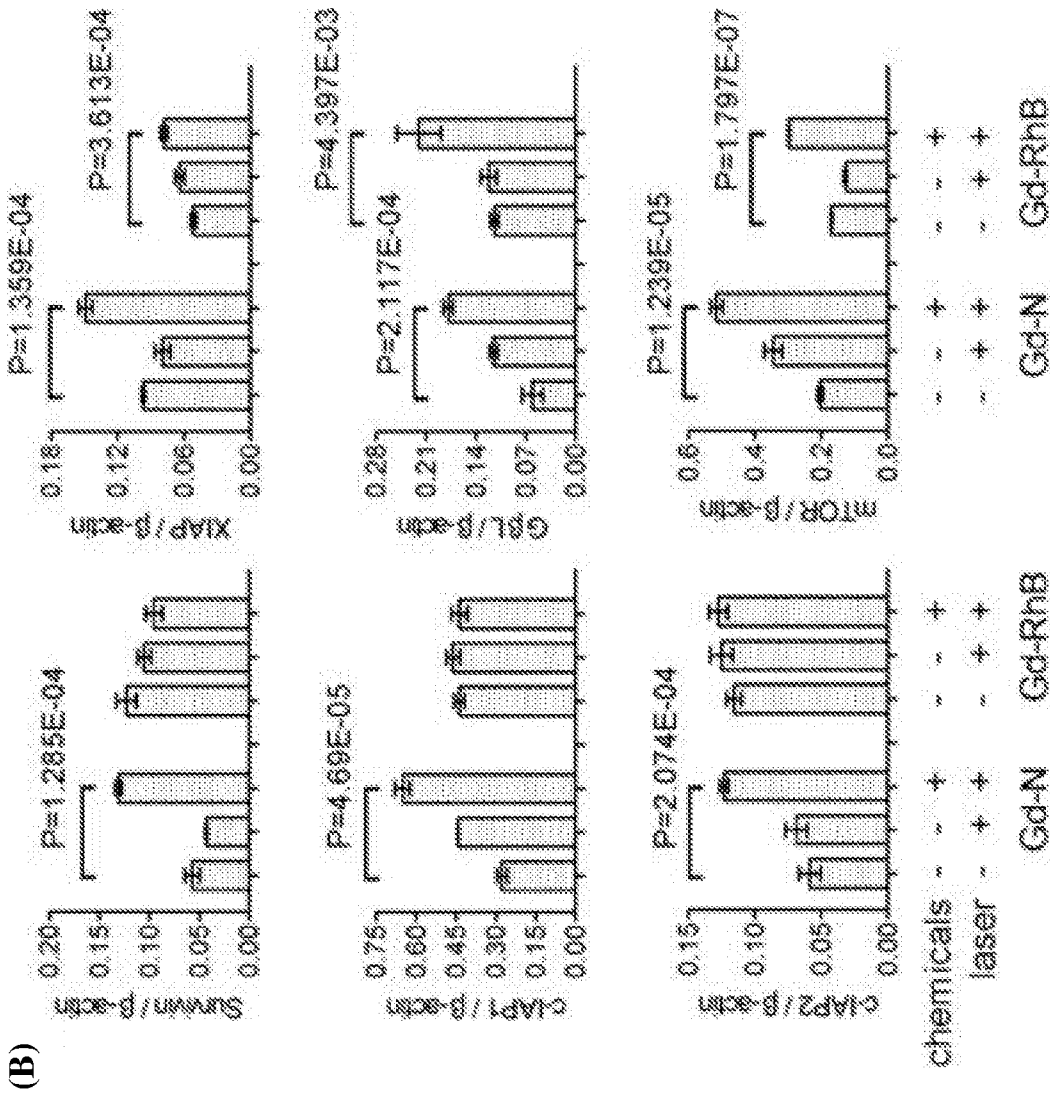


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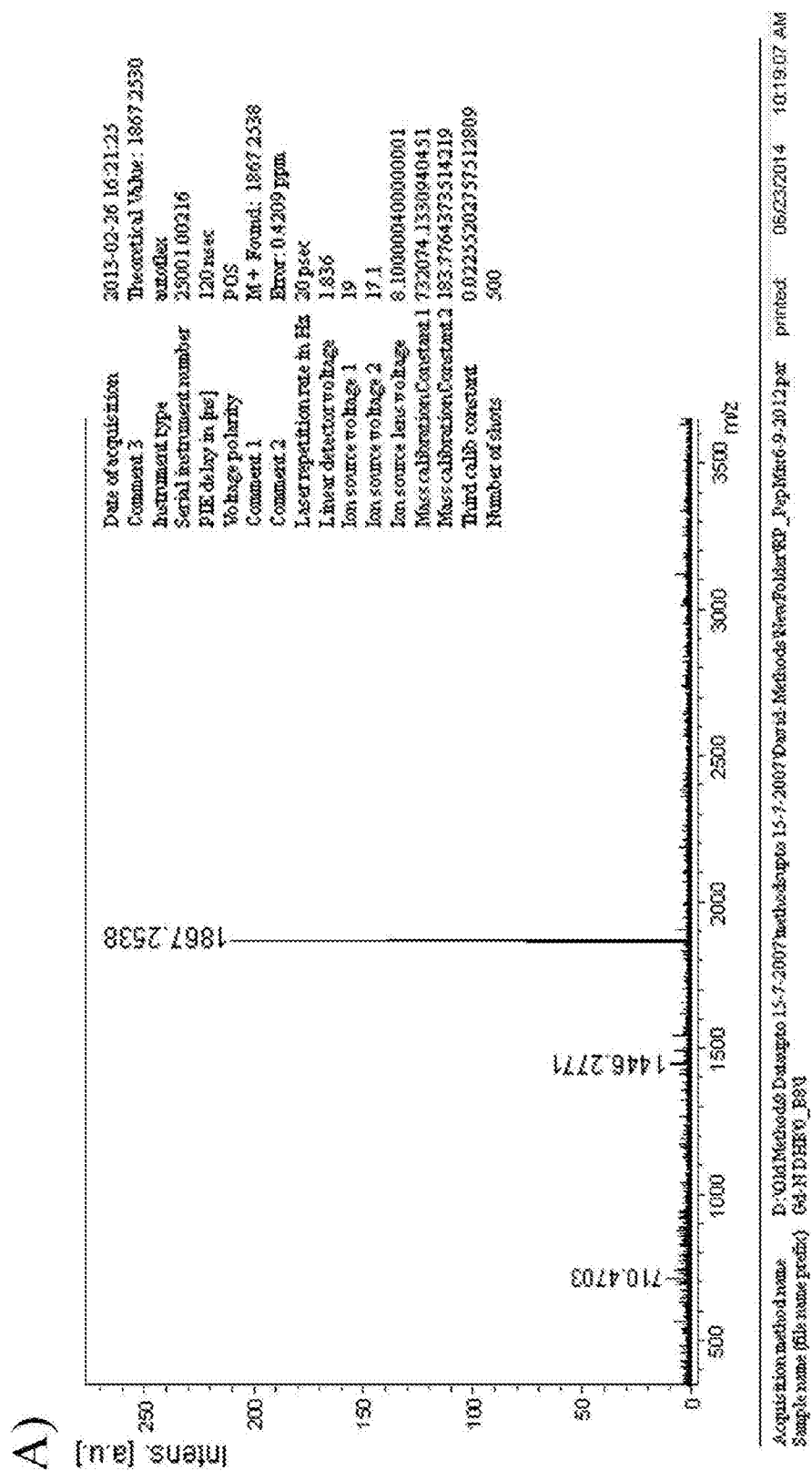


Figure 8

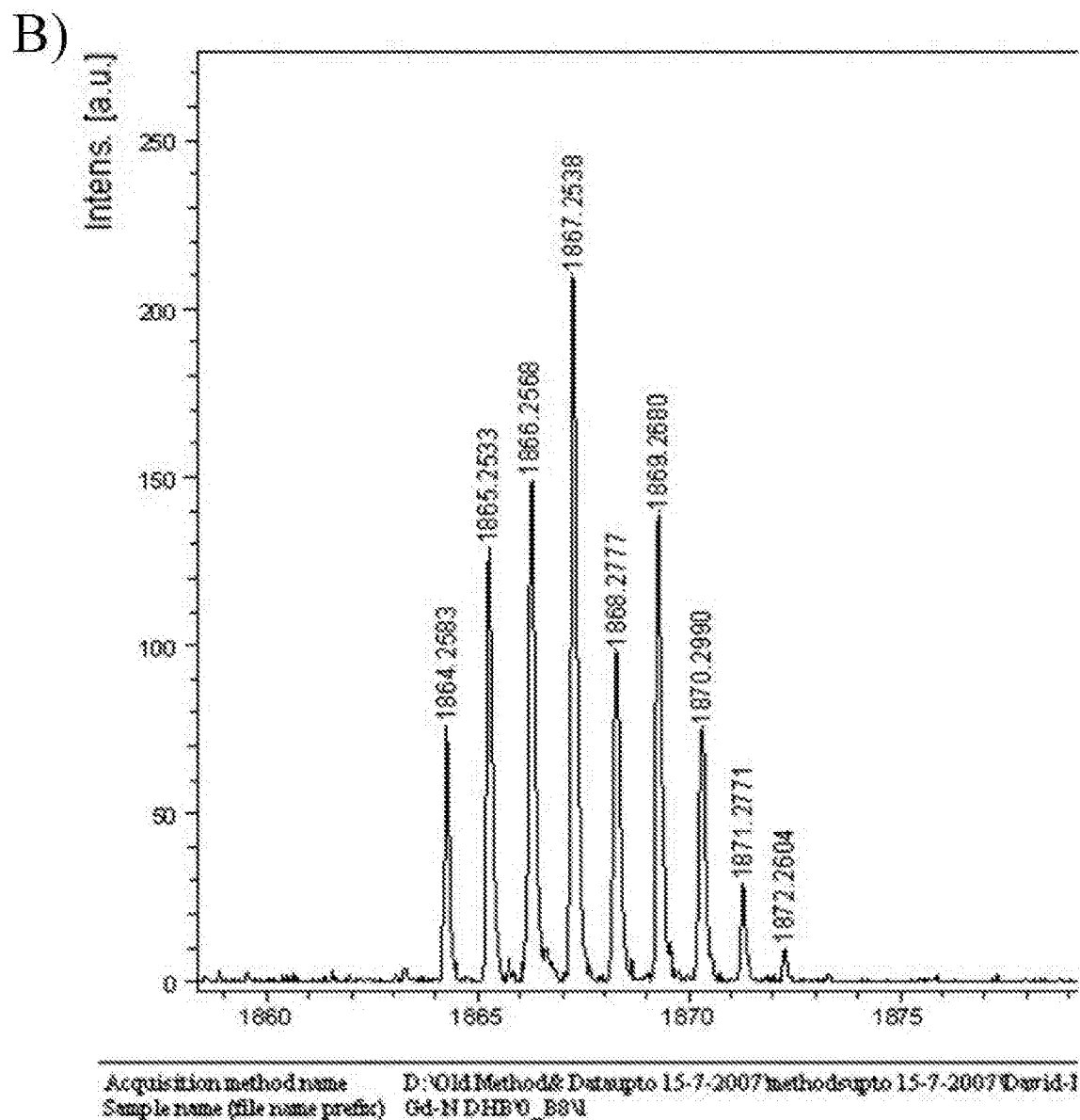
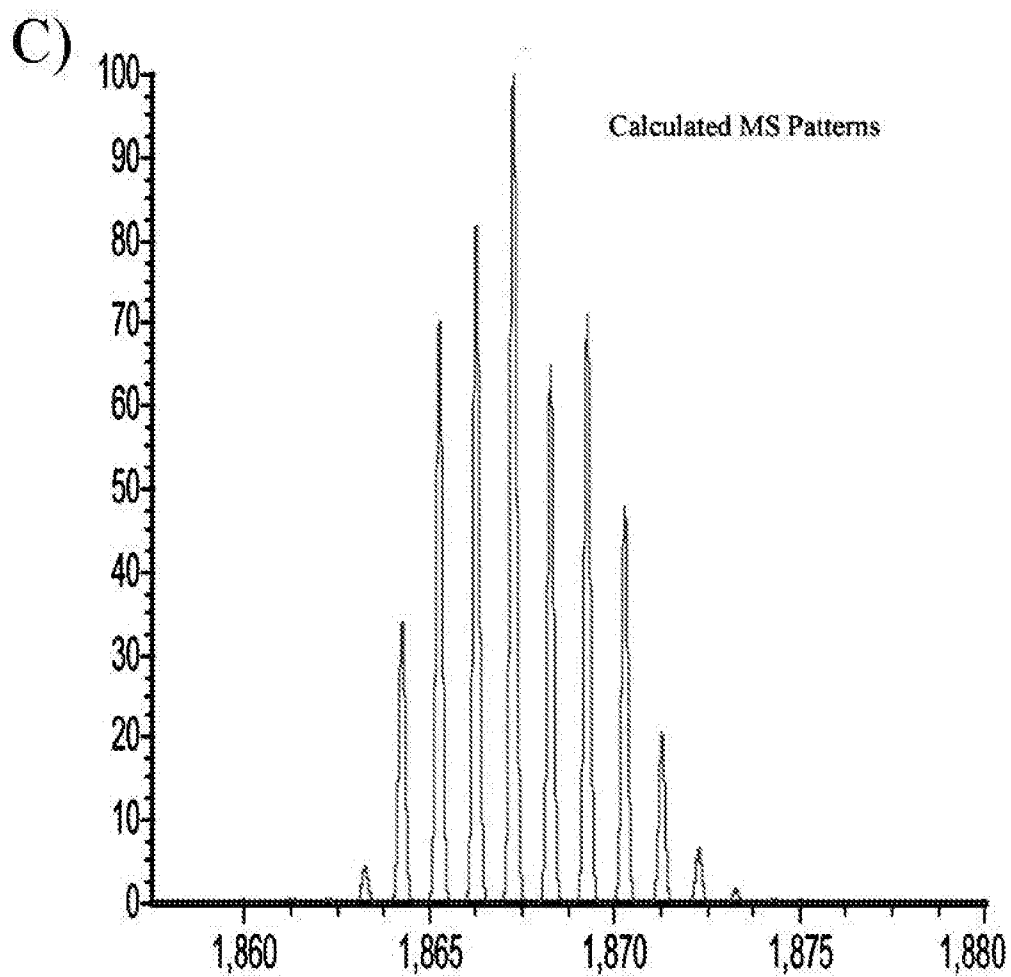
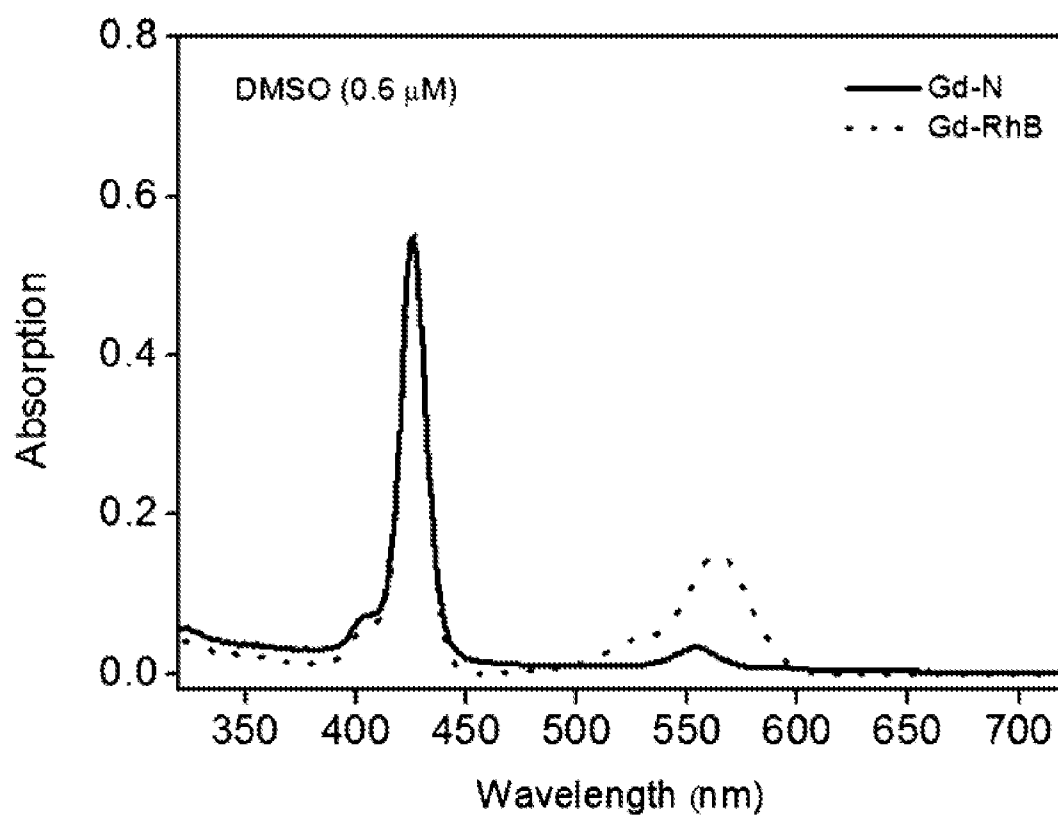


Figure 8 (continued)

**Figure 8 (continued)**

**Figure 9**

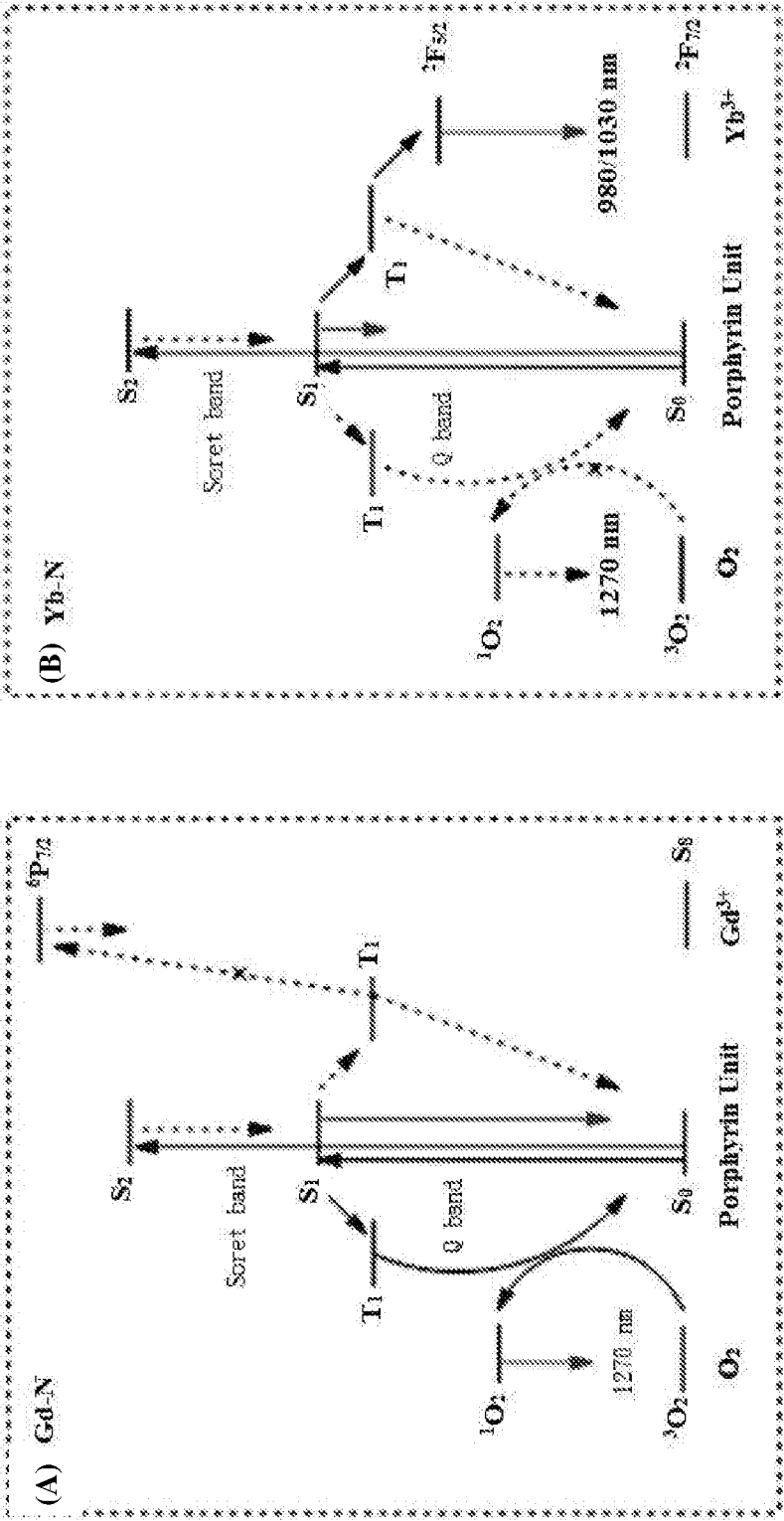
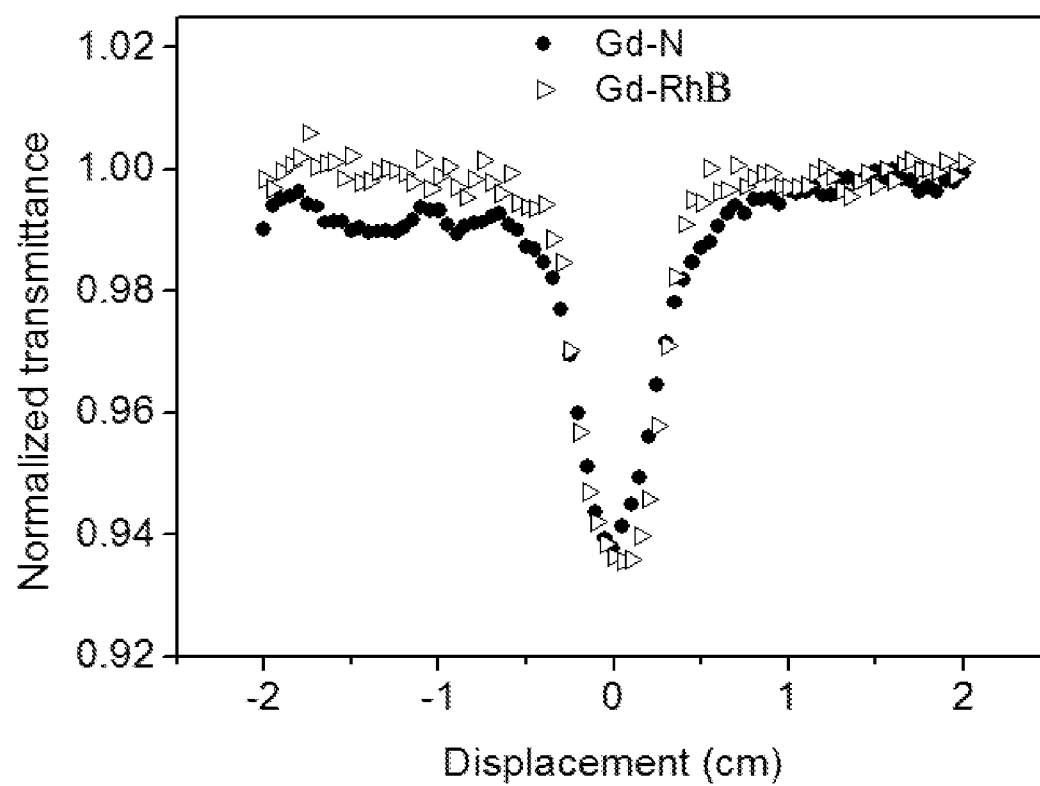


Figure 10

**Figure 11**

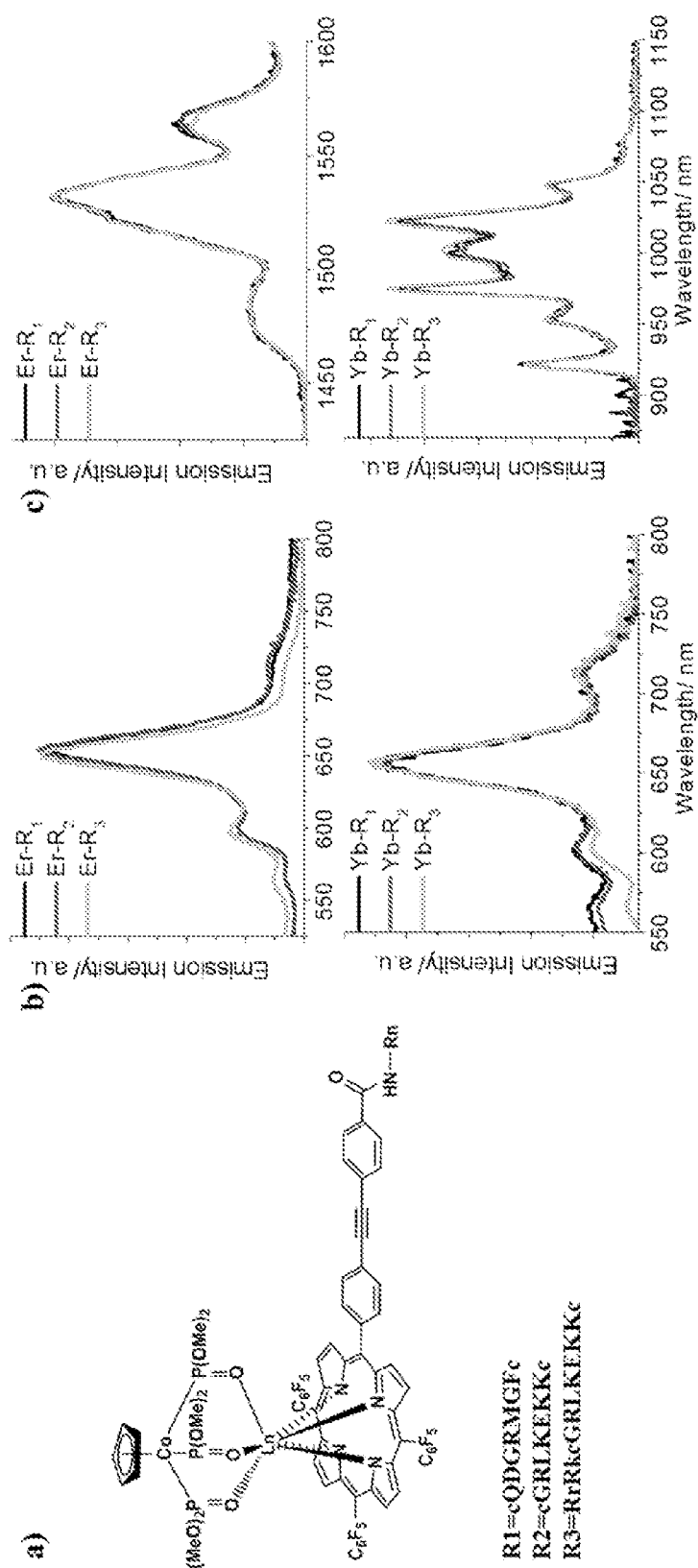


Figure 12

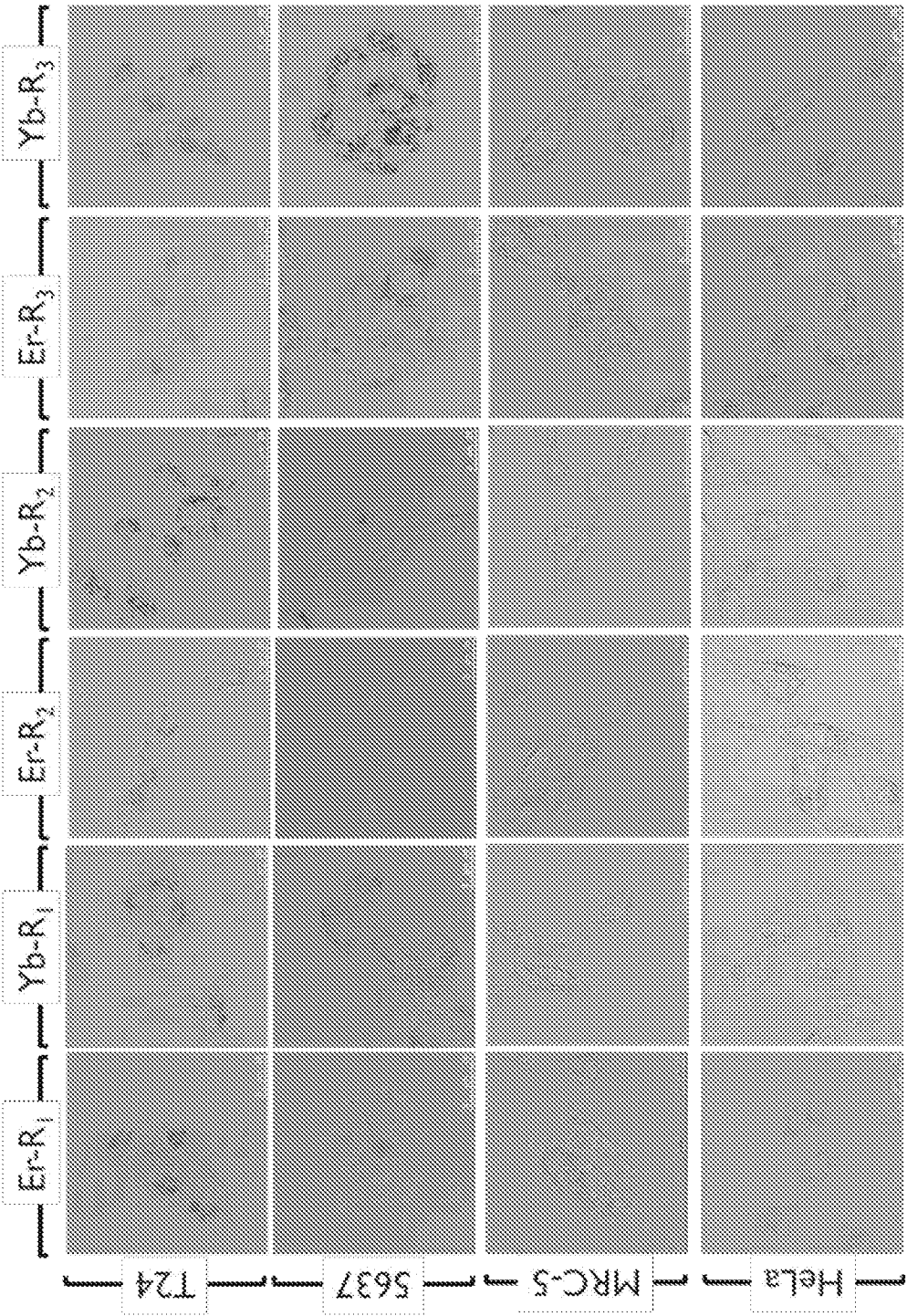


Figure 13

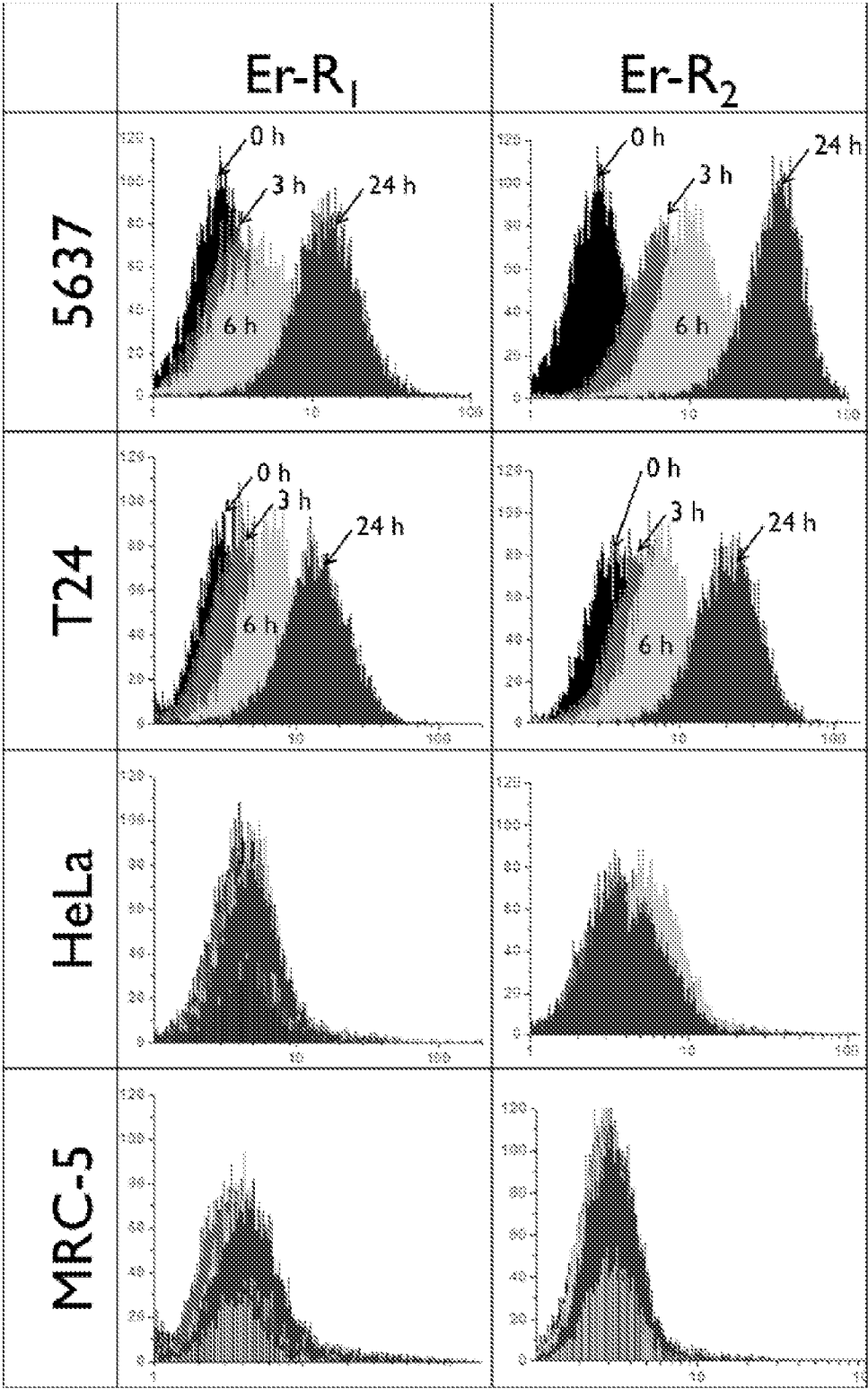


Figure 14

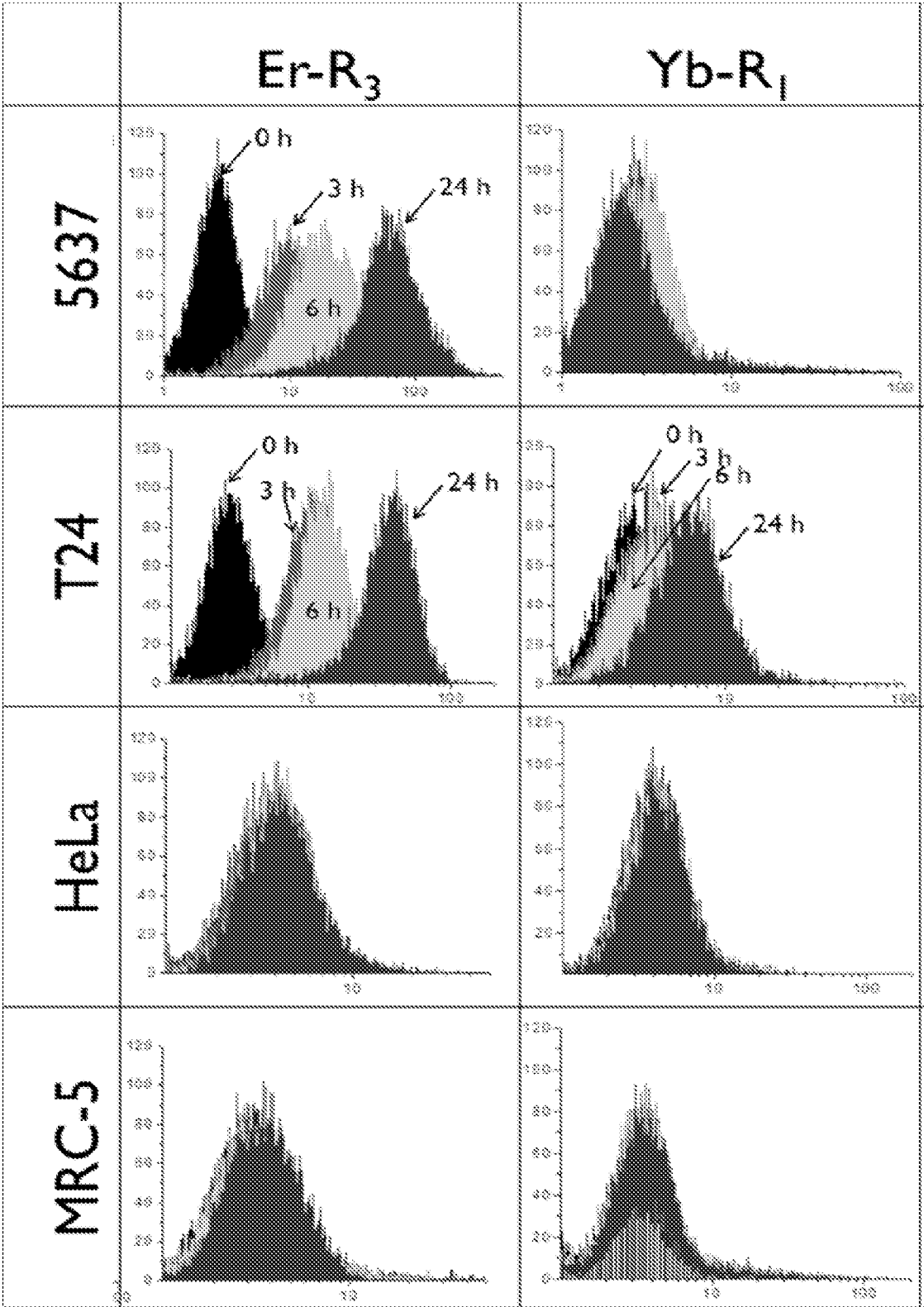


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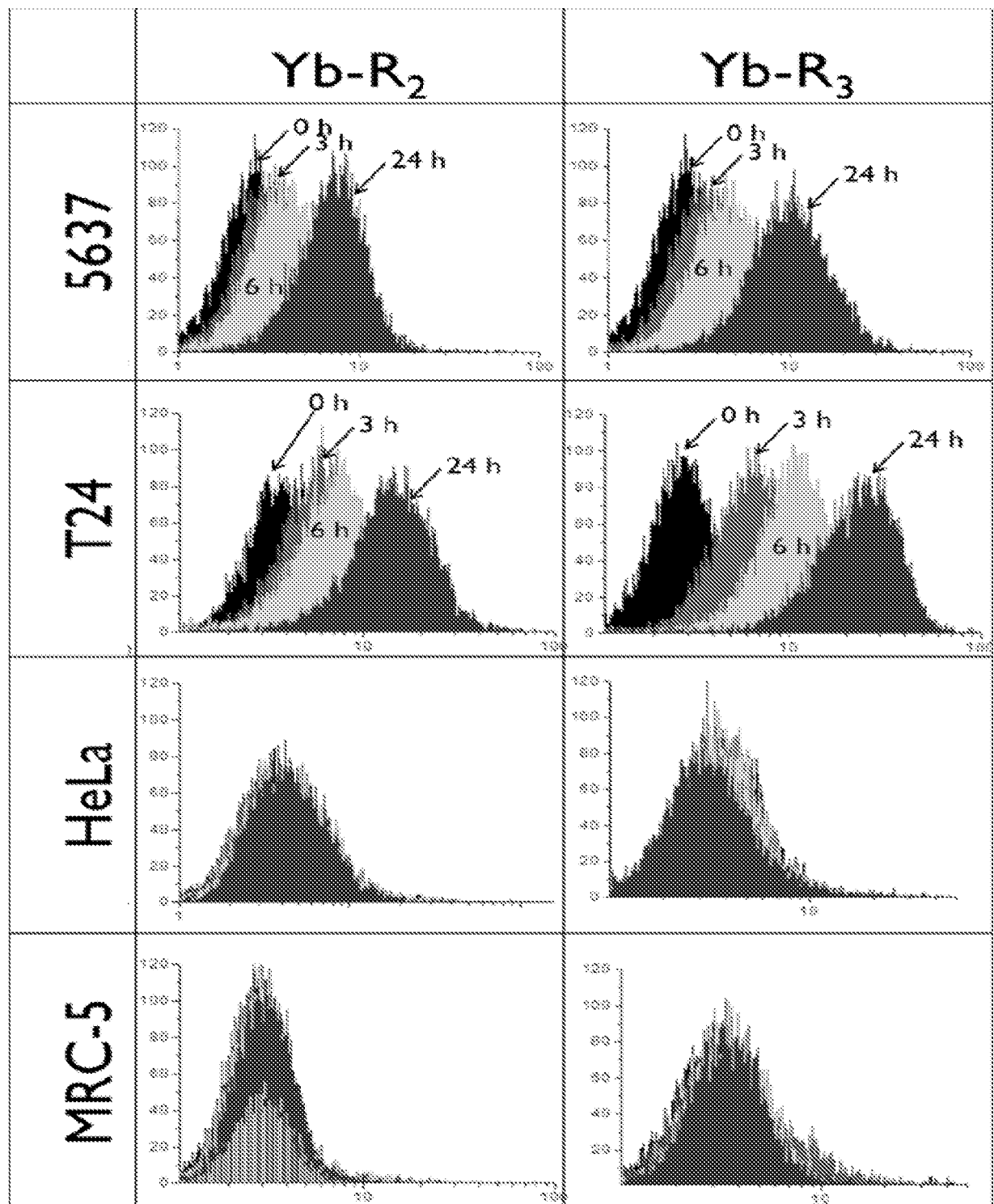


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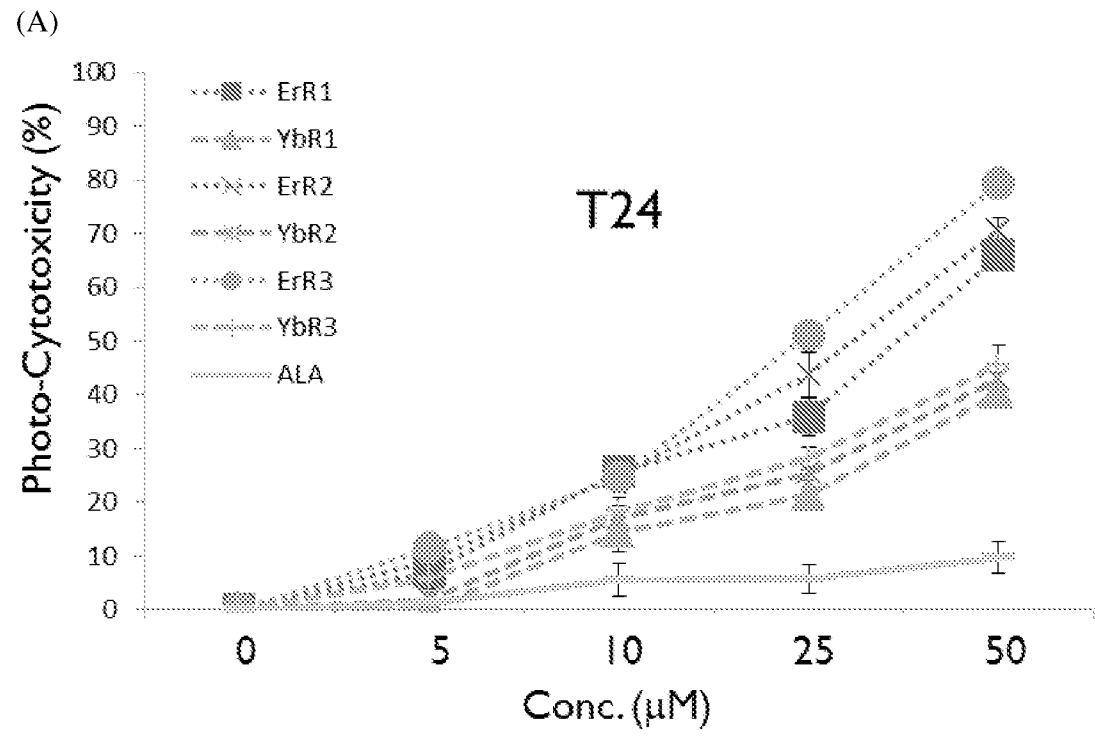


Figure 15

(B)

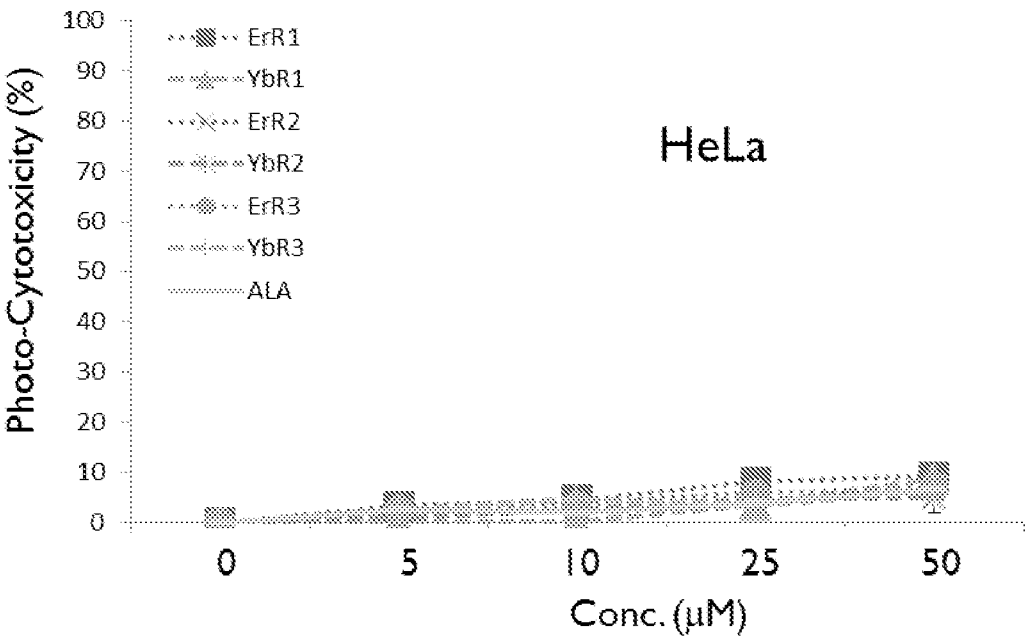
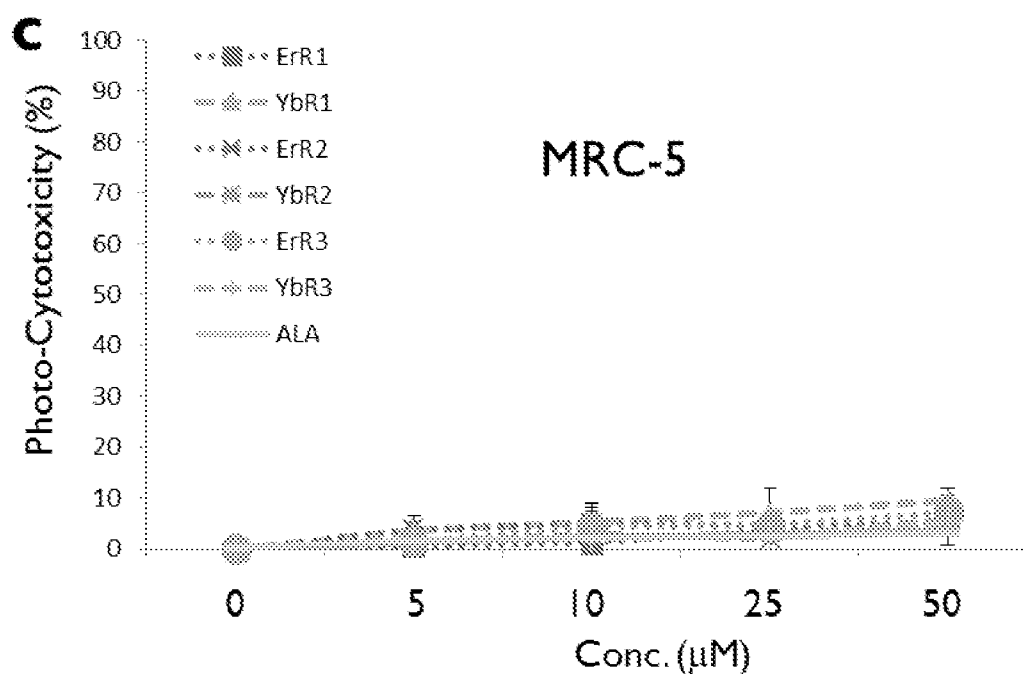


Figure 15 (continued)



(D)

Photo- IC_{50} /mM (Dark- IC_{50} /mM)	T24	HeLa	MRC-5
Er- R_1	38 (817)	271 (937)	489 (922)
Yb- R_1	63 (1414)	287 (1297)	264 (1382)
Er- R_2	35 (932)	392 (1184)	497 (1073)
Yb- R_2	60 (1551)	499 (1422)	434 (1601)
Er- R_3	31 (1044)	343 (1302)	345 (1127)
Yb- R_3	56 (1766)	393 (1683)	473 (1884)
ALA	260 (929)	375 (1043)	744 (1547)

Figure 15 (continued)

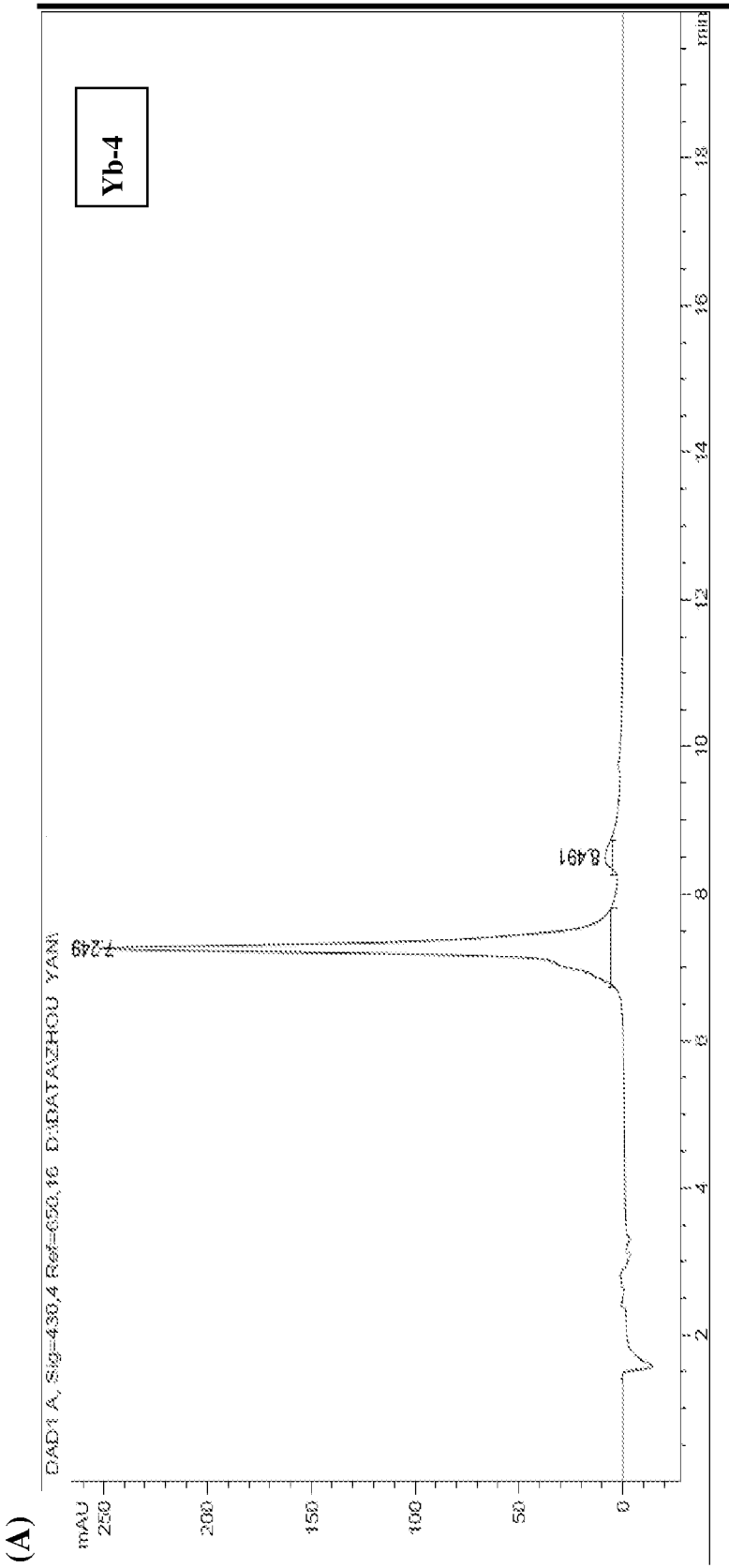


Figure 16

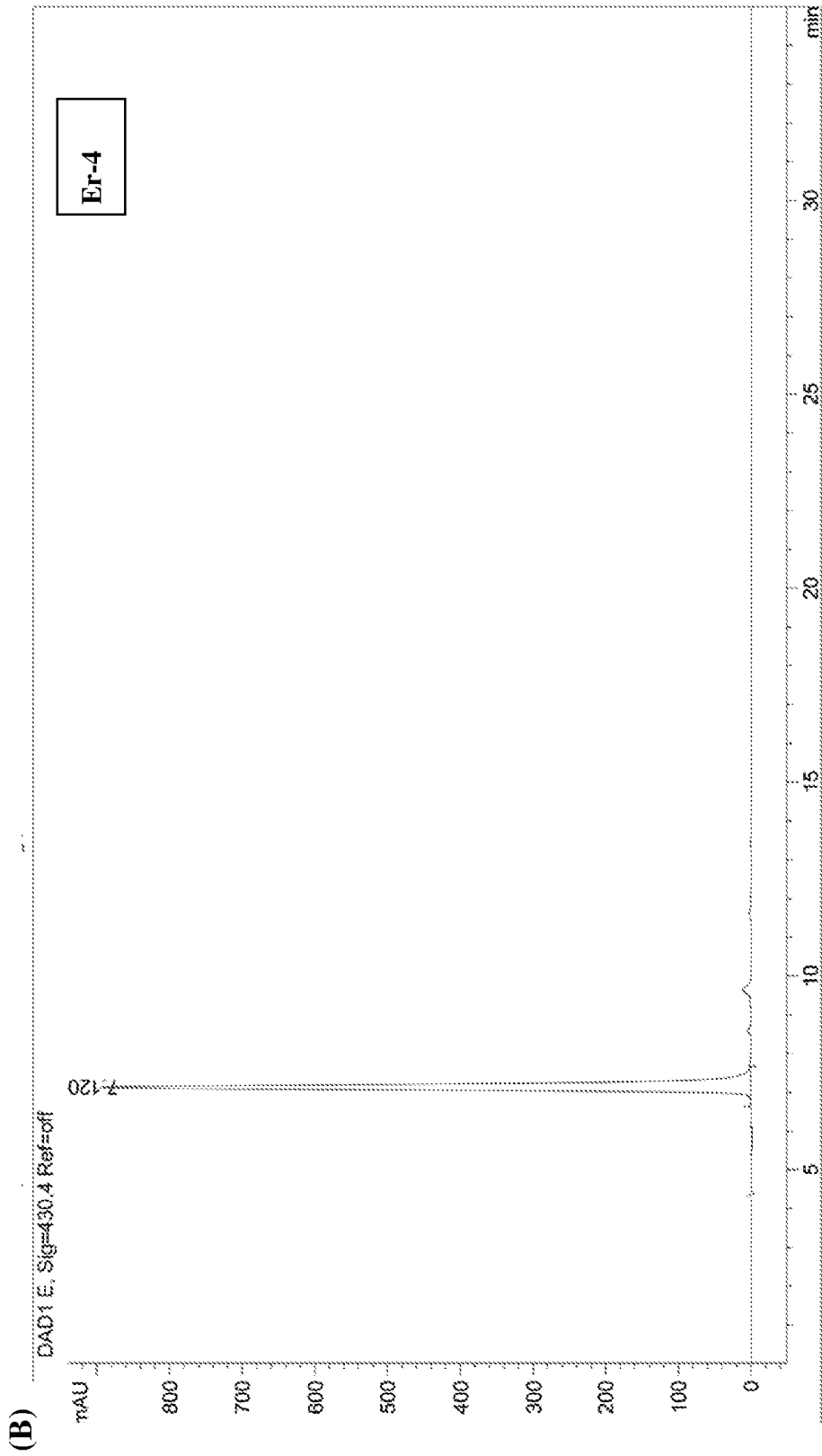


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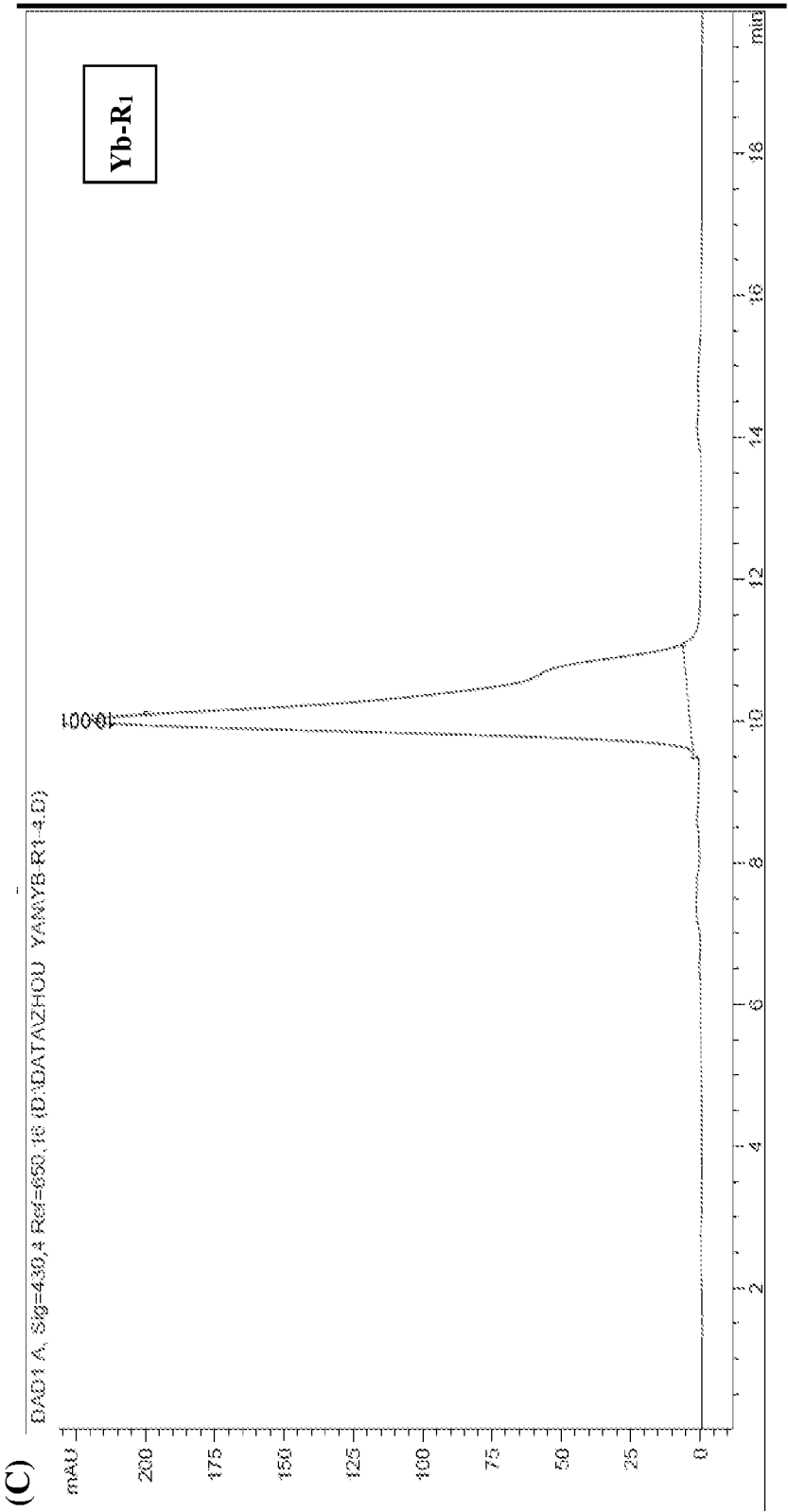


Figure 16 (continued)

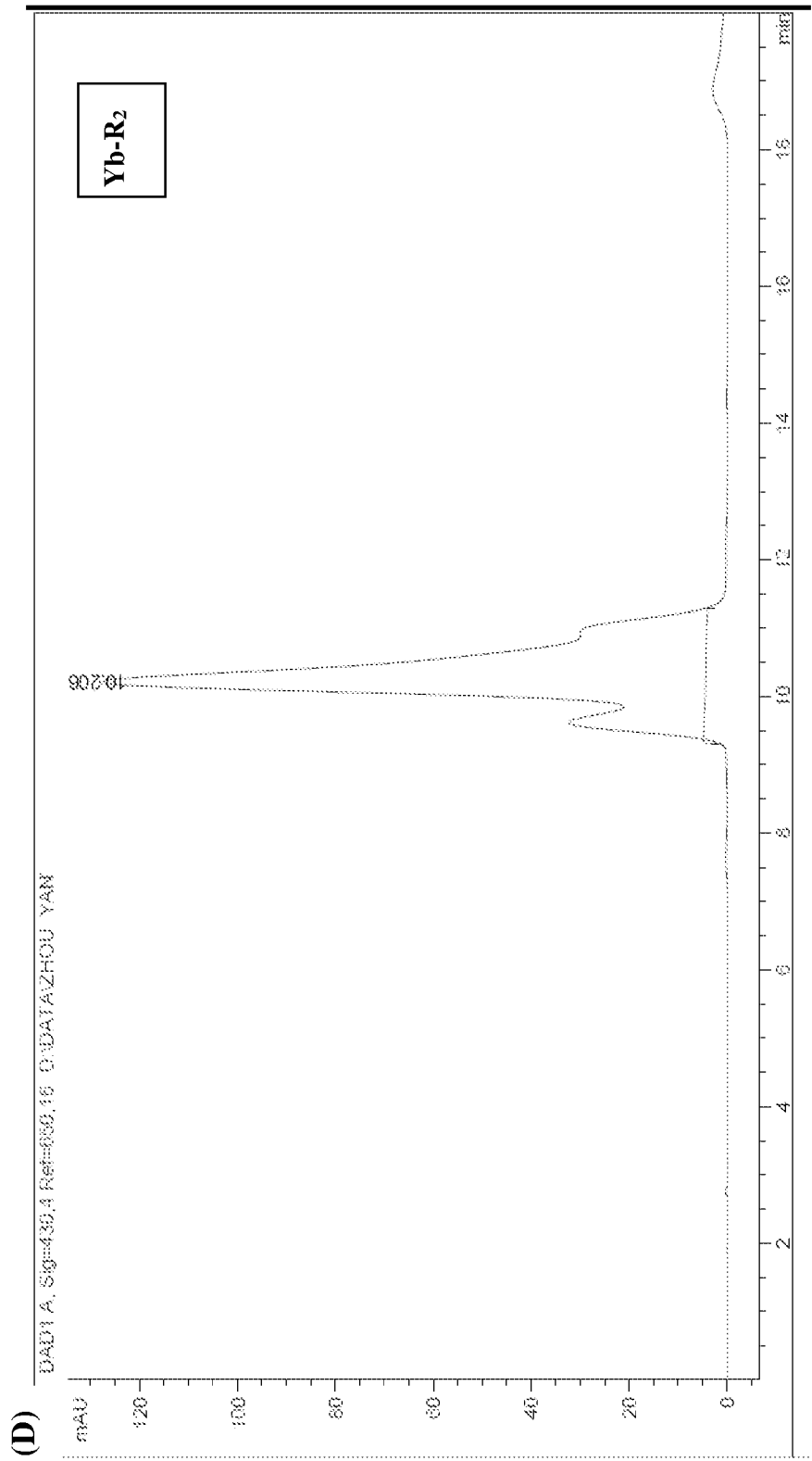


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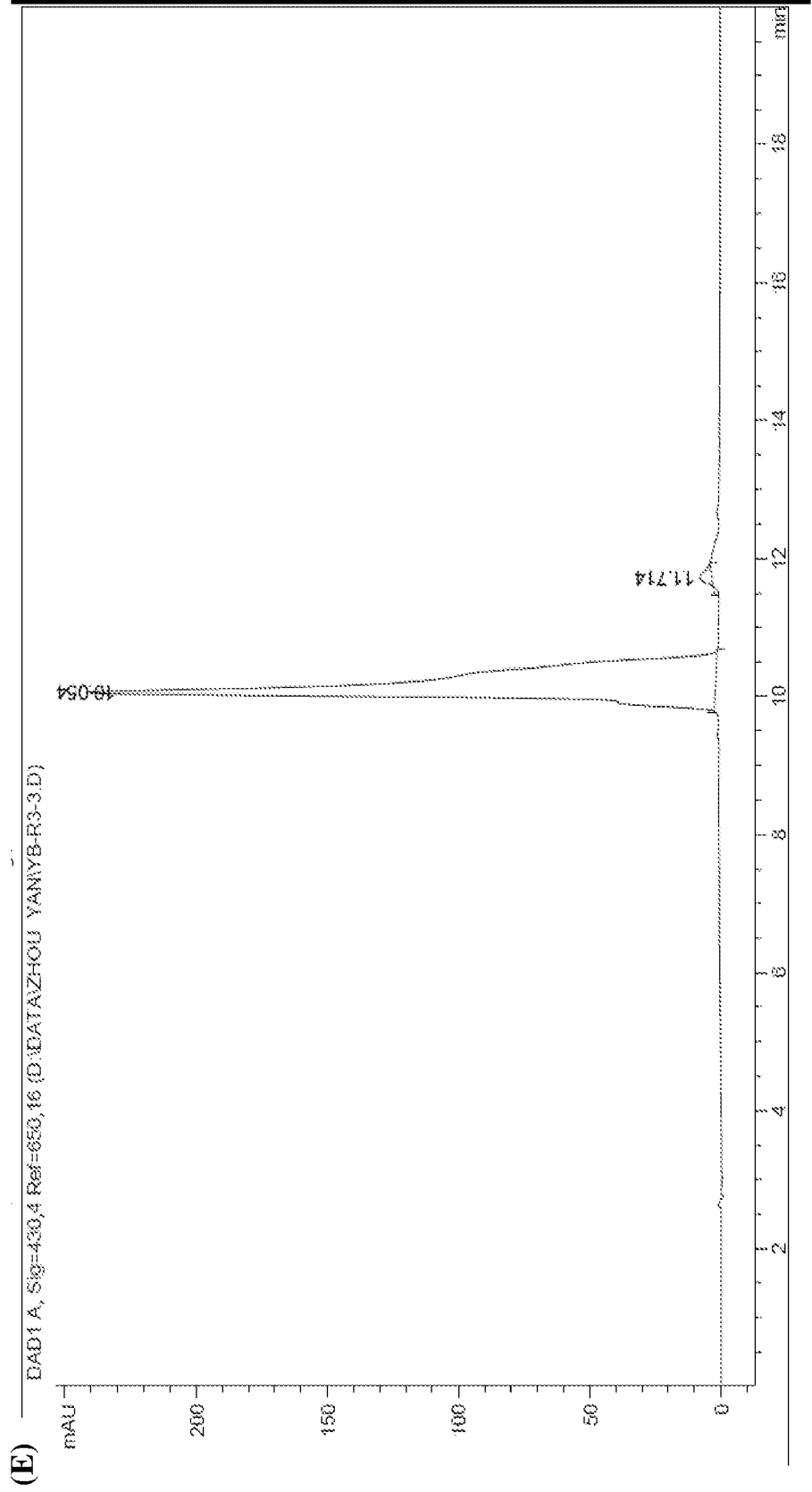


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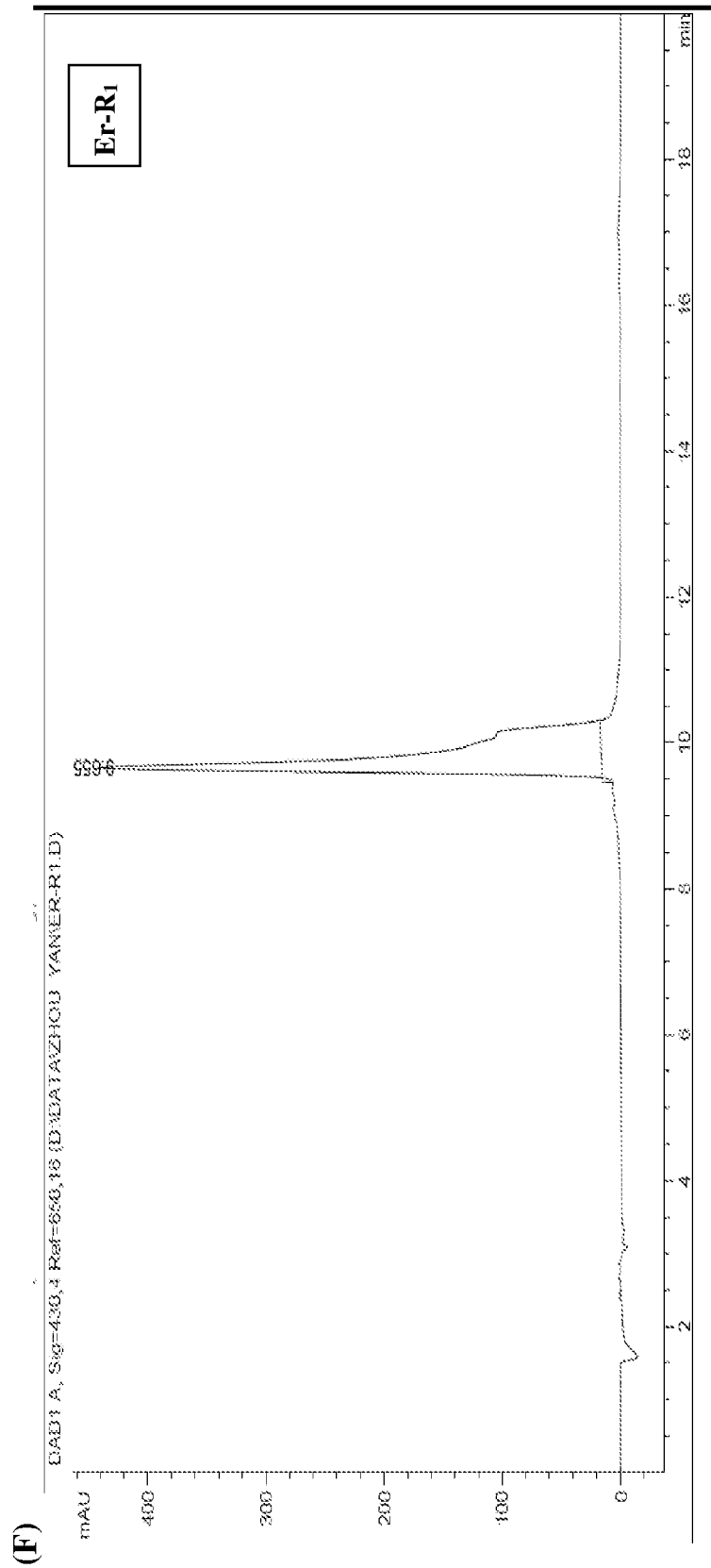


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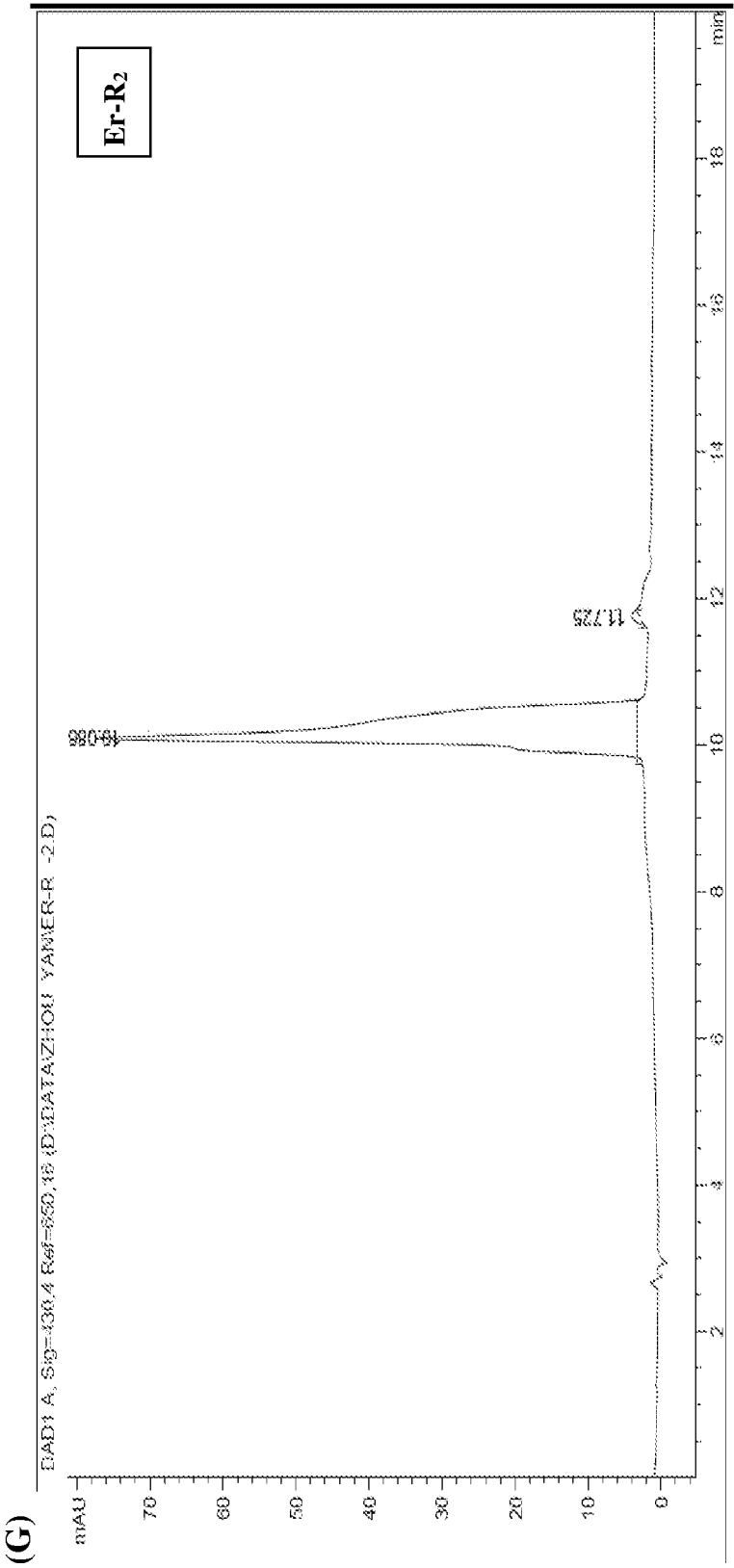


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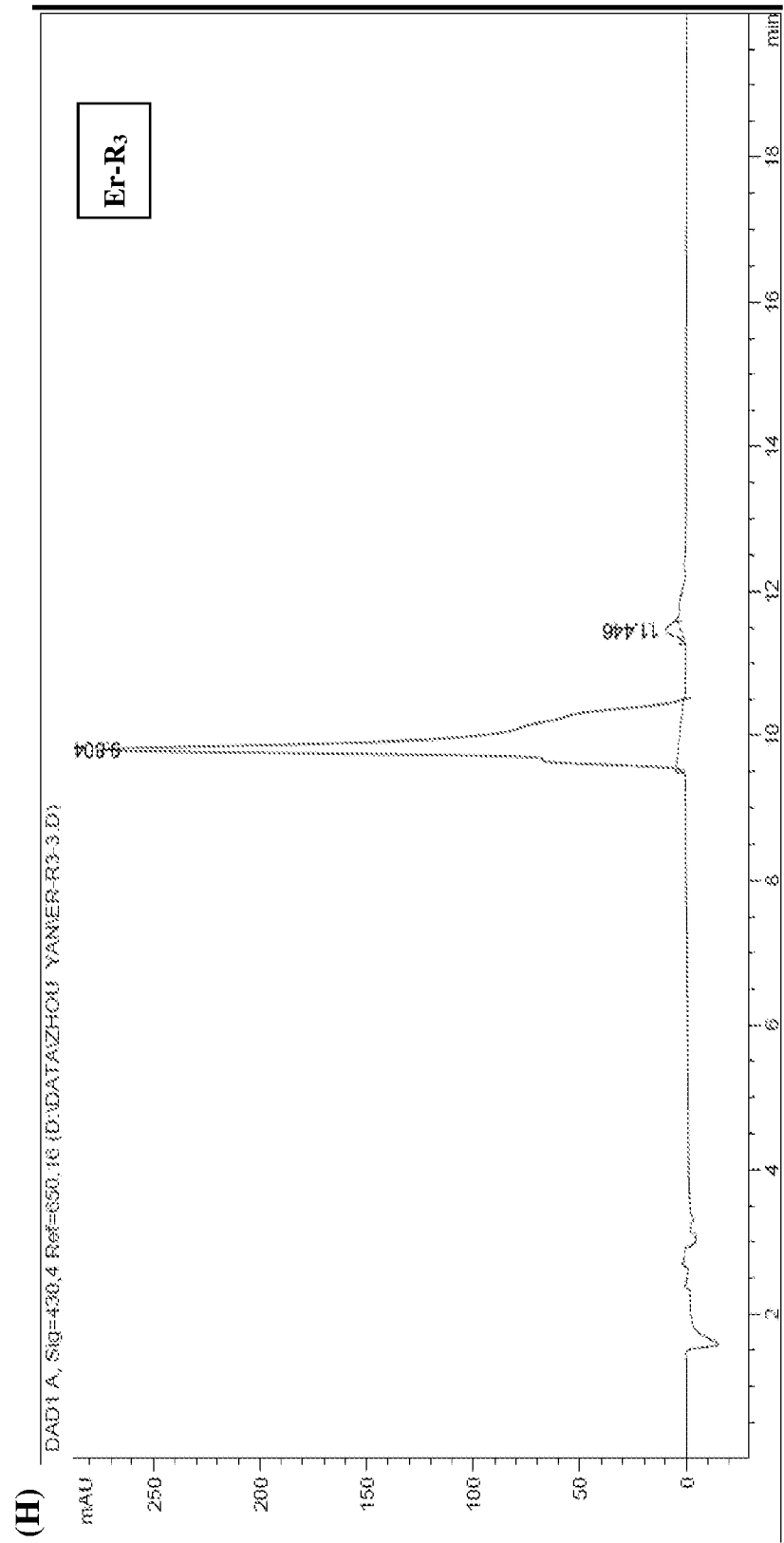


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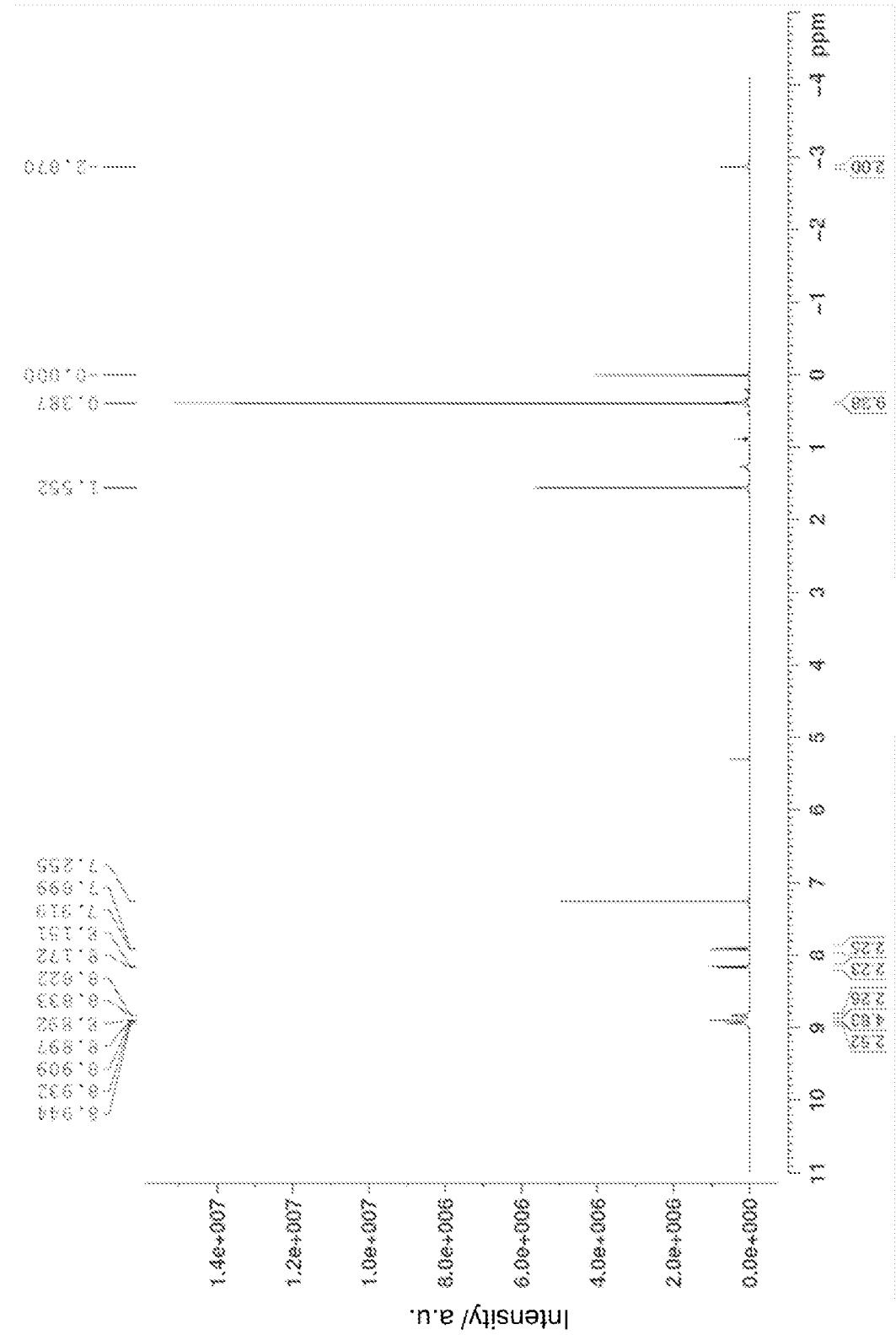


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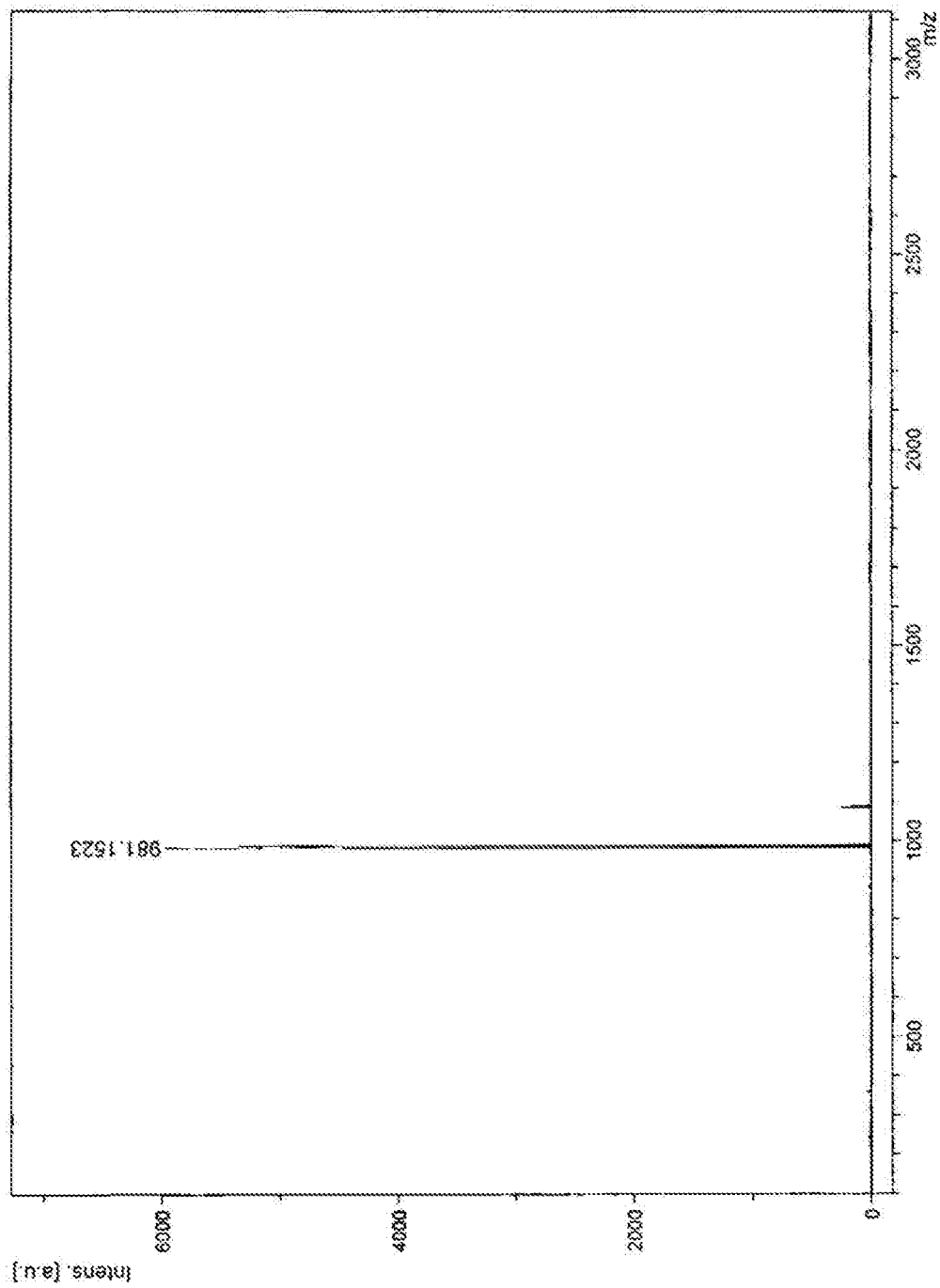


Figure 18

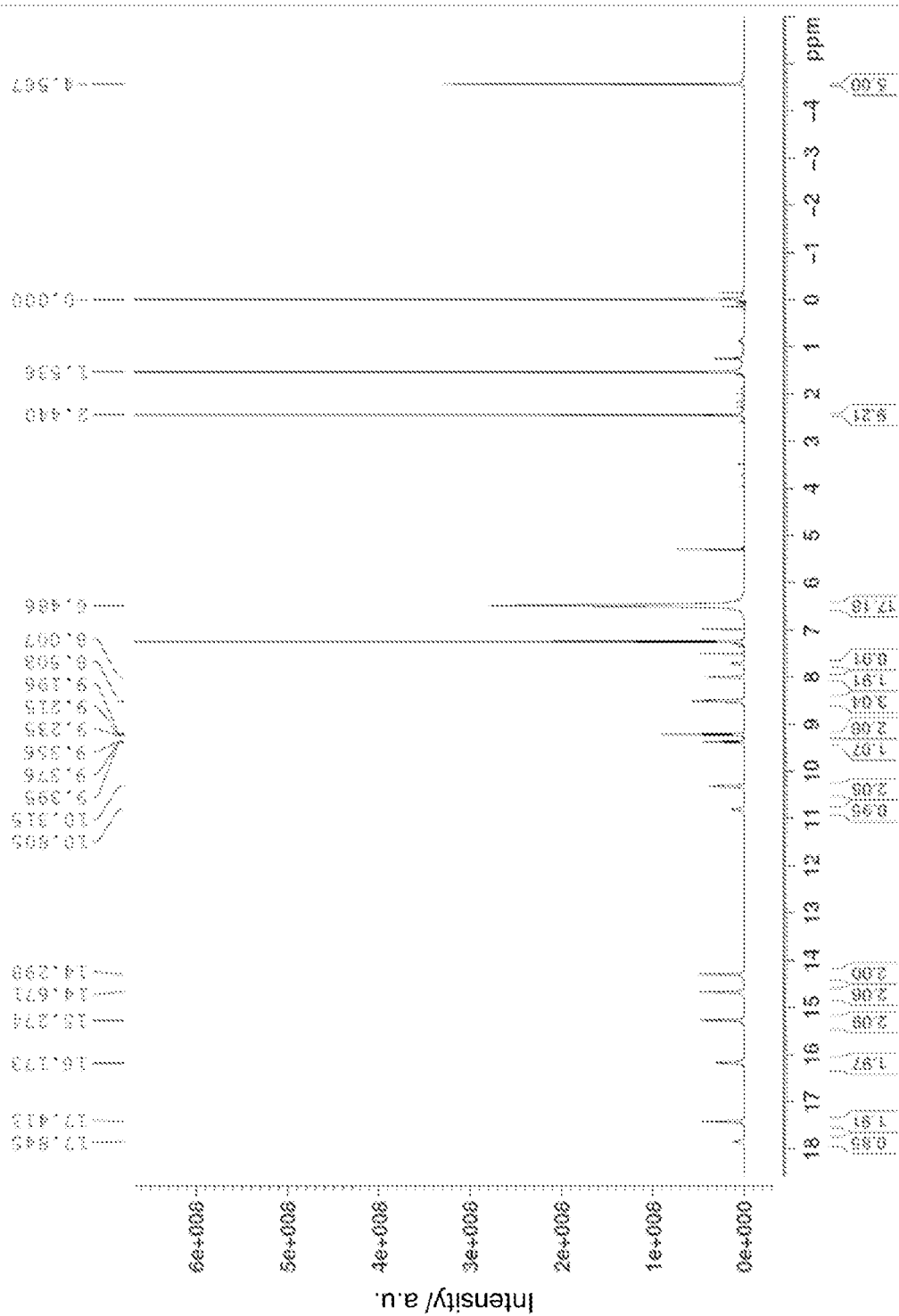


Figure 19

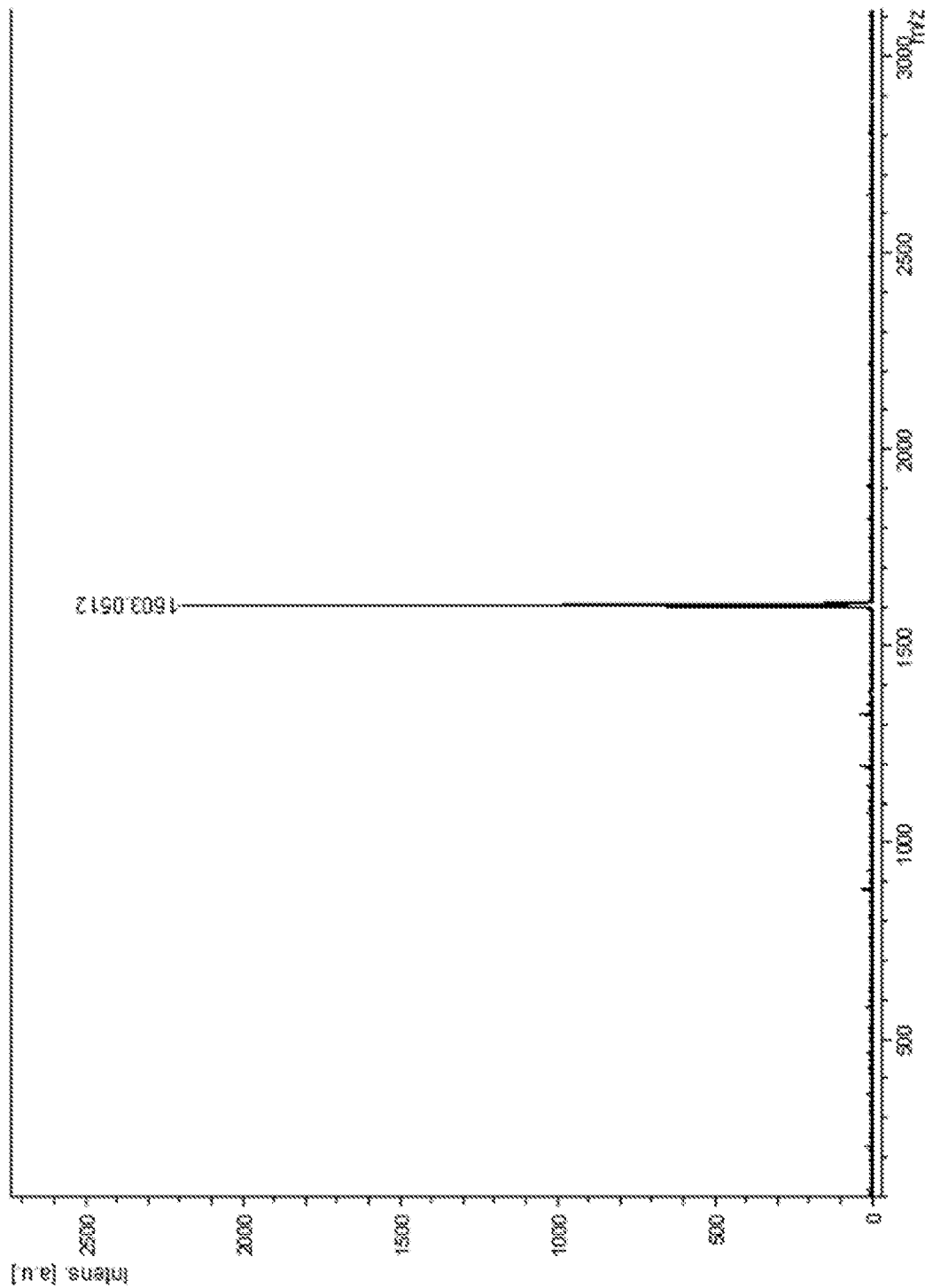


Figure 20

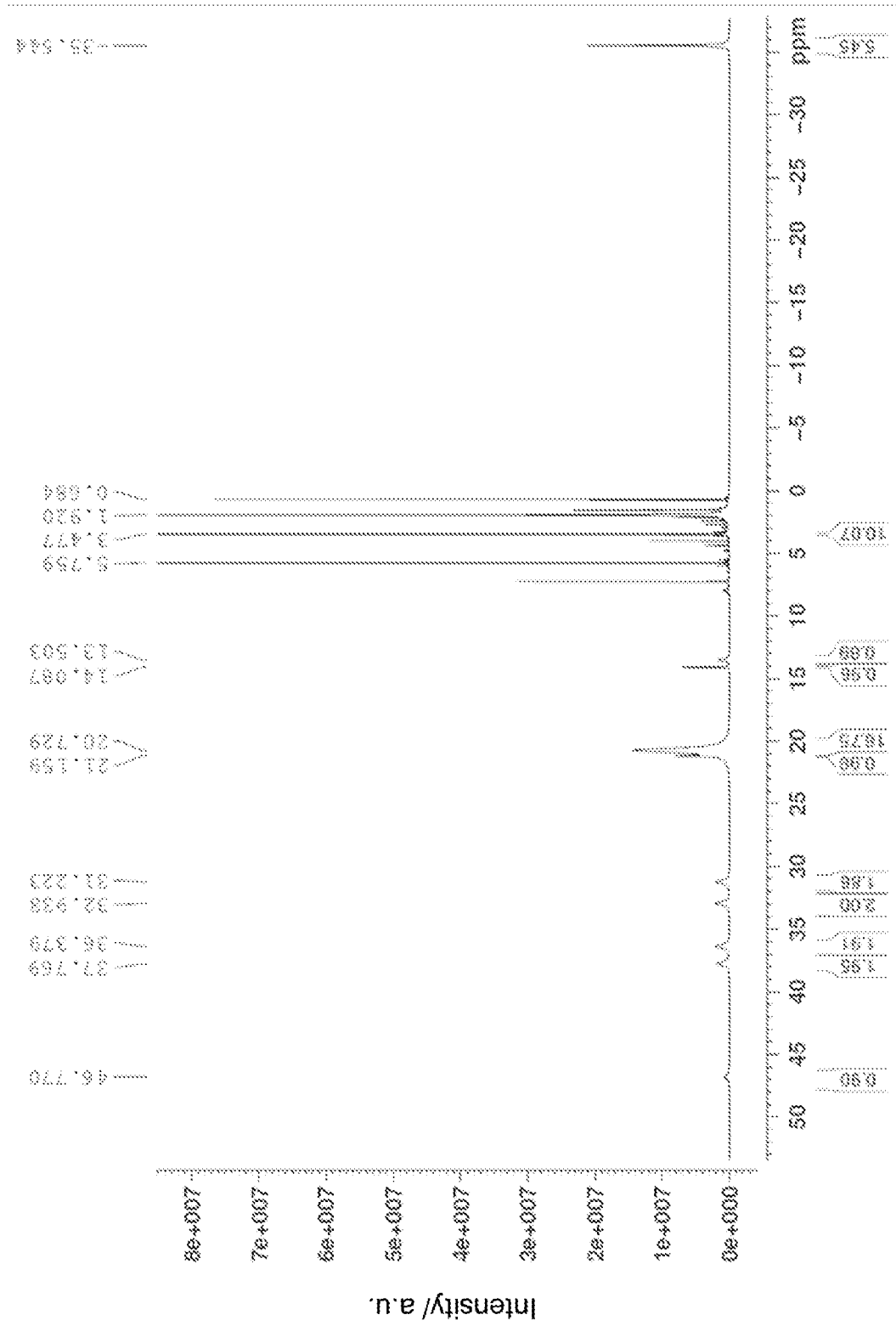


Figure 21

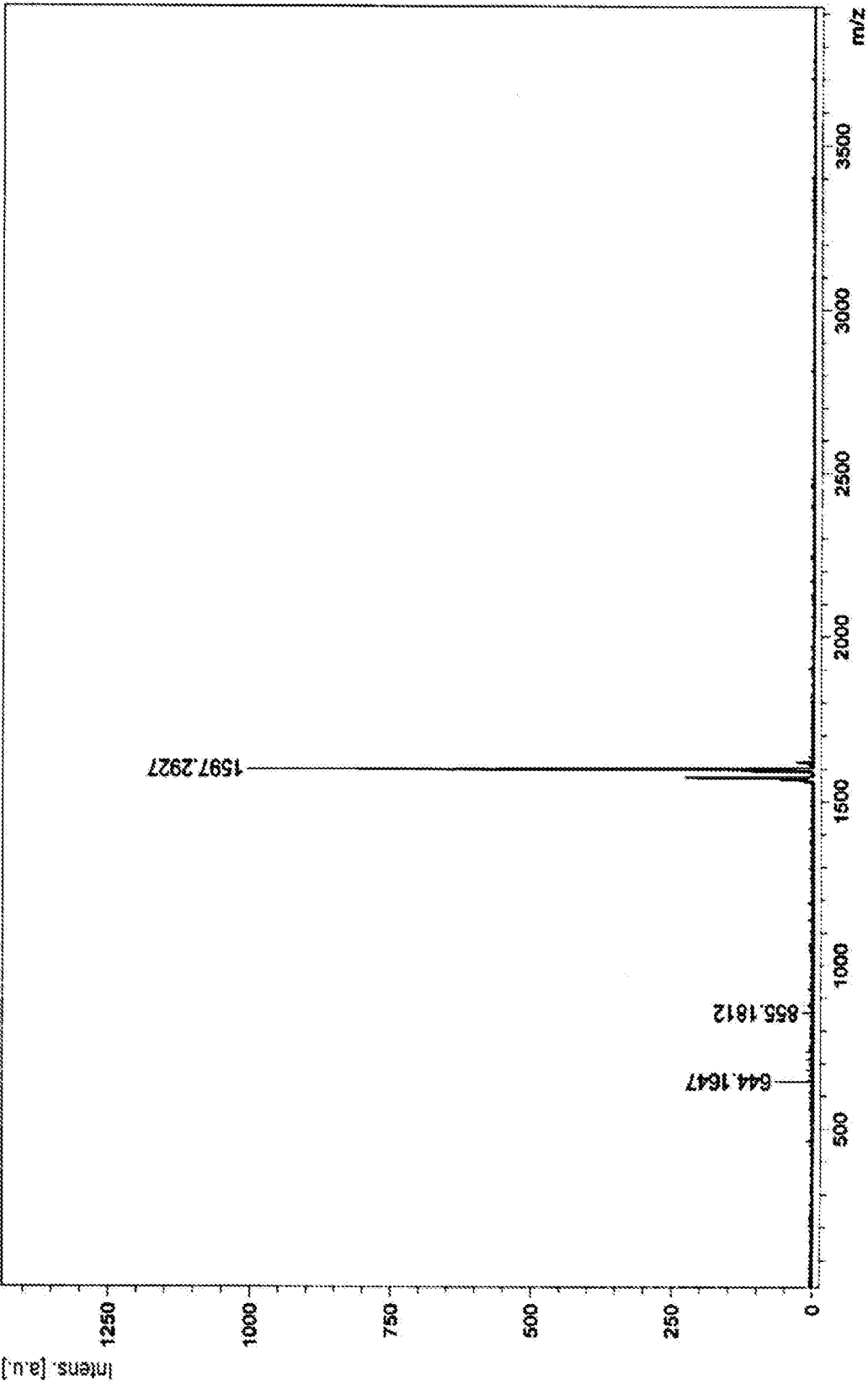


Figure 22

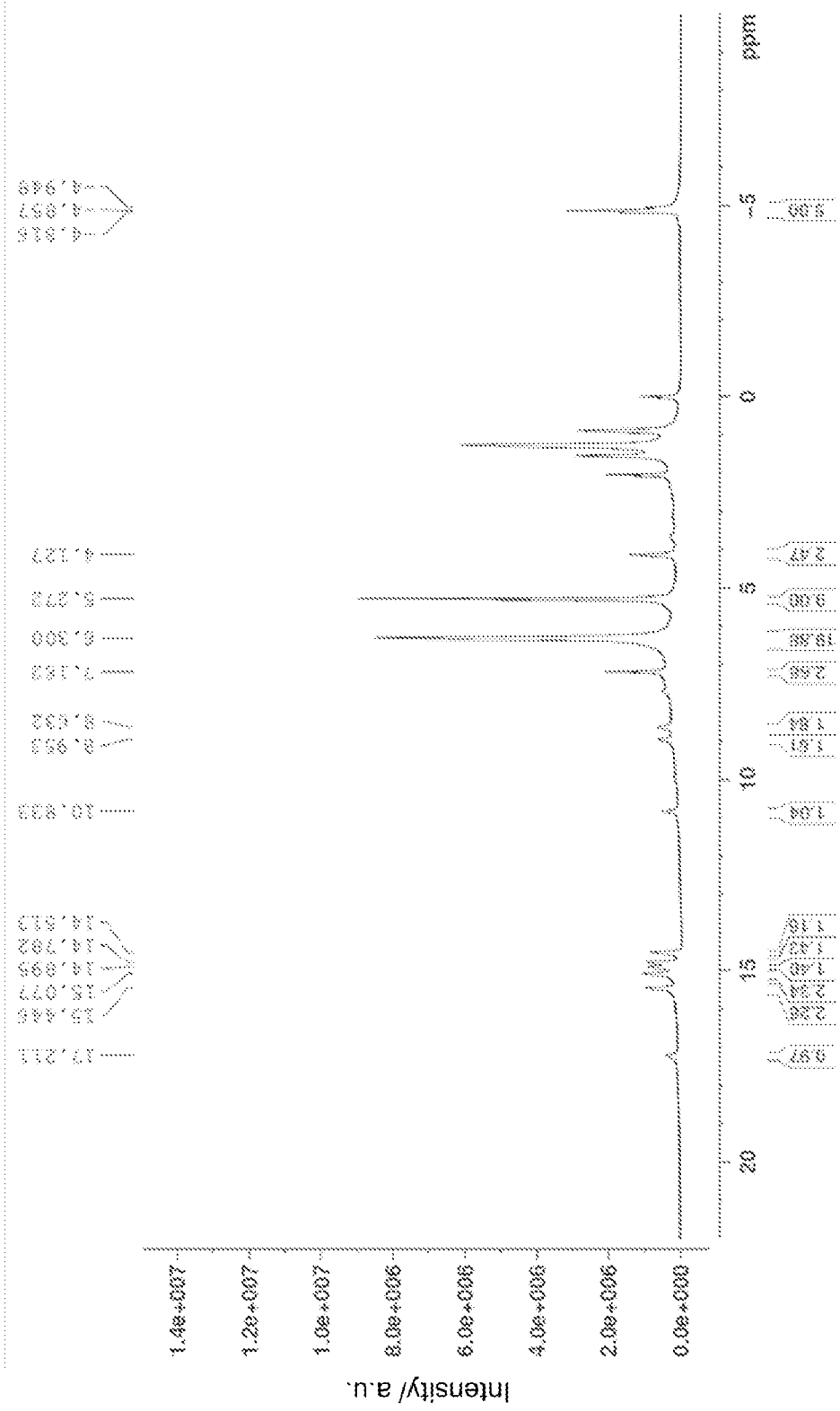


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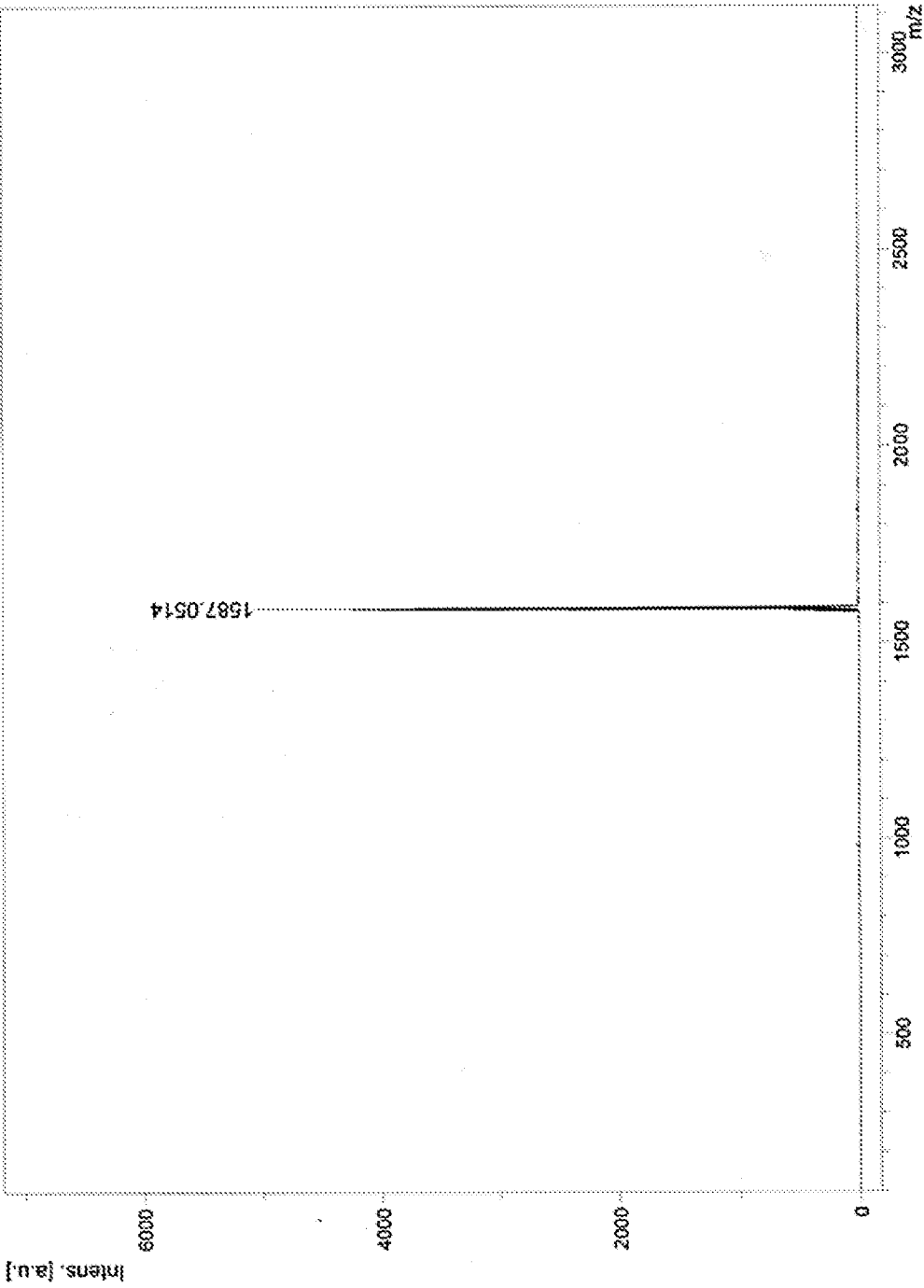


Figure 24

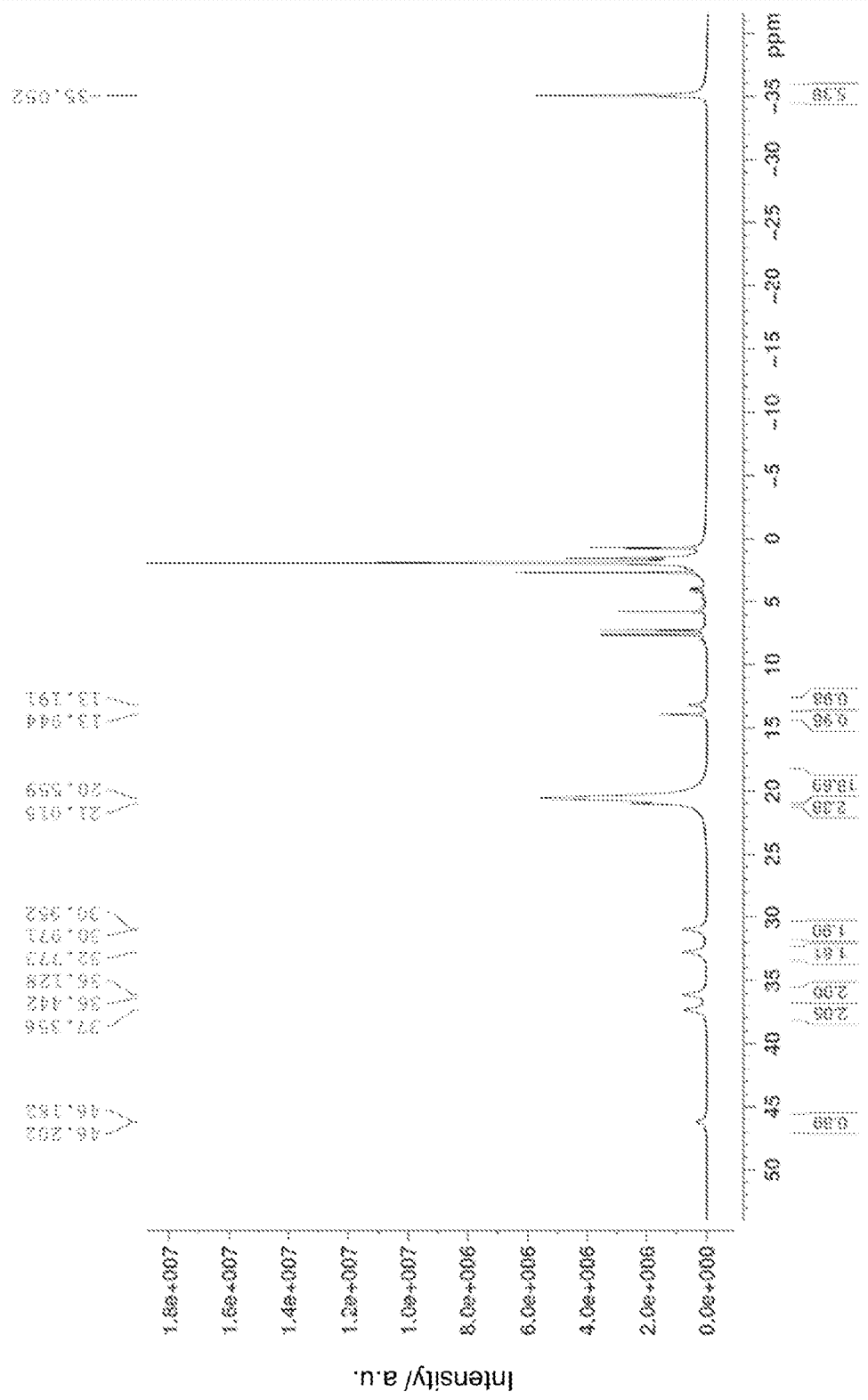


Figure 25

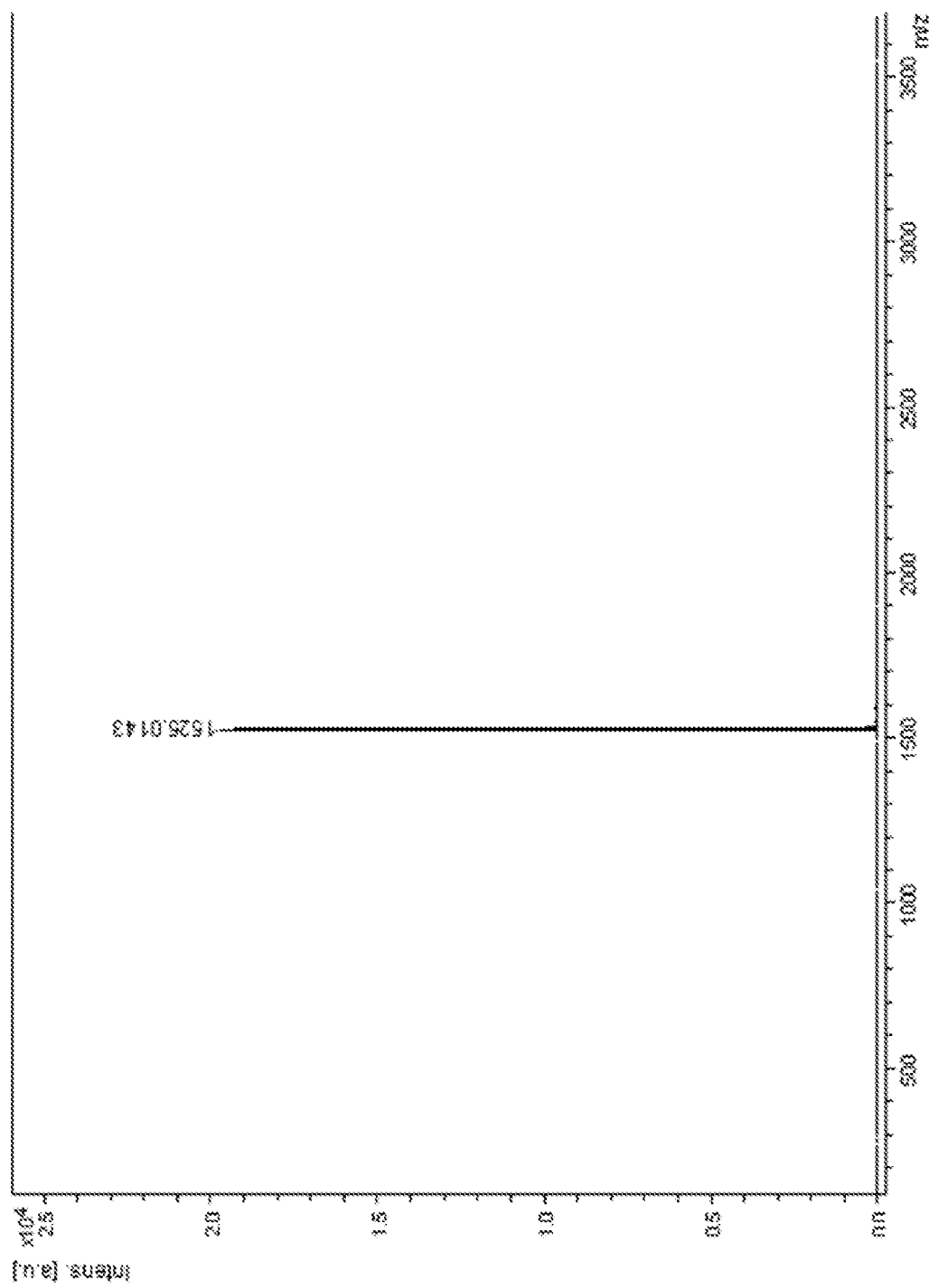


Figure 26

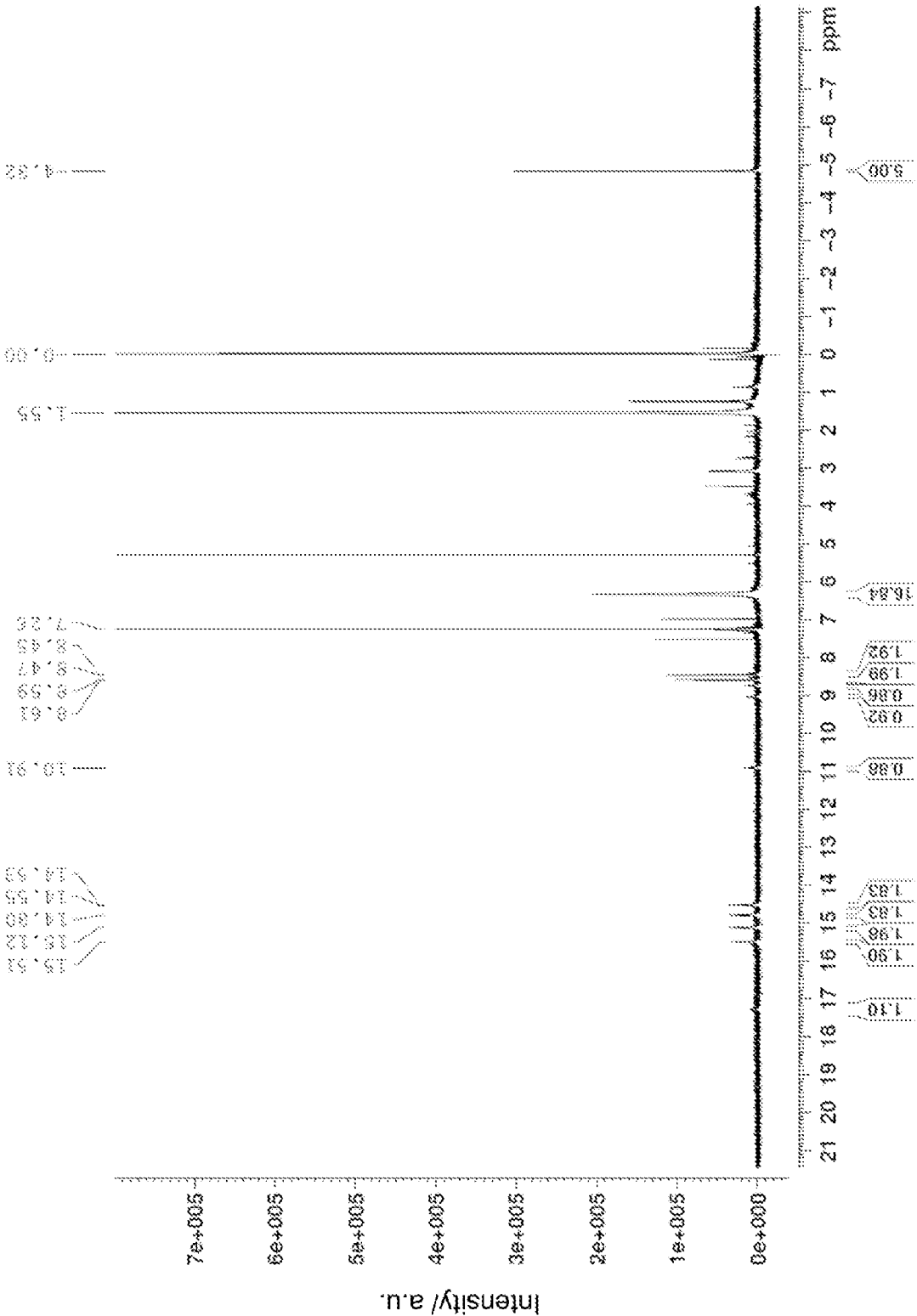


Figure 27

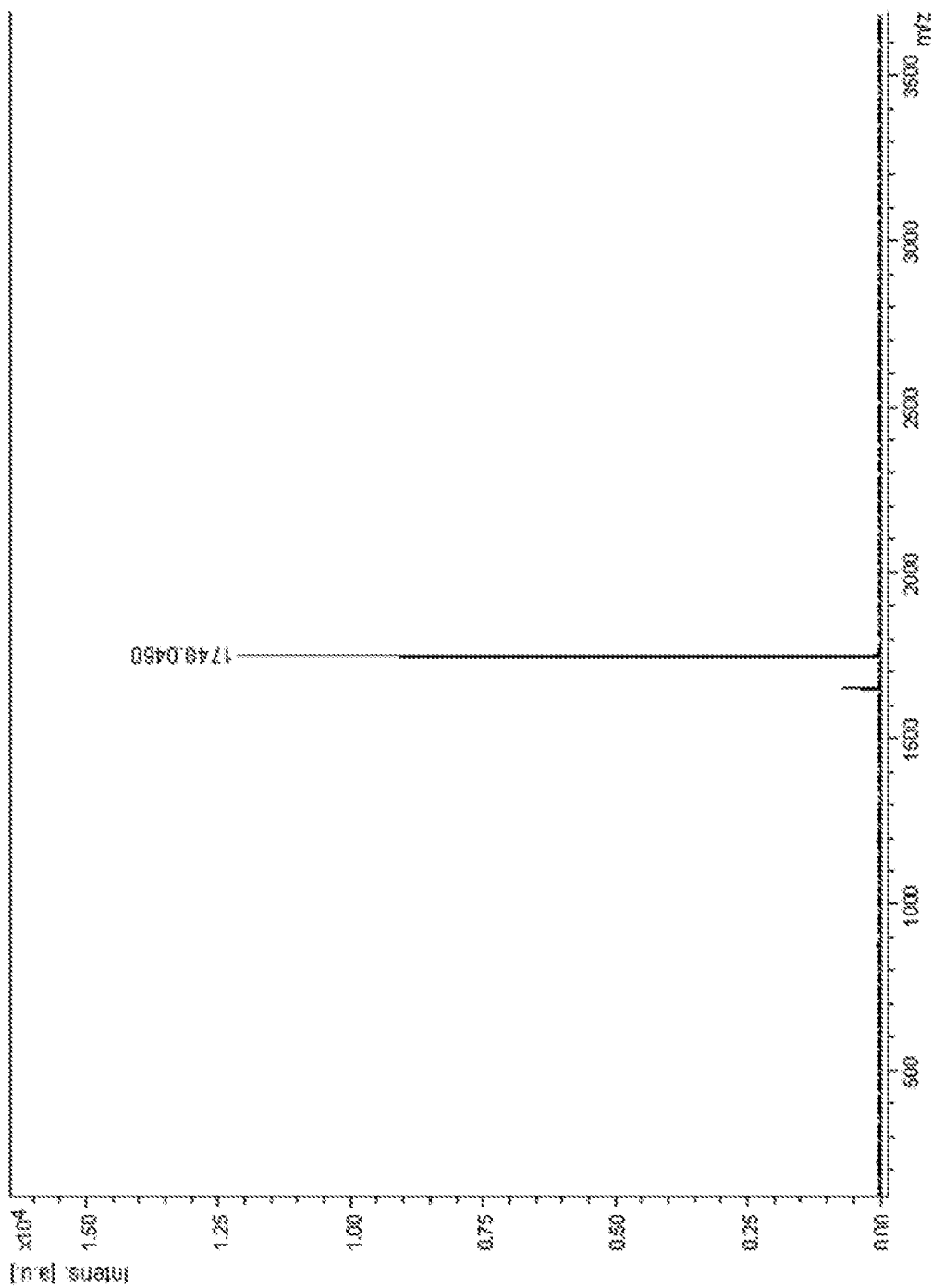
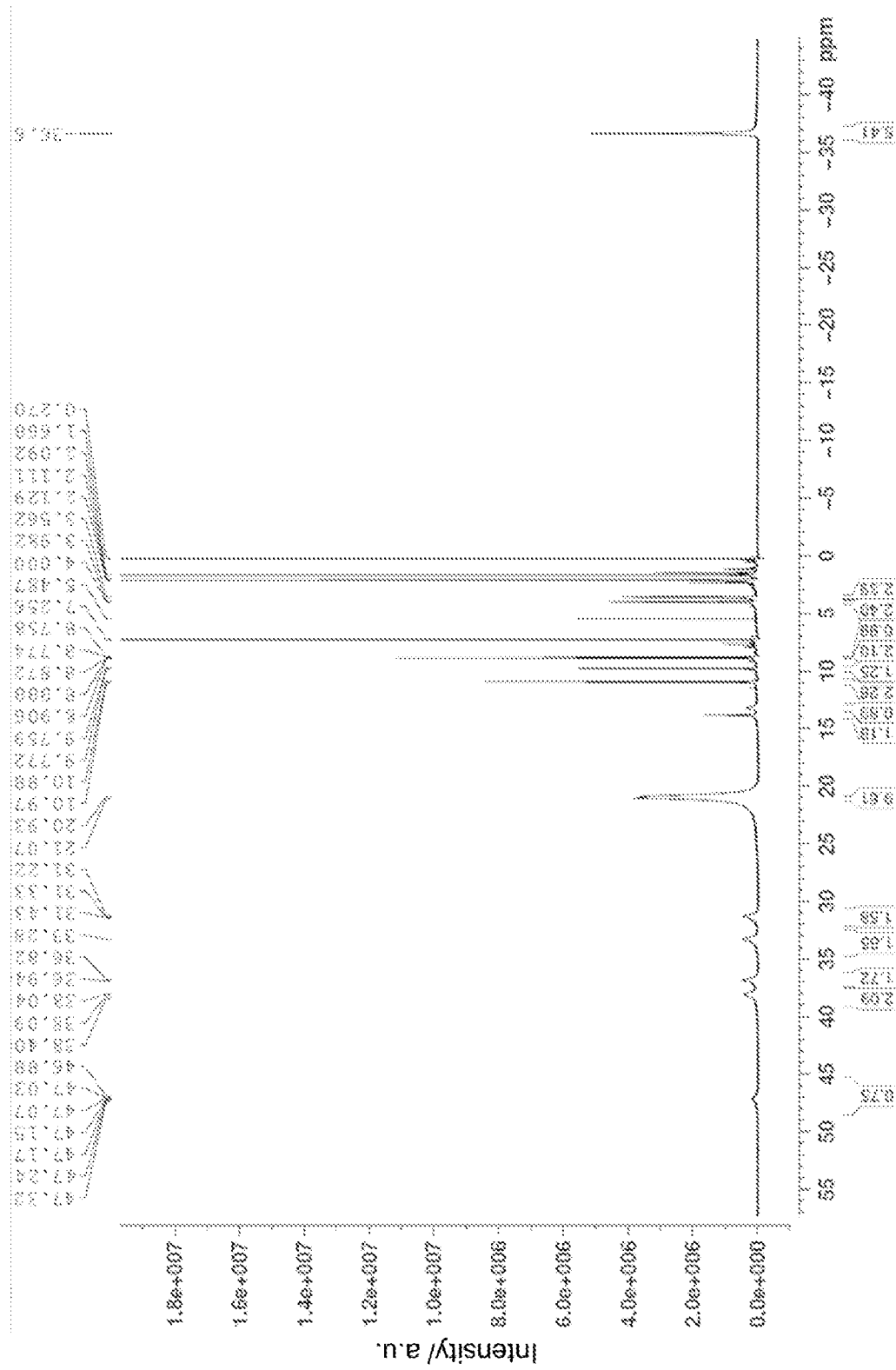


Figure 28



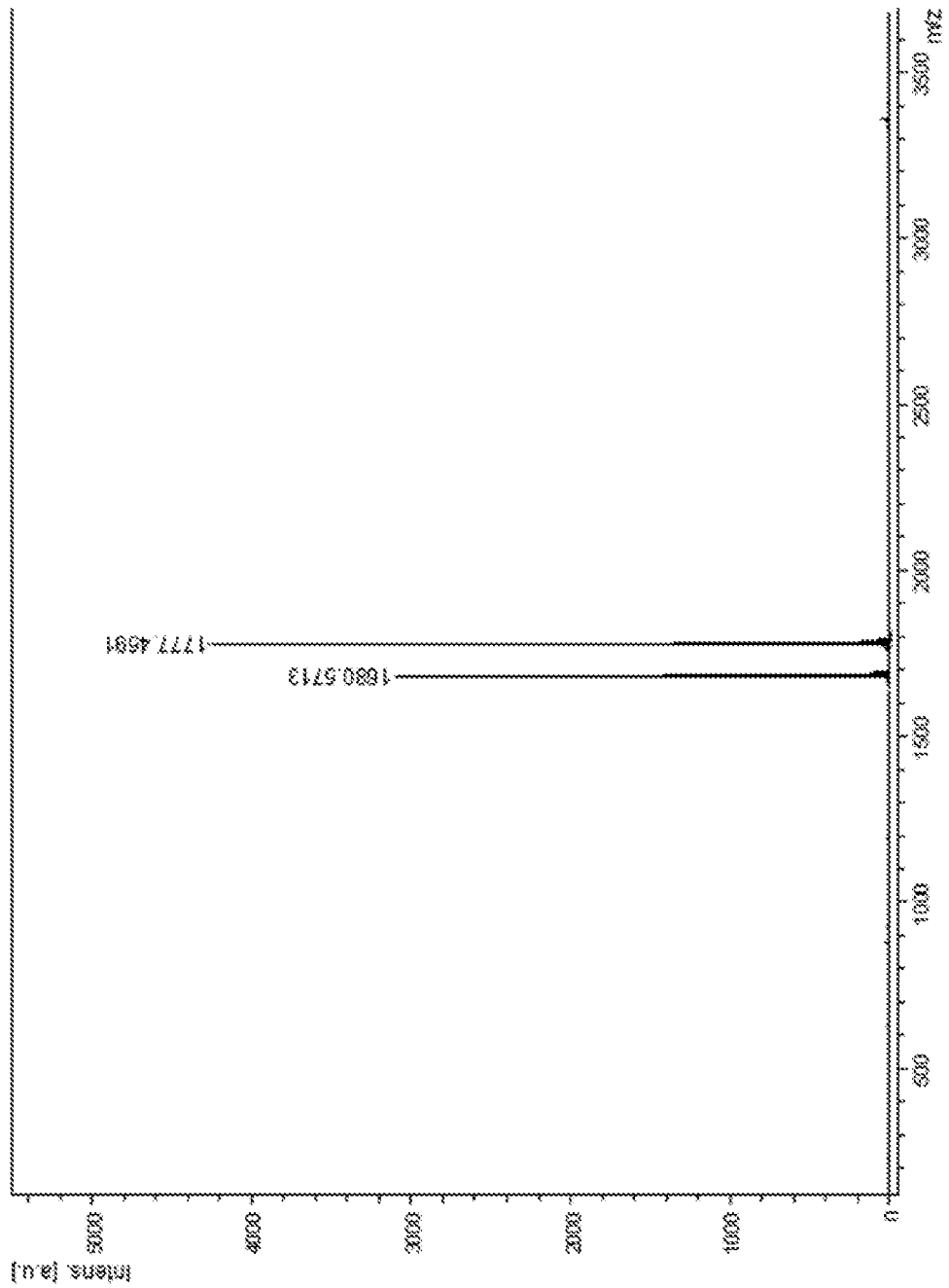


Figure 30

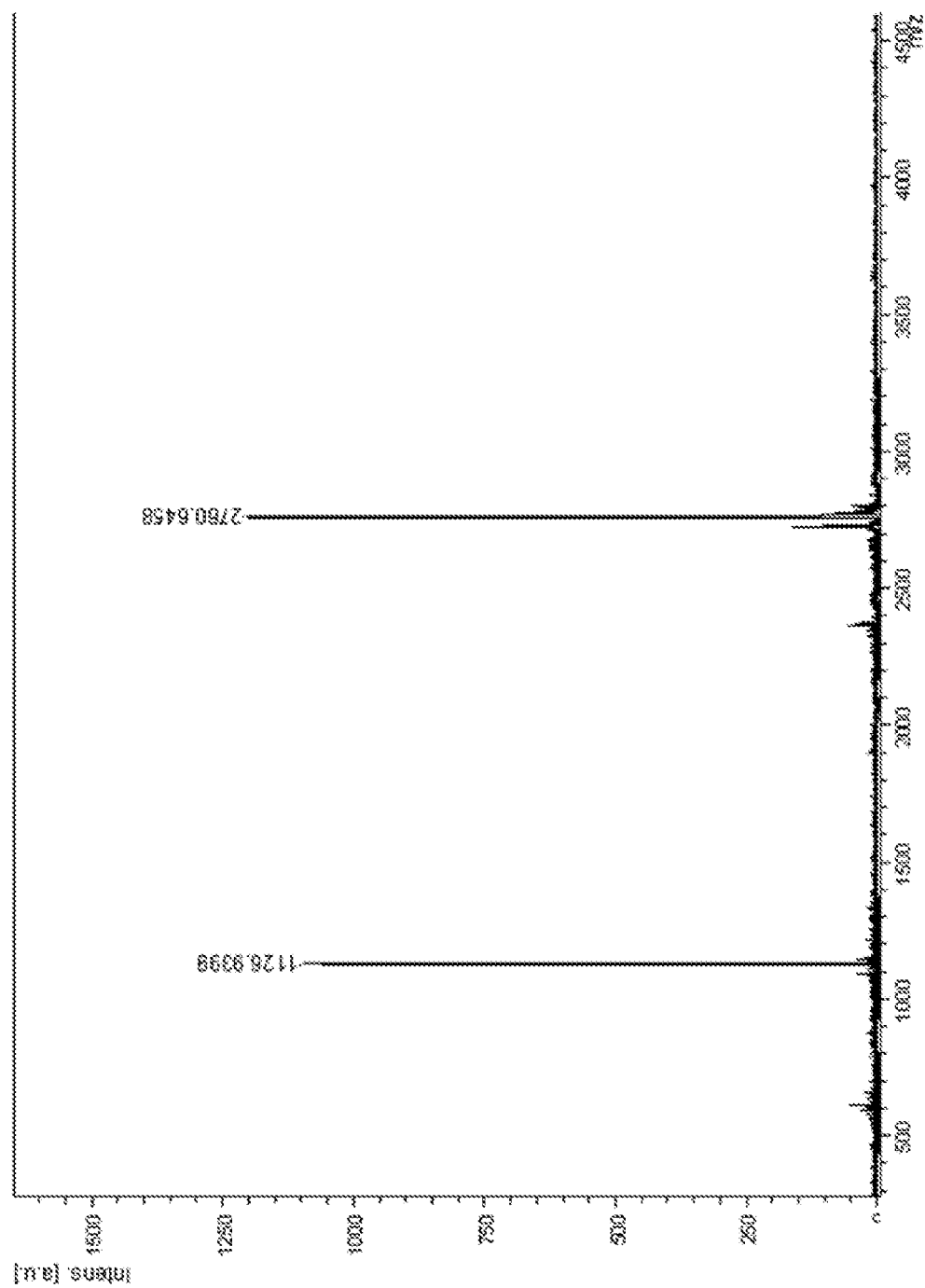


Figure 31

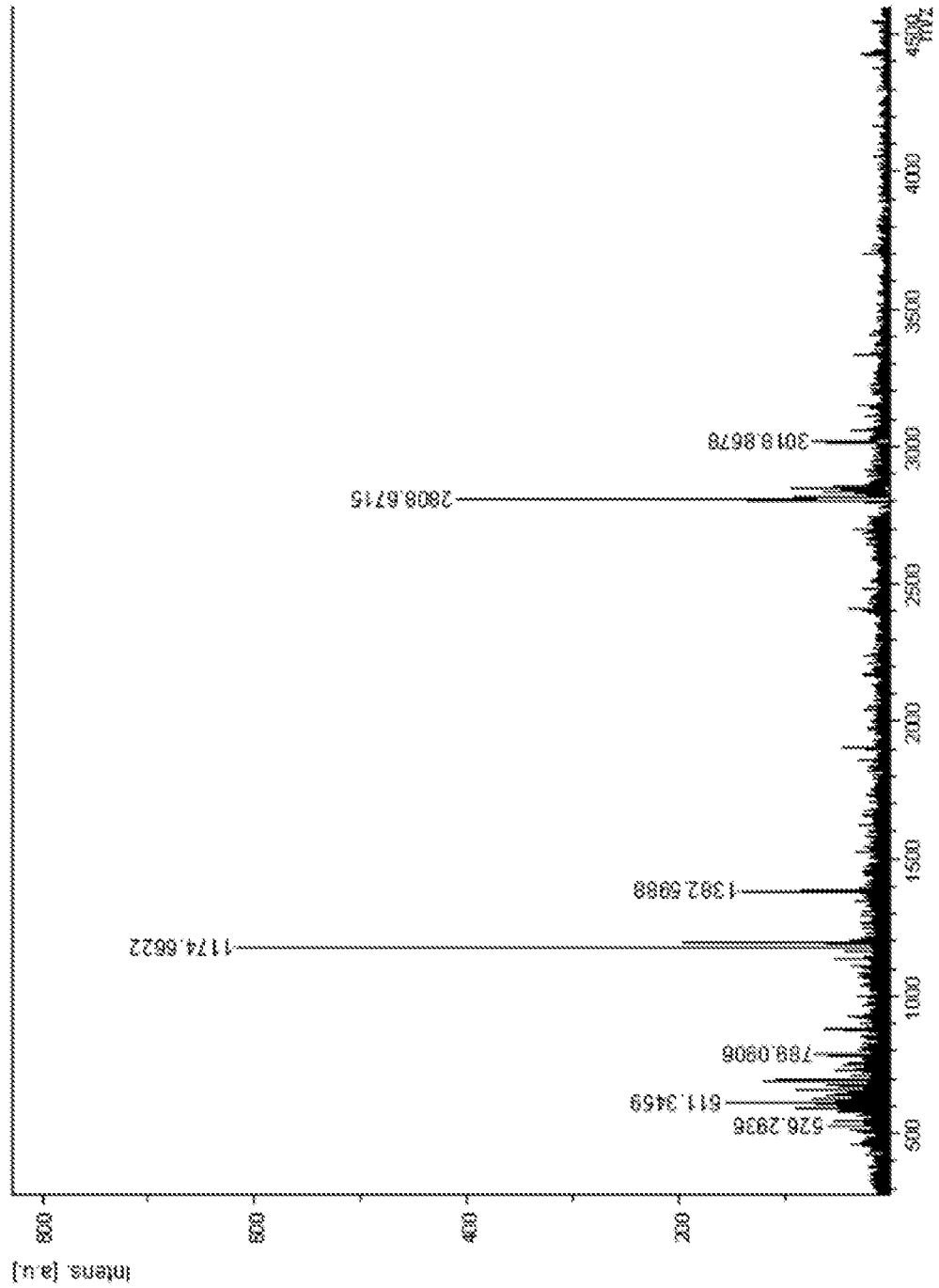


Figure 32

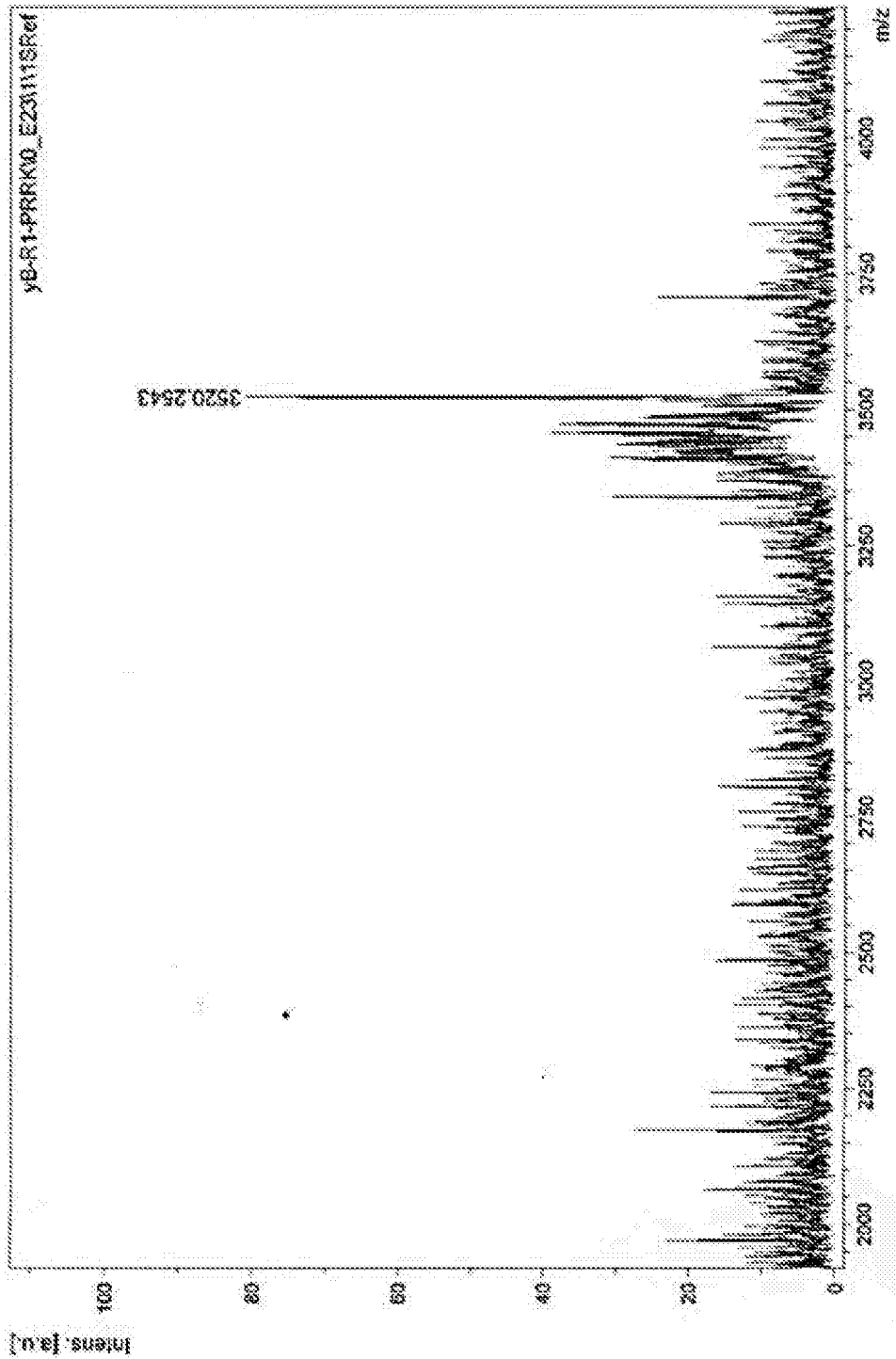


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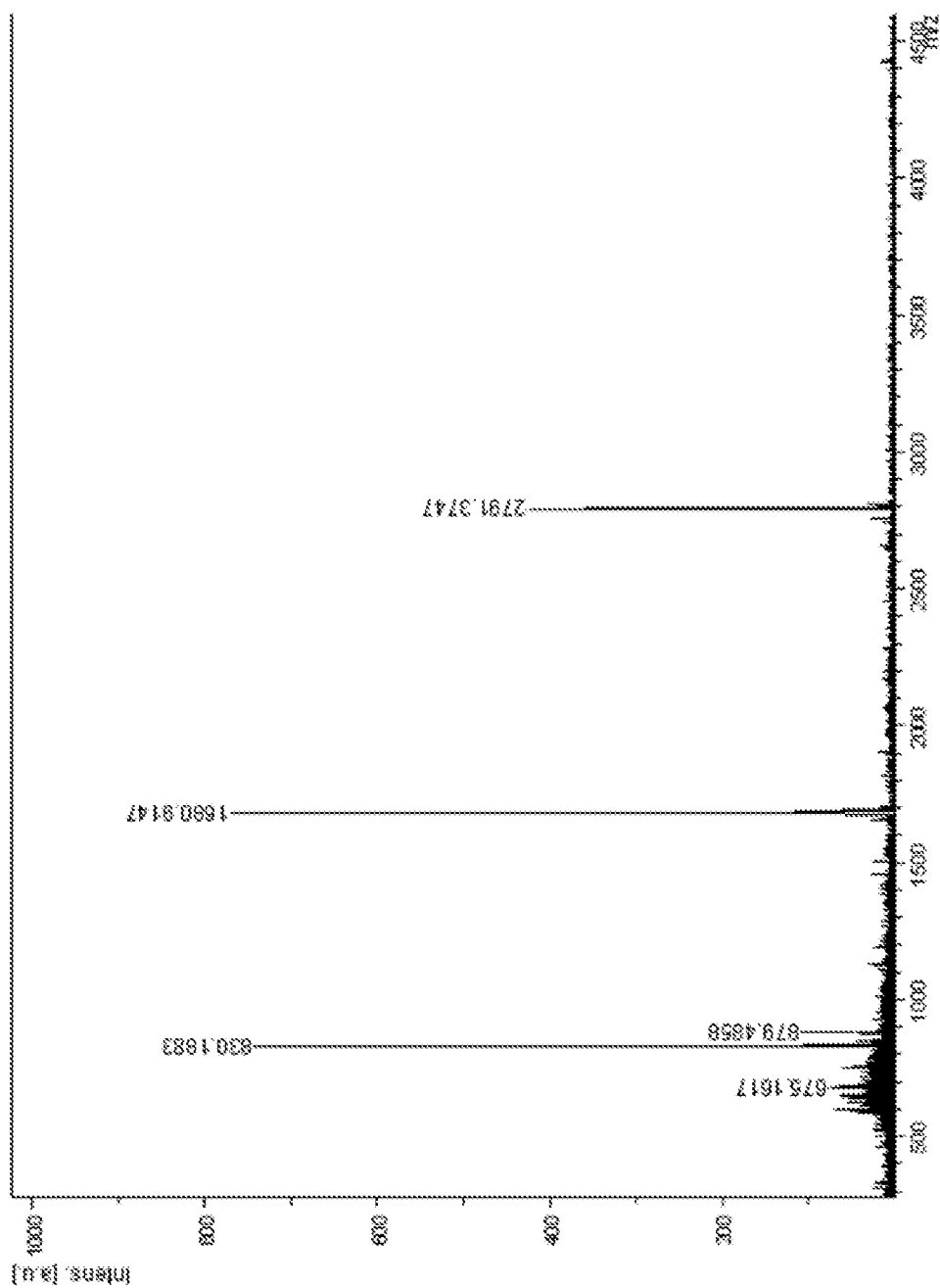


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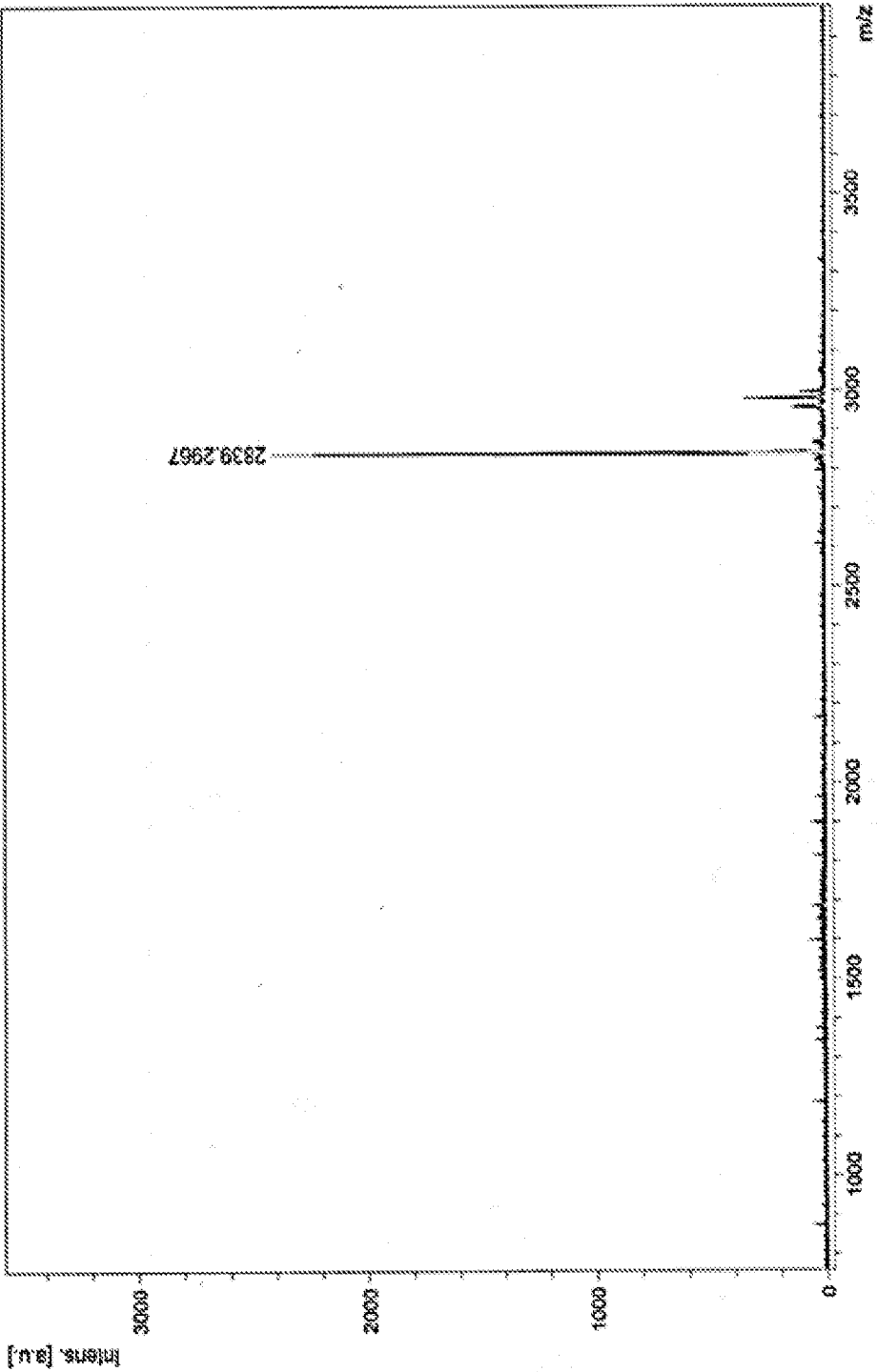


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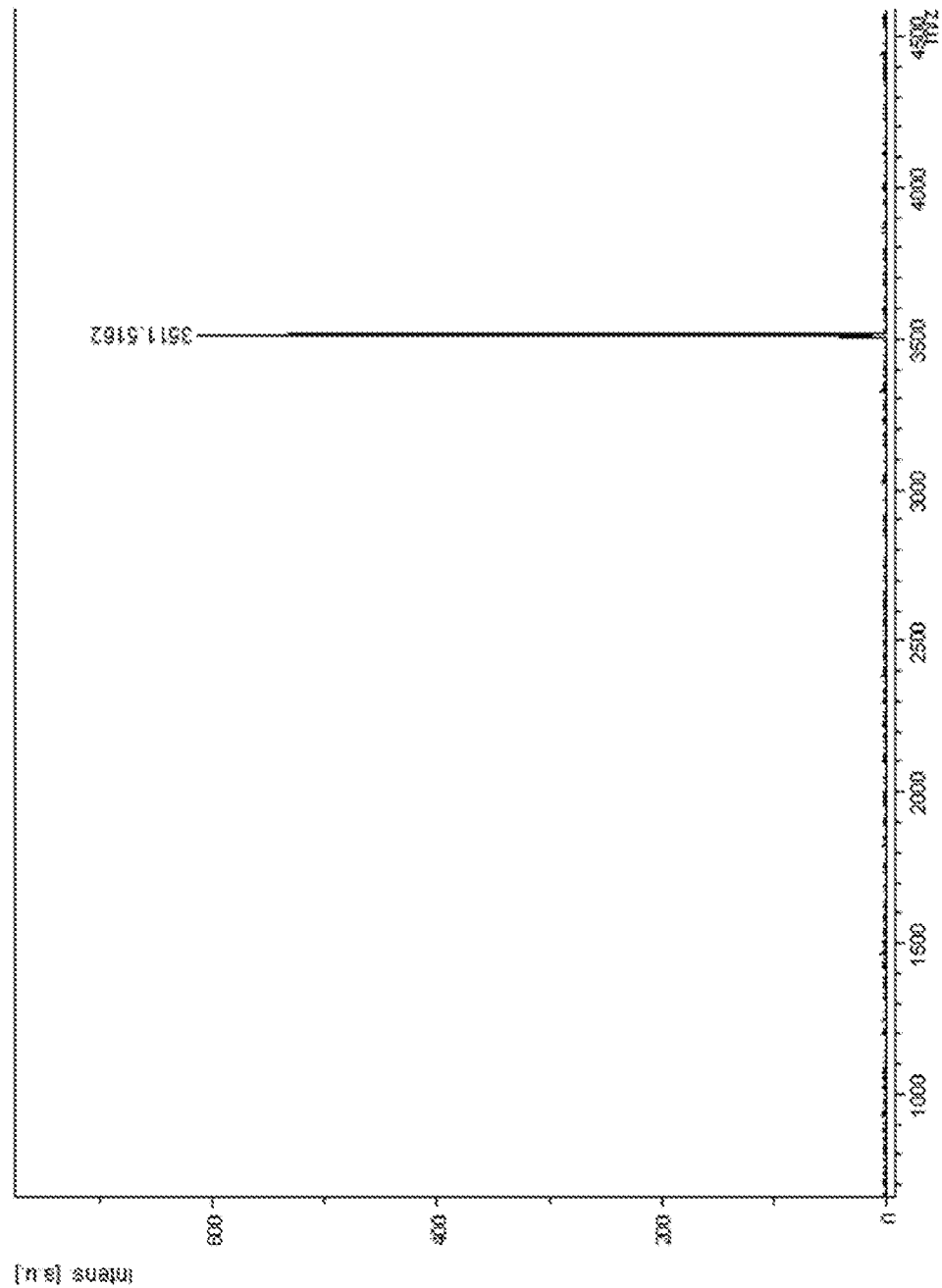


Figure 36

(A)

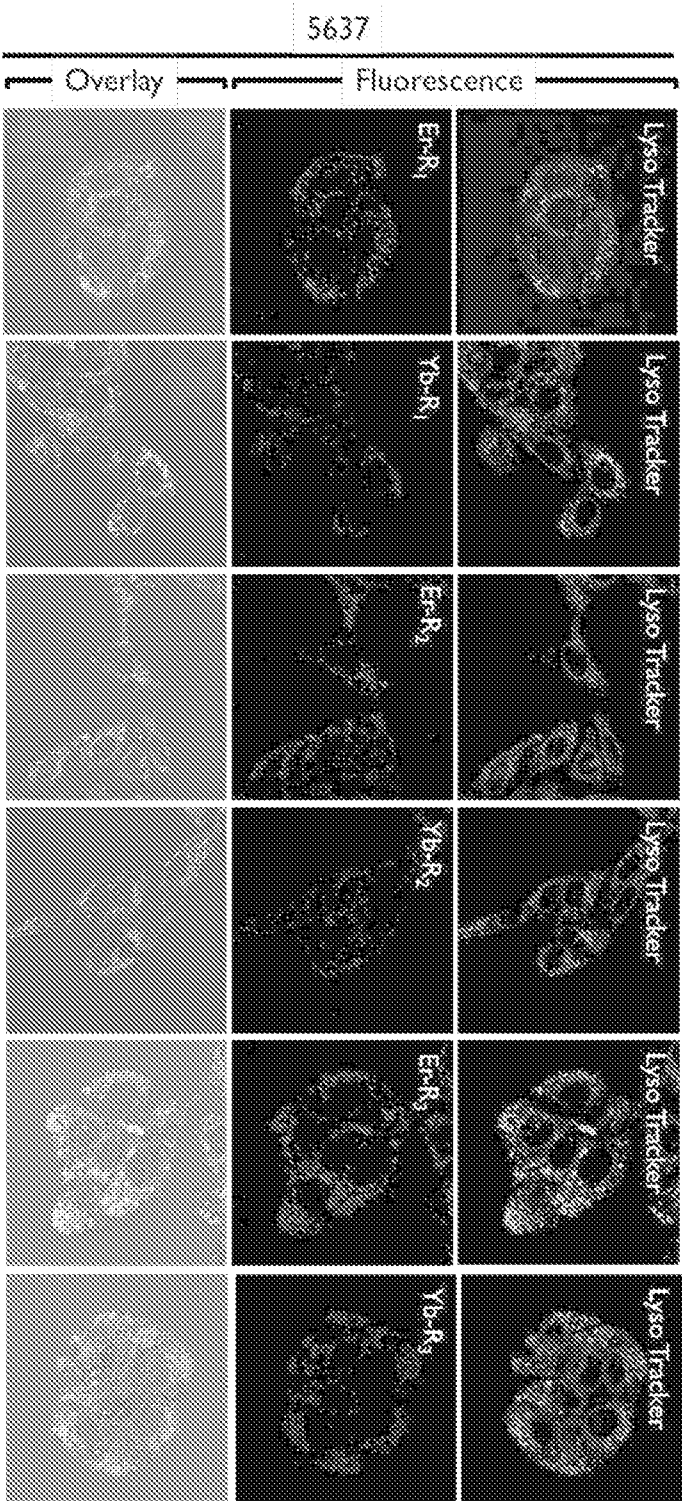


Figure 37

(B)

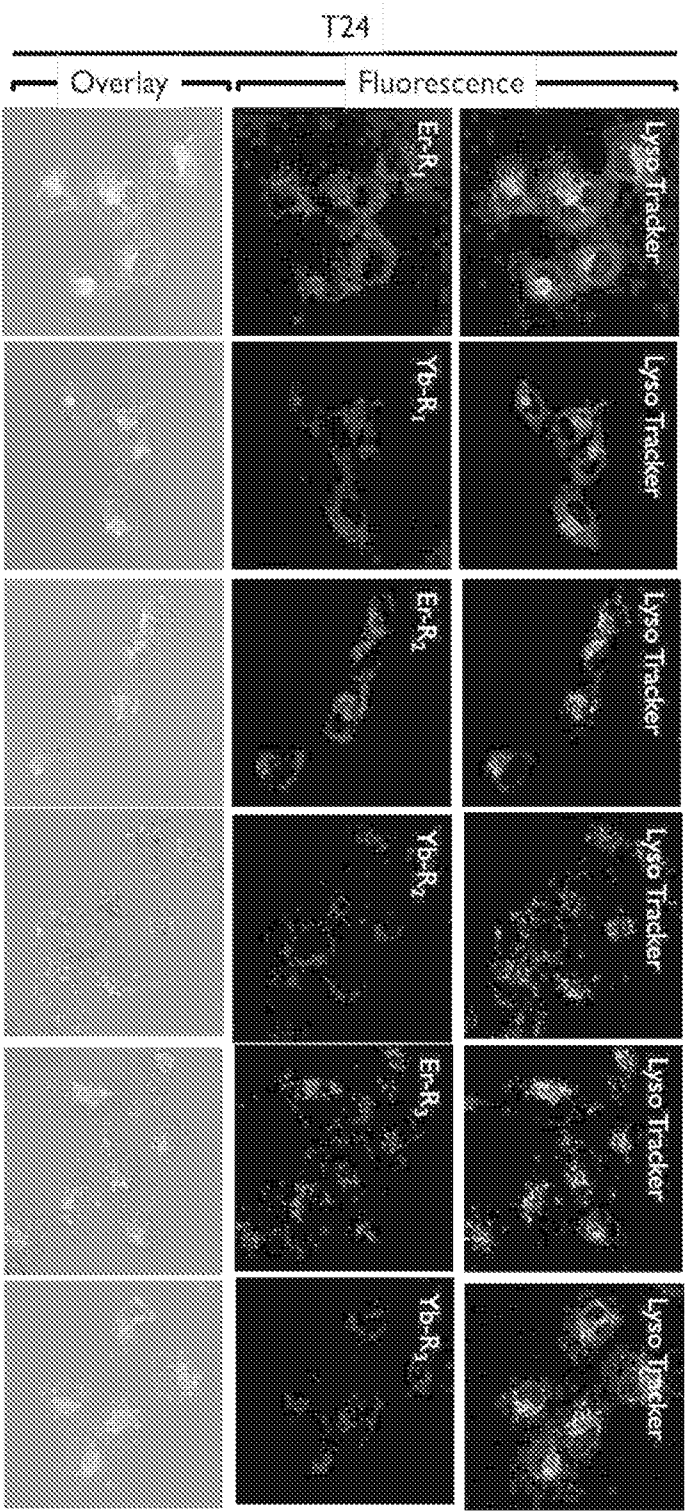


Figure 37 (continued)

(C)

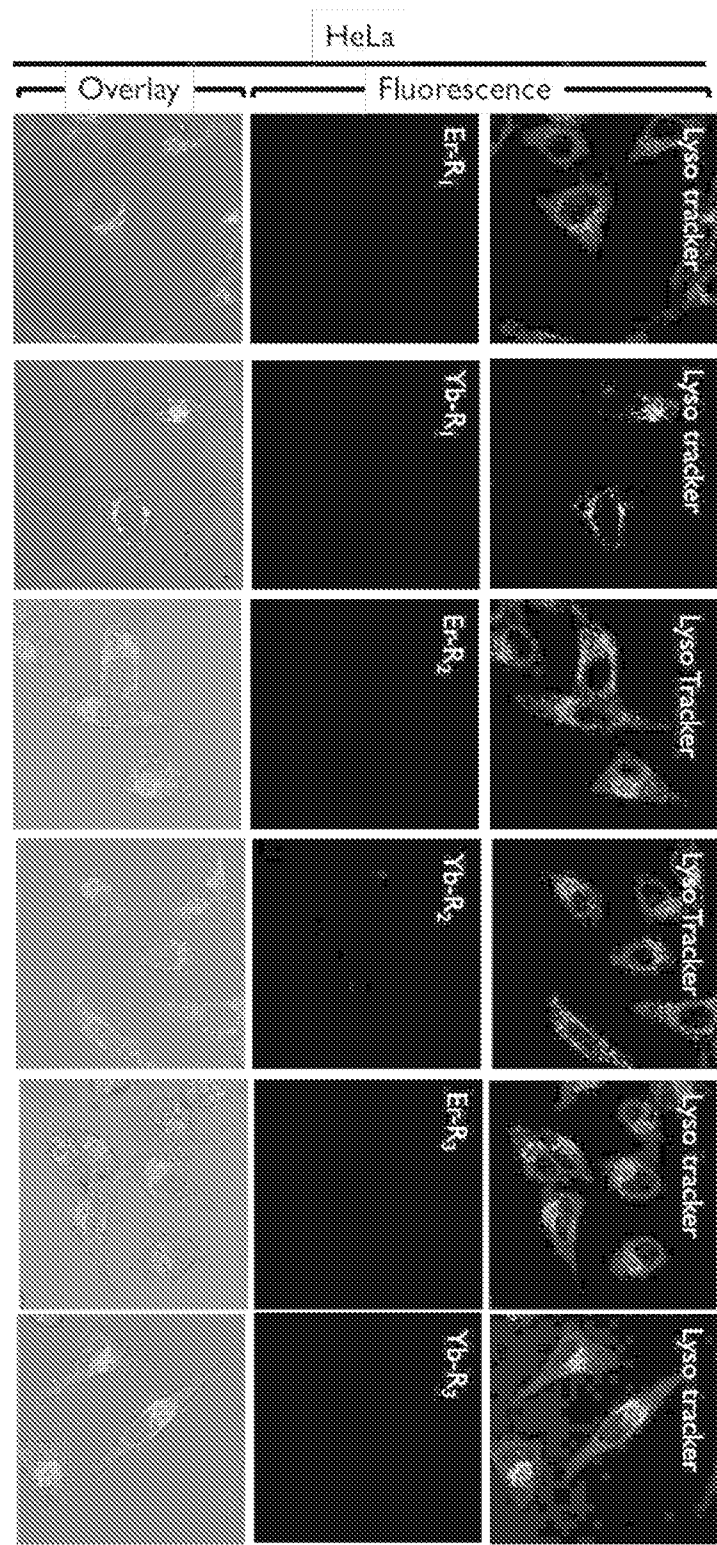


Figure 37 (continued)

(D)

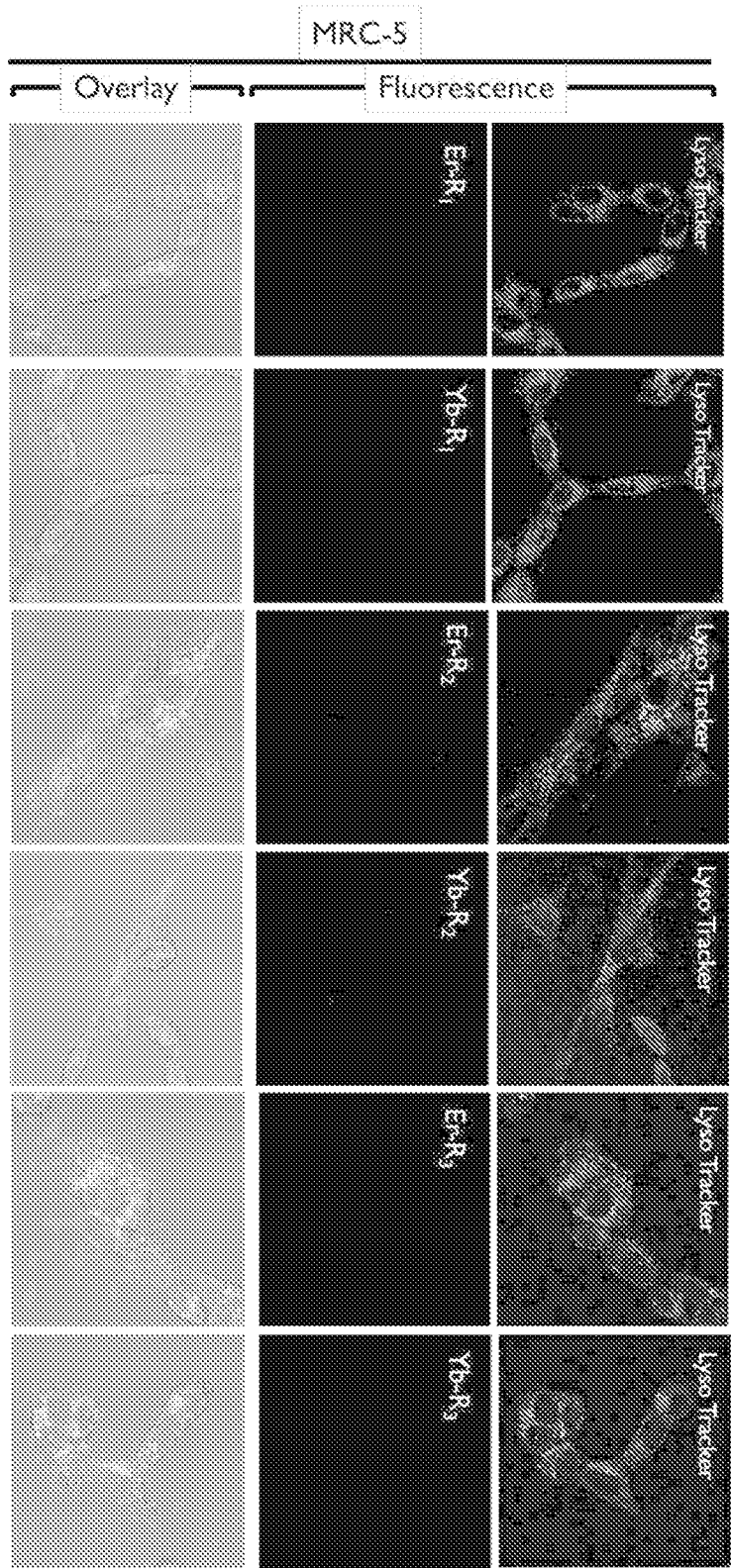


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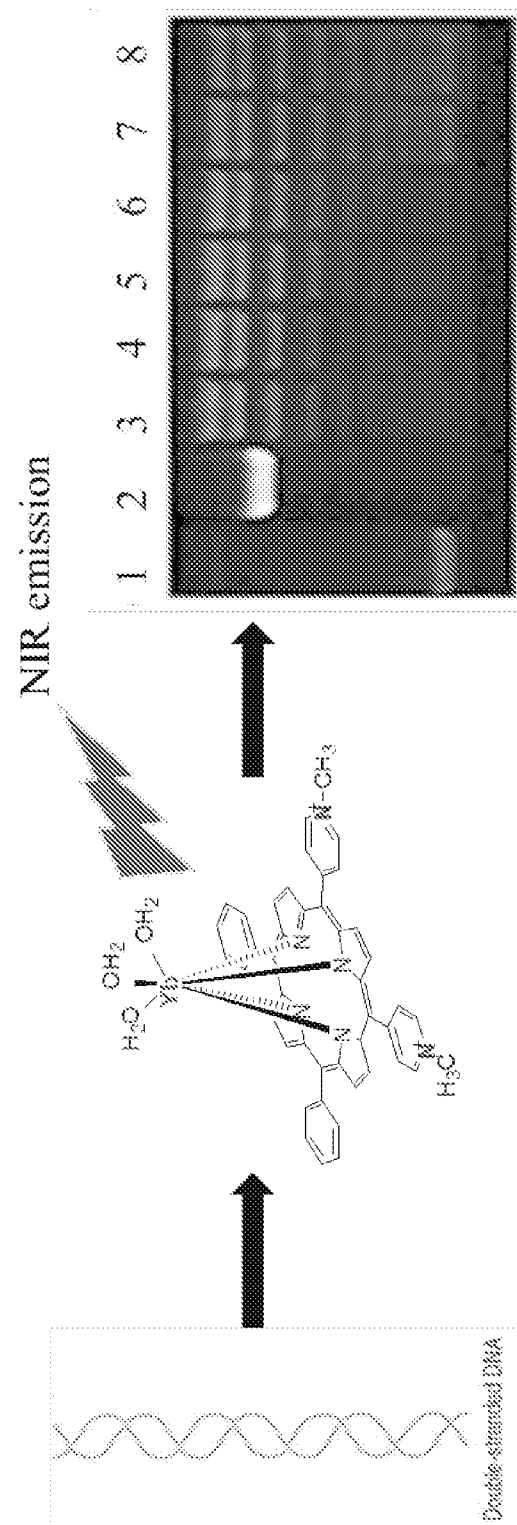


Figure 38A

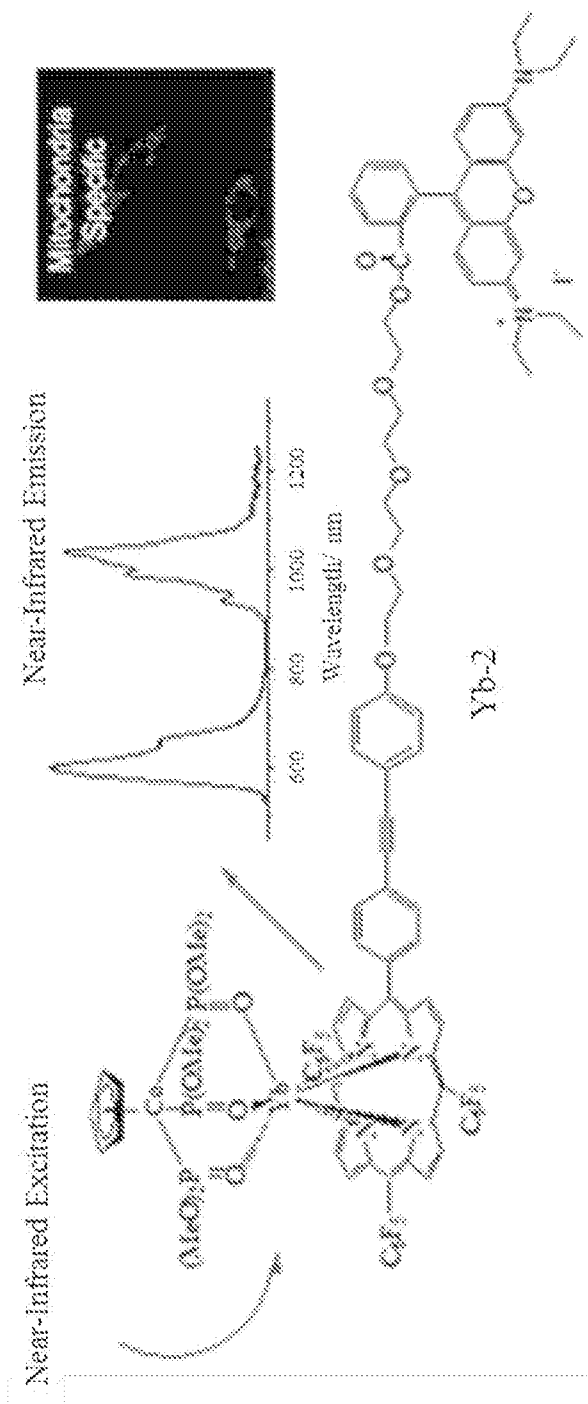


Figure 38B

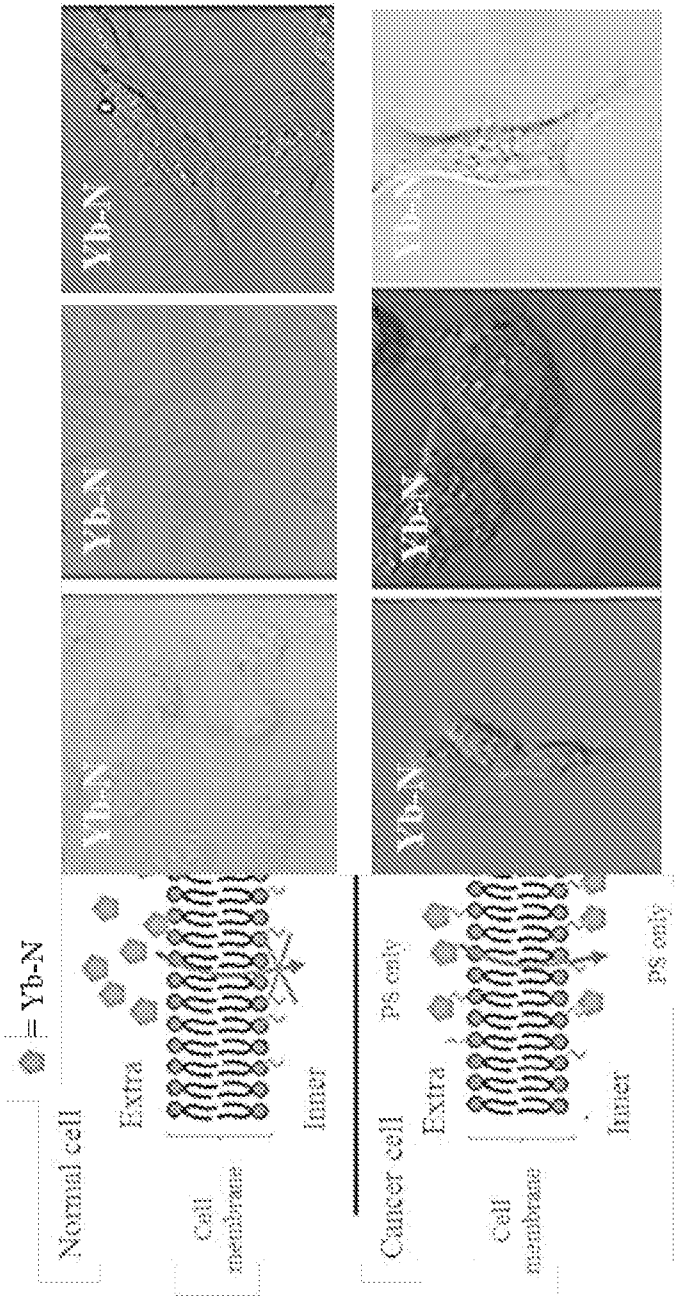


Figure 39A

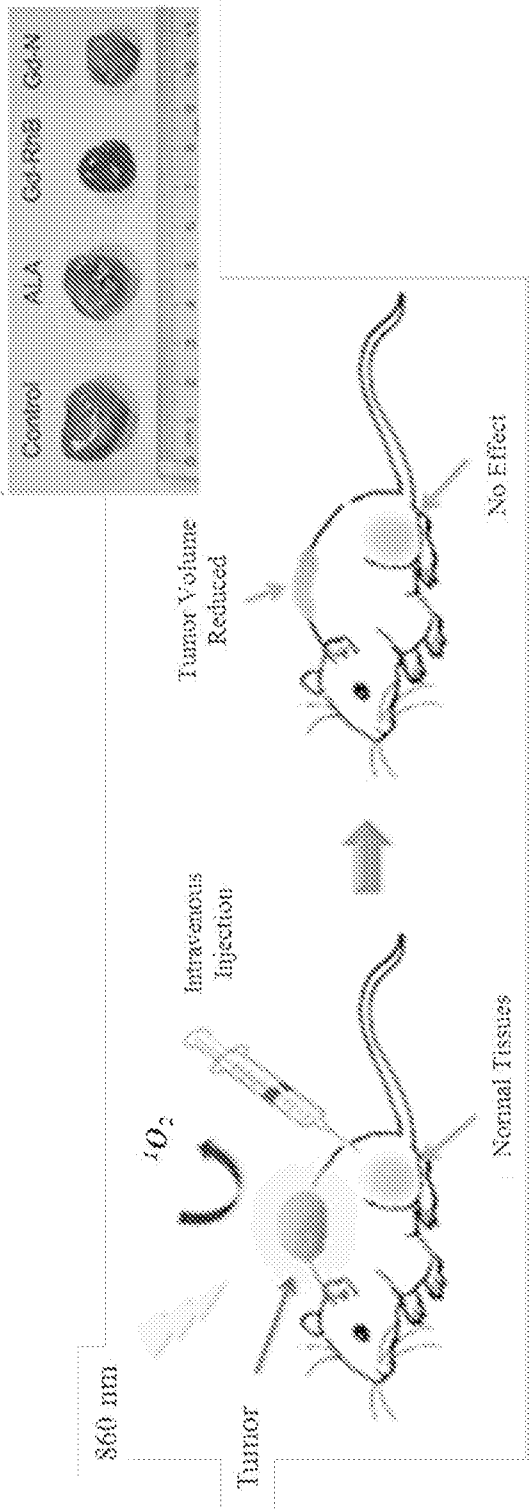


Figure 39B

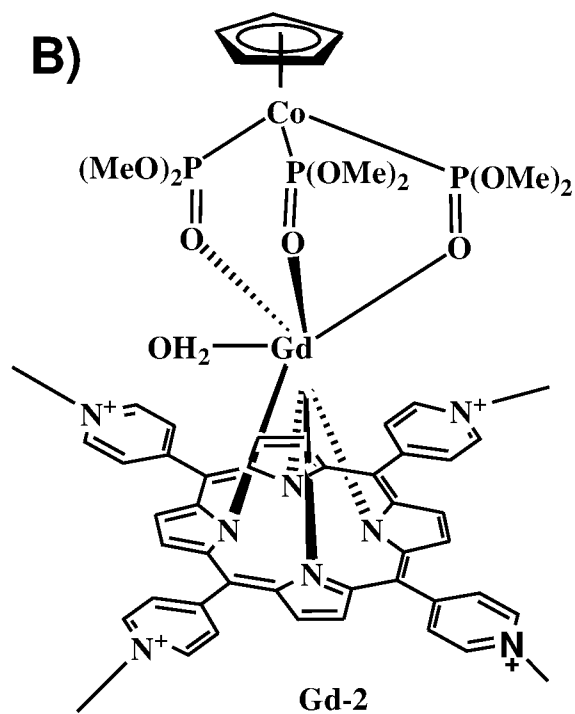
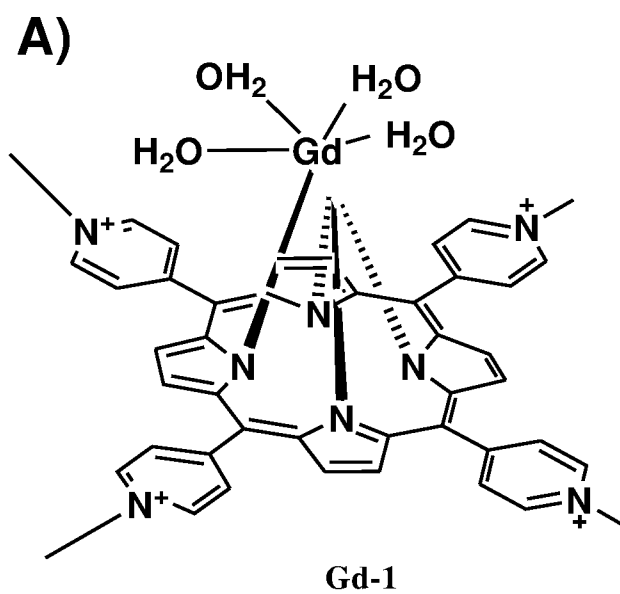
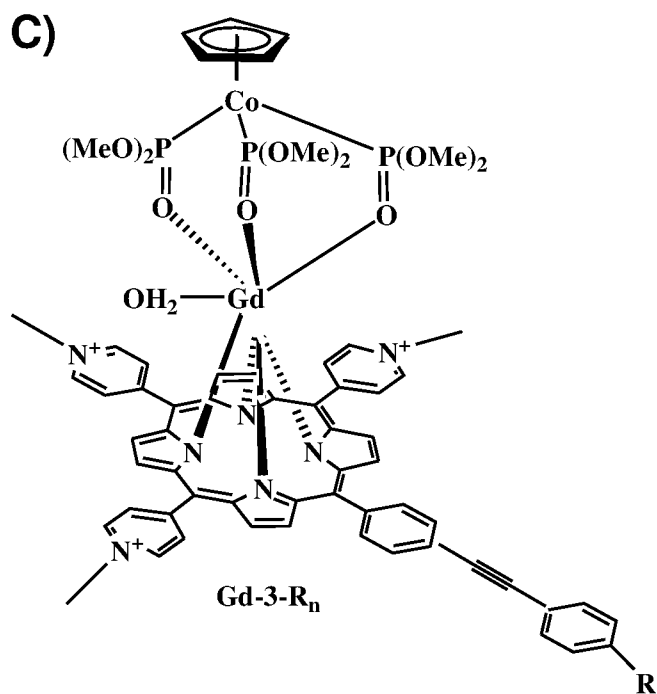


Figure 40



$\text{R}_n=\text{R}_1:\{\text{Ahx}\}-(\text{D-Cys})-\text{Gln}-\text{Asp}-\text{Gly}-\text{Arg}-\text{Met}-\text{Gly}-\text{Phe}-(\text{D-Cys})$

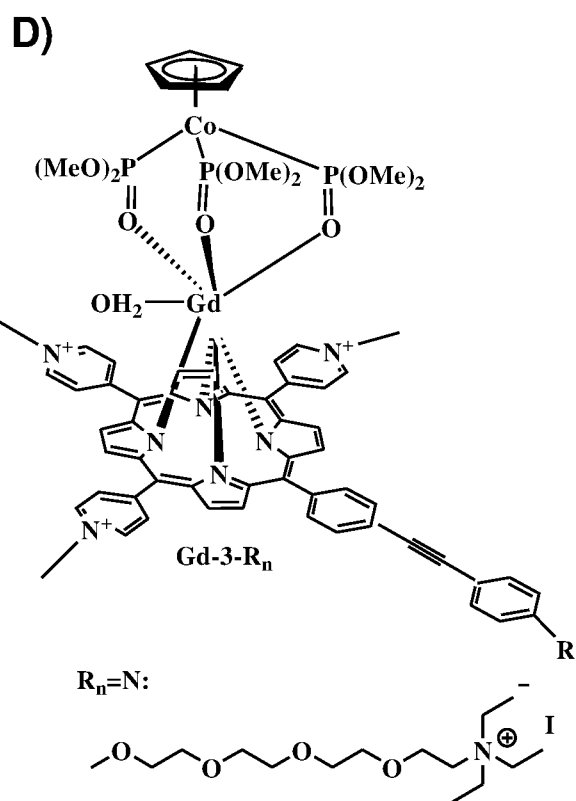


Figure 40 (continued)

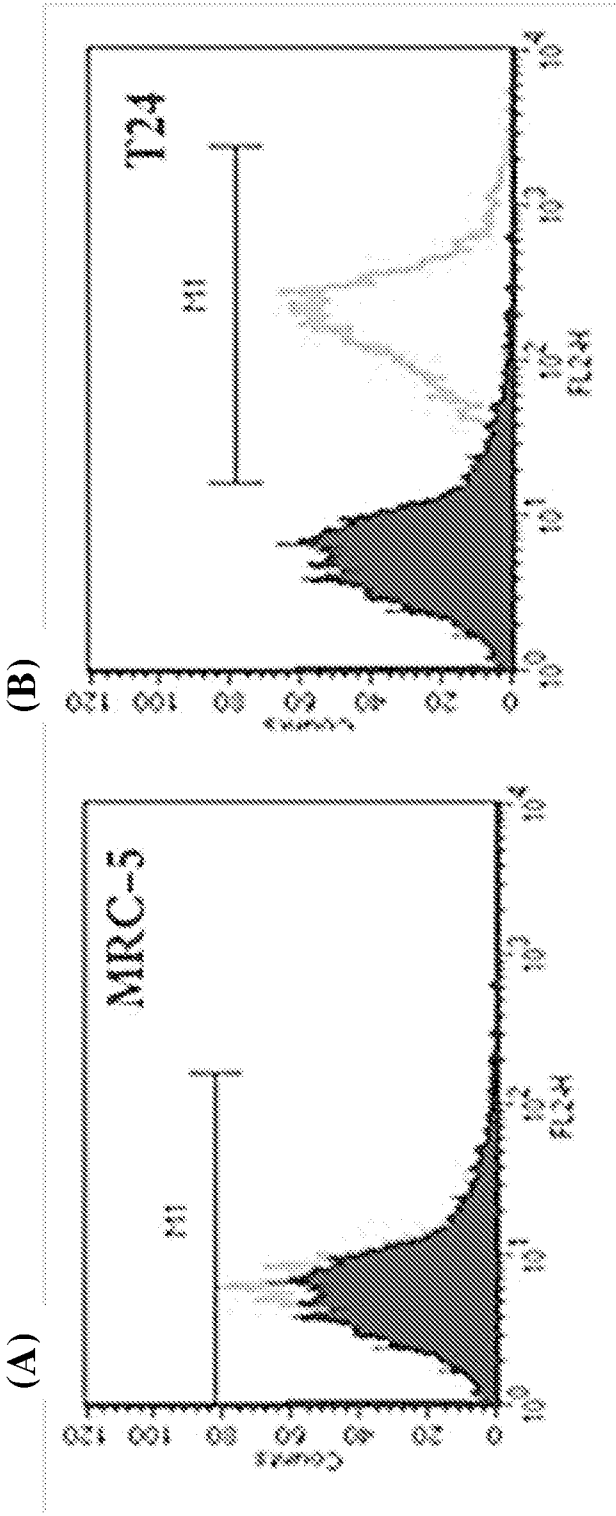


Figure 41

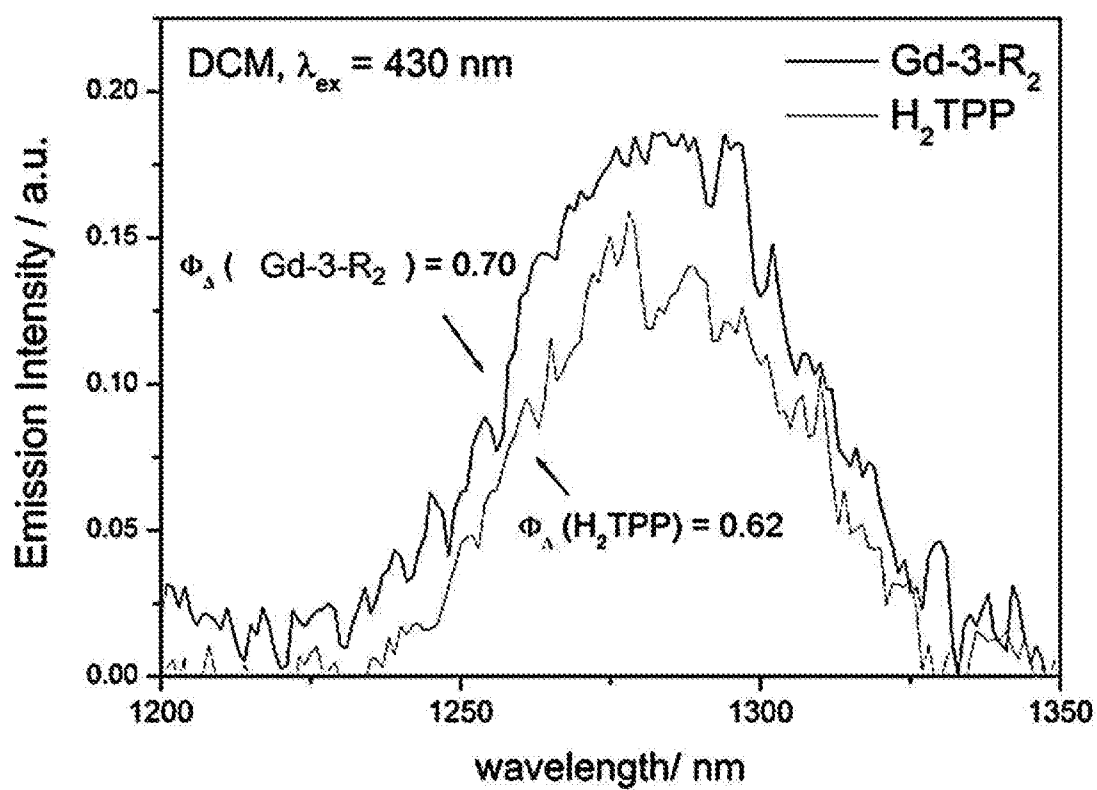


Figure 42

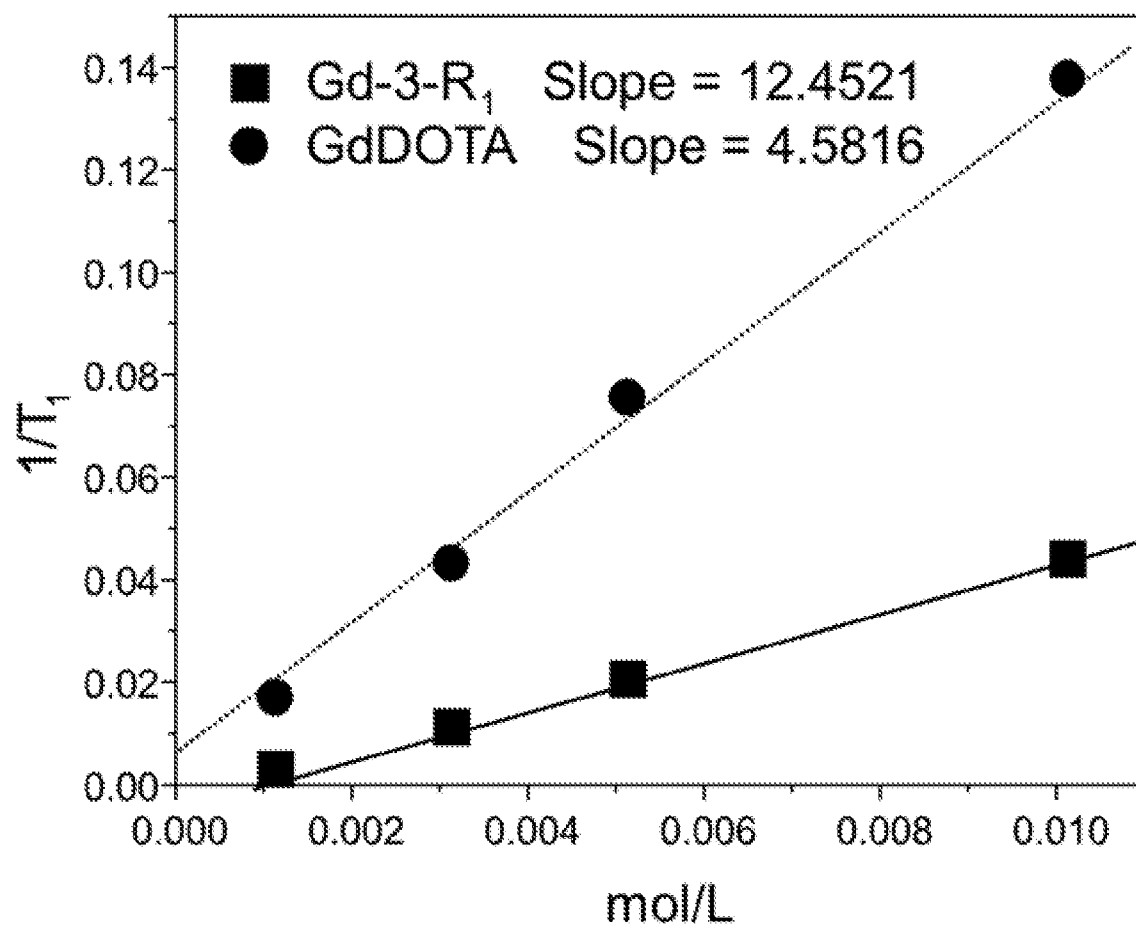


Figure 43

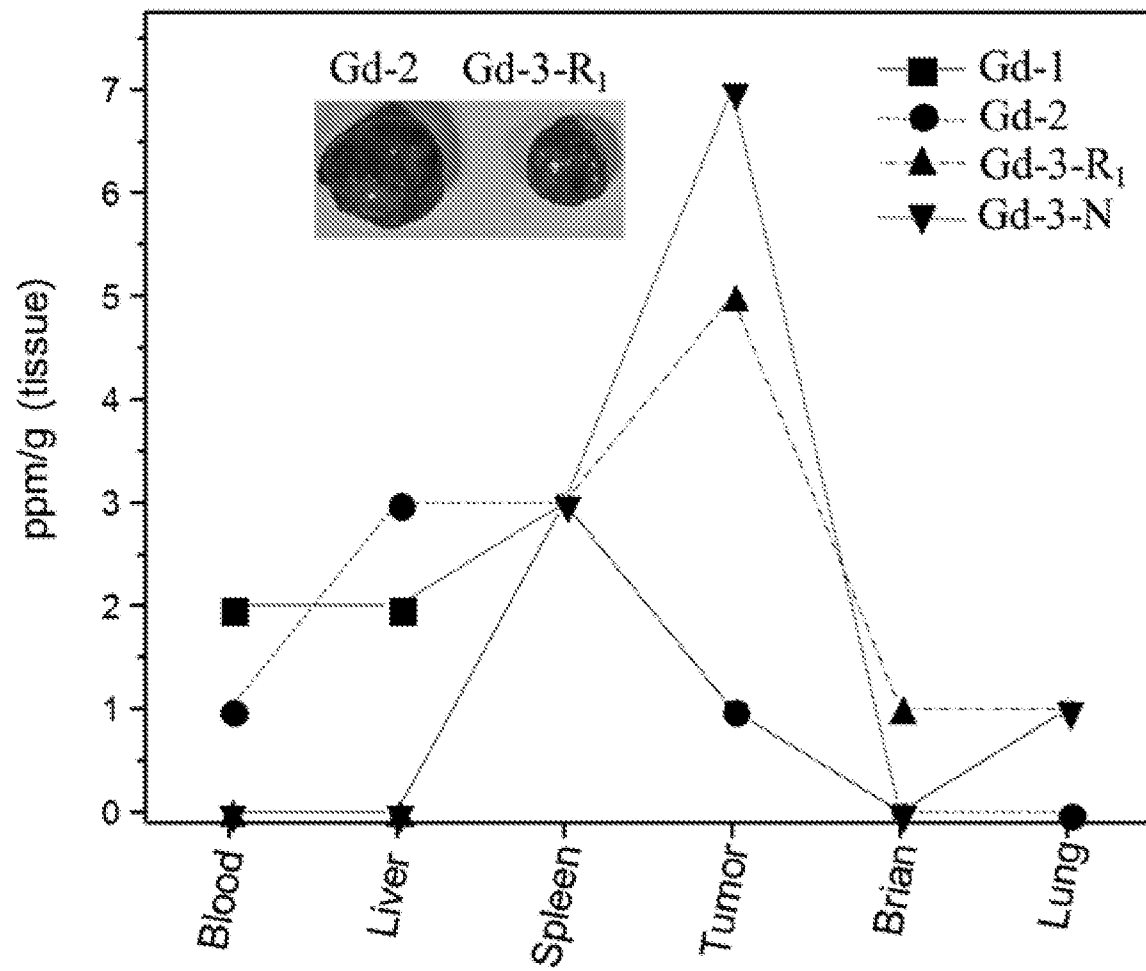


Figure 44

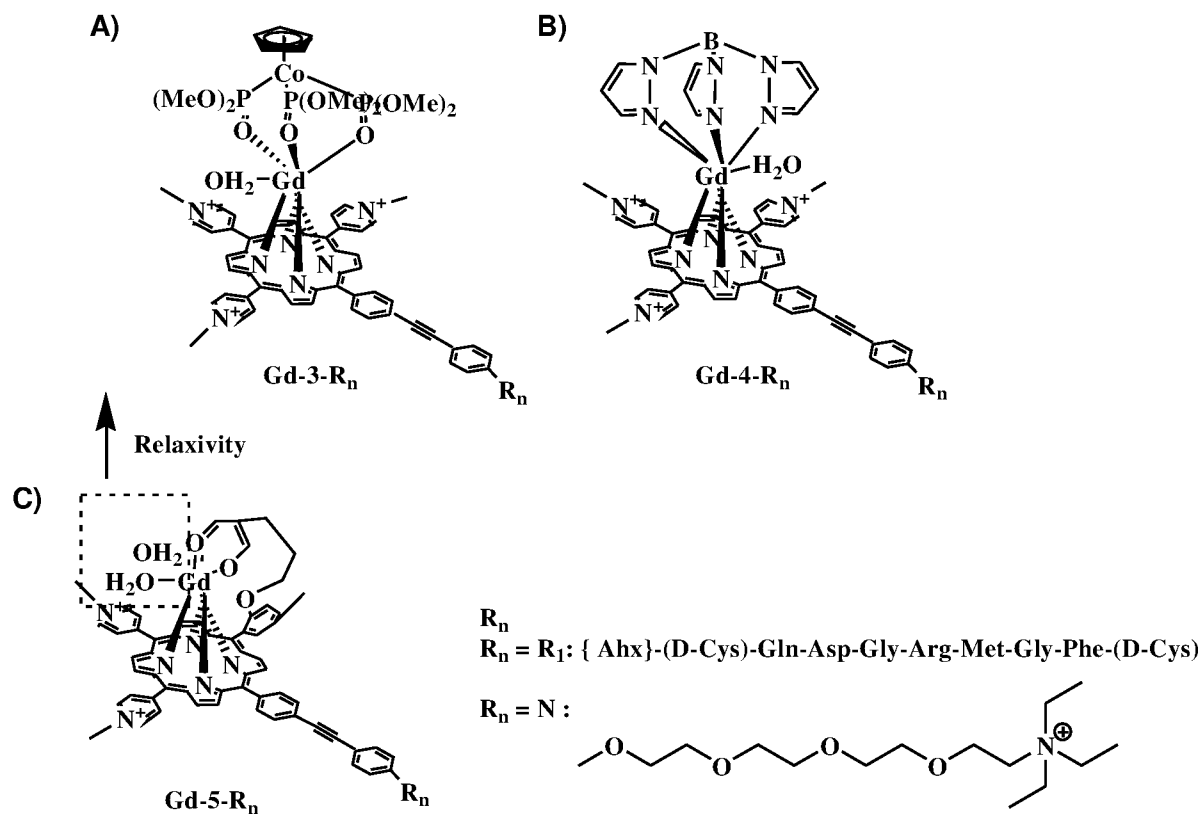


Figure 45

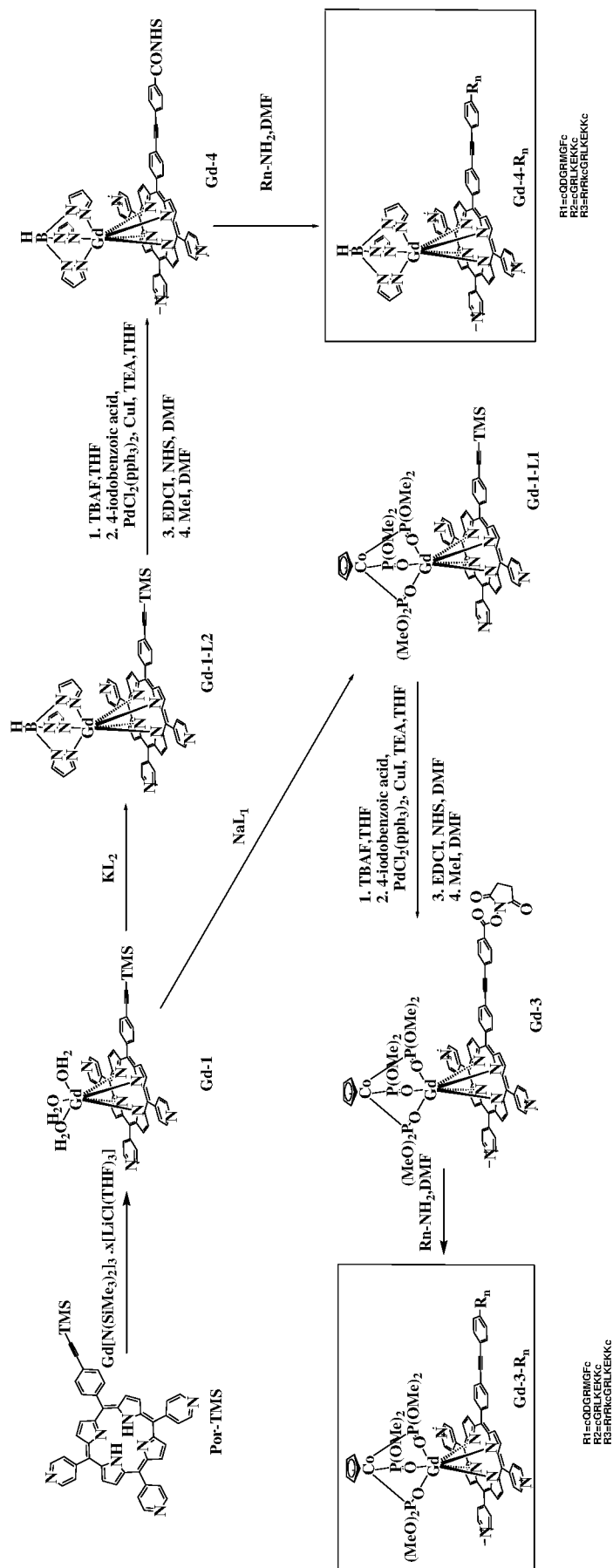


Figure 46

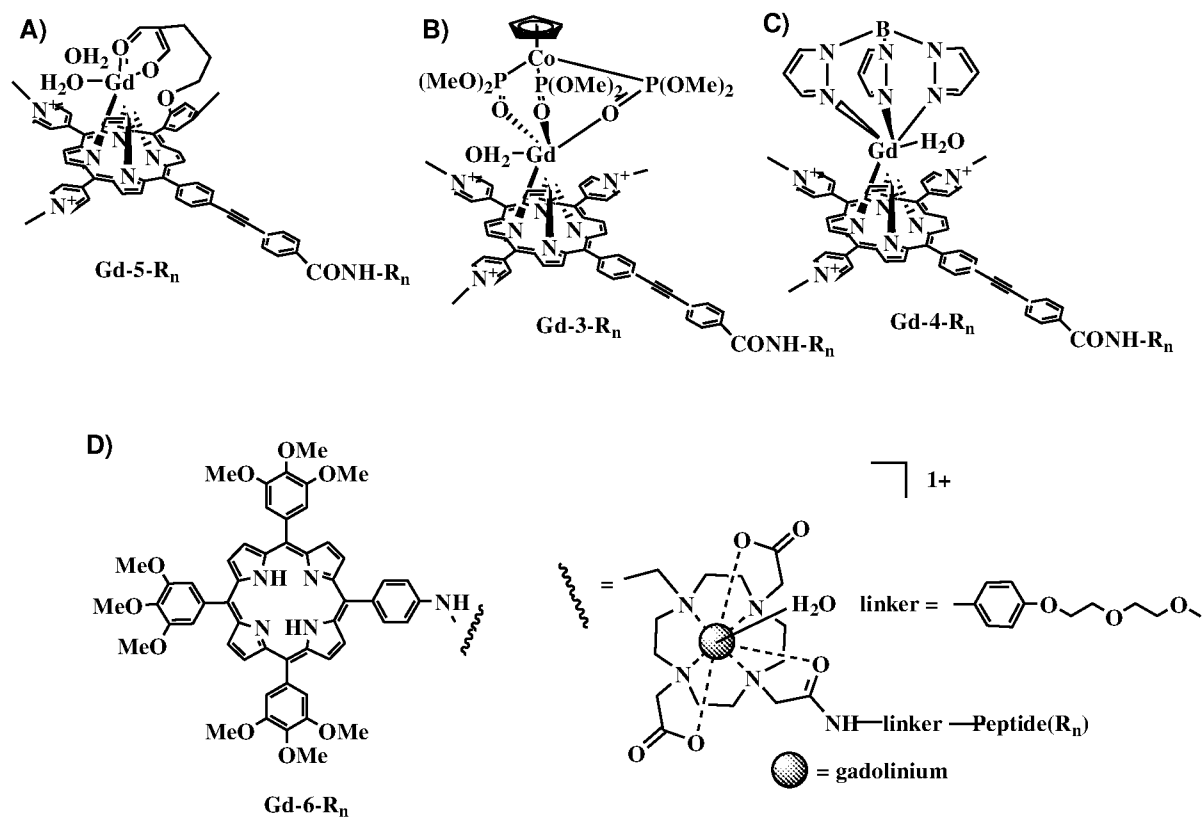

 R_n
 $n = 1: \{Ahx\}-(D-Cys)-Gln-Asp-Gly-Arg-Met-Gly-Phe-(D-Cys)$
 $n = 2: \{Ahx\}-(D-Cys)-Gly-Arg-Leu-Lys-Glu-Lys-Lys-(D-Cys)$
 $n = 3: Arg-Arg-(D-Arg)-Lys-\{Ahx\}-(D-Cys)-Gly-Arg-Leu-Lys-Glu-Lys-Lys-(D-Cys)$

Figure 47

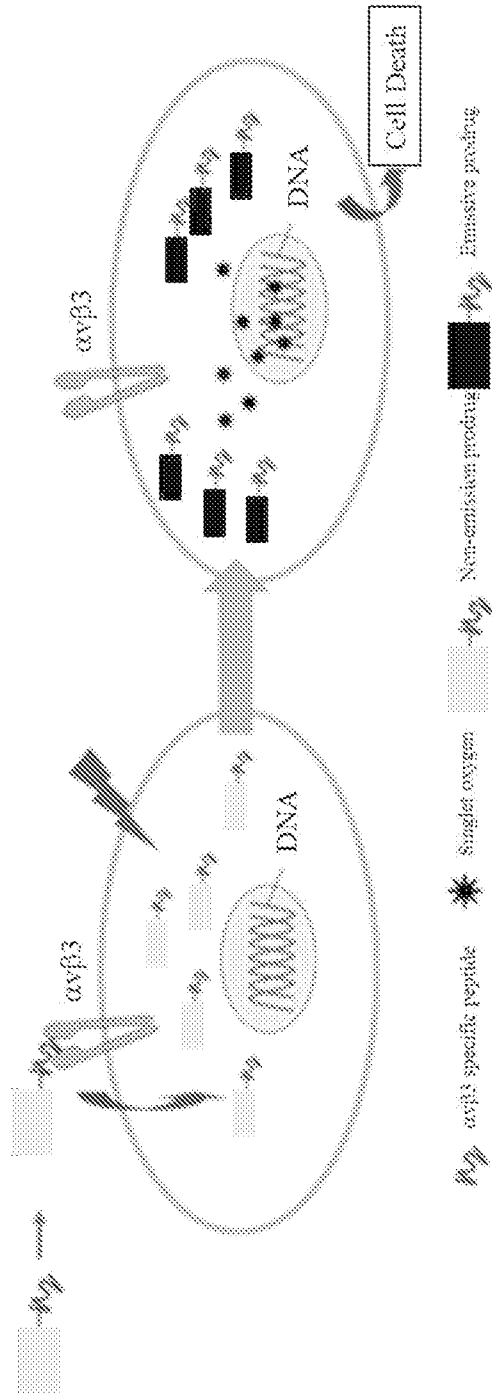
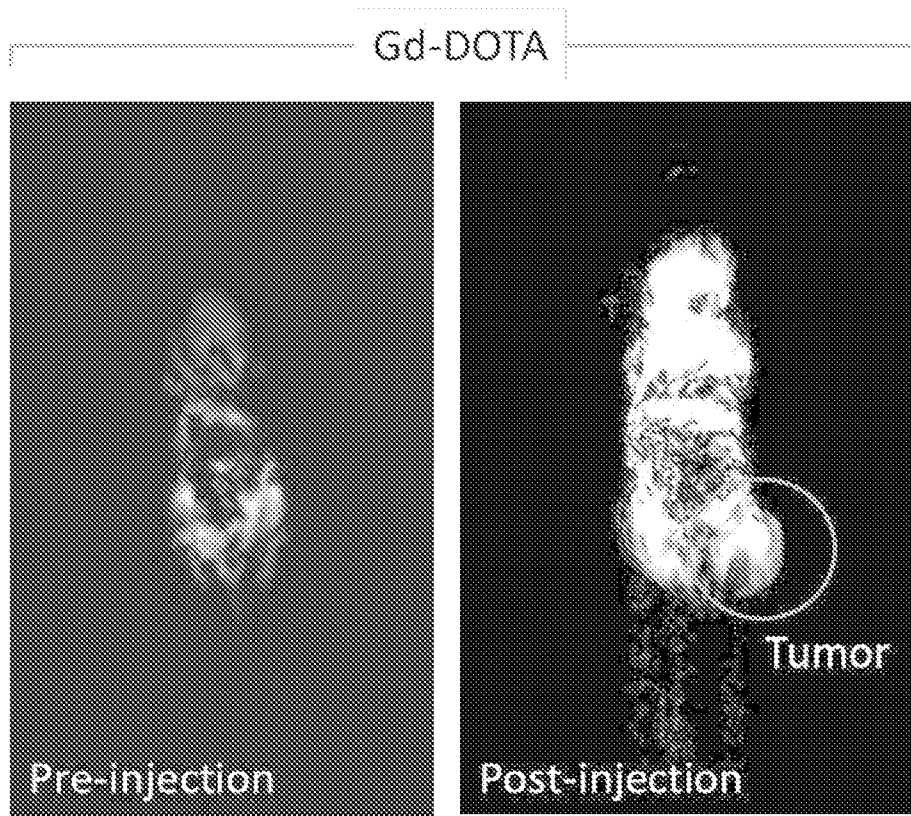
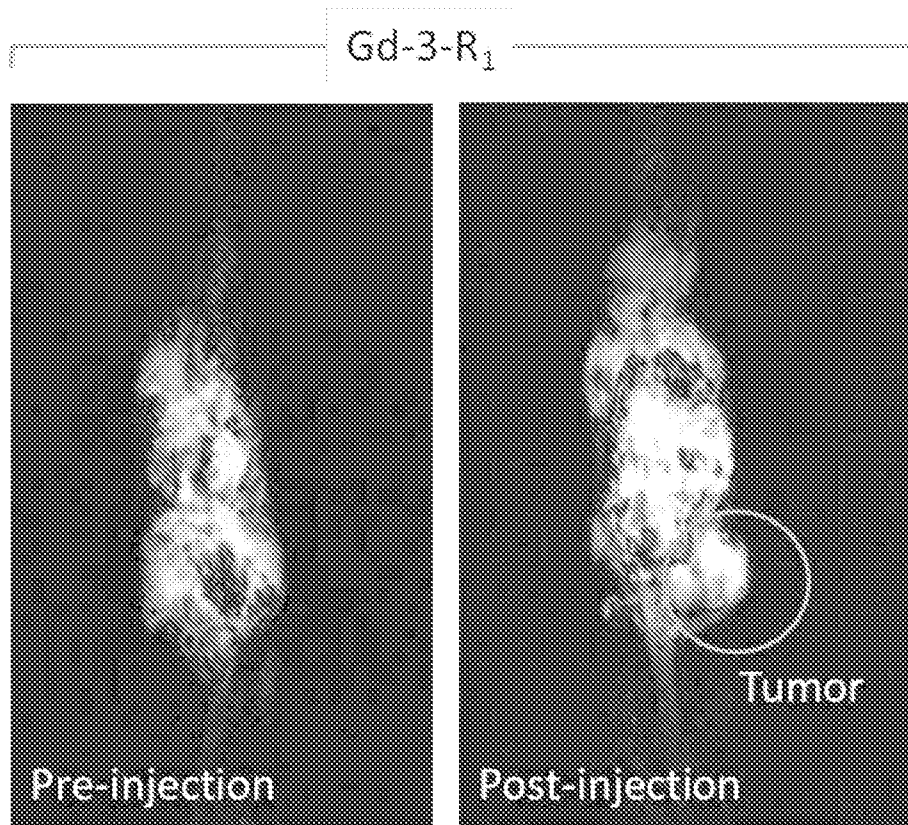


Figure 48

**Figure 49A**

**Figure 49B**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2017/104492

A. CLASSIFICATION OF SUBJECT MATTER C07F 5/00(2006.01)i; A61N 5/06(2006.01)i; A61K 41/00(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) C07F5/-;A61N5/-;A61K41/- Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPI:EPODOC;CAPAT;CAPLUS(STN);REGISTRY(STN);MARPART(STN);porphyrin+,Ln,Gd,cancer+,tumor+,tomour+, photodynam+,PDT		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	ZHOU, Y. et al. "αvβ3-Isoform specific erbium complexes highly specific for bladder cancer imaging and photodynamic therapy" <i>Chem. Commun.</i> , Vol. 53, No. 3, 06 December 2016 (2016-12-06), ISSN: 1359-7345, Pages 557-560	1-16
X	ZHU, X.J. et al. "Synthesis, Characterization, and DNA-Binding and -Photocleavage Properties of Water-Soluble Lanthanide Porphyrinate Complexes" <i>Chem. Eur. J.</i> , Vol. 17, No. 25, 09 May 2011 (2011-05-09), ISSN: 1521-3765, Pages 7041-7052	1,7-16,18
X	CAPLUS STN, AN:2013:59719, . "Beyond photodynamic therapy porphyrins and their derivatives as antitumor DNA-ligands,ACS,26 Apr.2013(26.04.2013),RN 1429332-76-2,Abstract" &Rossiiskii Bioterapevticheskii Zhurnal, (2011),10(4),47-53,	1,7-16
A	WO 2016070851 A1 (HONG KONG BAPTIST UNIVERSITY) 12 May 2016 (2016-05-12) Paragraphs [0004] and [0009],figure 1	1-17
☒ 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 another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 18 December 2017		Date of mailing of the international search report 09 January 2018
Name and mailing address of the ISA/CN STATE INTELLECTUAL PROPERTY OFFICE OF THE P.R.CHINA 6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088 China Facsimile No. (86-10)62019451		Authorized officer AN,Lingling Telephone No. (86-10)82246746

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2017/104492

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHANG, T. et al. "Porphyrin-based ytterbium complexes targeting anionic phospholipid membranes as selective biomarkers for cancer cell imaging" <i>Chem. Commun.</i> , Vol. 49, No. 65, 24 June 2013 (2013-06-24), ISSN: 1359-7345, Pages 7252-7254	1-17
A	ZHANG, T. et al. "In vivo selective cancer-tracking gadolinium eradicator as new-generation photodynamic therapy agent" <i>PNAS</i> , Vol. 111, No. 51, 01 December 2014 (2014-12-01), ISSN: 0027-8424, E5492-E5497	1-17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2017/104492

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 sequence listing filed or furnished:

a. (means)

☐

on paper

☒

in electronic form

b. (time)

☐

in the international application as filed

☒

together with the international application in electronic form

☐

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/CN2017/104492

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.: **8-16**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] Claims 8-16 are directed to methods for treatment of the human body by therapy. This search report has been established on the basis of the use of the said compositions for manufacture of medicaments thereof.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2017/104492

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2016070851	A1	12 May 2016	CN	107109219	A	29 August 2017
				US	9522925	B2	20 December 2016
				EP	3215586	A1	13 September 2017
				US	2016130284	A1	12 May 2016