



US 20170232119A1

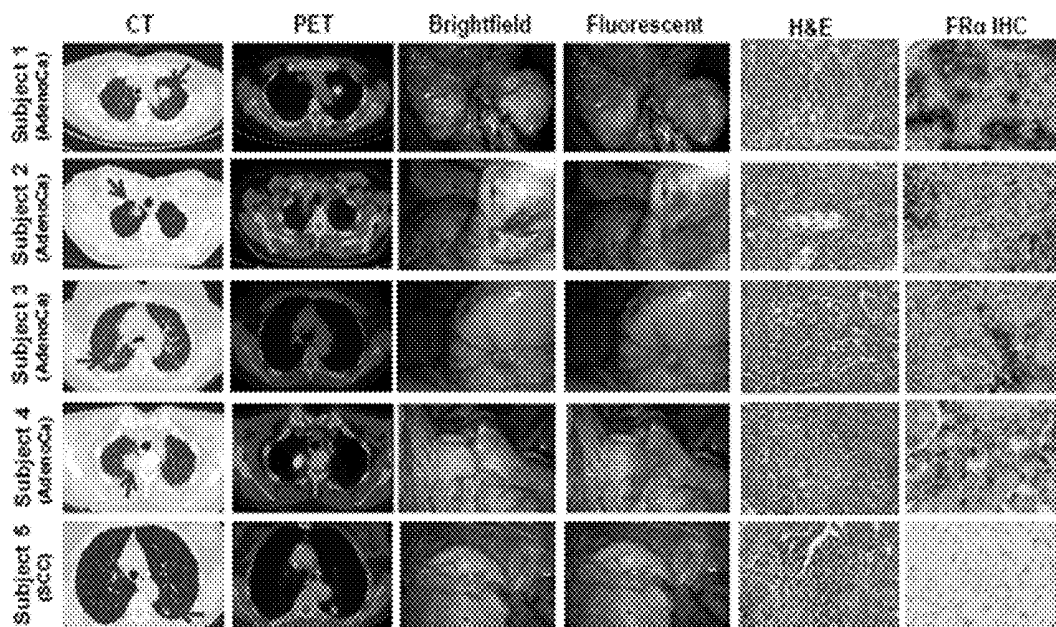
(19) **United States**(12) **Patent Application Publication**  
**Kularatne et al.**(10) **Pub. No.: US 2017/0232119 A1**(43) **Pub. Date: Aug. 17, 2017**(54) **SYNTHESIS AND COMPOSITION OF AMINO ACID LINKING GROUPS CONJUGATED TO COMPOUNDS USED FOR THE TARGETED IMAGING OF TUMORS**

(60) Provisional application No. 61/791,921, filed on Mar. 15, 2013.

**Publication Classification**(71) Applicant: **Purdue Research Foundation**, West Lafayette (IN)(51) **Int. Cl.**  
*A61K 49/00* (2006.01)  
*A61B 5/00* (2006.01)(72) Inventors: **Sumith A. Kularatne**, West Lafayette, IN (US); **Sunil Singhal**, Moorestown, NJ (US)(52) **U.S. Cl.**  
CPC ..... *A61K 49/0052* (2013.01); *A61K 47/48* (2013.01); *A61B 5/0071* (2013.01); *G01N 2021/6432* (2013.01)(21) Appl. No.: **15/584,752**(57) **ABSTRACT**(22) Filed: **May 2, 2017****Related U.S. Application Data**

(63) Continuation-in-part of application No. 15/259,719, filed on Sep. 8, 2016, which is a continuation-in-part of application No. 14/953,928, filed on Nov. 30, 2015, which is a continuation of application No. 14/715,799, filed on May 19, 2015, now Pat. No. 9,341,629, which is a continuation of application No. 14/207,130, filed on Mar. 12, 2014, now Pat. No. 9,061,057, which is a continuation of application No. 14/010,098, filed on Aug. 26, 2013, now Pat. No. 9,333,270.

The present disclosure relates to compounds that are useful as near-infrared fluorescence probes, wherein the compounds include i) a pteroyl ligand that binds to a target receptor protein, ii) a dye molecule, and iii) a linker molecule that comprises an amino acid or derivative thereof. The disclosure further describes methods and compositions for incorporating the compounds as used for the targeted imaging of non-small cell lung cancer (NSCLC) in human subjects. Conjugation of the amino acid linking groups increase specificity and detection of the compound. Methods and compositions for use thereof in diagnostic imaging are contemplated.



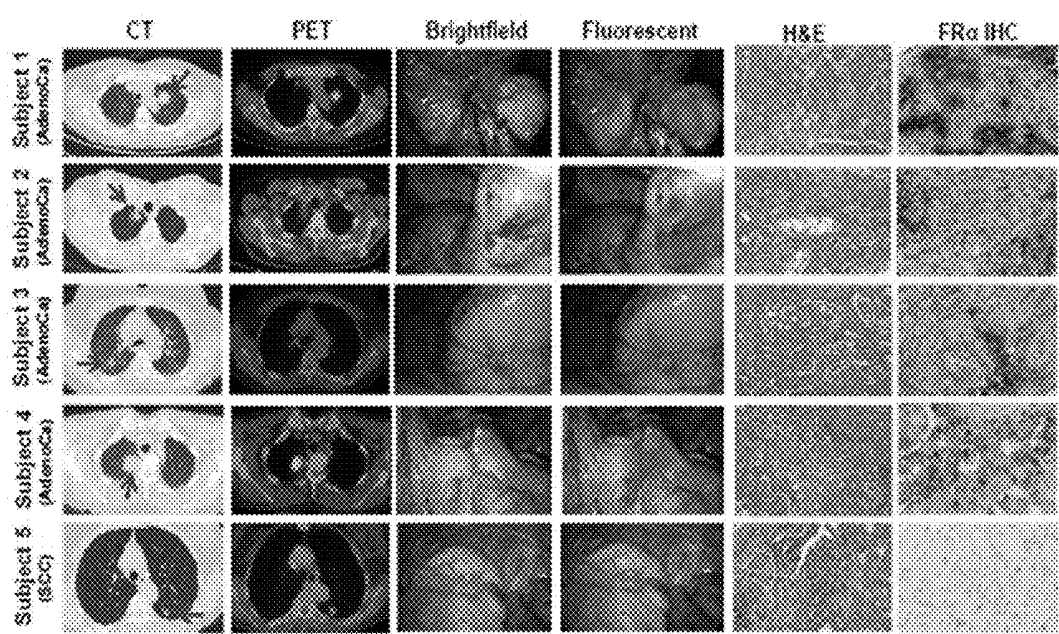


Figure 1.

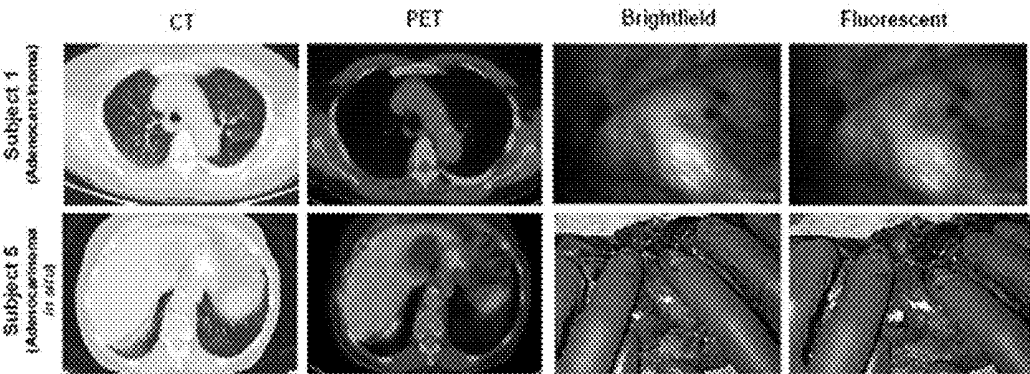


Figure 2.

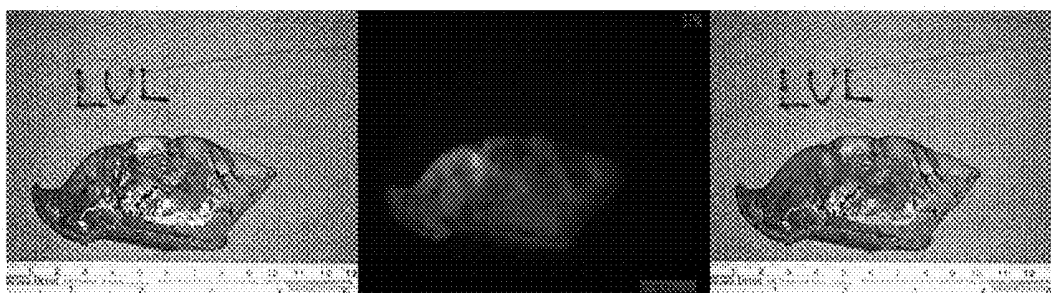


Figure 3.

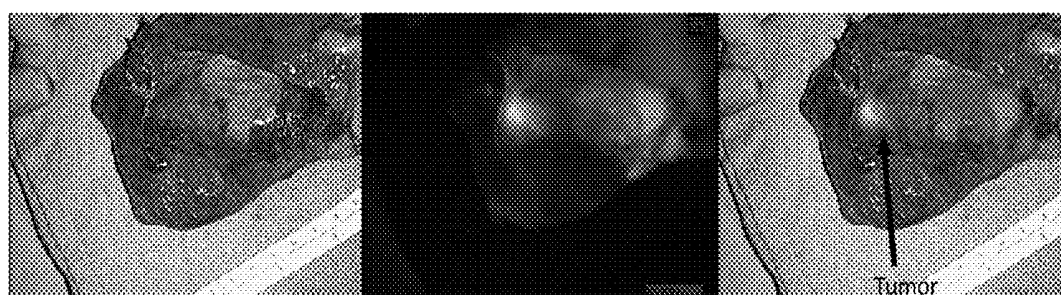


Figure 4.

## SYNTHESIS AND COMPOSITION OF AMINO ACID LINKING GROUPS CONJUGATED TO COMPOUNDS USED FOR THE TARGETED IMAGING OF TUMORS

### RELATED APPLICATIONS

[0001] The present patent application is a Continuation-in-Part of U.S. patent application Ser. No. 15/259,719, filed Sep. 8, 2016, which is a continuation-in-part of U.S. patent application Ser. No. 14/953,928, filed Nov. 30, 2015, which is a continuation of U.S. patent application Ser. No. 14/715,799, filed May 19, 2015, now U.S. Pat. No. 9,341,629, which is a continuation of U.S. patent application Ser. No. 14/207,130, filed Mar. 12, 2014, now U.S. Pat. No. 9,061,057, which is a continuation of U.S. patent application Ser. No. 14/010,098, filed Aug. 26, 2013, now U.S. Pat. No. 9,333,270, which is related to and claims priority benefit of U.S. Provisional Patent Application No. 61/791,921, filed Mar. 15, 2013, all of which are hereby incorporated by reference in their entirety into this disclosure.

### FIELD OF THE DISCLOSURE

[0002] The present disclosure is in the area of diagnostics. This disclosure provides methods of synthesizing and utilizing amino acid linking groups that are conjugated to a compound used for the targeted imaging of tumors. Conjugation of the amino acid linking groups increases specificity and detection of the compound. Methods and compositions for use thereof in diagnostic imaging are contemplated.

### BACKGROUND OF THE DISCLOSURE

[0003] Surgical removal of malignant disease constitutes one of the most common and effective therapeutic for primary treatment for cancer. Resection of all detectable malignant lesions results in no detectable return of the disease in approximately 50% of all cancer patients' and may extend life expectancy or reduce morbidity for patients in whom recurrence of the cancer is seen. Not surprisingly, surgical methods for achieving more quantitative cytoreduction are now receiving greater scrutiny.

[0004] Resection of all detectable malignant lesions results in no detectable return of the disease in approximately 50% of all cancer patients and may extend life expectancy or reduce morbidity for patients in whom recurrence of the cancer is seen. Given the importance of total resection of the malignant lesions, it is beneficial to ensure that the malignant lesions are accurately and completely identified. Identification of malignant tissue during surgery is currently accomplished by three methods. First, many tumor masses and nodules can be visually detected based on abnormal color, texture, and/or morphology. Thus, a tumor mass may exhibit variegated color, appear asymmetric with an irregular border, or protrude from the contours of the healthy organ. A malignant mass may also be recognized tactilely due to differences in plasticity, elasticity or solidity from adjacent healthy tissues. Finally, a few cancer foci can be located intraoperatively using fluorescent dyes that flow passively from the primary tumor into draining lymph nodes. In this latter methodology, fluorescent (sentinel) lymph nodes can be visually identified, resected and examined to determine whether cancer cells have metastasized to these lymph nodes.

[0005] During minimally invasive pulmonary resection, both limited visualization and tactile feedback can make localization of subcentimeter pulmonary nodules and ground-glass opacities (GGOs) challenging. Current techniques to improve intraoperative detection include ultrasound, radionuclide imaging, wire localization and intraoperative marking by bronchoscopy or CT]. There are, however, challenges associated with these approaches, most notably the need for prior knowledge regarding nodule location and the potential for patient morbidity. Furthermore, these approaches do little to assist the surgeon to perform other critical oncologic steps such as identifying synchronous disease or evaluating margin status.

[0006] Despite the recognition of the importance of removal of the tumor and the availability of certain identification techniques for visualizing tumor mass, many malignant nodules still escape detection, leading to disease recurrence and often death. Thus, there is a need for improved tumor identification. This motivation has led to the introduction of two new approaches for intraoperative visualization of malignant disease. In the first, a quenched fluorescent dye is injected systemically into the tumor-bearing animal, and release of the quenching moiety by a tumor-specific enzyme, pH change, or change in redox potential is exploited to activate fluorescence within the malignant mass selectively. In the second approach, a fluorescent dye is conjugated to a tumor-specific targeting ligand that causes the attached dye to accumulate in cancers that over-express the ligand's receptor. Examples of tumor targeting ligands used for this latter purpose include folic acid, which exhibits specificity for folate receptor (FR) positive cancers of the ovary, kidney, lung, endometrium, breast, and colon, and DUPA, which can deliver attached fluorescent dyes selectively to cells expressing prostate-specific membrane antigen (PSMA), i.e. prostate cancers and the neovasculature of other solid tumors. Beneficially, one folate-targeted fluorescent dye (folate-fluorescein or EC17) has been recently tested intra-operatively in human ovarian cancer patients. In this study, ~5× more malignant lesions were removed with the aid of the tumor-targeted fluorescent dye than without it, and all resected fluorescent lesions were confirmed by pathology to be malignant.

[0007] Conventional fluorescent techniques use probes in the visible light spectrum (~400-600 nm), which is not optimal for intra-operative image-guided surgery as it is associated with a relatively high level of nonspecific background light due to collagen in the tissues. Hence the signal to noise ratio from these conventional compounds is low. Moreover, the absorption of visible light by biological chromophores, in particular, hemoglobin, limits the penetration depth to a few millimeters. Thus, tumors that are buried deeper than a few millimeters in the tissue may remain undetected. Moreover, ionization equilibrium of fluorescein (pKa=6.4) leads to pH-dependent absorption and emission over the range of 5 to 9. Therefore, the fluorescence of fluorescein-based dyes is quenched at low pH (below pH 5).

[0008] For example, the potential use of EC17 dye for a more widespread use in optical imaging for the characterization and measurement diseased tissue in a clinical setting has been hampered by the major drawback of that the attached dye (fluorescein) emits fluorescence in the visible range. This makes EC17 and related dyes poor for in vivo use in tissues because tissues typically autofluorescence strongly in the visible range, and light penetrates tissue

poorly. Moreover, EC17 (folate-ethylenediamine—fluorescein isothiocyanate) consists a thiourea linker. It is well known that thiourea compounds have a low shelf life due to the instability of the thiourea linkage. Thus, a compound such as EC17 is not optimal for use in optical imaging because of this instability and the related decomposition of the thiourea bridge.

**[0009]** The combination of light absorption by hemoglobin in the visible light spectrum (<600 nm) and water and lipids in the IR range (>900 nm), offers an optical imaging window from approximately 650-900 nm in which the absorption coefficient of tissue is at a minimum. A suitable alternative to dyes that emit light in the visible range would be to develop dyes that can be used in the near infra-red (NIR) range because the light in the near infrared region induces very little autofluorescence and permeates tissue much more efficiently. Another benefit to near-IR fluorescent technology is that the background from the scattered light from the excitation source is greatly reduced since the scattering intensity is proportional to the inverse fourth power of the wavelength. Low background fluorescence is necessary for highly sensitive detection. Furthermore, the optically transparent window in the near-IR region (650 nm to 900 nm) in biological tissue makes NIR fluorescence a valuable technology for in vivo imaging and subcellular detection applications that require the transmission of light through biological components.

**[0010]** While the use of light in the NIR range for deeper tissue imaging is preferable to light in the visible spectrum, the NIR imaging dyes currently used in the art suffer from a number of challenges and disadvantages such as a susceptibility to photobleach, poor chemical stability, absorbance and emission spectra that fall within the same range as many physiological molecules (resulting in high background signal and autofluorescence). Moreover, most of the NIR dyes are not stable during the synthesis, especially conjugating to a ligand with an amine linker, leading to multiple unwanted side products. Therefore, taking ligand-targeted NIR imaging agent to clinic can be expensive. Thus, current imaging methods that utilize NIR fluorescent probes are not effective in deep tissue imaging (>5 mm from the surface), in quantifying fluorescence signal in mammalian tissues, or in production cost that increases preclinical-to-clinical translational time.

**[0011]** Two promising approaches to fluorescence-guided surgery are currently under intense investigation for use in the clinic. In one method, an activatable NIR fluorescent probe, which is minimally fluorescent in the steady state due to its proximity to an attached quencher, becomes highly fluorescent upon release of the quencher in malignant tissue. One of the most commonly used release mechanisms involves incorporation of a peptide sequence between the dye and the quencher that can be specifically cleaved by a tumor-enriched protease (i.e. cathepsins, caspases and matrix metalloproteinases). A major advantage of this strategy lies in the absence of fluorescence in tissues that lack the activating enzyme, allowing tissues along the excretion

pathway (e.g. kidneys, bladder, liver) to remain nonfluorescent unless they fortuitously express the cleaving enzyme. Such tumor-activated NIR dyes can also generate substantial fluorescence in the tumor mass as long as the malignant lesion is enriched in the cleaving protease and the released dye is retained in the tumor. The major disadvantage of this methodology arises from the poor tumor specificities of many of the relevant hydrolases (most of which are also expressed in healthy tissues undergoing natural remodeling or experiencing inflammation). Moreover, the abundance of the desired proteases may vary among tumor masses, leading to slow or no activation of fluorescence in some malignant lesions and rapid development of fluorescence in others.

**[0012]** Non-small cell lung cancer (NSCLC) is characterized by assessing the morphology of the tumor biopsy by histological analysis including immunohistochemical analysis. The most common histological subtypes of NSCLC are squamous cell carcinoma, large cell carcinoma, adenocarcinoma, neuroendocrine carcinoma, carcinoid tumors, metastatic tumors, and lymph node. There are several other subtypes that occur less frequently. As NSCLC arises from the epithelial cells of the lung of the central bronchi to terminal alveoli, the histological subtypes of NSCLC correlate with the site of origin, reflecting the variation in respiratory tract epithelium of the bronchi to alveoli.

**[0013]** Thus, there remains a need for a dye substance that can be used to specifically target diseased tissue and has increased stability and brightness for use in vivo for tissue imaging.

#### BRIEF SUMMARY OF THE DISCLOSURE

**[0014]** This disclosure provides a method for synthesizing amino acid linking groups that are conjugated to a compound used for the targeted imaging of tumors and lymph nodes. In certain embodiments, this disclosure relates to a compound or a salt derivative thereof, that comprises a folate or pteroyl ligand, a linking group, and a fluorescent dye. In certain embodiments, the linking group can be an amino acid, an isomer, a derivative, or a racemic mixture thereof. In other aspects, the fluorescent dye is selected from the group consisting of LS288, IR800, SP054, S0121, KODAK, S2076, and S0456.

**[0015]** In some aspects, this disclosure provides a method of conjugating an amino acid linking group to a fluorescent dye, wherein the amino acid can be tyrosine, serine, threonine, lysine, arginine, asparagine, glutamine, cysteine, selenocysteine, isomers, and the derivatives thereof. In certain embodiments, the amino acid, isomers, or the derivatives thereof, contain an —OH, —NH<sub>2</sub>, or —SH functional group that upon addition of the fluorescent dye in slight molar excess produces the conjugation of the fluorescent group with the amino acid, isomer, or the derivatives thereof. In other embodiments, the amino acid, isomers, or the derivatives thereof, contains an —OH functional group that upon synthesis generates an ether bond with the fluorescent dye that increases the brightness and detection of the compound.

In some embodiments, this disclosure relates to the conjugation of the amino acid linking group with the fluorescent dye, wherein the amino acid, isomers, or the derivatives thereof, contains an —SH, —SeH, —PoH, or —TeH functional group that upon synthesis generates a C—S, C—Se, C—Po, or C—Te bond with the fluorescent dye. In some aspects, this disclosure relates to the conjugation of the amino acid linking group to a fluorescent dye that has an absorption and emission maxima between about 500 nm and about 900 nm. In other aspects, the amino acid linking group is conjugated to a fluorescent dye that has an absorption and emission maxima between about 600 nm and about 800 nm.

**[0016]** In additional embodiments, this disclosure provides a method for conjugating the amino acid linking group to a folate ligand, wherein the amino acid linking group is tyrosine, serine, threonine, lysine, arginine, asparagine, glutamine, cysteine, selenocysteine, isomers or the derivatives thereof, and is conjugated to folate through a dipeptide bond. In additional aspects, this disclosure provides a method of conjugating the linking group with a folate ligand, wherein the linking group is tyrosine, serine, threonine, lysine, arginine, asparagine, glutamine, cysteine, selenocysteine, isomers, or the derivatives thereof, and is conjugated to folate through a homo-oligopeptide bond. In other embodiments, this disclosure relates to a method of conjugating a pteroyl ligand to an amino acid linking group, wherein the linking group is tyrosine, serine, threonine, lysine, arginine, asparagine, glutamine, cysteine, selenocysteine, isomers or the derivatives thereof. In certain aspects, the carboxylic acid of the linking group is bound to the alpha carbon of any amino acid, hence increasing the specificity of the com-

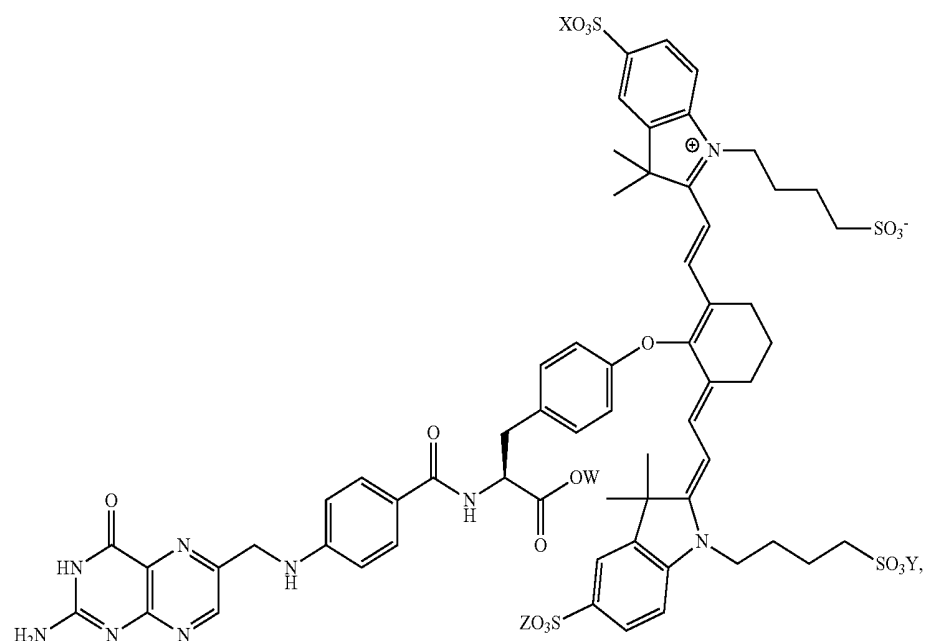
pound for targeted receptors. In some embodiments, the amino acid linking group contributes specificity to the compound, wherein the observed binding affinity of the compound to targeted receptors is folate receptor.

**[0017]** In additional aspects, the compound is highly selective for targeting to tumor cells expressing the target receptor.

**[0018]** In other embodiments, this disclosure relates to the use of a compound designated, Pte-Tyr-S0456 (OTL-0038) for image-guided surgery, tumor imaging, lymph node imaging, inflammatory diseases, atherosclerosis, infection diseases, forensic applications, mineral applications, dental, gel staining, DNA sequencing, nerve staining, or plastic surgery. In other aspects, the Pte-Tyr-S0456 derivative can be Pte-D-Tyr-S0456, Pte-homoTyr-S0456, Pte-beta-homo-Tyr-S0456, Pte-(NMe)-Tyr-S0456, Pte-Tyr(OMe)-S0456, Pte-Tyr(OBn)-S0456, Pte-NHNH-Tyr-OAc-S0456, salts, or derivatives thereof.

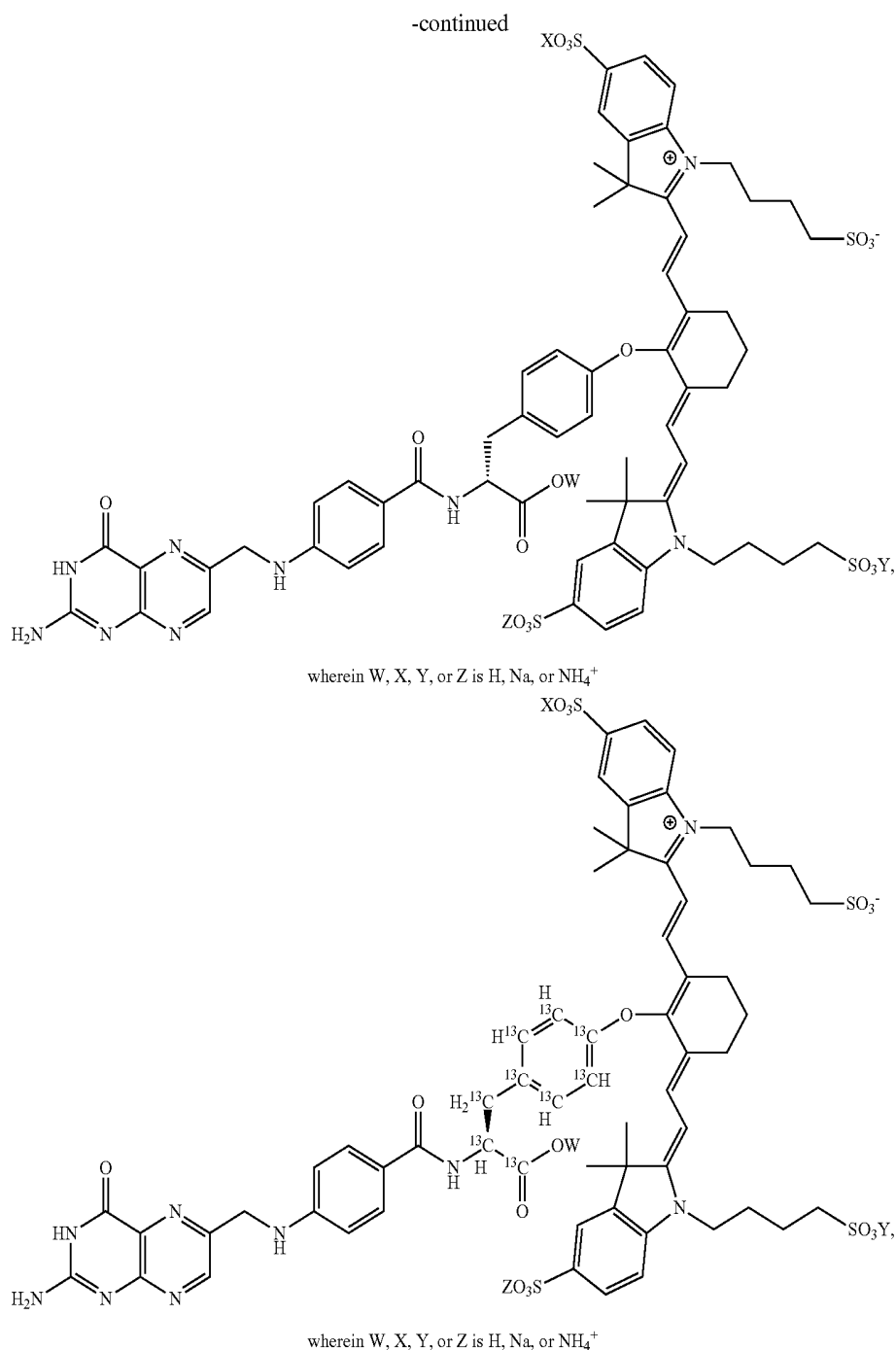
**[0019]** In other aspects, this disclosure provides a method of synthesizing the compound, wherein a protecting group is used to avoid undesired reactivity with groups other than the amino groups that might generate unwanted compounds. The methods provided in this disclosure produce a final compound with a yield of over 98% purity.

**[0020]** In certain aspects, this disclosure relates to a compound used for the targeted imaging of tumors, wherein the compound could be used for research, diagnostic, or therapeutic purposes. In other embodiments, this disclosure provides a composition comprising an imaging compound and a pharmaceutically acceptable carrier, excipient, diluents, or salts.



wherein W, X, Y, or Z is H, Na, or NH<sub>4</sub><sup>+</sup>





**[0021]** In certain aspects, this disclosure provides imaging of Non-small cell lung cancer (NSCLC) in human subjects. NSCLC is characterized by assessing morphology of the tumor biopsy by histological analysis including immunohistochemical analysis. The most common histological subtypes of NSCLC are squamous cell carcinoma, large cell carcinoma, adenocarcinoma, neuroendocrine carcinoma, carcinoid tumors, metastatic tumors, and lymph node. There are several other subtypes that occur less frequently. As NSCLC arises from the epithelial cells of the lung of the central bronchi to terminal alveoli, the histological subtypes of NSCLC correlate with site of origin, reflecting the variation in respiratory tract epithelium of the bronchi to alveoli.

#### BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

**[0022]** FIG. 1 illustrates the accumulations of OTL38 in FR $\alpha$  expressing pulmonary adenocarcinomas. Five subjects were enrolled in a pilot study involving IMI with OTL38 (0.025 mg/kg). Approximately 4 hours after intravenous delivery, subjects underwent minimally invasive pulmonary resection (VATS). Preoperative CT (Column 1) and PET (Column 2) scans are provided. Intraoperative brightfield (Column 3) and fluorescent overlay views (Column 4) during VATS resection. H&E (Column 5) and FR $\alpha$  IHC (Column 6) of resected tumors.

**[0023]** FIG. 2 illustrates the identification of synchronous nodules that are otherwise undetectable using OTL38. In two subjects (Subject 1 and Subject 5), synchronous disease was identified with IMI. In Subject 1, a 0.6 cm synchronous adenocarcinoma was identified in the left lower lobe. In Subject 5, a 0.8 cm adenocarcinoma in situ was identified in the left lower lobe. For each nodule, corresponding preoperative CT (Column 1) and PET (Column 2) images are provided. Intraoperative brightfield (Column 3) and fluorescent overlay views (Column 4) are also displayed.

**[0024]** FIG. 3 illustrates the accumulation of OTL38 in FR $\alpha$  expressing squamous cell carcinoma. Subject was enrolled in a pilot study involving IMI with OTL38 (0.025 mg/kg). Approximately 4 hours after intravenous delivery, subjects underwent minimally invasive pulmonary resection (VATS).

**[0025]** FIG. 4: OTL38 accumulates in FR $\alpha$  expressing carcinoid lung tumor. Subject was enrolled in a pilot study involving IMI with OTL38 (0.025 mg/kg). Approximately 4 hours after intravenous delivery, subjects underwent minimally invasive pulmonary resection (VATS).

#### DETAILED DESCRIPTION OF THE DISCLOSURE

**[0026]** Surgery is one of the best therapies for all the solid tumors, such as prostate, ovarian, lung, breast, colon, and pancreatic cancer. While surgery is effective in 50% of patients with solid tumors in the US, chemo- and radiotherapy alone are effective in less than 5% of all cancer patients. Over 700,000 patients undergo cancer surgery every year in the US and 40% of surgical patients have a recurrence of locoregional disease within 5 years. Despite major advances in the oncology field over the last decade, there remain significant hurdles to overcome in the field. For example, it remains difficult to achieve complete resection of the primary tumor with negative margins, removal of the lymph nodes harboring metastatic cancer cells and identification of satellite disease. Achieving improvements in these three cases not only improves disease clearance but also guides decisions regarding postoperative chemotherapy and radiation. While non-targeted fluorescent dyes have been shown to passively accumulate in some tumors, the resulting tumor-to-background ratios are often poor and the boundaries between malignant and healthy tissues can be difficult to define. Although ligand-targeted fluorescence dyes (e.g., EC17: Folate-EDA-FITC) have been used for imaging a tissue, those dyes have been ineffective as they would not penetrate deep tissue and hence only identified the specific cells on the surface of a tissue rather than deeper within the tissue sample. In addition, it has been shown that the excitation and emission spectra of these previous fluorescence dyes was such that it produced significant background noise such that the targeted tissue was not easily detected. In addition, as discussed in the background above, fluorescein-based dyes have the disadvantages of low shelf-life stability. EC17 easily decomposes as a result of the instability of the thiourea bridge in that compound. In addition, as EC17 uses fluorescein which has the drawback of a relatively high level of nonspecific background noise from collagen in the tissues surrounding the imaging site. Moreover, the absorption of visible light by biological chromophores, in particular hemoglobin, further limits the usefulness of dyes that incorporate fluorescein. This means that conventional dyes cannot readily detect tumors that may be buried deeper than a

few millimeters in the tissue. Furthermore, fluorescence from fluorescein is quenched at low pH (below pH 5)

**[0027]** In order for a dye material to be useful in detecting and guiding surgery or providing other tissue imaging it is important to overcome these drawbacks.

**[0028]** Several criteria were considered in preparation of conjugates including near infrared dyes. Ease of synthesis and chemical stability were primary chemical attributes. Spectral properties, such as absorption and emission spectra and quantum yield, were considered. Several biological properties were evaluated, such as binding affinity in cell studies, whole body animal imaging using mice with tumors, and biodistribution. Specifically for biodistribution, several aspects were considered including dead mice after 2 hours per oral distribution, live mice imaging and dose escalation. Finally, safety considerations were taken including Maximum Tolerance Dose (MTD), ImmunoHistoChemical (IHC) analysis, and general clinical pathology analysis.

**[0029]** The present disclosure provides pteroyl conjugates of near infrared dyes that are stable, fluoresce in the infrared range, and penetrate deep into targeted tissue to produce a specific and bright identification of areas of tissue that express folate receptor. More specifically, the pteroyl conjugates are linked to the near infrared dyes through an amino acid linker. Even more specifically, it has been found that where the amino acid linker is tyrosine or a derivative of tyrosine, the intensity of the fluorescence of the dye is maintained or even enhanced.

**[0030]** An amino acid is defined as including an amine functional group linked to a carboxylic acid functional group, and a side-chain specific to each amino acid. An alpha-amino acid is any compound of the general formula  $R^5CH(NH_2)COOH$  ( $\alpha$ -amino acid), wherein  $R^5$  is selected from the group consisting of H or any known amino acid side chain.

**[0031]** A beta amino acid is defined as including an amine functional group linked at a beta carbon and a carboxylic acid functional group linked at the alpha carbon. A beta homo-amino acid is defined as including an amine functional group linked at a beta carbon, a carboxylic acid functional group linked at the alpha carbon and a side-chain starting at either the alpha carbon or the beta carbon wherein the side-chain is bound to another amino acid.

**[0032]** Naturally occurring amino acids can be divided into the following four groups: (1) acidic amino acids, (2) basic amino acids, (3) neutral polar amino acids, and (4) neutral nonpolar amino acids. Representative amino acids within these various groups include, but are not limited to: (1) acidic (negatively charged) amino acids such as aspartic acid and glutamic acid; (2) basic (positively charged) amino acids such as arginine, histidine, and lysine; (3) neutral polar amino acids such as glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; and (4) neutral nonpolar (hydrophobic) amino acids such as alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine.

**[0033]** Conserved substitution for an amino acid within a naturally occurring amino acid sequence can be selected from other members of the group to which the naturally occurring amino acid belongs. For example, the aliphatic side chains group of amino acids is glycine, alanine, valine, leucine, and isoleucine. Conserved substitution of naturally occurring amino acid valine includes the use of glycine, alanine, leucine, or isoleucine.

**[0034]** The aliphatic-hydroxyl side chain group of amino acids is serine and threonine. The amide-containing side chain group of amino acids is asparagine and glutamine. The aromatic side chain group of amino acids is phenylalanine, tyrosine, and tryptophan. The basic side chain group of amino acids is lysine, arginine, and histidine. The sulfur-containing side chain group of amino acids having is cysteine and methionine. Examples of naturally conservative amino acids substitutions are valine for leucine, serine for threonine, phenylalanine for tyrosine, lysine for arginine, cysteine for methionine, and asparagine for glutamine.

**[0035]** In preferred embodiments, it is shown herein that such pteroyl conjugates specifically target to tumor cells within a tissue. Moreover, the intensity of the fluorescence is greater than the intensity of previously observed with other near infrared dyes that are targeted with folate for folate receptor positive tumors. This increased intensity allows the targeting and clear identification of smaller areas of biological samples (e.g., smaller tumors) from a tissue being monitored. Also, the increased intensity of the compounds of the present disclosure provides the added advantage that lower doses/quantities of the dye can be administered and still produces meaningful results. Thus, the compounds of the present disclosure lead to more economical imaging techniques. Moreover, there is an added advantage that a lower dose of the compounds of the disclosure as compared to conventional imaging compounds minimizes the toxicity and other side effects that are attendant with the administration of foreign materials to a body.

**[0036]** Furthermore, identification of small tumors will lead to a more accurate and more effective resection of the primary tumor to produce negative margins, as well as accurate identification and removal of the lymph nodes harboring metastatic cancer cells and identification of satellite disease. Each of these advantages positively correlates with a better clinical outcome for the patient being treated.

**[0037]** In specific experiments, it was found that use of amino acids other than tyrosine as the linker resulted in the loss of near infrared fluorescence. Specifically, note the synthetic pathway lead to undesired by-product 4 as major product that does not have NIR properties

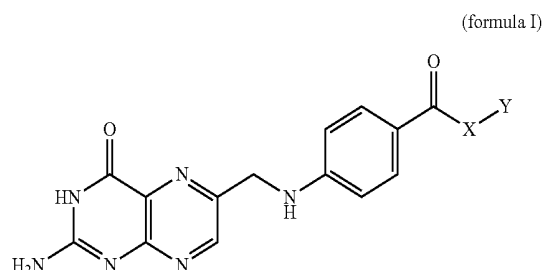
**[0038]** However, it is contemplated that in addition to tyrosine and tyrosine derivatives, a pteroyl conjugate of a near infrared dye with cysteine or cysteine derivatives also may be useful. Furthermore, it is contemplated that a direct linkage of the pteroyl or folate moiety to the dye or linkage of the dye to pteric acid or folic acid through an amine linker also produces a loss of intensity of the fluorescence from the conjugate whereas the presence of the tyrosine or tyrosine derivative as the linking moiety between the pteroyl (targeting moiety) and the near infrared dye (the fluorescing moiety) is beneficial to maintain or enhance the fluorescence of the conjugated compound. Tyrosine-based compounds of the disclosure do not require an extra amine linker to conjugate the S0456 because conjugation through the phenol moiety of the tyrosine leads to enhanced fluorescence.

**[0039]** The compounds can be used with fluorescence-mediated molecular tomographic imaging systems, such as those designed to detect near-infrared fluorescence activation in deep tissues. The compounds provide molecular and tissue specificity, yield high fluorescence contrast, brighter fluorescence signal, and reduce background autofluorescence, allowing for improved early detection and molecular target assessment of diseased tissue in vivo (e.g., cancers).

The compounds can be used for deep tissue three-dimensional imaging, targeted surgery, and methods for quantifying the amount of a target cell type in a biological sample.

**[0040]** Compounds

**[0041]** In an aspect the disclosure relates to compounds comprising the formula: Formula (I):



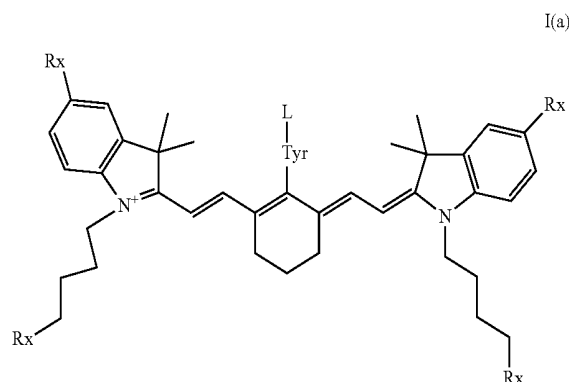
**[0042]** wherein:

**[0043]** X is an amino acid or a derivative thereof, and

**[0044]** Y is a dye that has a fluorescence excitation and emission spectra in the near infra-red range and said compound maintains or enhances the fluorescence of Y.

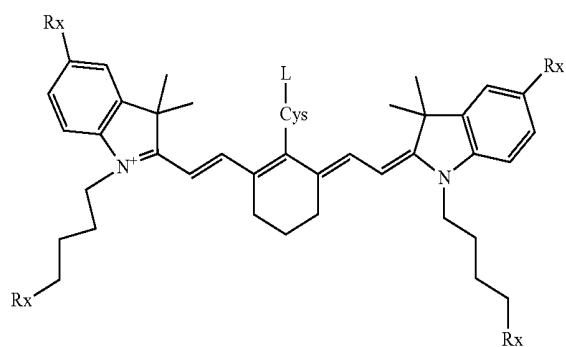
**[0045]** In some embodiments, the amino acid or amino acid derivative induces a shift in the electronic emission spectrum, the electronic absorption spectrum, or both the electronic emission and absorption spectrum, relative to the electronic spectra of the unmodified dye molecule. Suitably, the shift in the electronic spectrum is a bathochromic shift (i.e., shift to longer wavelength/lower frequency) that helps to improve the detection of the compound in the near infrared (NIR) spectral window and/or reduce the amount of background signal, auto-fluorescence, interferences from the tissue surrounding the area being visualized. More specifically, this shift in electronic spectrum is particularly observed with NIR dyes that comprise electronegative atoms that are incorporated into the 6-membered ring. Thus, in certain embodiments, the amino acid or amino acid (X) derivative comprises an electron-rich moiety such as, for example, oxygen, sulfur, or nitrogen. Non-limiting examples of such amino acids can include cysteine, methionine, threonine, serine, tyrosine, phenylalanine, tryptophan, histidine, lysine, arginine, aspartic acid, glutamic acid, asparagine, and glutamine, or derivatives thereof.

**[0046]** In embodiments of this aspect, the disclosure provides compounds of Formulas (I)a, (I)b, (I)c, and (I)d:

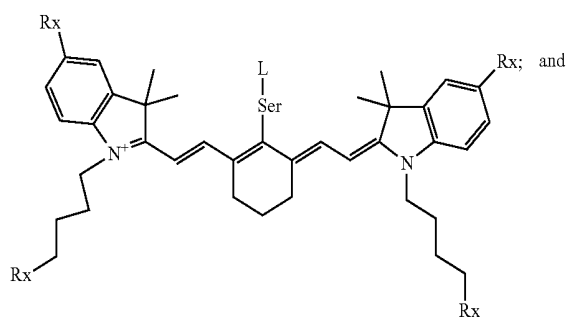


-continued

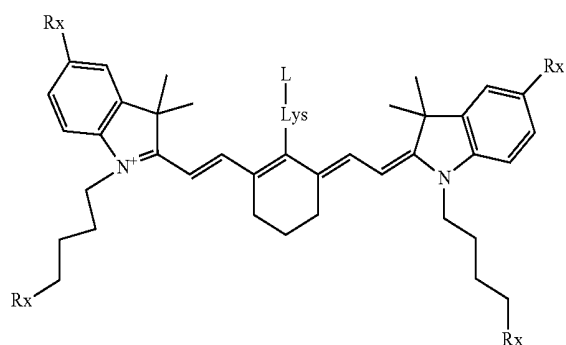
I(b)



I(c)

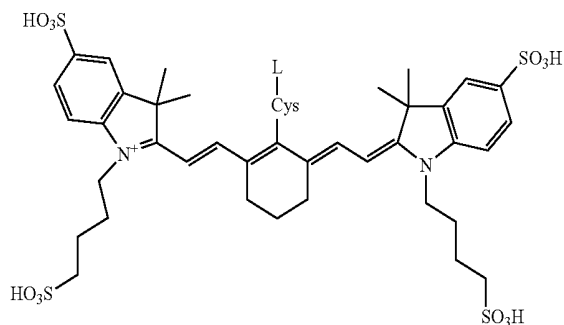


I(d)

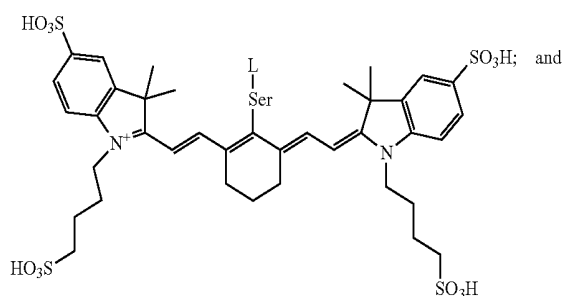


-continued

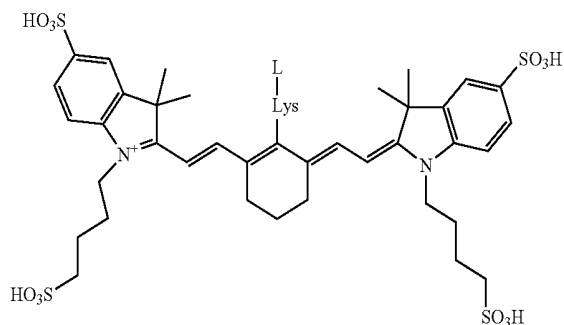
(I)b1



(I)c1



(I) d1

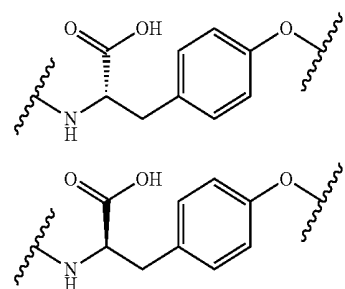
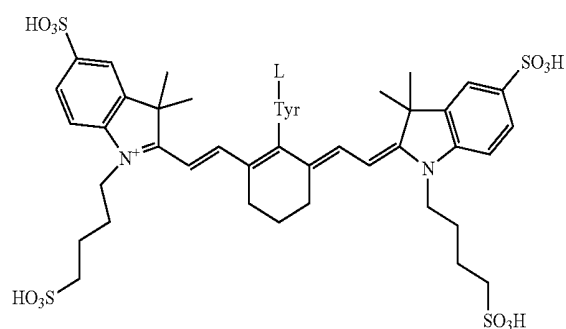


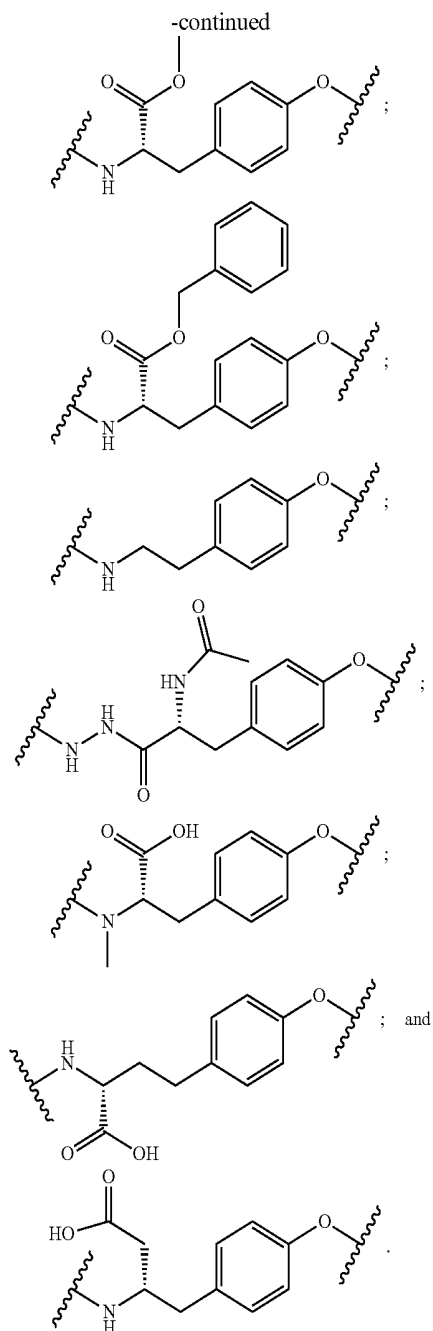
**[0047]** wherein the Tyr, Cys, Ser, and Lys groups indicate a tyrosine, a cysteine, a serine, and a lysine amino acid residue, respectively, or derivatives thereof, and L is preferably a pteroyl or folate and Rx each comprises an independently selected solubilizing group that is optionally absent.

**[0048]** Wherein the Tyr, Cys, Ser, and Lys groups indicate a tyrosine, a cysteine, a serine, and a lysine amino acid residue, respectively, or derivatives thereof, and L is preferably a pteroyl or folate. Preferably, L is pteroyl.

**[0049]** In specific preferred embodiments the disclosure provides a compound of Formula I(a), wherein Tyr is selected from the group consisting of:

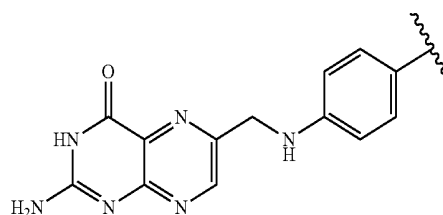
(I)a1





**[0050]** Suitably, the compounds disclosed herein have a maximum light absorption wavelengths in the near infrared region of between about 650 nm and 1000 nm, for example, and preferably, at approximately 800 nm.

**[0051]** In specific preferred embodiments, the compounds disclosed herein include a ligand (L) that is effective to target the compound to a particular cell or tissue type and allow for imaging of that targeted cell or tissue. It is preferable the L is either pteroyl moiety or folate moiety and more preferable that L is pteroyl moiety. However, it is contemplated that the skilled person may use some other ligand L to target the compounds to a particular cell surface protein or receptor protein of interest. In specific and preferred embodiments, the ligand comprises pteroyl:



#### **[0052]** Methods of Use

**[0053]** As noted hereinabove, there is a need for near infrared dye compounds that specifically target to regions within a tissue. This is so that the compounds may be used in imaging techniques and to assist in the diagnosis and therapeutic intervention of disease. As discussed in detail above, the compounds provided herein are useful as dyes and imaging agents in the NIR region of the light spectrum. As such, the compounds have broad applicability to any number of imaging, diagnostic, and targeted therapeutic methods.

**[0054]** In specific embodiments, the present disclosure relates to methods that incorporate at least one of the compounds disclosed herein (e.g., of Formula I, I(a), I(b), I(c), and/or I(d)). can be used to specifically and sensitively identify tumors within a tissue. More specifically, the identified tumors may then be therapeutically resected through surgical methods. In this manner, the compounds of the present disclosure may be useful in fluorescence-guided surgical resection of tumors, lymph nodes, and the like. Alternatively, the compounds of the present disclosure may readily be used in whole body imaging in which the compound is administered to a subject, and the localization of the fluorescence facilitates identification of a tumor site.

**[0055]** In this manner, the compounds of the present disclosure can be used for the in vivo identification of diseased tissue in a subject in need thereof. The disclosure method includes irradiating an in vivo body part of the subject containing diseased tissue with light having at least one excitation wavelength in the near infrared range from about 600 nm to about 1000 nm. Fluorescence emanating from a compound of the present disclosure administered to the subject and which has specifically bound to and/or been taken up by the diseased tissue in the body part, in response to the at least one excitation wavelength is directly viewed to determine the location and/or surface area of the diseased tissue in the subject.

**[0056]** Light having a wavelength range from 600 nm and 850 nm lies within the near infrared range of the spectrum, in contrast to visible light, which lies within the range from about 401 nm to 500 nm. Therefore, the excitation light used in practice of the disclosure diagnostic methods will contain at least one wavelength of light to illuminates the tissue at the infrared wavelength to excite the compounds in order that the fluorescence obtained from the area having uptake of the compounds of the present disclosure is clearly visible and distinct from the auto-fluorescence of the surrounding tissue. The excitation light may be monochromatic or polychromatic. In this manner, the compounds of the present disclosure are advantageous as they eliminate the need for the use of filtering mechanisms that would be used to obtain a desired diagnostic image if the fluorescent probe is one that fluoresces at wavelengths below 600 nm. In this manner, the compounds of the present disclosure avoid obscured diag-

nostic images that are produced as a result of excitation light of wavelengths that would be reflected from healthy tissue and cause loss of resolution of the fluorescent image.

**[0057]** Operating rooms for surgical procedures can be equipped with an overhead light that produces wavelengths of light in the optical emitting spectrum useful in the practice of disclosure diagnostic methods, such as lamps that produce light in the appropriate wavelength. Such a light can be utilized in the practice of the disclosure diagnostic methods merely by turning out the other lights in the operating room (to eliminate extraneous light that would be visibly reflected from tissue in the body part under investigation) and shining the excitation light of near infrared wavelength into the body cavity or surgically created opening so that the fluorescent image received directly by the eye of the observer (e.g., the surgeon) is predominantly the fluorescent image emanating from the fluorophore(s) in the field of vision. Light emanating from a source in the 600 nm and 850 nm range, preferably 750 nm-850 nm range would be used in accomplishing the goal of direct visualization by the observer so that light reflecting from the body part, other than that from the fluorescing moiety(ies), is minimized or eliminated.

**[0058]** Accordingly, in disclosure diagnostic methods, the diseased tissue (and bound or taken-up targeting construct) is "exposed" to the excitation light (e.g., by surgically created opening or endoscopic delivery of the light to an interior location. The disclosed method is particularly suited to in vivo detection of diseased tissue located at an interior site in the subject, such as within a natural body cavity or a surgically created opening, where the diseased tissue is "in plain view" (i.e., exposed to the human eye) to facilitate a procedure of biopsy or surgical excision of the area that has been highlighted by uptake of the compounds of the present disclosure. As the precise location and/or surface area of the tumor tissue are readily determined by the uptake of the compounds of the present disclosure, the methods employing the compounds of the present disclosure provide a valuable guide to the surgeon, who needs to "see" in real time the exact outlines, size, etc. of the mass to be resected as the surgery proceeds.

**[0059]** Thus, in specific embodiments, the present disclosure entails optical imaging of a biological tissue that expresses a folate receptor by contacting the tissue with a composition comprising compounds of the present disclosure (e.g., compounds of Formula I) and allowing time for the compound in the composition to distribute within the tissue and interact with the site of folate receptor. After a sufficient time for such interaction has passed, the tissue is illuminated with an excitation light to cause the compound in the composition to fluoresce. The fluorescence is then detected as and where such fluorescence is observed is an area that contains the folate receptor.

**[0060]** In like manner, the compounds of the present disclosure are used to identify a target cell type in a biological sample by contacting the biological sample with such compounds for a time and under conditions that allow for binding of the compound to at least one cell of the target cell type. The bound compound is then optically detected such that presence of fluorescence of the near infrared wavelength emanating from the bound, targeted compound of the present disclosure indicated that the target cell type is present in the biological sample. This method thus provides an image of the targeted cell type in the tissue being

assessed. Most preferably, the targeted cell type is a tumor cell or a lymph node to which a tumor cell has spread.

**[0061]** These methods advantageously provide an improved method of performing image guided surgery on a subject as the administration of a composition comprising the compound of the disclosure under conditions and for a time sufficient for said compound to accumulate at a given surgical site will assist a surgeon in visualizing the tissue to be removed. Preferably the tissue is a tumor tissue and illuminating the compound that has been taken up by the tissue facilitates visualization of the tumor by the near infrared fluorescence of the compound using infrared light. With the aid of the visualization facilitated by the targeting of the compound of the disclosure to the site of the tumor, surgical resection of the areas that fluoresce upon excitation by infrared light allows an improved and accurate removal of even small tumors.

**[0062]** It should be understood that in any of the surgical methods of the disclosure the compounds of the present disclosure may be administered before the surgical incision takes place or even after the surgical cavity and site of the tumor have been revealed by the surgery.

**[0063]** If the putative diseased site is a natural body cavity or surgically produced interior site, an endoscopic device can be optionally used to deliver the excitation light to the site, to receive fluorescence emanating from the site within a body cavity, and to aid in formation of a direct image of the fluorescence from the diseased tissue. For example, a lens in the endoscopic device can be used to focus the detected fluorescence as an aid in formation of the image. As used herein, such endoscope-delivered fluorescence is said to be "directly viewed" by the practitioner and the tissue to which the targeting construct binds or in which it is taken up must be "in plain view" to the endoscope since the light used in the disclosure diagnostic procedure will not contain wavelengths of light that penetrate tissue, such as wavelengths in the near infrared range. Alternatively, the excitation light may be directed by any convenient means into a body cavity or surgical opening containing a targeting construct administered as described herein and the fluorescent image so produced can be directly visualized by the eye of the observer without aid from an endoscope. With or without aid from any type of endoscopic device, the fluorescent image produced by the disclosure method is such that it can be viewed without the aid of an image processing device, such as a CCD camera, TV monitor, photon collecting device, and the like.

**[0064]** It is contemplated that the diagnostic or imaging methods of the present disclosure allow the surgeon/practitioner to contemporaneously see/view/visualize diseased or abnormal tissue through a surgical opening to facilitate a procedure of biopsy or surgical excision. As the location and/or surface area of the diseased tissue are readily determined by the diagnostic procedure of the disclosure employing the compounds described herein, the disclosure method is a valuable guide to the surgeon, who needs to know the exact outlines, size, etc. of the mass, for example, for resection as the surgery proceeds. In particular, it is noted that the compounds of the disclosure fluorescence in the near infrared range to a greater intensity than those previously described. As such, advantageously, it is contemplated that less of the compound will be needed to achieve diagnostic imaging. Also, the compounds of the present disclosure

penetrate deep into the tumor, and hence the disclosure advantageously allows a greater accuracy that the tumor has been removed.

**[0065]** The present disclosure provides methods for utilizing a diagnostic procedure during surgery in a subject in need thereof by administering to the subject a composition comprising a compound of the present disclosure and irradiating an in vivo body part of the subject containing diseased tissue with light having at least one excitation wavelength in the range from about 600 nm to about 850 nm, directly viewing fluorescence emanating from a targeting construct administered to the subject that has specifically bound to and/or been taken up by the diseased tissue in the body part, wherein the targeting construct fluoresces in response to the at least one excitation wavelength, determining the location and/or surface area of the diseased tissue in the subject, and removing at least a portion of the tumor tissue.

**[0066]** In yet another embodiment, the present disclosure provides methods for in vivo diagnosis of tumor tissue in a subject in need thereof. In this embodiment, the disclosure method comprises contacting samples of tumor cells obtained from the subject in vitro with a plurality of detectably labeled compounds, each of which binds to or is selectively taken up by a distinct tumor type, determining which of the compounds is bound to or taken up by the sample tumor cells, administering a diagnostically effective amount of at least one biologically compatible fluorescing targeting construct containing a compound of the present disclosure that has been determined to bind to and/or be taken up by the sample tumor cells and a fluorophore responsive to at least one wavelength of light in the range from about 600 nm to about 850 nm, and diagnosing the location and/or surface area of the tumor tissue in the in vivo body part by directly viewing fluorescence emanating from the targeting construct bound or taken up in the tumor tissue upon irradiation thereof with light providing the at least one excitation wavelength for the fluorescent targeting construct.

**[0067]** In some embodiments, a single type of fluorescent moiety is relied upon for generating fluorescence emanating from the irradiated body part (i.e., from the fluorescent targeting construct that binds to or is taken up by diseased tissue) and subjecting the targeting construct with a source of light from the near infrared spectrum.

**[0068]** In other embodiments, it is contemplated that a plurality (i.e., two, three, four, or more) targeting constructs are used to obtain a diagnostic image. Such additional targeting constructs may be additional compounds of the present disclosure distinct from the first such compound. Alternatively, the additional targeting constructs may comprise the dyes described herein but with the pteroyl moiety being replaced by a ligand for another receptor other than folate receptor. In still other embodiments, the additional targeting moieties may be other fluorescing targeting constructs (e.g., antibodies, or biologically active fragments thereof, having attached fluorophores) that bind to other receptors or antigens on the tumor or tissue (e.g., a site of atherosclerosis, infection, cardiovascular diseases, neurodegenerative diseases, immunologic diseases, autoimmune diseases, respiratory diseases, metabolic diseases, inherited diseases, infectious diseases, bone diseases, and environmental diseases or the like) to be imaged. Any additional targeting moiety that specifically targets the tumor or specific site on the tissue may be used provided that it is specific

for the site to be monitored. The purpose of the additional fluorescing targeting construct is to increase the intensity of fluorescence at the site to be monitored thereby aiding in the detection of diseased or abnormal tissue in the body part. For example, a given tumor may have numerous markers and in addition to the compounds of the present disclosure a cocktail of fluorescent moieties is provided which are specific for that given tumor such that the signal emanating from the tumor is generated by more than one compound or fluorescent moiety that has targeted and become localized to the tumor site of interest.

**[0069]** In practice, the skilled person would administer a compound of the present disclosure either alone or as part of a cocktail of targeting detectable moieties and allow these compounds and targeting moieties to bind to and/or be taken up by any targeting tissue that may be present at the site under investigation and then provide a supply of the light source. Typically, the compounds of the present disclosure and any additional targeting moieties will be administered prior to surgery for a time and in compositions that allow the fluorescent compounds of the present disclosure as well as any additional fluorescent constructs to be taken up by the target tissue.

**[0070]** Those of skill in the art will be able to devise combinations of successively administered fluorescing targeting constructs, each of which specifically binds to the target site. It is preferable that all of the fluorescing targeting constructs used in such cocktails to identify the target tissue comprise fluorophores that fluoresce within the same wavelength band or at the same wavelength as does the compound of the present disclosure (e.g. a fluorescing sensitive to near infrared wavelength of light in the compounds of the present disclosure) to minimize the number of different light sources that need to be employed to excite simultaneous fluorescence from all of the different targeting constructs used in practice of the disclosure method. However, it is contemplated that the additional targeting moieties other than the compounds of the present disclosure may fluoresce in response to the irradiating light at a different color (i.e., has a different wavelength) than that of the fluorescent compounds of the present disclosure. The difference in the colors of the fluorescence emanating from the compounds of the present disclosure and those of the additional targeting compounds may aid the observer in determining the location and size of the diseased tissue. In some examples, it may be desirable to include fluorophores in targeting constructs targeted to target normal tissue and the compounds of the present disclosure to target diseased tissue such that the contrast between the diseased tissue and normal tissue is further enhanced to further aid the observer in determining the location and size of the target tissue. The use of such additional fluorophores and targeting agents in addition to the compounds of the present disclosure provides the advantage that any natural fluorescence emanating from normal tissue is obscured by the fluorescence emanating from fluorophore(s) in supplemental targeting constructs targeted to the normal tissue in the body part. The greater the difference in color between the fluorescence emanating from normal and target tissue, the easier it is for the observer to visualize the outlines and size of the target tissue. For instance, targeting a fluorescing targeting construct comprising a fluorophore producing infrared light from the compounds of the present disclosure to the target tissue (i.e., abnormal tissue) and a fluorophore producing green light to

healthy tissue aids the observer in distinguishing the target tissue from the normal tissue. Those of skill in the art can readily select a combination of fluorophores that present a distinct visual color contrast.

**[0071]** The spectrum of light used in the practice of the disclosure method is selected to contain at least one wavelength that corresponds to the predominant excitation wavelength of the targeting construct, or of a biologically compatible fluorescing moiety contained within the targeting construct. Generally the excitation light used in practice of the disclosure method comprises at least one excitation wavelength of light in the near infrared wavelength range from about 600 nm to about 850 nm

**[0072]** However, when a combination of targeting ligands that fluoresce at different wavelengths is used in practice of the disclosure, the spectrum of the excitation light must be broad enough to provide at least one excitation wavelength for each of the fluorophores used. For example, it is particularly beneficial when fluorophores of different colors are selected to distinguish normal from diseased tissue, that the excitation spectrum of the light(s) includes excitation wavelengths for the fluorophores targeted to normal and target tissue.

**[0073]** As noted herein the compounds of the present disclosure are specifically targeted to the folate receptor by way of pteroyl or folate ligand being part of the compounds of the present disclosure. In embodiments where an additional targeting moiety is used, the targeting construct of such an additional targeting moiety is selected to bind to and/or be taken up specifically by the target tissue of interest, for example to an antigen or other surface feature contained on or within a cell that characterizes a disease or abnormal state in the target tissue. As in other diagnostic assays, it is desirable for the targeting construct to bind to or be taken up by the target tissue selectively or to an antigen associated with the disease or abnormal state; however, targeting constructs containing ligand moieties that also bind to or are taken up by healthy tissue or cell structures can be used in the practice of the disclosure method so long as the concentration of the antigen in the target tissue or the affinity of the targeting construct for the target tissue is sufficiently greater than for healthy tissue in the field of vision so that a fluorescent image representing the target tissue can be clearly visualized as distinct from any fluorescence coming from healthy tissue or structures in the field of vision.

**[0074]** For example, colon cancer is often characterized by the presence of carcinoembryonic antigen (CEA), yet this antigen is also associated with certain tissues in healthy individuals. However, the concentration of CEA in cancerous colon tissue is often greater than is found in healthy tissue so that an anti-CEA antibody could be used as a ligand moiety in the practice of the disclosure. In another example, deoxyglucose is taken up and utilized by healthy tissue to varying degrees, yet its metabolism in healthy tissues, except for certain known organs, such as the heart, is substantially lower than in tumor. The known pattern of deoxyglucose consumption in the body can, therefore, be used to aid in determination of those areas wherein unexpectedly high uptake of deoxyglucose signals the presence of tumor cells.

**[0075]** The disease or abnormal state detected by the disclosure method can be any type characterized by the presence of a known target tissue for which a specific binding ligand is known. For example, various heart con-

ditions are characterized by the production of necrotic or ischemic tissue or production of atherosclerotic tissue for which specific binding ligands are known. As another illustrative example, breast cancer is characterized by the production of cancerous tissue identified by monoclonal antibodies to CA15-3, CA19-9, CEA, or HER2/neu. It is contemplated that the target tissue may be characterized by cells that produce either a surface antigen for which a binding ligand is known or an intracellular marker (i.e. antigen) since many targeting constructs penetrate the cell membrane. Representative disease states that can be identified using the disclosure method include such various conditions as different types of tumors, bacterial, fungal and viral infections, and the like. As used herein "abnormal" tissue includes precancerous conditions, necrotic or ischemic tissue, and tissue associated with connective tissue diseases, and auto-immune disorders, and the like. Further, examples of the types of target tissue suitable for diagnosis or examination using the disclosure method include cardiac, breast, ovarian, uterine, lung, endothelial, vascular, gastrointestinal, colorectal, prostatic tissue, endocrine tissue, and the like, as well as combinations of any two or more thereof.

**[0076]** Simply by way of example, antigens for some common malignancies and the body locations in which they are commonly found are known to those of skill in the art, and targeting ligands, such as antibodies or for these antigens or indeed ligands where the antigens are receptors are known in the art. For example, CEA (carcinoembryonic antigen) is commonly found in tumors from the colon, breast, and lung; PSA (prostate specific antigen, or sometimes referred to as prostate specific membrane antigen (PSMA)) is specific for prostate cancer; CA-125 is commonly found in tumors of ovarian cancer origin, CA 15-3, CA19-9, MUC-1, Estrogen receptor, progesterone receptor and HER2/neu are commonly found in breast cancer tumors, alpha-feto protein is found in both testicular cancer and hepatic cancer tumors, beta-human chorionic gonadotropin is found testicular cancer and choriocarcinoma, both estrogen receptor and progesterone receptor also are found in uterine cancer tumors and epidermal growth factor receptor is commonly found in tumors from bladder cancer. Other tumor-specific ligands and markers are well known to those of skill in the art. In preferred embodiments, the present disclosure employs folate or pteroyl moieties for targeting the folate receptor and PMSA target moieties for targeting the dyes to prostate cancer cells.

**[0077]** It is contemplated that any of these commonly known markers of tumors can be targeted either using the dyes described herein (by switching out the pteroyl moiety for a moiety that specifically targets these markers) or alternatively, these markers can be targeted in addition and in combination with the folate receptor that is being targeted using the compounds of the present disclosure. As discussed previously, it may be particularly advantageous to have targeting moieties to several different markers on a given tumor to serve as a diagnostic cocktail in which several markers are targeted to more brightly and clearly visualize the tumor.

**[0078]** In addition to chemical compounds, the targeting moieties in such cocktails may include a protein or polypeptide, such as an antibody, or biologically active fragment thereof, preferably a monoclonal antibody. The supplemental fluorescing targeting construct(s) used in practice of the disclosure method may also be or comprise polyclonal or



monoclonal antibodies tagged with a fluorophore. The term “antibody” as used in this disclosure includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')<sub>2</sub>, and Fv that are capable of binding the epitopic determinant. Methods of making these fragments are known in the art. (See for example, Harlow & Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference). As used in this disclosure, the term “epitope” means any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three-dimensional structural characteristics, as well as specific charge characteristics.

**[0079]** In addition to antibodies, the cocktails may comprise compounds in which the ligand moiety attached to the fluorescent targeting construct is selected from among the many biologically compatible compounds that bind with specificity to receptors and/or are preferentially taken up by tumor cells, and can be used as the ligand moiety in the disclosure targeting constructs. Compounds that are preferentially “taken up” by tumor cells may enter the cells through surface or nuclear receptors (e.g., hormone receptors), pores, hydrophilic “windows” in the cell lipid bilayer, and the like.

**[0080]** Illustrative of this class of compounds to target tumors are somatostatin, somatostatin receptor-binding peptides, deoxyglucose, methionine, and the like. Particularly useful somatostatin receptor-binding peptides are a long-acting, octapeptide analog of somatostatin, known as octreotide (D-phenylalanyl-L-cysteinyl-L-phenylalanyl-D-tryptophyl-L-lysyl-L-threonyl-N-[2-hydroxy-1-(hydroxymethyl)propyl]-L-cysteinamide cyclic (2→7)-disulfide), lanreotide, an oral formulation of octreotide, P829, P587, and the like. Somatostatin-binding peptides are disclosed in U.S. Pat. No. 5,871,711, and methods for linking such peptides covalently to a radioisotope through their carboxyl terminal amino acid under reducing conditions are disclosed in U.S. Pat. No. 5,843,401, which are both incorporated herein by reference in their entireties. One of skill in the art can readily adapt such teachings for the preparation of fluorescence-sensitive somatostatin receptor-binding peptides by substituting the fluorescing moieties of this disclosure in the place of a radioisotope.

**[0081]** Somatostatin and somatostatin receptor-binding peptides are particularly effective for use as the tumor-targeting ligand moiety in the targeting construct when the disease state is a neuroendocrine or endocrine tumor. Examples of neuroendocrine tumors that can be diagnosed using the disclosure method include adenomas (GH-producing and TSH-producing), islet cell tumors, carcinoids, undifferentiated neuroendocrine carcinomas, small cell and non-small cell lung cancer, neuroendocrine and/or intermediate cell carcinomas, neuroendocrine tumors of ovary, cervix, endometrium, breast, kidney, larynx, paranasal sinuses, and salivary glands, meningiomas, well differentiated glioma-derived tumors, pheochromocytomas, neuroblastomas, ganglioneuro(blasto)mas, paragangliomas, papillary, follicular and medullary carcinomas in thyroid cells, Merkel cell carcinomas, and melanomas, as well as granulomas and lymphomas. These tumor cells are known to have somatostatin receptors and can be targeted using somatostatin or soma-

tostatin receptor binding peptides as the tumor-targeting ligand in the disclosure fluorescent targeting construct.

**[0082]** Vasointestinal peptide (VIP), which is used in VIP receptor scintigraphy (I. Virgolini, *Eur. J. Clin. Invest.* 27(10):793-800, 1997, is also useful in the disclosure method for diagnosis of small primary adenocarcinomas, liver metastases and certain endocrine tumors of the gastrointestinal tract.

**[0083]** Another molecule illustrative of the tumor-targeting ligands that are preferentially taken up by tumors is deoxyglucose, which is known to be preferentially taken up in a variety of different types of tumors. Illustrative of the types of tumors that can be detected using deoxyglucose as the tumor-targeting ligand include melanoma, colorectal and pancreatic tumors, lymphoma (both HD and NHL), head and neck tumors, myeloma, cancers of ovary, cancer, breast, and brain (high grade and pituitary adenomas), sarcomas (grade dependent), hepatoma, testicular cancer, thyroid (grade dependent) small cell lung cancer, bladder and uterine cancer, and the like.

**[0084]** Other tumor-targeting compounds that can be used in cocktails of the present disclosure include L-aminocyclobutane-1-carboxylic acid and L-methionine. L-methionine is an essential amino acid that is necessary for protein synthesis. It is known that malignant cells have altered methionine metabolism and require an external source of methionine.

**[0085]** Additional examples of biologically compatible tumor-targeting compounds that bind with specificity to tumor receptors and/or are preferentially taken up by tumor cells include mammalian hormones, particularly sex hormones, neurotransmitters, and compounds expressed by tumor cells to communicate with each other that are preferentially taken up by tumor cells, such as novel secreted protein constructs arising from chromosomal aberrations, such as transfers or inversions within the clone.

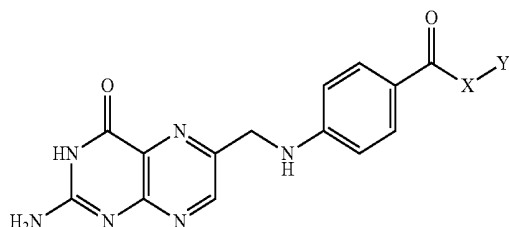
**[0086]** Hormones, including sex hormones, cell growth hormones, cytokines, endocrine hormones, erythropoietin, and the like also serve well as tumor targeting moieties. As is known in the art, some tumor types express receptors for hormones, for example, estrogen, progesterone, androgens, such as testosterone, and the like. Such hormones are preferentially taken up by tumor cells, for example, via specific receptors.

**[0087]** The targeting constructs and supplemental targeting constructs used in practice of the disclosure method can be administered by any route known to those of skill in the art, such as topically, intraarticularly, intracisternally, intraocularly, intraventricularly, intrathecaly, intravenously, intramuscularly, and intraperitoneally.

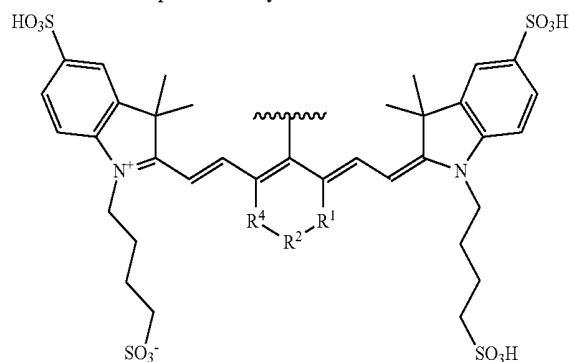
**[0088]** Some embodiments, this disclosure provides imaging of Non-small cell lung cancer (NSCLC) in human subjects. NSCLC is characterized by assessing morphology of the tumor biopsy by histological analysis including immunohistochemical analysis. The most common histological subtypes of NSCLC are squamous cell carcinoma, large cell carcinoma, adenocarcinoma, neuroendocrine carcinoma, carcinoid tumors, metastatic tumors, and lymph node. There are several other subtypes that occur less frequently. As NSCLC arises from the epithelial cells of the lung of the central bronchi to terminal alveoli, the histological subtypes of NSCLC correlate with the site of origin, reflecting the variation in respiratory tract epithelium of the bronchi to alveoli.

[0089] Some embodiments are methods of imaging a carcinoma that expresses a folate receptor in a subject in need thereof comprising

a. administering to the subject an effective amount of a compound capable of binding to a cancerous cell having the formula:



or a pharmaceutically acceptable salt or isotope thereof, wherein: X is a single amino acid or a single amino acid derivative thereof, wherein the single amino acid or single amino acid derivative contains an —OH, —NH<sub>2</sub>, or —SH functional group, and Y is a dye that has a fluorescence excitation and emission spectra in the near infra-red range, wherein Y is represented by the formula:

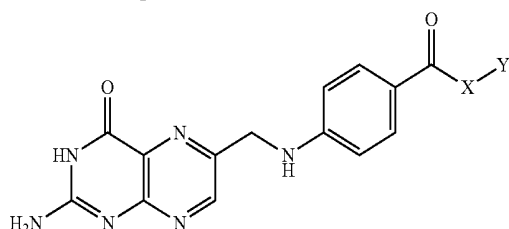


wherein, R<sup>1</sup> is independently selected from the group consisting of O, S, N and C, and R<sup>2</sup> is independently selected from the group consisting of CH<sub>2</sub> and CH<sub>2</sub>CH<sub>2</sub>, and the compound enhances the fluorescence of the dye, Y; and b. fluorescent imaging of an area in the subject's body where the compound binds to a cancerous cell wherein the carcinoma is selected from the group consisting of cervical cancer, ovarian cancer, breast cancer, leukemia cancer, and lung cancer.

[0090] In some embodiments, the subject is a human.

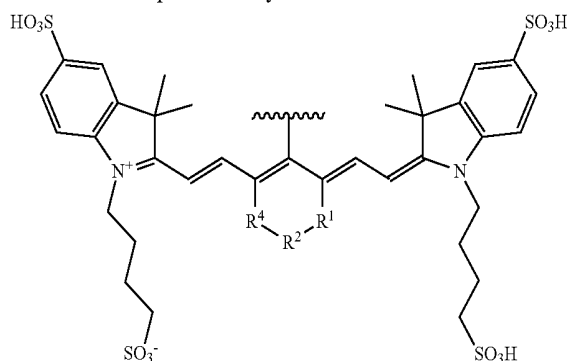
[0091] Some embodiments are methods of in vivo identification of diseased tissue in a subject in need thereof, comprising

[0092] (ii) administering a compound to the subject, wherein the compound has the formula:



or a pharmaceutically acceptable salt or isotope thereof, wherein: X is a single amino acid or a single amino acid

derivative thereof, wherein the single amino acid or single amino acid derivative contains an —OH, —NH<sub>2</sub>, or —SH functional group, and Y is a dye that has a fluorescence excitation and emission spectra in the near infra-red range, wherein Y is represented by the formula:



wherein, R<sup>1</sup> is independently selected from the group consisting of O, S, N and C, and R<sup>2</sup> is independently selected from the group consisting of CH<sub>2</sub> and CH<sub>2</sub>CH<sub>2</sub>, and the compound enhances the fluorescence of the dye, Y;

[0093] (ii) irradiating an area of subject's body containing diseased tissue with at least one excitation wavelength in the near infrared range;

[0094] (iii). fluorescent imaging the compound in the area of the subject's body wherein the compound has bound to or been taken up by the diseased tissue in the subject's body, in response to the at least one excitation wavelength, and

[0095] (iii) determining the location or surface area of the diseased tissue in the subject.

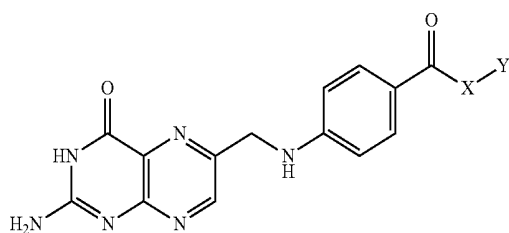
[0096] In some embodiments, the near infra red range in step (ii) is from about 600 nm to about 1000 nm. In yet another embodiment the near infra red range in step (ii) is from about 600 nm to about 850 nm. In some embodiments, the excitation light is monochromatic or polychromatic. In some embodiments, the fluorescence image is visible and distinct from the auto-fluorescence of the surrounding tissue. In some embodiments, the diseased tissue is located at an interior site in the subject. In other embodiments, the diseased tissue is selected from the group consisting of cardiac, breast, ovarian, uterine, lung, endothelial, vascular, gastrointestinal, colorectal, prostatic tissue, endocrine tissue, and combinations thereof.

[0097] In some embodiments, the subject has a preoperative diagnosis of pulmonary adenocarcinoma. In yet another embodiment the preoperative diagnosis was determined by transthoracic needle or transbronchial needle aspiration.

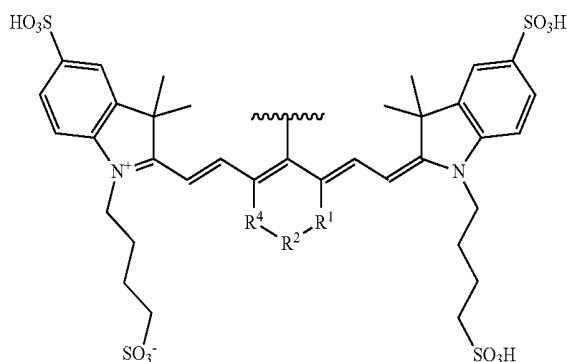
[0098] Some embodiments comprise the additional step of using an endoscopic device to deliver the excitation light to the site, to receive fluorescence emanating from the site within a body cavity, and to aid in formation of a direct image of the fluorescence from the diseased tissue.

[0099] Some embodiments are methods for utilizing a diagnostic procedure during surgery in a subject in need thereof, comprising:

[0100] (i) administering to the subject a composition comprising a compound wherein the compound having the formula:



or a pharmaceutically acceptable salt or isotope thereof, wherein: X is a single amino acid or a single amino acid derivative thereof, wherein the single amino acid or single amino acid derivative contains an —OH, —NH<sub>2</sub>, or —SH functional group, and Y is a dye that has a fluorescence excitation and emission spectra in the near infra-red range, wherein Y is represented by the formula:



wherein, R<sup>1</sup> is independently selected from the group consisting of O, S, N and C, and R<sup>2</sup> is independently selected from the group consisting of CH<sub>2</sub> and CH<sub>2</sub>CH<sub>2</sub>, and the compound enhances the fluorescence of the dye, Y;

[0101] (ii) irradiating an area of the subject's body containing diseased tissue with light having at least one excitation wavelength in the range from about 600 nm to about 850 nm;

[0102] (iii) fluorescence imaging the compound in the area of the subject's body wherein the compound has bound to or been taken up by the diseased tissue in the body part, wherein the targeting construct fluoresces in response to the at least one excitation wavelength;

[0103] (iv) determining the location or surface area of the diseased tissue in the subject; and

[0104] (v) removing at least a portion of the tumor tissue.

[0105] In some of these embodiments, the subject is a human. In some embodiments, the diseased tissue being identified includes non-small cell lung cancer. In another embodiment, the non-small cell lung cancer is selected from the group consisting of lung adenocarcinoma, squamous cells carcinoma, large cell lung carcinoma, neuroendocrine carcinoma, carcinoid tumors, metastatic tumors, and lymph node.

[0106] In some embodiments, the diagnostic procedure is minimally invasive pulmonary resection.

[0107] In some embodiments, the tumor tissue is an FR expressing tumor.

[0108] In some embodiments, preoperative CT and PET were obtained in the subject.

[0109] In some embodiments, the tumor size was determined to be in the range of 1.5 to 4.3 cm.

[0110] In some embodiments, the compound is administered in the range of about 1.79 to about 2.58 mg. In some embodiments, the compound is administered about 3 to about 6 hours prior to resection. In yet another embodiment, the compound is administered intravenously.

[0111] In some embodiments, a lobe of interest or any other lobe of the subject is imaged in situ using an NIR thorascopic camera. In a further embodiment, the NIR thorascopic camera is included in an NIR imaging system including a 10 mm, 30° thorascopic; an excitation light source of 785 nm; and emission filters selecting for light ranging from about 800 nm to about 835 nm. In yet a further embodiment, in situ fluorescence is appreciated through the pleural surface with a tumor-to-background ratio range of about 2.7 to about 4.2.

[0112] In some embodiments the imaging with the targeting construct reveals nodules. In a further embodiment, the nodule revealed is a synchronous nodule. In a further embodiment, the nodule revealed is a synchronous adenocarcinoma. In yet a further embodiment the synchronous adenocarcinoma is a subcentimeter carcinoma. In yet a further embodiment the subcentimeter carcinoma is about 0.6 cm in length or diameter.

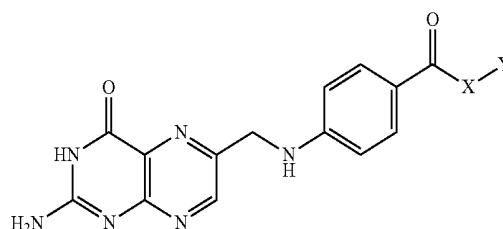
[0113] In some embodiments, the compound accumulates in FRα expressing tumors.

[0114] In some embodiments, the method improves identification of subcentimeter pulmonary adenocarcinomas.

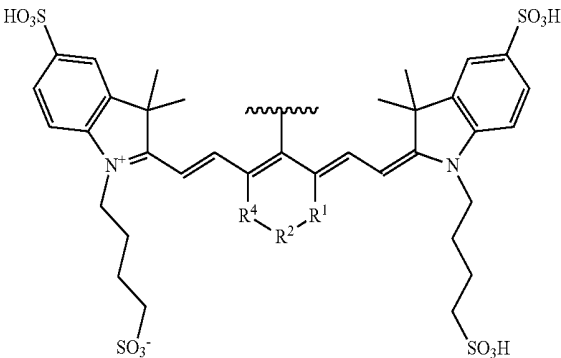
[0115] In some embodiments, the identification of synchronous nodules aids the method of treatment of diseased tissue in the subject.

[0116] Some embodiments are methods for in vivo diagnosis of tumor tissue in a subject in need thereof, comprising:

[0117] (i) administering a diagnostically effective amount of at least one biologically compatible fluorescing targeting construct containing a compound that has been determined to bind to or be taken up by the sample tumor cells and a fluorophore responsive to at least one wavelength of light in the range from about 600 nm to about 850 nm, wherein the compound having the formula:



or a pharmaceutically acceptable salt or isotope thereof, wherein: X is a single amino acid or a single amino acid derivative thereof, wherein the single amino acid or single amino acid derivative contains an —OH, —NH<sub>2</sub>, or —SH functional group, and Y is a dye that has a fluorescence excitation and emission spectra in the near infra-red range, wherein Y is represented by the formula:



wherein, R<sup>1</sup> is independently selected from the group consisting of O, S, N and C, and R<sup>2</sup> is independently selected from the group consisting of CH<sub>2</sub> and CH<sub>2</sub>CH<sub>2</sub>, and the compound enhances the fluorescence of the dye, Y; and

[0118] (ii) contacting samples of tumor cells obtained from the subject with a plurality of detectably labeled compounds, each of which binds to or is selectively taken up by a distinct tumor type; and

[0119] (iii) diagnosing the location or surface area of the tumor tissue in the subject's body part by directly viewing fluorescence emanating from the targeting construct bound or taken up in the tumor tissue upon irradiation thereof with light providing the at least one excitation wavelength for the fluorescent targeting construct.

[0120] In some embodiments, the subject is a human.

[0121] In some embodiments, the method includes the step identifying the compounds is bound to or taken up by the sample tumor cells.

EXAMPLES

[0122] The examples are intended to illustrate, but in no way limit, the scope of the invention.

Example 1: Pilot Study of Intraoperative Molecular Imaging (IMI) with OTL38 in Subjects Undergoing Pulmonary Resection

[0123] Experimental Procedure: A pilot study of IMI with OTL38 was approved by the University of Pennsylvania Institutional Review Board. All subjects (n=5) provided

informed consent and were recruited between January 2015 and April 2015. Subjects had previously undergone CT scanning with 1 mm slice thickness that was reviewed by a specialized thoracic radiologist to confirm the presence of a pulmonary nodule and identify other suspicious nodules. Three subjects had a preoperative diagnosis of pulmonary adenocarcinoma as determined by transthoracic needle or transbronchial needle aspiration. Subjects also underwent preoperative positron-emission tomography (PET). Study participants received 0.025 mg/kg of OTL38 intravenously 3-6 hours prior to resection. During minimally invasive pulmonary resection, surgeons utilized white-light (also known as brightfield imaging) and finger palpation through port-site incisions to confirm the lesion in the lobe of interest. Nodules were then imaged using an optimized NIR imaging system consisting of a 10 mm, 30° thoracoscope; an excitation light source of 785 nm; and emission filters selecting for light ranging from 800 nm to 835 nm (Vision-sense, Philadelphia, Pa.). Additionally, surgeons evaluated the remainder of the thorax using white-light thoracoscopy and IMI to inspect the ipsilateral lung for additional nodules. All specimens underwent pathologic examination by a specialized lung pathologist. The presence of FRα expression was determined by FRα immunohistochemistry as described above.

[0124] Statistics: Within the pilot study, given the small number of subjects (n=5), data are presented as mean (range) unless otherwise noted. All comparisons were made using Stata Statistical Software: Release 14 (College Station, Tex.: StataCorp LP). A p-value of 0.05 or less was considered statistically significant.

[0125] Results and Discussion:

[0126] OTL38 Accumulates in FRα Expressing Pulmonary Adenocarcinomas

[0127] To evaluate feasibility of OTL38 based IMI in humans, a pilot study involving 5 subjects was executed. Five subjects (n=3 female) with a mean age of 74.6 years (range, 67 and 79 years) were enrolled after meeting inclusion criteria. All subjects had a diagnosis of a solitary pulmonary nodule, with subjects 1, 2 and 3 having a preoperative diagnosis of pulmonary adenocarcinoma. Pre-operative CT and PET were obtained in all subjects and mean tumor size was determined to be 2.7 cm (range, 1.5 to 4.3 cm) and mean standardized uptake value (SUV) was 6.7 (range, 1.6 to 11.8). A full summary of subject and tumor characteristics is provided in Table 1.

TABLE 1

| Clinical and Histopathologic Characteristics of NSCLC Subjects Involved in a Pilot Study of IMI with OTL38 |             |        |          |           |           |            |       |                              |              |               |                 |                                                                                                                                                                                                                                                    |
|------------------------------------------------------------------------------------------------------------|-------------|--------|----------|-----------|-----------|------------|-------|------------------------------|--------------|---------------|-----------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| ID                                                                                                         | Age (years) | Gender | Location | Histology | Size (cm) | SUV by PET | Stage | Total OTL0038 Delivered (mg) | Time (hours) | Adverse Event | Fluorescent/TBR | Impact of MI with OTL0038                                                                                                                                                                                                                          |
| 1                                                                                                          | 79          | F      | LUL      | AC        | 2.5       | 8.9        | IIIA* | 2.58                         | 3.3          | no            | yes/3.6         | Identification of a 0.6 cm synchronous adenocarcinoma in the Left Lower Lobe (FIG. 6). This finding upstaged subject from Stage IA (T1N0) to Stage IIIA (T4N0). Upstaging altered operative plan and need for postoperative adjuvant chemotherapy. |

TABLE 1-continued

| Clinical and Histopathologic Characteristics of NSCLC Subjects Involved in a Pilot Study of IMI with OTL38 |             |        |          |           |           |            |       |                              |              |               |                 |                                                                                                                                                                                                    |
|------------------------------------------------------------------------------------------------------------|-------------|--------|----------|-----------|-----------|------------|-------|------------------------------|--------------|---------------|-----------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| ID                                                                                                         | Age (years) | Gender | Location | Histology | Size (cm) | SUV by PET | Stage | Total OTL0038 Delivered (mg) | Time (hours) | Adverse Event | Fluorescent/TBR | Impact of MI with OTL0038                                                                                                                                                                          |
| 2                                                                                                          | 78          | M      | RUL      | AC        | 4.3       | 2.8        | IB    | 2.16                         | 5.0          | no            | yes/4.2         | None                                                                                                                                                                                               |
| 3                                                                                                          | 67          | F      | RUL      | AC        | 1.5       | 1.6        | IA    | 1.79                         | 3.01         | no            | yes/2.7         | This patient did not have a preoperative biopsy and PET scan was negative; however, IMI with OTL0038 showed strong fluorescence. Final Pathology revealed AC with FR $\alpha$ expression (FIG. 5). |
| 4                                                                                                          | 71          | M      | RUL      | AC        | 3.5       | 11.8       | IIIA  | 2.18                         | 4.8          | no            | yes/3.1         | None                                                                                                                                                                                               |
| 5                                                                                                          | 77          | F      | LLL      | SCC       | 1.7       | 8.7        | IA    | 1.54                         | 5.6          | no            | no/1.1          | Known SCC did not fluoresce (FIG. 5); however, IMI identified a 0.8 cm synchronous Adenocarcinoma in situ (FIG. 6) in the Left Lower Lobe.                                                         |

\*subject upstaged after additional nodules identified with use of IMI

SUV—Standardized Uptake Value

LUL—Left Upper Lobe,

RUL—Right Upper Lobe,

LLL—Left Lower Lobe,

AC—Adenocarcinoma,

SCC—Squamous Cell Carcinoma

**[0128]** Subjects received an average of 2.05 mg (range, 1.79 to 2.58 mg) of the study drug 4.3 hours (range, 3.0 to 5.6 hours) prior to resection and imaging (Table 1). No adverse events were observed during drug delivery, intraoperatively or postoperatively.

**[0129]** During minimally invasive pulmonary resection, the operating surgeon manipulated the lobe to localize the preoperatively described nodule. Once the nodule was identified, the lobe was imaged in situ using a NIR thoroscopic camera. In 4 out of 5 subjects (80%), in situ fluorescence was appreciated through the pleural surface with a mean TBR 3.4 of (range, 2.7 to 4.2) (FIG. 1). Upon pathologic review, 4 of 4 (100%) of fluorescent nodules were found to be pulmonary adenocarcinoma with FR $\alpha$  expression. The non-fluorescent nodule (TBR=1.1) was found to be a squamous cell carcinoma with absent FR $\alpha$  expression (FIG. 2).

**[0130]** In two subjects, IMI with OTL38 revealed nodules that were not identified on review of preoperative imaging or with traditional intraoperative techniques (finger palpation and visualization). First, in Subject 1, in addition to the previously known left upper lobe nodule, a synchronous 0.6 cm adenocarcinoma was located in the left lower lobe (TBR=2.4) (FIG. 2). Based on these findings, the operative plan changed from a planned a left upper lobectomy to a wedge resection of each nodule. Further, identification of the synchronous nodule upstaged the subject from Stage IA(T1N0) to Stage IIIA(T4N0) and resulted in chemotherapy being offered to this subject. Next, in Subject 5, the preoperatively identified left lower lobe nodule was non-fluorescent (squamous cell carcinoma); however, during completion lobectomy, an incidental left lower lobe nodule measuring 0.8 cm was identified (TBR=2.7) (FIG. 2). On final pathology, this nodule was found to be adenocarcinoma in situ.

**[0131]** A final observation involved Subject 3. This subject had a preoperative CT demonstrating a 1.5 cm right upper lobe which was not PET avid. During resection with IMI, however, this tumor displayed high levels of fluorescence (TBR=2.7). Pathologic evaluation confirmed that this nodule was a pulmonary adenocarcinoma with FR $\alpha$  expression.

**[0132]** FIG. 3 shows imaging of squamous cell carcinoma using OTL38 and FIG. 4 shows imaging of carcinoid lung tumor. Both of these data shows that OTL38 can be used to image FR-positive cancer and can be used in image-guided surgery.

**[0133]** These findings suggest that IMI with OTL38 is safe and feasible. Additionally, these preliminary data suggest IMI may improve accurate identification of subcentimeter pulmonary adenocarcinomas that may otherwise be undetectable. These advances can impact both intraoperative and postoperative patient care.

**[0134]** In a pilot study of IMI with OTL38, feasibility and safety was observed. IMI with OTL38 accurately identified 4 of 4 pulmonary adenocarcinomas detected on preoperative imaging. Further, IMI with OTL38 allowed for identification of additional subcentimeter neoplastic processes in 2 of 5 subjects.

## CONCLUSIONS

**[0135]** OTL38 is associated with excellent optical properties and reproducibly accumulates in FR $\alpha$  expressing tumors. This novel technology may be a valuable diagnostic and therapeutic tool useful in surgical candidates with NSCLC and other FR $\alpha$  expressing malignancies.

**[0136]** The most suitable route for administration will vary depending upon the disease state to be treated, or the location of the suspected condition or tumor to be diagnosed. For example, for treatment of inflammatory conditions and various tumors, local administration, including administra-

tion by injection directly into the body part to be irradiated by the excitation light (e.g., intracavitarily) provides the advantage that the targeting construct (e.g., fluorescently tagged antibodies) can be administered in a high concentration without risk of the complications that may accompany systemic administration thereof.

[0137] The compounds of the present disclosure, as well as any additional targeting constructs used in diagnostic cocktails comprising the compounds of the present disclosure, are administered in an “effective amount” for diagnosis. An effective amount is the quantity of a targeting construct necessary to aid in direct visualization of any target tissue located in the body part under investigation in a subject. A “subject” as the term is used herein is contemplated to include any mammal, such as a domesticated pet, farm animal, or zoo animal, but preferably is a human. Amounts effective for diagnostic use will, of course, depend on the size and location of the body part to be investigated, the affinity of the targeting construct for the target tissue, the type of target tissue, as well as the route of administration. Local administration of the targeting construct will typically require a smaller dosage than any mode of systemic administration, although the local concentration of the targeting construct may, in some cases, be higher following local administration than can be achieved with safety upon systemic administration.

[0138] Since individual subjects may present a wide variation in severity of symptoms and each targeting construct has its unique diagnostic characteristics, including, affinity of the targeting construct for the target, rate of clearance of the targeting construct by bodily processes, the properties of the fluorophore contained therein, and the like, the skilled practitioner will weigh the factors and vary the dosages accordingly.

[0139] The compounds of the present disclosure, as well as cocktails comprising these compounds, can be formulated as a sterile injectable suspension according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1-4, butanediol. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed, including synthetic mono- or diglycerides, fatty acids (including oleic acid), naturally occurring vegetable oils like sesame oil, coconut oil, peanut oil, cottonseed oil, etc., or synthetic fatty vehicles like ethyl oleate, or the like. Buffers, preservatives, antioxidants, and the like, can be incorporated as required, or, alternatively, can comprise the formulation.

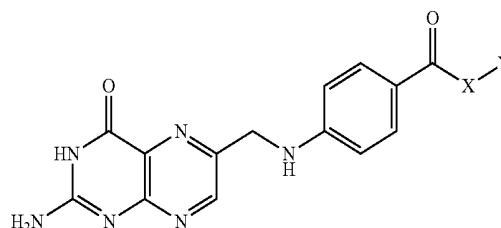
[0140] It will be apparent to those skilled in the art that various changes may be made in the disclosure without departing from the spirit and scope thereof, and therefore, the disclosure encompasses embodiments in addition to those specifically disclosed in the specification, but only as indicated in the appended claims.

[0141] The examples that follow are merely provided for the purpose of illustrating particular embodiments of the disclosure and are not intended to be limiting to the scope of the appended claims. As discussed herein, particular features of the disclosed compounds and methods can be modified in various ways that are not necessary to the operability or advantages they provide. For example, the compounds can incorporate a variety of amino acids and amino acid deriva-

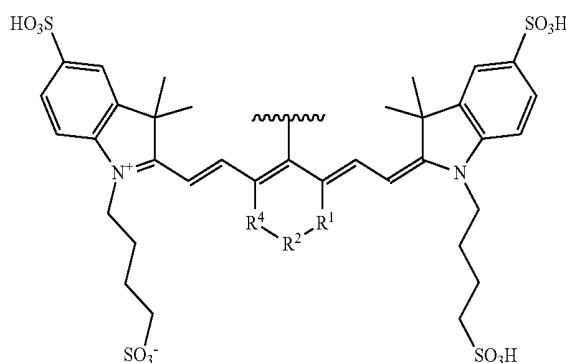
tives as well as targeting ligands depending on the particular use for which the compound will be employed. One of skill in the art will appreciate that such modifications are encompassed within the scope of the appended claims.

1. A method of imaging a carcinoma that expresses a folate receptor in a subject in need thereof comprising

a. administering to the subject an effective amount of a compound capable of binding to a cancerous cell having the formula:



or a pharmaceutically acceptable salt or isotope thereof, wherein: X is a single amino acid or a single amino acid derivative thereof, wherein the single amino acid or single amino acid derivative contains an —OH, —NH<sub>2</sub>, or —SH functional group, and Y is a dye that has a fluorescence excitation and emission spectra in the near infra-red range, wherein Y is represented by the formula:



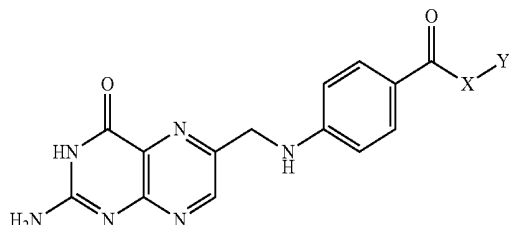
wherein, R<sup>1</sup> is independently selected from the group consisting of O, S, N and C, and R<sup>2</sup> is independently selected from the group consisting of CH<sub>2</sub> and CH<sub>2</sub>CH<sub>2</sub>, and the compound enhances the fluorescence of the dye, Y; and

b. fluorescent imaging of an area in the subject's body where the compound binds to a cancerous cell wherein the carcinoma is selected from the group consisting of cervical cancer, ovarian cancer, breast cancer, leukemia cancer, and lung cancer.

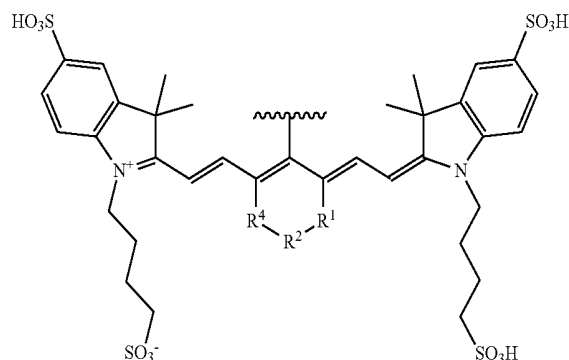
2. The method of claim 1 wherein the subject is a human.

3. A method of in vivo identification of diseased tissue in a subject in need thereof, comprising

- (ii) administering a compound to the subject, wherein the compound has the formula:



or a pharmaceutically acceptable salt or isotope thereof, wherein: X is a single amino acid or a single amino acid derivative thereof, wherein the single amino acid or single amino acid derivative contains an —OH, —NH<sub>2</sub>, or —SH functional group, and Y is a dye that has a fluorescence excitation and emission spectra in the near infra-red range, wherein Y is represented by the formula:



wherein, R<sup>1</sup> is independently selected from the group consisting of O, S, N and C, and R<sup>2</sup> is independently selected from the group consisting of CH<sub>2</sub> and CH<sub>2</sub>CH<sub>2</sub>, and the compound enhances the fluorescence of the dye, Y;

- (ii) irradiating an area of subject's body containing diseased tissue with at least one excitation wavelength in the near infrared range;
- (iii). fluorescent imaging the compound in the area of the subject's body wherein the compound has bound to or been taken up by the diseased tissue in the subject's body, in response to the at least one excitation wavelength, and
- (iii) determining the location or surface area of the diseased tissue in the subject.

4. The method of claim 3 wherein the near infra red range in step (ii) is from about 600 nm to about 1000 nm.

5. The method of claim 3 wherein the near infra red range in step (ii) is from about 600 nm to about 850 nm.

6. The method of claim 3 wherein the excitation light is monochromatic or polychromatic.

7. The method of claim 3 wherein fluorescence image is visible and distinct from the auto-fluorescence of the surrounding tissue.

8. The method of claim 3 wherein the diseased tissue is located at an interior site in the subject.

9. The method of claim 3 wherein the diseased tissue is selected from the group consisting of cardiac, breast, ovar-

ian, uterine, lung, endothelial, vascular, gastrointestinal, colorectal, prostatic tissue, endocrine tissue, and combinations thereof.

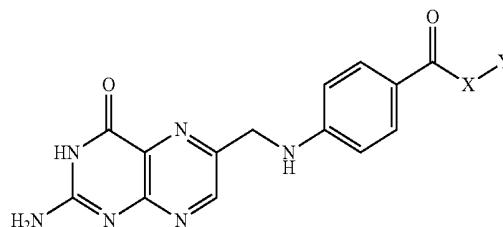
10. The method of claim 3 wherein the subject has a preoperative diagnosis of pulmonary adenocarcinoma.

11. The method of claim 10 wherein the preoperative diagnosis was determined by transthoracic needle or trans-bronchial needle aspiration.

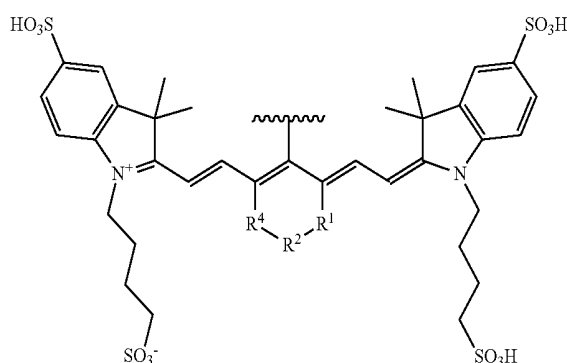
12. The method of claim 10 further comprising the step of using an endoscopic device to deliver the excitation light to the site, to receive fluorescence emanating from the site within a body cavity, and to aid in formation of a direct image of the fluorescence from the diseased tissue.

13. A method for utilizing a diagnostic procedure during surgery in a subject in need thereof, comprising:

- (i) administering to the subject a composition comprising a compound wherein the compound having the formula:



or a pharmaceutically acceptable salt or isotope thereof, wherein: X is a single amino acid or a single amino acid derivative thereof, wherein the single amino acid or single amino acid derivative contains an —OH, —NH<sub>2</sub>, or —SH functional group, and Y is a dye that has a fluorescence excitation and emission spectra in the near infra-red range, wherein Y is represented by the formula:



wherein, R<sup>1</sup> is independently selected from the group consisting of O, S, N and C, and R<sup>2</sup> is independently selected from the group consisting of CH<sub>2</sub> and CH<sub>2</sub>CH<sub>2</sub>, and the compound enhances the fluorescence of the dye, Y;

- (ii) irradiating an area of the subject's body containing diseased tissue with light having at least one excitation wavelength in the range from about 600 nm to about 850 nm;

(iii) fluorescence imaging the compound in the area of the subject's body wherein the compound has bound to or been taken up by the diseased tissue in the body part,

wherein the targeting construct fluoresces in response to the at least one excitation wavelength;

(iv) determining the location or surface area of the diseased tissue in the subject; and

(v) removing at least a portion of the tumor tissue.

14. The method of claim 13 wherein the subject is a human.

15. The method of claim 13 wherein the diseased tissue being identified includes non-small cell lung cancer.

16. The method of claim 15 wherein the non-small cell lung cancer is selected from the group consisting of lung adenocarcinoma, squamous cells carcinoma, large cell lung carcinoma, neuroendocrine carcinoma, carcinoid tumors, metastatic tumors, and lymph node.

17. The method of claim 13 wherein the diagnostic procedure is minimally invasive pulmonary resection.

18. The method of claim 13 wherein the tumor tissue is an FR expressing tumor.

19. The method of claim 13 wherein preoperative CT and PET were obtained in the subject.

20. The method of claim 19 wherein the tumor size was determined to be in the range of 1.5 to 4.3 cm.

21. The method of claim 13 wherein the compound of step (i) is administered in the range of about 1.79 to about 2.58 mg.

22. The method of claim 13 wherein the compound of step (i) is administered about 3 to about 6 hours prior to resection.

23. The method of claim 13 wherein the compound of step (i) is administered intravenously.

24. The method of claim 13 wherein a lobe of the subject is imaged in situ using a NIR thorascopic camera.

25. The method of claim 24 wherein the camera is included in a NIR imaging system including an 10 mm, 30° thoracoscope; an excitation light source of 785 nm; and emission filters selecting for light ranging from about 800 nm to about 835 nm.

26. The method of claim 25 wherein in situ fluorescence is appreciated through the pleural surface with a tumor-to-background ratio range of about 2.7 to about 4.2.

27. The method of claim 13 wherein the method reveals nodules.

28. The method of claim 27 wherein the nodule a synchronous nodule.

29. The method of claim 27 wherein the nodule a synchronous adenocarcinoma.

30. The method of claim 29 wherein the synchronous adenocarcinoma is a subcentimeter carcinoma.

31. The method of claim 30 wherein the subcentimeter carcinoma is about 0.6 cm in length or diameter.

32. The method of claim 13 wherein the compound accumulates in FR $\alpha$  expressing tumors.

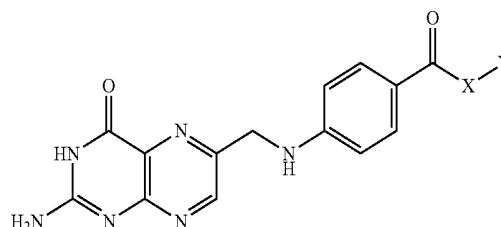
33. The method of claim 30 wherein the identification of subcentimeter pulmonary adenocarcinomas is improved.

34. The method of claim 28 wherein the identification of synchronous nodules aids the method of treatment of diseased tissue in the subject.

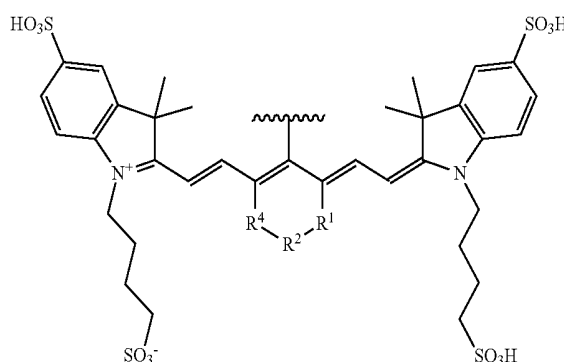
35. A method for in vivo diagnosis of tumor tissue in a subject in need thereof, comprising:

(i) administering a diagnostically effective amount of at least one biologically compatible fluorescing targeting construct containing a compound that has been deter-

mined to bind to or be taken up by the sample tumor cells and a fluorophore responsive to at least one wavelength of light in the range from about 600 nm to about 850 nm, wherein the compound having the formula:



or a pharmaceutically acceptable salt or isotope thereof, wherein: X is a single amino acid or a single amino acid derivative thereof, wherein the single amino acid or single amino acid derivative contains an —OH, —NH<sub>2</sub>, or —SH functional group, and Y is a dye that has a fluorescence excitation and emission spectra in the near infra-red range, wherein Y is represented by the formula:



wherein, R<sup>1</sup> is independently selected from the group consisting of O, S, N and C, and R<sup>2</sup> is independently selected from the group consisting of CH<sub>2</sub> and CH<sub>2</sub>CH<sub>2</sub>, and the compound enhances the fluorescence of the dye, Y; and

(ii) contacting samples of tumor cells obtained from the subject with a plurality of detectably labeled compounds, each of which binds to or is selectively taken up by a distinct tumor type; and

(iii) diagnosing the location or surface area of the tumor tissue in the subject's body part by directly viewing fluorescence emanating from the targeting construct bound or taken up in the tumor tissue upon irradiation thereof with light providing the at least one excitation wavelength for the fluorescent targeting construct.

36. The method of claim 34 wherein the subject is a human.

37. The method of claim 34 further comprising the step of identifying the compounds is bound to or taken up by the sample tumor cells.

\* \* \* \* \*



|                |                                                                                                                                                      |         |            |
|----------------|------------------------------------------------------------------------------------------------------------------------------------------------------|---------|------------|
| 专利名称(译)        | 氨基酸连接基团的合成和组成与用于肿瘤靶向成像的化合物缀合                                                                                                                         |         |            |
| 公开(公告)号        | <a href="#">US20170232119A1</a>                                                                                                                      | 公开(公告)日 | 2017-08-17 |
| 申请号            | US15/584752                                                                                                                                          | 申请日     | 2017-05-02 |
| [标]申请(专利权)人(译) | 普渡研究基金会                                                                                                                                              |         |            |
| 申请(专利权)人(译)    | 普渡研究基金会                                                                                                                                              |         |            |
| 当前申请(专利权)人(译)  | 普渡研究基金会                                                                                                                                              |         |            |
| [标]发明人         | KULARATNE SUMITH A<br>SINGHAL SUNIL                                                                                                                  |         |            |
| 发明人            | KULARATNE, SUMITH A.<br>SINGHAL, SUNIL                                                                                                               |         |            |
| IPC分类号         | A61K49/00 A61B5/00                                                                                                                                   |         |            |
| CPC分类号         | A61K47/48 A61B5/0071 A61K49/0052 G01N2021/6439 G01N2021/6432 A61K49/0032 G01N33/57411 G01N33/57415 G01N33/57423 G01N33/57426 G01N33/57449 G01N33/582 |         |            |
| 优先权            | 61/791921 2013-03-15 US                                                                                                                              |         |            |
| 外部链接           | <a href="#">Espacenet</a> <a href="#">USPTO</a>                                                                                                      |         |            |

摘要(译)

本公开涉及可用作近红外荧光探针的化合物，其中所述化合物包括i) 与靶受体蛋白结合的蝶酰配体，ii) 染料分子，和iii) 包含氨基酸的连接分子。或其衍生物。本公开进一步描述了用于掺入用于人受试者中的非小细胞肺癌 ( NSCLC ) 的靶向成像的化合物的方法和组合物。氨基酸连接基团的缀合增加了化合物的特异性和检测。考虑了用于诊断成像的方法和组合物。

