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(54) Title: METHOD OF DETECTING HUMAN SERUM ALBUMIN IN BIOLOGICAL FLUIDS

(57) Abstract: A method for human serum albumin (HSA) detection in a biological fluid using small molecule, fluorescent compounds having aggregation-induced emission (AIE) characteristics includes contacting the biological fluid with a fluorescent compound to provide a mixture, irradiating the mixture with ultraviolet light, and determining a presence of human serum albumin when an observable emission is detected. The fluorescent compound binds with human serum albumin in the biological fluid and the binding of the compound with the human serum albumin produces the emission. The biological fluid can be urine. The compounds can be water soluble and can include tetrazole-tagged AIEgens. The present methods can be performed using conventional fluorescent spectrometers, normally present in hospitals, or using an HSA detection device. The HSA detection device is portable and can be used by a patient at home.



Method of Detecting Human Serum Albumin in Biological Fluids

FIELD

The present subject matter relates generally to use of a series of fluorescent compounds having aggregation-induced emission (AIE) characteristics for detecting human serum albumin in biological fluids.

BACKGROUND

Early diagnosis and/or daily monitoring of a chronic disease can often facilitate successful treatment and/or inhibition of the disease. Renal damage is an example of a common chronic disease, especially prevalent in the elderly, patients with diabetes, high blood pressure, urinary tract infection, and/or acute pancreatitis, and patients who are taking medicine which may have a detrimental impact on kidney function. Urine, a non-invasively accessible biological fluid, is an ideal analyte to monitor for early diagnosis of renal damage.

Human serum albumin (HSA) is the major protein in blood plasma. HSA is typically not present in urine unless the kidney malfunctions and glomerular permeability becomes abnormally high. Therefore, urine HSA concentration is a closely-related parameter for indicating renal conditions. For example, a urine HSA concentration ranging from 30 mg/L to 300 mg/L, known as microalbuminuria, can be an early renal disease signal. A urine HSA concentration exceeding 300 mg/L can indicate albuminuria.

Traditionally, the sulphosalicylic acid test and Heller's test have been used for fast-qualitative HSA detection. These tests indicate the presence of urine proteins upon precipitation formation. Colorimetric methods, such as Bradford and Lowry assays, have also been used in protein detection. Many of these tests, however, typically lack accuracy, sensitivity, and/or fail to quantify the proteins. Typically, additional procedures, such as microchip electrophoresis, capillary electrophoresis, high performance liquid chromatography, enzymatic immunoassay, or turbidimetric inhibition immunoassay, are required for quantification. These methods are often very complicated, time-consuming, and expensive.

Currently, dry chemistry test paper is the most commonly-used product for detecting urine protein presence and providing a rough estimate of the amount of urine proteins present based on a color comparison between the used strip and a color scale. The strip includes a pH-sensitive dye, such as tetrabromophenol blue, along with an acid buffer to maintain a constant pH

value. The amino-group-rich urine proteins can deprotonate the indicator to induce a color change. Although this method is fast, it lacks accuracy and a capability to provide a concentration range of the urine proteins.

Colloidal gold immuno(chromatographic) strip is also a widely used semi-quantification method. A red test line indicates the presence of urine HSA only when the analyte-gold particle complexes aggregate on the test line to a certain degree. The gold particle labeling is attributed to electrostatic interaction, however, which is not a stable-enough physical adsorption process. Further, this method is relatively expensive and insufficiently sensitive.

A fluorescence method for detecting proteins can be more cost-effective, more sensitive, less complex and more stable than many other conventional methods of detecting proteins. Generally, if a fluorescent probe turns on fluorescence only after binding with HSA, the HSA concentration should be proportional to fluorescence intensity within the linear dynamic range.

While many HSA-specific fluorescent probes have been reported, prior fluorescent probes are seldom used with real urine samples. For example, prior probes described in CN106153942, CN105572095A, and CN105838355A are based on the mechanism of twisted intramolecular charge transfer (TICT). “Push-pull” structures including a strong electron donor (e.g., a dimethylamine group), a rotatable bridge (e.g., a double bond), and a strong electron acceptor (e.g., a malononitrile group) are typically not very emissive in a polar aqueous solution but emissive in the relatively non-polar HSA cavity. As such, these structures can achieve long-wavelength emission. However, they are mostly water-insoluble, and an organic co-solvent is required in order to dissolve the probes for detection. This can be detrimental for retaining protein conformation. In addition, because this kind of probe is responsive to polarity, the fluorescence may vary as the pH of a urine sample changes.

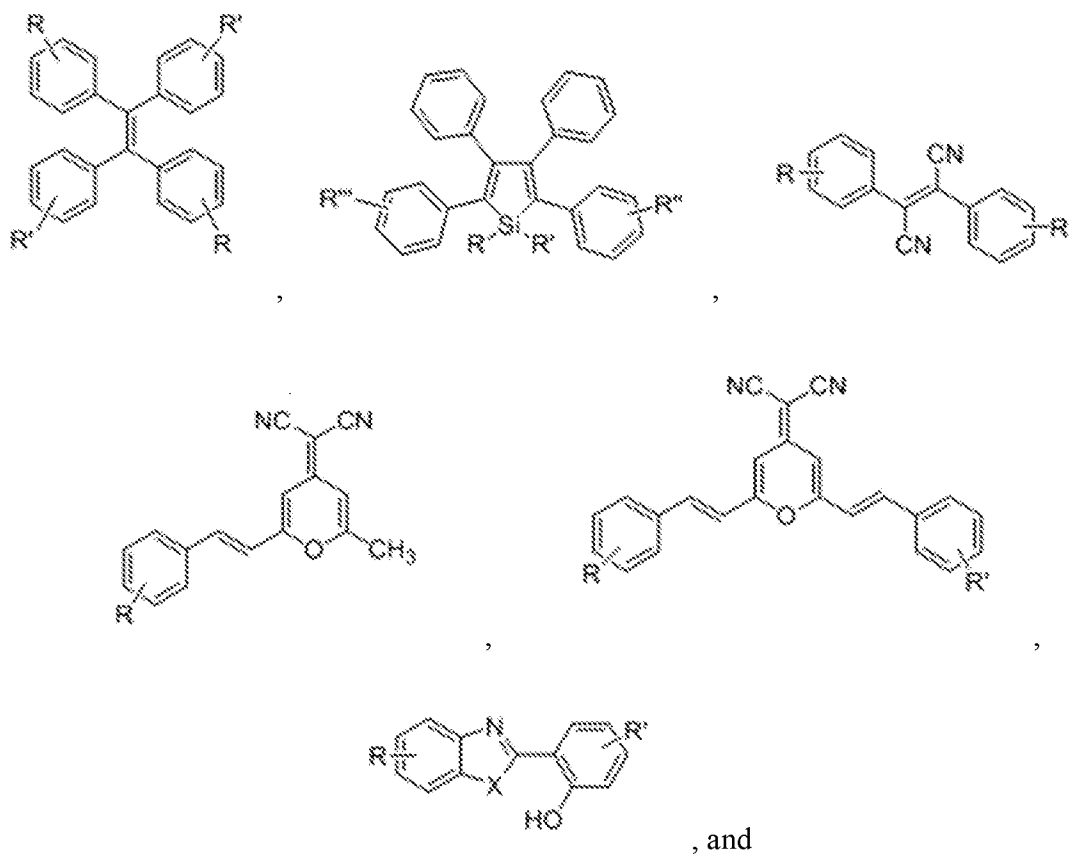
Accordingly, a method of HSA detection in a biological fluid which overcomes these challenges is highly desirable.

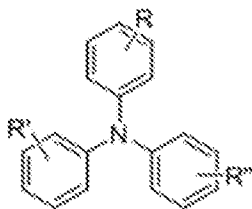
SUMMARY

The present subject matter contemplates a method for human serum albumin (HSA) detection in a biological fluid using a small molecule, fluorescent compound having aggregation-induced emission (AIE) characteristics. The method can include contacting the biological fluid with the fluorescent compound to provide a mixture, irradiating the mixture with ultraviolet light, and determining a presence of human serum albumin when an observable emission is

detected. The fluorescent compound is non-emissive when HSA is not present in the biological fluid. When HSA is present in the biological fluid, the fluorescent compound binds with HSA and the binding of the compound with the HSA produces the emission. The biological fluid can be urine. The compounds can include tetrazole-tagged AIEgens. The compounds are water soluble. The present methods can be performed using conventional fluorescent spectrometers, normally present in hospitals, or using an HSA detection device described herein. The HSA detection device is portable and can be used by a patient at home.

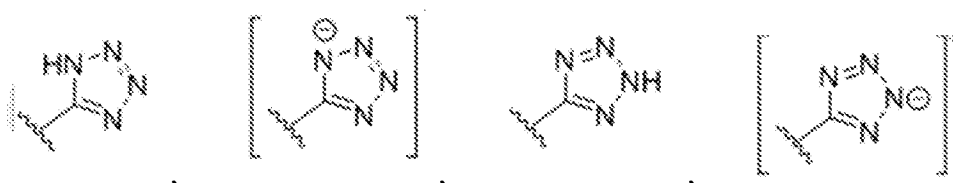
An exemplary fluorescent compound has a backbone structural formula selected from the group consisting of:





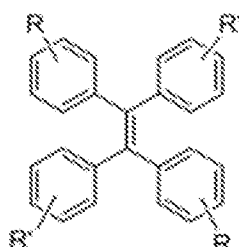
wherein X is O or S;

wherein at least one of R, R', R'' or R''' is independently selected from the group consisting of

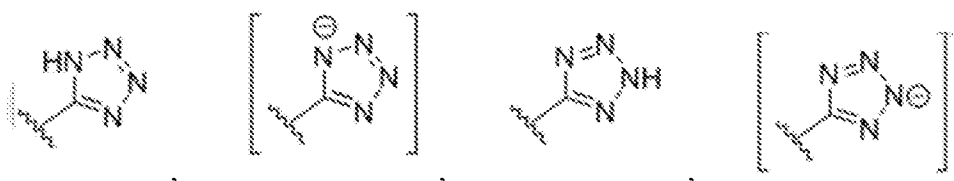


and wherein all other of the R, R', R'', and, R''' groups are selected from the group consisting of H, heteroatom, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

In an embodiment, the compound has the following backbone structural formula:



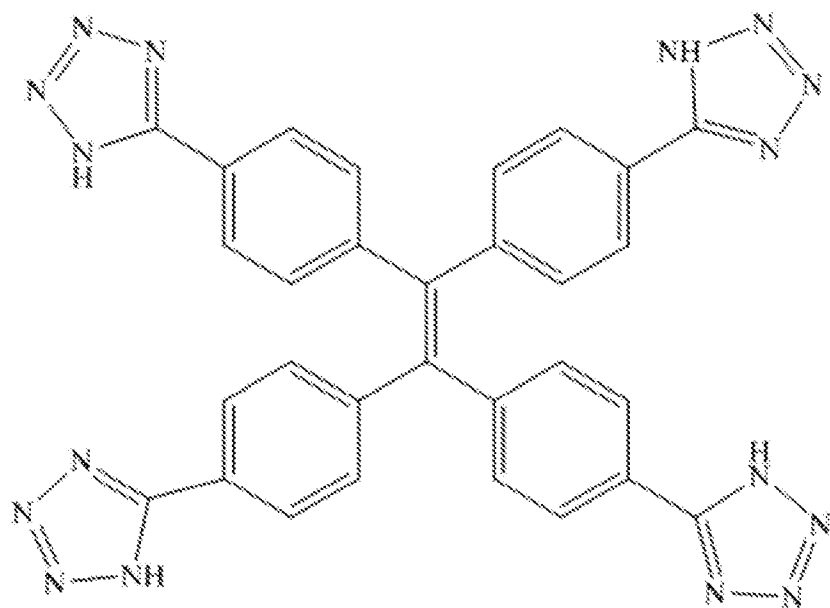
wherein at least two of the R and R' groups are independently selected from the group consisting of



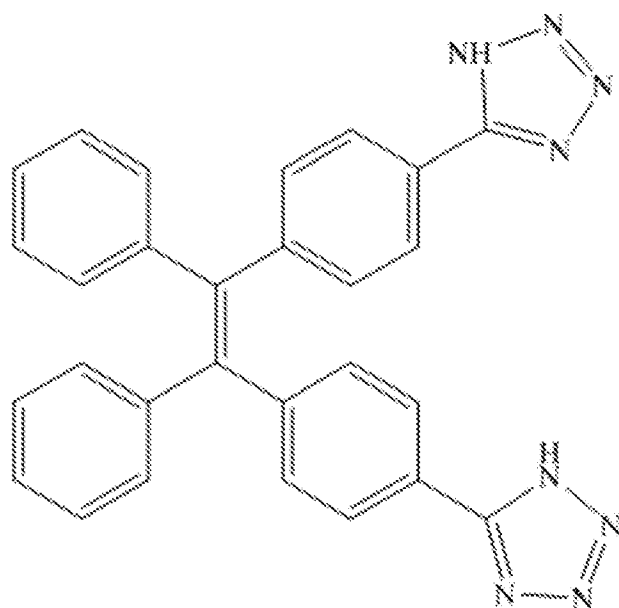
and wherein all other of the R and R' groups are selected from the group consisting of H, heteroatom, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and

heteroaryl.

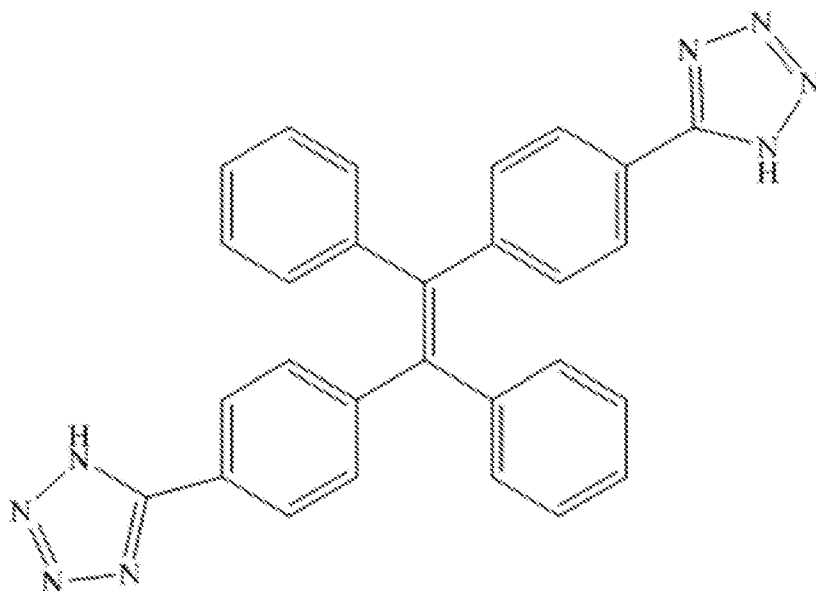
In an embodiment, the compound is a compound selected from the group consisting of:



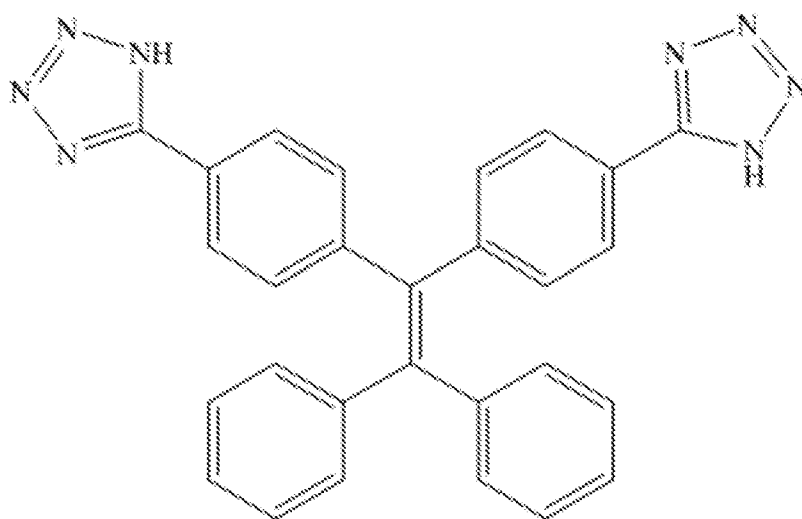
TPE-4TA,



TPE-2TA-a,



TPE-2TA-b, and



TPE-2TA-c.

BRIEF DESCRIPTION OF DRAWINGS

Various embodiments will now be described in detail with reference to the accompanying drawings.

Fig. 1 depicts (A) fluorogenic detection of HSA in pH 7.4 phosphate buffered saline (PBS) buffer including [TPE-4TA] = 5 μ M; λ_{ex} = 370 nm; (B) the corresponding peak

fluorescence intensity of (A), where I_0 equals the intensity of [HSA]=0mg/L; (C) fluorogenic detection of HSA in pH 7.4 phosphate buffered saline (PBS) buffer including [TPE-2TA-a] = 5 μ M; λ_{ex} = 370 nm; (D) the corresponding peak fluorescence intensity of (C), where I_0 equals the intensity of [HSA]=0mg/L; (E) fluorogenic detection of HSA in pH 7.4 phosphate buffered saline (PBS) buffer including [TPE-2TA-b] = 5 μ M; λ_{ex} = 370 nm; (F)) the corresponding peak fluorescence intensity of (E), where I_0 equals the intensity of [HSA]=0mg/L; (G) fluorogenic detection of HSA in pH 7.4 phosphate buffered saline (PBS) buffer including [TPE-2TA-c] = 5 μ M; λ_{ex} = 370 nm; and (H) the corresponding peak fluorescence intensity of (G), where I_0 equals the intensity of [HSA]=0mg/L;.

Fig. 2 depicts (A) fluorescence of TPE-4TA in a pH gradient, where [TPE-4TA] = 5 μ M; (B) fluorescence intensity change of TPE-4TA and the corresponding HSA-TPE-4TA mixture (top line) at 490 nm in a pH gradient, where [TPE-4TA] = 5 μ M, [HSA] = 0.5 μ M; (C) fluorescence response of the TPE-4TA probe towards biomolecules in PBS, where [TPE-4TA] = 5 μ M, [Biomolecules] = 1 mg/mL. I_0 : intensity of the blank probe solution at 490 nm; (D) effects of common components in urine on the fluorescence intensity of AIEgen-HSA complex at 490 nm, where [TPE-4TA] = 5 μ M, [HSA] = 5 μ M, [urine component] = 10 mg/mL, in PBS buffer. I_0 : the intensity of the blank TPE-4TA solution.

Fig. 3 depicts (A) ITC calorimetric curves during the titration of HSA with serial injections of TPE-4TA. [TPE-4TA] = 0.07 mM, [HSA] = 0.001 mM, at 25°C; (B) the fitting curve of integration of peak area of each injection with sequential binding sties model; (C) the Job plot for determination of the binding stoichiometry of TPE-4TA and HSA, [TPE-4TA] + [HSA] kept constant as 2mM in pH 7.4 PBS.

Fig. 4 depicts a graph of HSA concentrations of reference urine samples determined by turbidimetric inhibition immunoassay method (X-axis) versus corresponding fluorescence intensity at 490 nm using albumin assay including TPE-4TA (Y-axis), where I_0 equals the fluorescence intensity at 490 nm of urine blanks in the absence of the TPE-4TA probe.

Fig. 5A depicts a top view of the HSA detection device according to the present teachings.

Fig. 5B depicts a side view of the HSA detection device according to the present teachings.

DETAILED DESCRIPTION

Definitions

The following definitions are provided for the purpose of understanding the present subject matter and for constructing the appended patent claims.

It is noted that, as used in this specification and the appended claims, the singular forms “a”, “an”, and “the” include plural references unless the context clearly dictates otherwise.

The term “ λ_{ex} ” as used herein refers to excitation wavelength.

The phrase “aggregation caused quenching” or “ACQ” as used herein refers to the phenomenon wherein the aggregation of π -conjugated fluorophores significantly decreases the fluorescence intensity of the fluorophores. The aggregate formation is said to “quench” light emission of the fluorophores.

The phrase “aggregation induced emission” or “AIE” as used herein refers to the phenomenon manifested by compounds exhibiting significant enhancement of light-emission upon aggregation in the amorphous or crystalline (solid) states whereas they exhibit weak or almost no emission in dilute solutions.

“Emission intensity” as used herein refers to the magnitude of fluorescence/phosphorescence normally obtained from a fluorescence spectrometer or fluorescence microscopy measurement; “fluorophore” or “fluorogen” as used herein refer to a molecule which exhibits fluorescence; “luminogen” or “luminophore” as used herein refer to a molecule which exhibits luminescence; and “AIEgen” as used herein refers to a molecule exhibiting AIE characteristics.

As used herein, “halo” or “halogen” refers to fluoro, chloro, bromo, and iodo.

As used herein, “alkyl” refers to a straight-chain or branched saturated hydrocarbon group. Examples of alkyl groups include methyl (Me), ethyl (Et), propyl (e.g., n-propyl and z'-propyl), butyl (e.g., n-butyl, z'-butyl, sec-butyl, tert-butyl), pentyl groups (e.g., n-pentyl, z'-pentyl, -pentyl), hexyl groups, and the like. In various embodiments, an alkyl group can have 1 to 40 carbon atoms (i.e., C1-40 alkyl group), for example, 1-30 carbon atoms (i.e., C1-30 alkyl group). In some embodiments, an alkyl group can have 1 to 6 carbon atoms, and can be referred to as a “lower alkyl group”. Examples of lower alkyl groups include methyl, ethyl, propyl (e.g., n-propyl and z'-propyl), and butyl groups (e.g., n-butyl, z'-butyl, sec-butyl, tert-butyl). In some

embodiments, alkyl groups can be substituted as described herein. An alkyl group is generally not substituted with another alkyl group, an alkenyl group, or an alkynyl group.

As used herein, “alkenyl” refers to a straight-chain or branched alkyl group having one or more carbon-carbon double bonds. Examples of alkenyl groups include ethenyl, propenyl, butenyl, pentenyl, hexenyl, butadienyl, pentadienyl, hexadienyl groups, and the like. The one or more carbon-carbon double bonds can be internal (such as in 2-butene) or terminal (such as in 1-butene). In various embodiments, an alkenyl group can have 2 to 40 carbon atoms (i.e., C2-40 alkenyl group), for example, 2 to 20 carbon atoms (i.e., C2-20 alkenyl group). In some embodiments, alkenyl groups can be substituted as described herein. An alkenyl group is generally not substituted with another alkenyl group, an alkyl group, or an alkynyl group.

As used herein, “heteroatom” refers to an atom of any element other than carbon or hydrogen and includes, for example, nitrogen, oxygen, silicon, sulfur, phosphorus, and selenium.

As used herein, “aryl” refers to an aromatic monocyclic hydrocarbon ring system or a polycyclic ring system in which two or more aromatic hydrocarbon rings are fused (i.e., having a bond in common with) together or at least one aromatic monocyclic hydrocarbon ring is fused to one or more cycloalkyl and/or cycloheteroalkyl rings. An aryl group can have 6 to 24 carbon atoms in its ring system (e.g., C6-24 aryl group), which can include multiple fused rings. In some embodiments, a polycyclic aryl group can have 8 to 24 carbon atoms. Any suitable ring position of the aryl group can be covalently linked to the defined chemical structure. Examples of aryl groups having only aromatic carbocyclic ring(s) include phenyl, 1-naphthyl (bicyclic), 2-naphthyl (bicyclic), anthracenyl (tricyclic), phenanthrenyl (tricyclic), pentacenyl (pentacyclic), and like groups. Examples of polycyclic ring systems in which at least one aromatic carbocyclic ring is fused to one or more cycloalkyl and/or cycloheteroalkyl rings include, among others, benzo derivatives of cyclopentane (i.e., an indanyl group, which is a 5,6-bicyclic cycloalkyl/aromatic ring system), cyclohexane (i.e., a tetrahydronaphthyl group, which is a 6,6-bicyclic cycloalkyl/aromatic ring system), imidazoline (i.e., a benzimidazolyl group, which is a 5,6-bicyclic cycloheteroalkyl/aromatic ring system), and pyran (i.e., a chromenyl group, which is a 6,6-bicyclic cycloheteroalkyl/aromatic ring system). Other examples of aryl groups include benzodioxanyl, benzodioxolyl, chromanyl, indolinyl groups, and the like. In some embodiments, aryl groups can be substituted as described herein. In some embodiments, an aryl group can have one or more halogen substituents, and can be referred to as a “haloaryl” group. Perhaloaryl

groups, i.e., aryl groups where all of the hydrogen atoms are replaced with halogen atoms (e.g., -C₆F₅), are included within the definition of "haloaryl". In certain embodiments, an aryl group is substituted with another aryl group and can be referred to as a biaryl group. Each of the aryl groups in the biaryl group can be substituted as disclosed herein.

As used herein, "heteroaryl" refers to an aromatic monocyclic ring system containing at least one ring heteroatom selected from oxygen (O), nitrogen (N), sulfur (S), silicon (Si), and selenium (Se) or a polycyclic ring system where at least one of the rings present in the ring system is aromatic and contains at least one ring heteroatom. Polycyclic heteroaryl groups include those having two or more heteroaryl rings fused together, as well as those having at least one monocyclic heteroaryl ring fused to one or more aromatic carbocyclic rings, non-aromatic carbocyclic rings, and/or non-aromatic cycloheteroalkyl rings. A heteroaryl group, as a whole, can have, for example, 5 to 24 ring atoms and contain 1-5 ring heteroatoms (i.e., 5-20 membered heteroaryl group). The heteroaryl group can be attached to the defined chemical structure at any heteroatom or carbon atom that results in a stable structure. Generally, heteroaryl rings do not contain O-O, S-S, or S-O bonds. However, one or more N or S atoms in a heteroaryl group can be oxidized (e.g., pyridine N-oxide thiophene S-oxide, thiophene S,S-dioxide). Examples of heteroaryl groups include, for example, the 5- or 6-membered monocyclic and 5-6 bicyclic ring systems shown below: where T is O, S, NH, N-alkyl, N-aryl, N-(arylalkyl) (e.g., N-benzyl), SiH₂, SiH(alkyl), Si(alkyl)₂, SiH(arylalkyl), Si(arylalkyl)₂, or Si(alkyl)(arylalkyl). Examples of such heteroaryl rings include pyrrolyl, furyl, thienyl, pyridyl, pyrimidyl, pyridazinyl, pyrazinyl, triazolyl, tetrazolyl, pyrazolyl, imidazolyl, isothiazolyl, thiazolyl, thiadiazolyl, isoxazolyl, oxazolyl, oxadiazolyl, indolyl, isoindolyl, benzofuryl, benzothienyl, quinolyl, 2-methylquinolyl, isoquinolyl, quinoxalyl, quinazolyl, benzotriazolyl, benzimidazolyl, benzothiazolyl, benzisothiazolyl, benzisoxazolyl, benzoxadiazolyl, benzoxazolyl, cinnolyl, 1H-indazolyl, 2H-indazolyl, indoliziny, isobenzofuryl, naphthyridinyl, phthalazinyl, pteridinyl, purinyl, oxazolopyridinyl, thiazolopyridinyl, imidazopyridinyl, furopyridinyl, thienopyridinyl, pyridopyrimidinyl, pyridopyrazinyl, pyridopyridazinyl, thienothiazolyl, thienoxazolyl, thienoimidazolyl groups, and the like. Further examples of heteroaryl groups include 4,5,6,7-tetrahydroindolyl, tetrahydroquinolyl, benzothienopyridinyl, benzofuropyridinyl groups, and the like. In some embodiments, heteroaryl groups can be substituted as described herein.

As used herein, a "donor" material refers to an organic material, for example, an organic

nanoparticle material, having holes as the majority current or charge carriers.

As used herein, an "acceptor" material refers to an organic material, for example, an organic nanoparticle material, having electrons as the majority current or charge carriers.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the presently described subject matter pertains.

Where a range of values is provided, for example, concentration ranges, percentage ranges, or ratio ranges, it is understood that each intervening value, to the tenth of the unit of the lower limit, unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the described subject matter. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and such embodiments are also encompassed within the described subject matter, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the described subject matter.

Throughout the application, descriptions of various embodiments use "comprising" language. However, it will be understood by one of skill in the art, that in some specific instances, an embodiment can alternatively be described using the language "consisting essentially of" or "consisting of".

For purposes of better understanding the present teachings and in no way limiting the scope of the teachings, unless otherwise indicated, all numbers expressing quantities, percentages or proportions, and other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term "about". Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained. At the very least, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

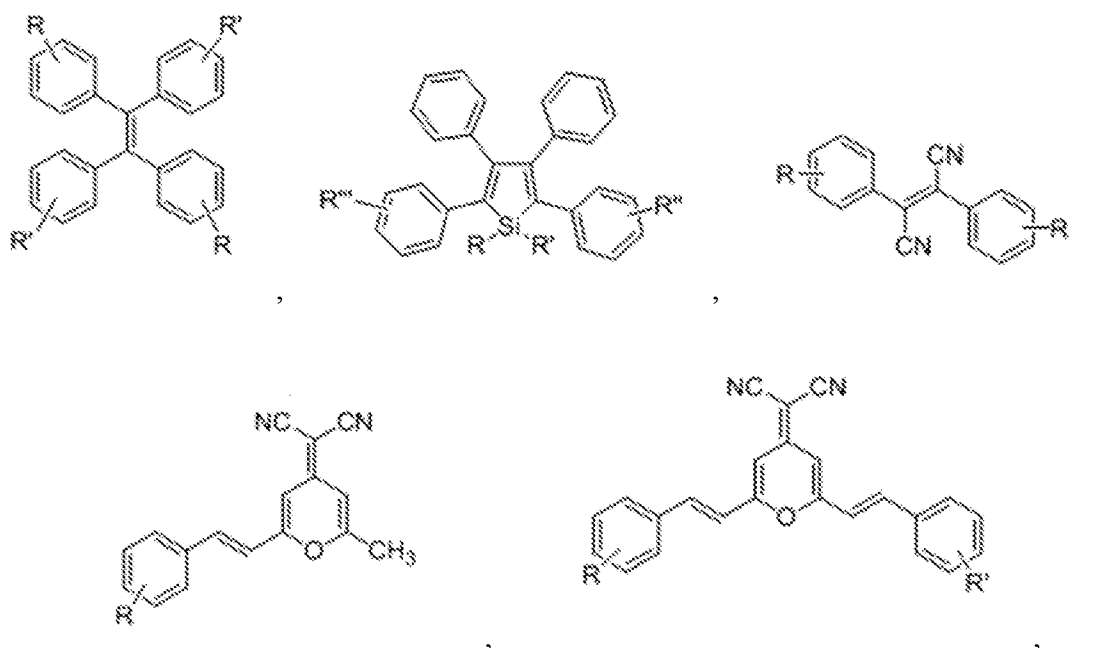
Human Serum Albumin (HSA) detection

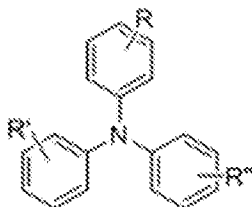
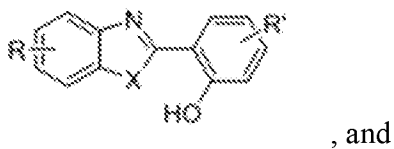
The present subject matter contemplates a method for human serum albumin (HSA) detection in a biological fluid using a small molecule, fluorescent compound having aggregation-induced emission (AIE) characteristics. The fluorescent compound having aggregation-induced

emission (AIE) characteristics is also referred to herein as “AIEgen.” The method can include contacting the biological fluid with the fluorescent compound to provide a mixture, irradiating the mixture with ultraviolet light, and determining a presence of human serum albumin when an observable emission is detected. The method can be carried out using any suitable instrument for fluorescence spectroscopy, e.g., a UV lamp and any suitable portable UV reader. The fluorescent compound binds with human serum albumin in the biological fluid. The binding of the compound with the human serum albumin produces the emission. The biological fluid can be urine. A volume ratio of the biological fluid to the fluorescent compound can range from about 1:6 to about 1:12. The compounds can include tetrazole-tagged AIEgens. The compounds are water soluble.

The present methods can be performed using conventional fluorescent spectrometers, normally present in hospitals, or using an HSA detection device described herein. The HSA detection device is portable and can be used by a patient at home.

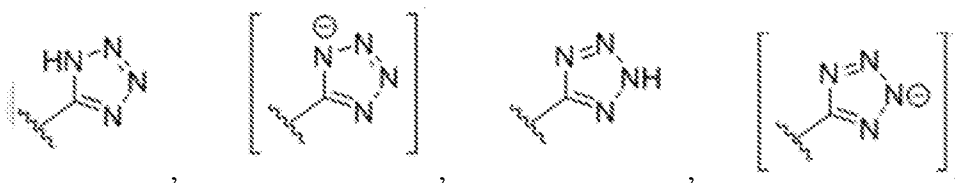
An exemplary fluorescent compound has a backbone structural formula selected from the group consisting of:





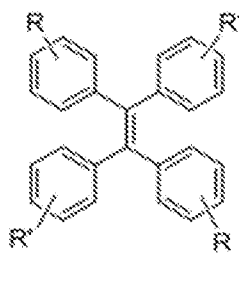
wherein X is O or S;

wherein at least one of R, R', R'' or R''' is independently selected from the group consisting of

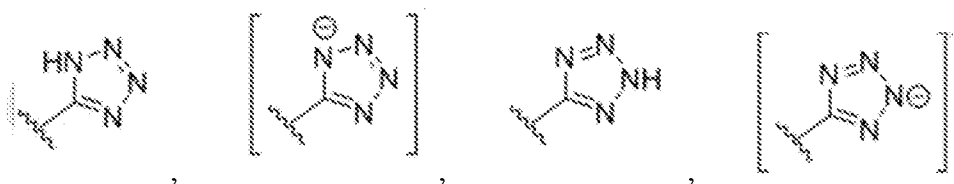


and wherein all other of the R, R', R'', and, R''' groups are selected from the group consisting of H, heteroatom, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

In an embodiment, the compound has the following backbone structural formula:

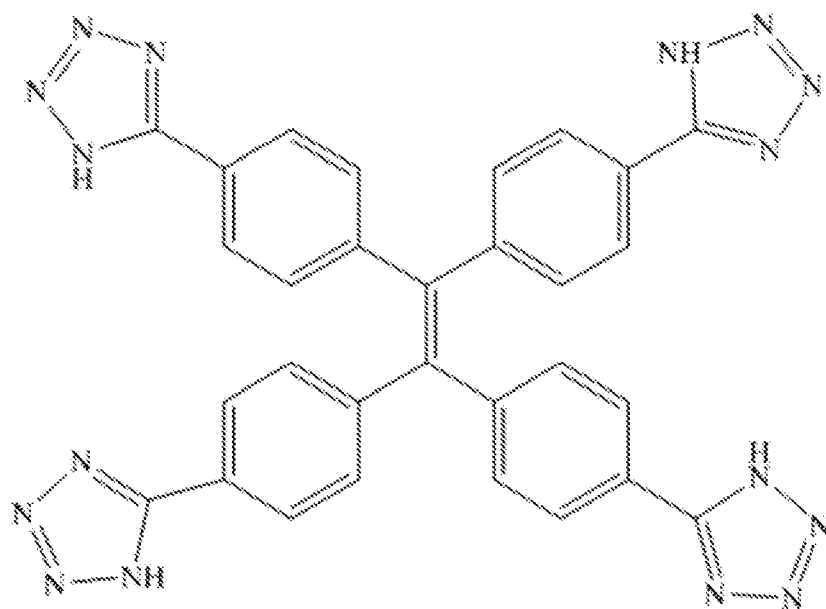


wherein at least two of the R and R' groups are independently selected from the group consisting of

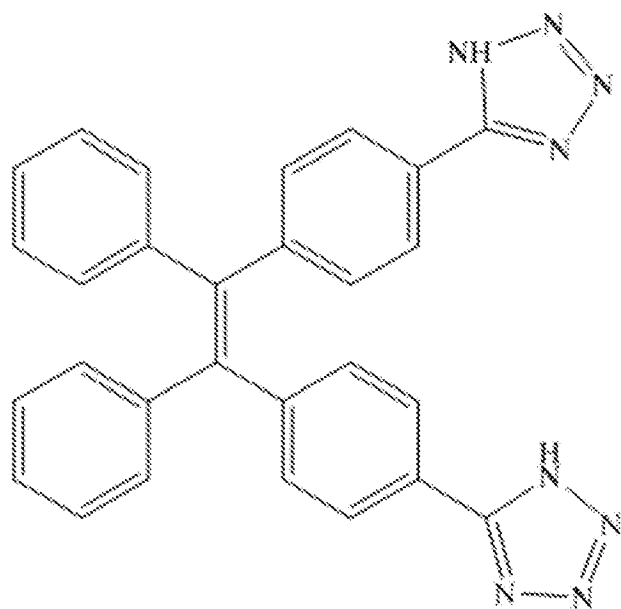


and wherein all other of the R and R' groups are selected from the group consisting of H, heteroatom, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

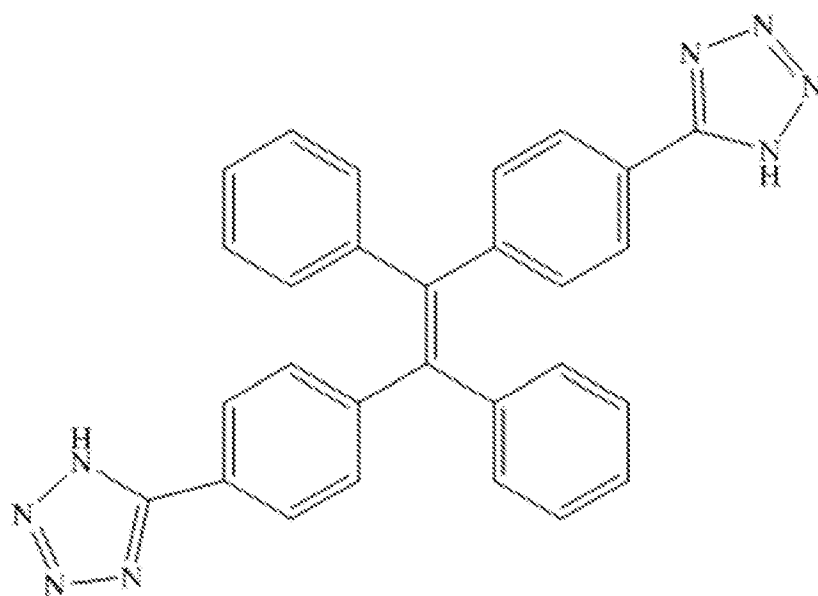
In an embodiment, the compound is a compound selected from the group consisting of:



TPE-4TA,

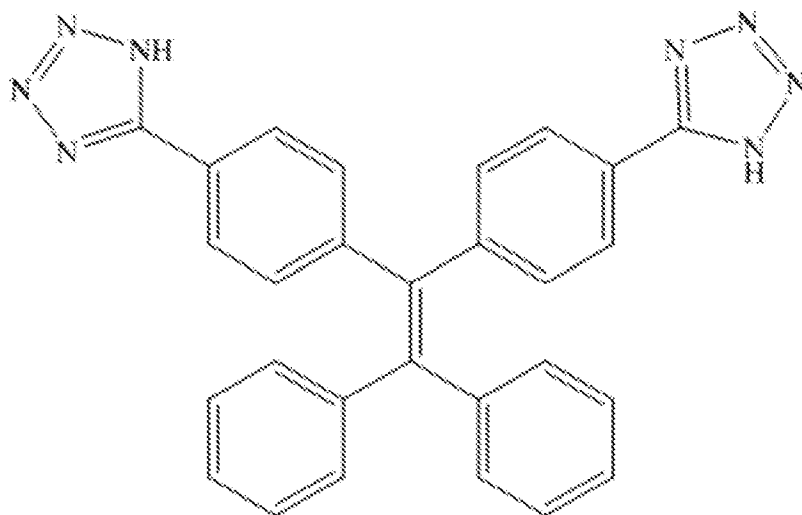


TPE-2TA-a,



TPE-2TA-b,

and

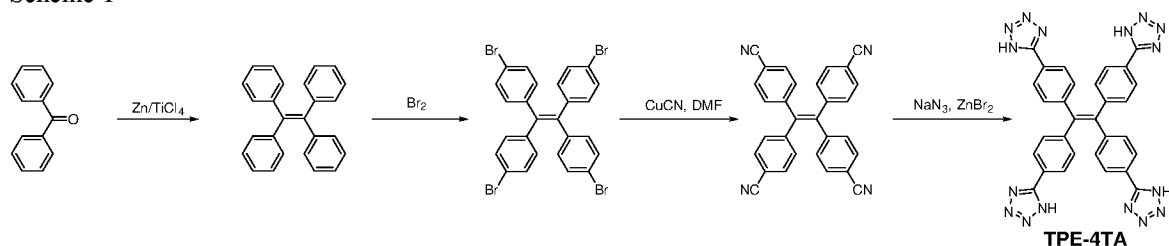


TPE-2TA-c.

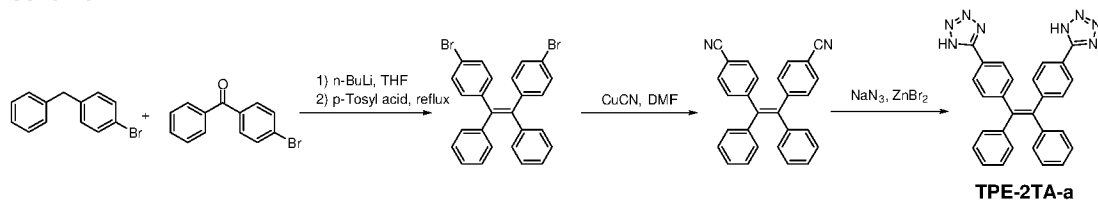
Method of Synthesis

Exemplary reaction schemes for synthesizing four of the fluorescent compounds, TPE-4TA, TPE,2TA-a, TPE-2TA-b, TPE,2TA-c, are provided below:

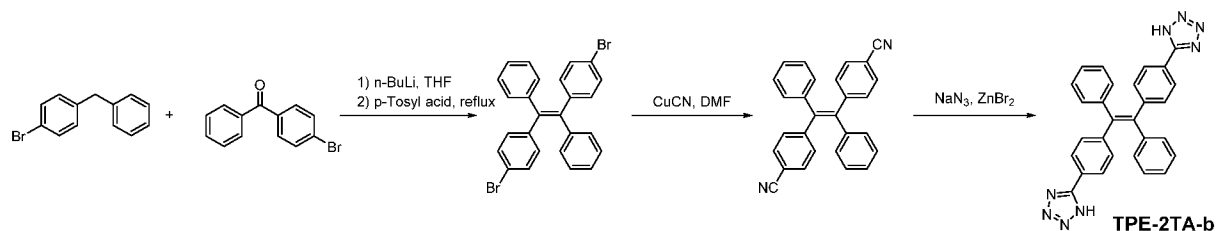
Scheme 1



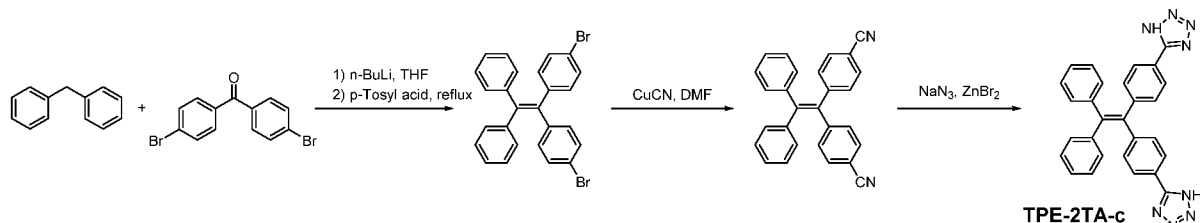
Scheme 2



Scheme 3



Scheme 4



According to an embodiment, tetrabrominated tetraphenylethylene (TPE) is obtained by addition of liquid bromide to TPE which is synthesized by McMurry coupling of benzophenone. Dibrominated TPEs can be synthesized from diphenylmethane/1-benzyl-4-bromobenzene and corresponding brominated biphenyl ketone in accordance with the procedure reported in *J Org Chem* 2007, 72, 8054. These brominated TPE derivatives undergo nucleophilic attack of CN^- , resulting in substitution of the -Br group with the -CN group. These CN-derivatized TPEs can be cycloadded with azido ions (N_3^-) to provide the tetrazole-derivatized TPEs.

Properties of Fluorescent Compounds for HSA detection

The fluorescent compounds described herein have aggregation-induced emission (AIE) characteristics. The fluorescent compounds are water soluble. The fluorescent compounds are non-emissive in urine samples without HSA due to their highly flexible intramolecular rotation and vibration, which favor non-radiative decay. In urine samples with HSA, the compounds bind with HSA and, thereby, become fixed into a relatively rigid conformation with restriction of intramolecular motion (RIM). Once bound to the HSA, the compounds emit strong fluorescence.

The fluorescent compounds for HSA detection are nearly non-emissive in PBS buffers with generally neutral pH (for example pH=7.4). After addition of HSA into the PBS buffer solution, however, the fluorescent compounds bind with HSA and the resulting HSA-AIEgen complexes can show an intense fluorescence at 370 nm light activation. Figs. 1A, 1C, 1E, and 1G show the fluorogenic detection of HSA in phosphate buffered saline (PBS) including **TPE-4TA**, **TPE-2TA-a**, **TPE-2TA-b**, and **TPE-2TA-c**, respectively, where $\lambda_{\text{ex}} = 370$ nm. Figs. 1B, 1D, 1F, and 1H show the corresponding peak fluorescence intensities for Figs. 1A, 1C, 1E, and

1G, where I_0 equals the intensity of $[HSA]=0\text{mg/L}$. As shown in the figures, the intensity of light depends on the concentration of HSA. For the fluorescent compound **TPE-4TA**, for example, the detection limit is below 30 mg/L of HSA, which is clinically regarded as indicative of a borderline state of microalbuminuria.

The dissociation constant of a HSA-**TPE-4TA** complex was determined by isothermal titration calorimetry (ITC) to be at micromolar levels. Fig. 3A shows ITC curves during titration of HSA with serial injections of **TPE-4TA**, where $[TPE-4TA] = 0.07\text{ mM}$, $[HSA] = 0.001\text{ mM}$, at 25° . Fig. 3B shows the fitting curve of integration of peak area of each injection with sequential binding sites model. **TPE-4TA** has a high binding affinity of $K=2.16 \times 10^6\text{M}^{-1}$. Fig. 3C shows the Job plot for determination of the binding stoichiometry of **TPE-4TA** and HSA, where $[TPE-4TA] + [HSA]$ are each kept constant at 2mM in pH 7.4 PBS.

Fig. 2A shows the fluorescence of **TPE-4TA** in a pH gradient. As shown, the probe remains in dark or non-emissive, where $\text{pH}>5$ PBS, $[TPE-4TA] = 5\text{ }\mu\text{M}$. Fig. 2B shows the fluorescence intensity change of **TPE-4TA** (bottom line) and the corresponding HSA-**TPE-4TA** mixture (top line) at 490 nm in a pH gradient, where $[TPE-4TA] = 5\text{ }\mu\text{M}$ and $[HSA] = 0.5\text{ }\mu\text{M}$.

The presence of biomolecules generally does not interfere with fluorescence intensity of the fluorescent compounds. Fig. 2C shows the fluorescence response of the **TPE-4TA** probe towards biomolecules in PBS, where $[TPE-4TA] = 5\text{ }\mu\text{M}$, $[\text{Biomolecules}] = 1\text{ mg/mL}$. and I_0 is the intensity of the blank probe solution at 490 nm. Fig. 2D shows the effects of common components in urine on the fluorescence intensity of AIEgen-HSA complex at 490 nm, where $[TPE-4TA] = 5\text{ }\mu\text{M}$, $[HSA] = 5\text{ }\mu\text{M}$, $[\text{urine component}] = 10\text{ mg/mL}$, in PBS buffer, and I_0 is the intensity of the blank **TPE-4TA** solution.

The fluorescent compounds exhibit high sensitivity, high binding affinity, good detection limits, high quantum yield, and can be effectively used in real urine samples.

Determining Human Serum Albumin Levels in a Biological Fluid

A method of determining human serum albumin concentration in a biological fluid or in a biological fluid test sample, can include detecting for presence of human serum albumin in the biological fluid or the biological fluid test sample, as described above, measuring the fluorescence intensity of the emission detected, and determining the concentration of human serum albumin in the biological fluid based on a predetermined correlation between a known concentration of human serum albumin in a biological fluid reference sample and a fluorescence

intensity produced when the fluorescent compound is added to the biological fluid reference sample. The biological fluid test sample can be human urine from one individual and the biological fluid reference sample can be human urine from another individual.

The predetermined correlation can be determined by obtaining a plurality of human urine reference samples from a population, determining the human serum albumin concentration in each of the human urine reference samples using a conventional method, e.g., a turbidimetric inhibition immunoassay. After determining the human serum albumin concentration for each human urine reference sample, the correlation can be determined by separating each urine reference sample into a first portion and a second portion, adding the fluorescent compound to the first portion of each reference sample to provide a plurality of target samples, adding phosphate buffer to the second portion of each reference sample to provide a plurality of blank samples, obtaining fluorescence intensity of the target sample and the blank sample for each reference urine sample at a maximum wavelength emission, subtracting the fluorescence intensity of the blank sample from the fluorescence intensity of the respective target sample for each human urine reference sample to provide a reference fluorescence intensity for each reference human urine sample, and plotting a linear curve of known human serum albumin concentration of each human urine reference sample against the corresponding reference intensity to provide a calibration curve. The calibration curve can provide the predetermined correlation. The maximum wavelength emission can be the emission peak of the AIEgen-HSA complex. The emission peak of TPE-4TA is 490 nm.

An unknown HSA concentration of a target urine sample from a patient can be obtained by obtaining the fluorescence intensity of the target urine sample, referring to a calibration curve of reference fluorescence intensities associated with known HSA concentration, plotting the fluorescence intensity of the target sample on the calibration curve, and using a fitted line plot to calculate the unknown HSA concentration in the target sample. The reference fluorescence intensities can include the fluorescence intensities of reference human urine samples with known HSA concentration, or just the fluorescence intensities of prepared HSA solutions with different known HSA concentrations. The prepared HSA solutions can be prepared by dissolving HSA in phosphate-buffered saline to provide a stock HSA solution and preparing serial dilutions of the HSA stock solution to provide prepared HSA solutions with different known HSA concentrations. According to an embodiment, an unknown HSA concentration of a new urine

sample from a patient can be obtained by measuring the fluorescence intensity of the sample, finding a matching reference fluorescence intensity on the calibration curve, then identifying the HSA concentration that corresponds with the matching reference fluorescence intensity on the calibration curve. The matching reference fluorescence intensity can be identical to or approximately the same as the measured fluorescence intensity. This method for determining HSA concentration of a urine sample is easier, faster, and more cost-effective than other instrumental analysis.

Human Serum Albumin Detection Device

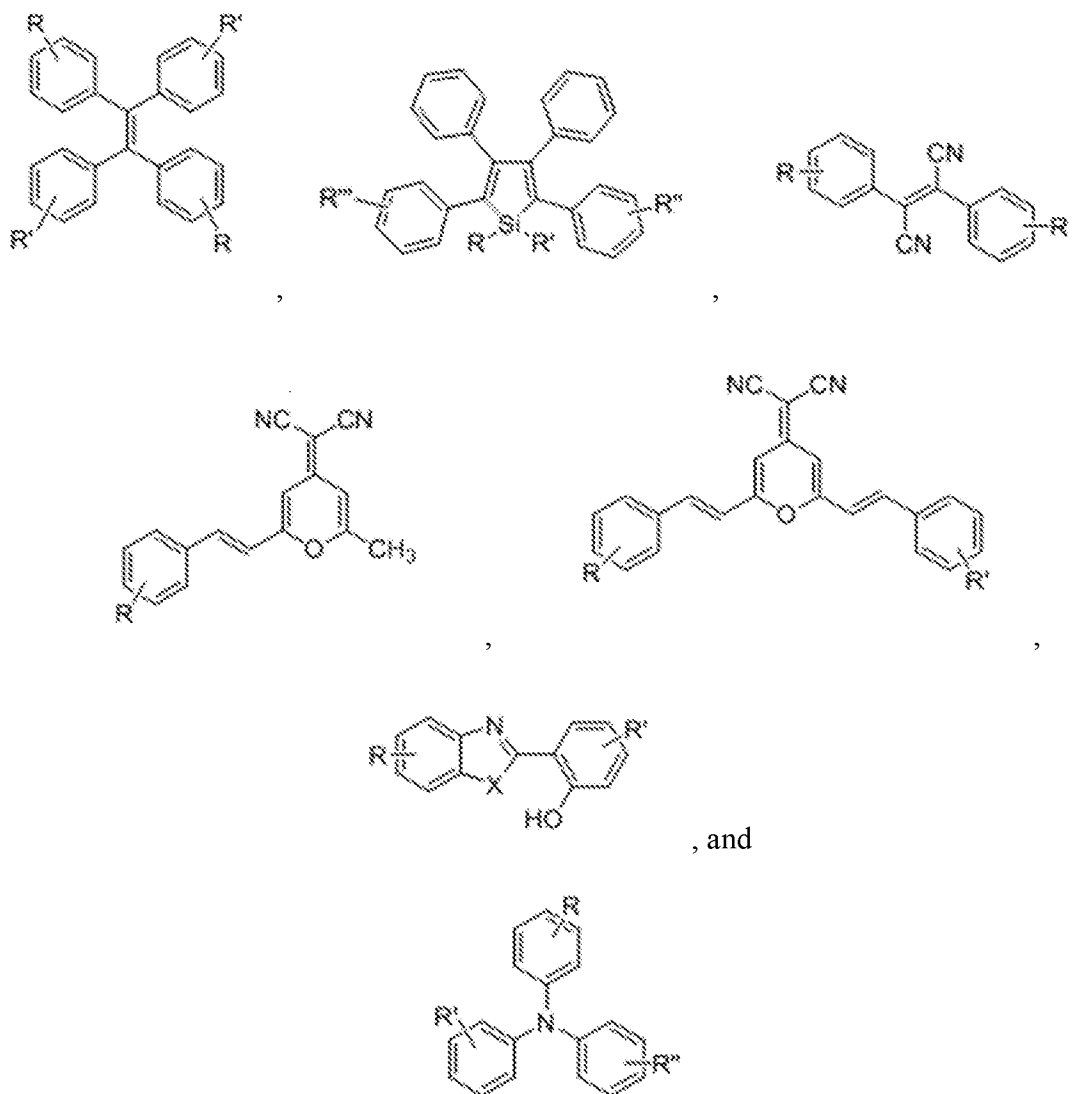
The present methods can be performed using conventional fluorescent spectrometers, normally present in hospitals, or using an HSA detection device. The HSA detection device can be an immuno(chromatographic) strip which analyzes a test sample as it flows laterally therein, thereby providing a type of lateral flow test. The HSA detection device includes one or more fluorescent compound(s) having aggregation induced emission properties as a conjugate dye. The HSA detection device can be portable and can be used by a patient at home to determine human serum albumin levels in the patient's urine.

An exemplary HSA detection device according to the present teachings is shown in Figs. 5A-5B. The HSA detection device, designated 10 in the figures, includes a hollow housing 15 defined by a top wall, a bottom wall, and a plurality of side walls connecting the top wall and the bottom wall. A sample inlet window 20 is defined within the top wall of the hollow housing. A first detection window 25a and a second detection window 25b are defined within the top wall and spaced from the sample inlet window 20. A sample pad 35 is disposed within the housing 15 below the sample inlet window 20. A conjugate pad 40 is disposed within the housing 15 adjacent the sample pad 35. The conjugate pad 40 includes at least one fluorescent compound capable of aggregation-induced emission. An incubation pad 45 is disposed within the housing 15, adjacent the conjugate pad 40 on a side opposite the sample pad 35. A first detection pad 50 is disposed within the housing 15, adjacent the incubation pad 45 and below the first detection window 25a. The first detection pad 50 includes a test line 30 including albumin-specific antibodies. A second detection pad 55 is disposed within the housing 15, spaced from the first detection pad 50 and below the second detection window 25b. The second detection pad 55 includes a control line 33 comprising human serum albumin. An absorbent pad 60 can be disposed adjacent the second detection pad 55 on the side of the second detection pad 55

opposite the side of the second detection pad 55 facing the first detection pad 50 for sample residue absorption. The absorbent pad 60 can be adjacent other sides of the second detection pad 55 so long as it is not also adjacent the first detection pad 50. The sample pad, conjugate pad, incubation pad, and first and second detection pads can be formed from a cellulose membrane or other suitable material. The cellulose membrane can be, for example, a cellulose acetate membrane or a glass cellulose membrane. The absorbent pad can be formed from any suitable water absorbing material, such as filter paper. A portable fluorescent reader (not shown) or UV light device can be coupled to the housing to provide excitation and record emission. For quantification or semi-quantification, a UV lamp for irradiating the test line can be sufficient, as the resulting emission can be visible to the naked eye. A reader including an excitation system (light source) for irradiating the test line and a detection system (camera, transducer, or a smartphone camera with an appropriate application for emission detection) can be useful for more accurate quantification. The results can be transmitted to and displayed on the device 10 directly or recorded and analyzed by the smartphone.

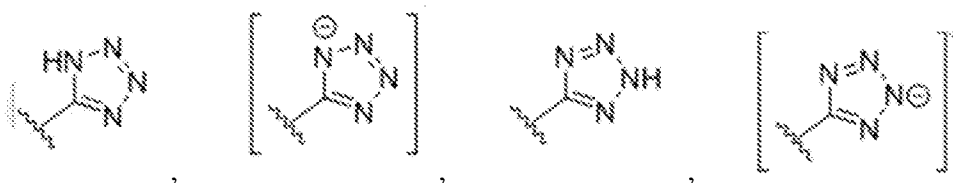
In use, a urine test sample can be dispensed on the sample pad 35 through the sample inlet window 20. The urine flows from the sample pad 35 to the conjugate pad 40. The AIEgen deposited on the conjugate pad 40 can dissolve in the urine and bind with HSA in the urine. Then, the urine including the AIEgen flows through the incubation pad 45 to the first detection pad 50. Once the HSA is captured by the antibodies forming the test line 30, the test line 30 becomes fluorescent. The intensity of fluorescence can indicate the protein concentration in the sample. The HSA concentration should be proportional to the fluorescence intensity. The control line 33 should also be fluorescent to confirm the validity.

The at least one fluorescent compound capable of aggregation-induced emission on the conjugate pad 40 can have a backbone structural formula selected from the group consisting of:



wherein X is O or S;

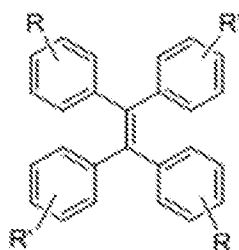
wherein at least one of R, R', R'' or R''' is independently selected from the group consisting of



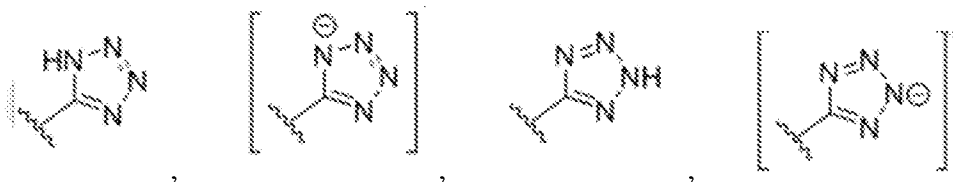
and wherein all other of the R, R', R'', and, R''' groups are selected from the group consisting of H, heteroatom, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl,

aryl, and heteroaryl.

In an embodiment, the fluorescent compound has the following backbone structural formula:

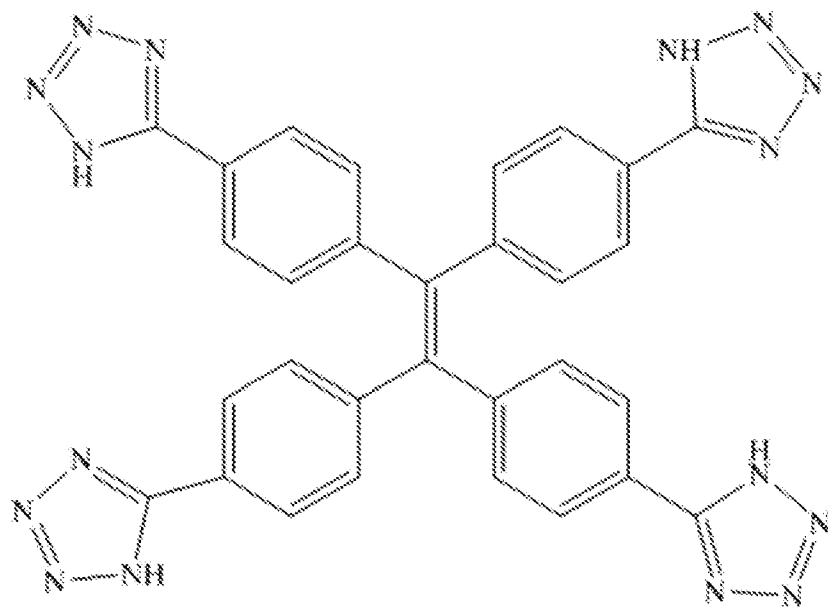


wherein at least two of the R and R' groups are independently selected from the group consisting of

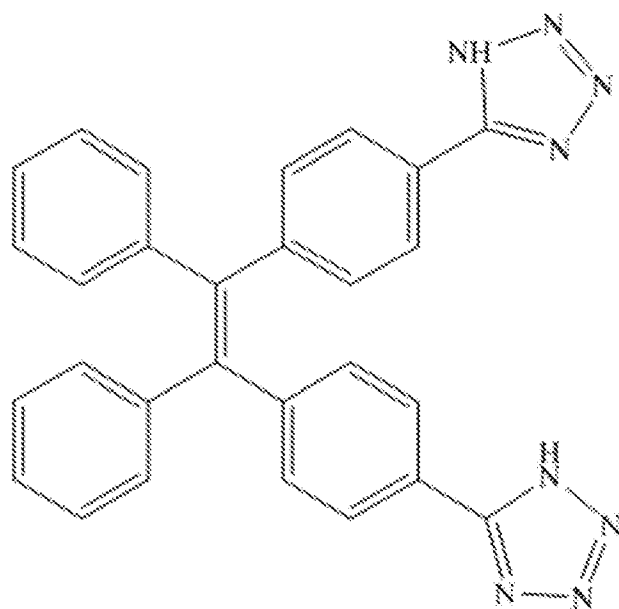


and wherein all other of the R and R' groups are selected from the group consisting of H, heteroatom, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

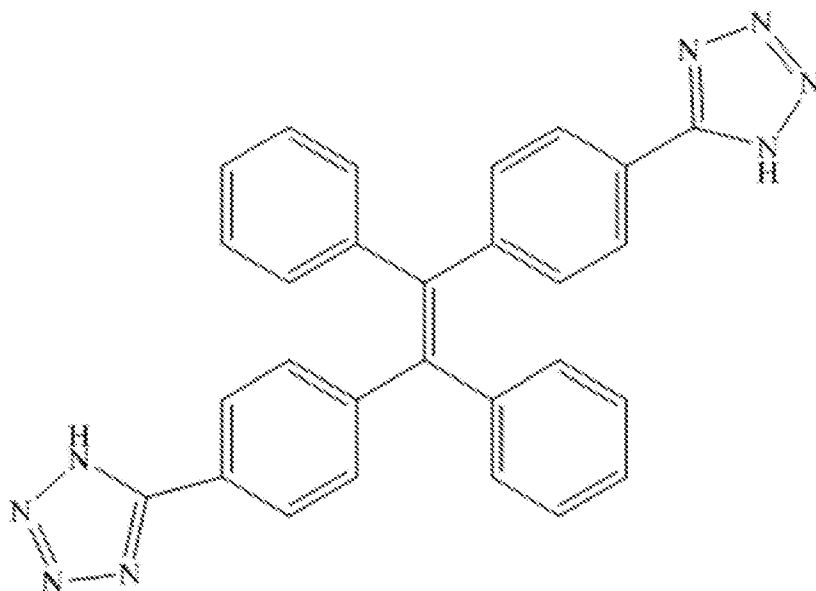
In an embodiment, the compound is a compound selected from the group consisting of:



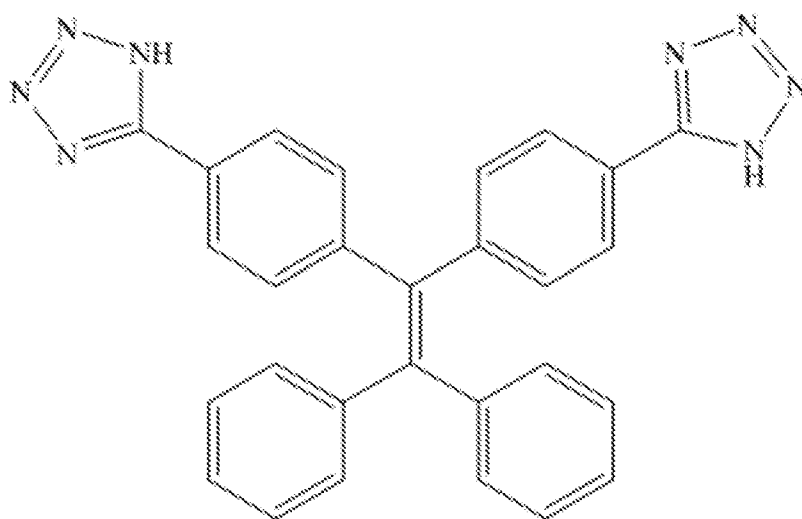
TPE-4TA,



TPE-2TA-a,



TPE-2TA-b, and



TPE-2TA-c.

The present teachings are illustrated by the following examples.

EXAMPLE

Real Urine Sample Testing

To examine the feasibility of quantifying urine albumin using the present fluorescent compounds, real urine samples were utilized. 100 urine samples were taken from patients from Southern Medical University and numerically identified to respect the confidentiality of the

patients. The samples were obtained from diverse individuals, e.g., people of different gender, age, disease state, etc., and collected at different times, e.g., morning, evening, etc. The protein concentrations of these samples were primarily determined with turbidimetric inhibition immunoassay ranging from 10-2000mg/L, covering the healthy (<30mg/L), microalbuminuria (30-300mg/L) and albuminuria (>300mg/L) conditions.

AIEgens were added to the samples to provide urine-AIEgen mixtures. The working concentration of AIEgen in the solutions was 50 μ M. The volume ratio of urine/AIEgen mixture ranged from 1:6 to 1:12. Upon excitation, fluorescence intensity at the wavelength of maximum emission of the urine-AIEgen mixture (I) and fluorescence intensity of urine-PBS buffer blank (I_0) were recorded using a fluorescence spectrometer. (I- I_0), values were linearly plotted against the known concentration of each sample to provide a calibration curve (Fig. 4). The HSA concentration of urine samples having an unknown HSA concentration can be determined by measuring the fluorescence intensity of the urine sample, then referring to the calibration curve to calculate the concentration.

The present subject matter being thus described, it will be apparent that the same may be modified or varied in many ways. Such modifications and variations are not to be regarded as a departure from the spirit and scope of the present subject matter, and all such modifications and variations are intended to be included within the scope of the following claims.

CLAIMS

We Claim:

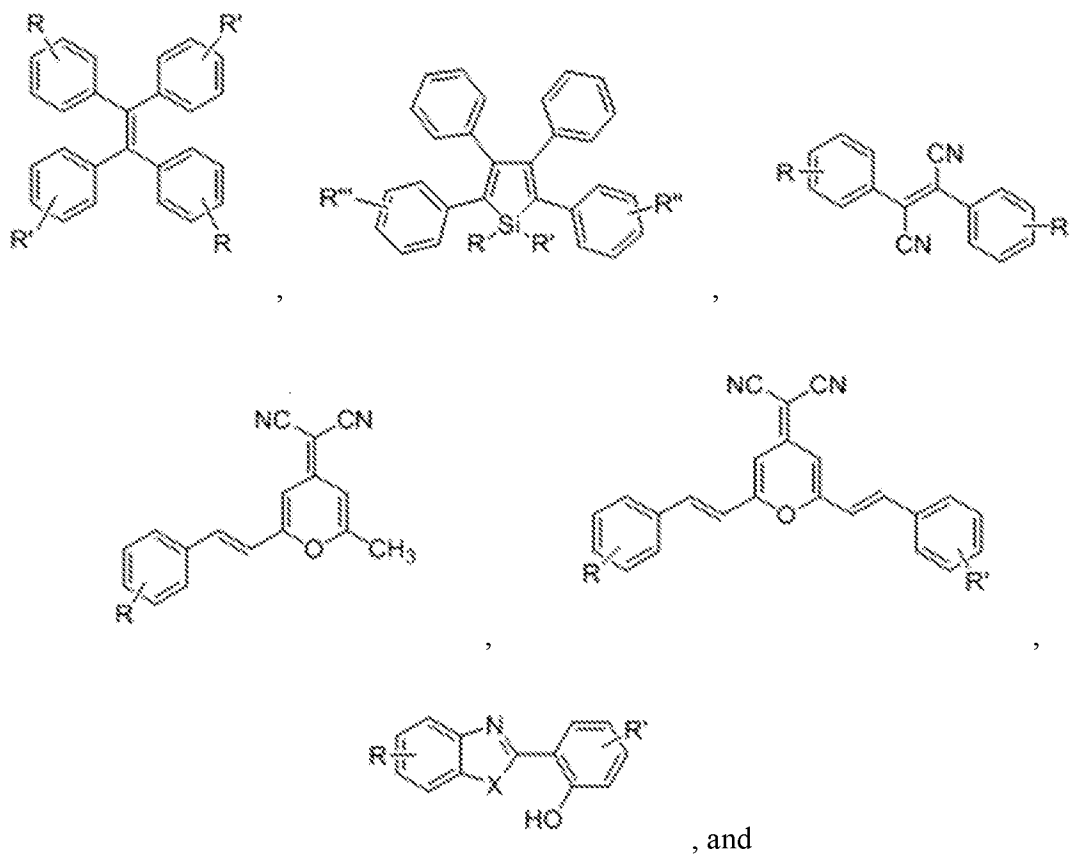
1. A method for detecting for presence of human serum albumin in a biological fluid test sample, comprising:

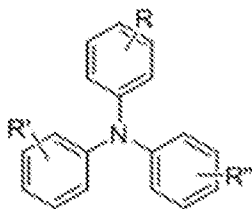
contacting the biological fluid test sample with a fluorescent compound to provide a mixture;

irradiating the mixture with ultraviolet light; and

determining a presence of human serum albumin when an observable emission is detected, wherein

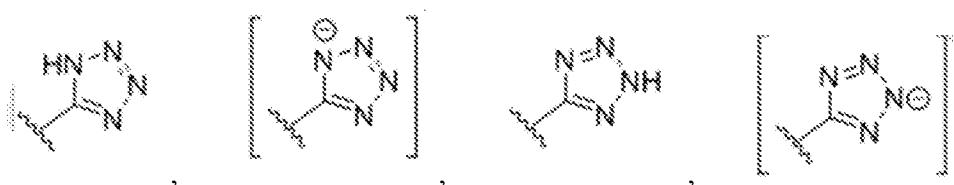
the fluorescent compound has a backbone structural formula selected from the group consisting of :





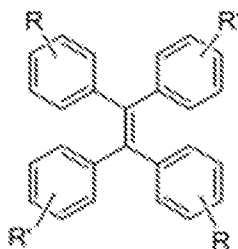
wherein X is O or S;

wherein at least one of R, R', R'' or R''' is independently selected from the group consisting of

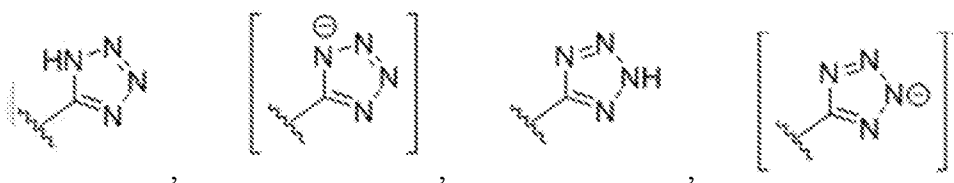


and wherein all other of the R, R', R'', and, R''' groups are selected from the group consisting of H, heteroatom, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

2. The method of claim 1, wherein the compound has the following backbone structural formula:

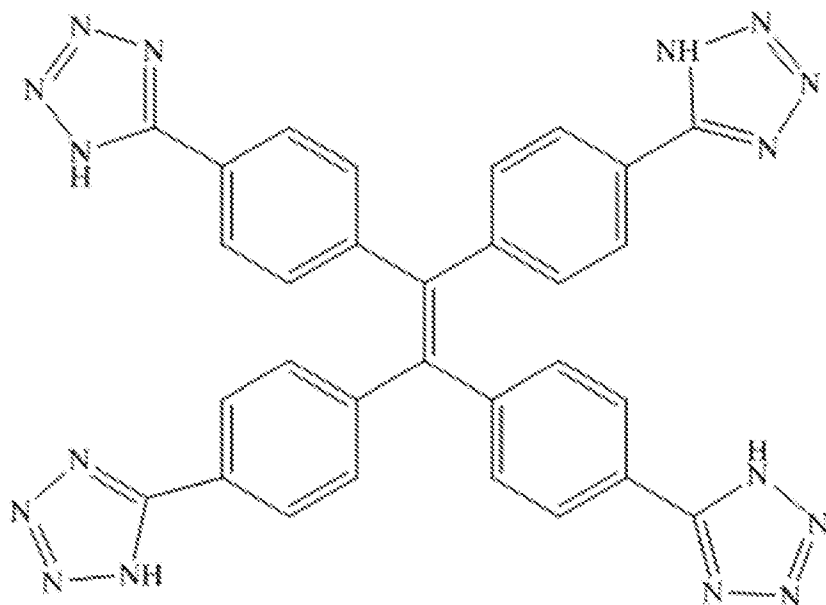


wherein at least two of the R and R' groups are independently selected from the group consisting of

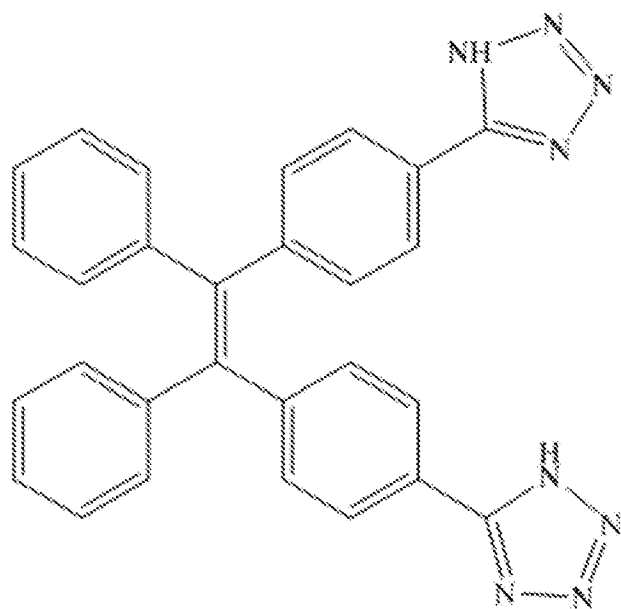


and wherein all other of the R and R' groups are selected from the group consisting of H, heteroatom, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

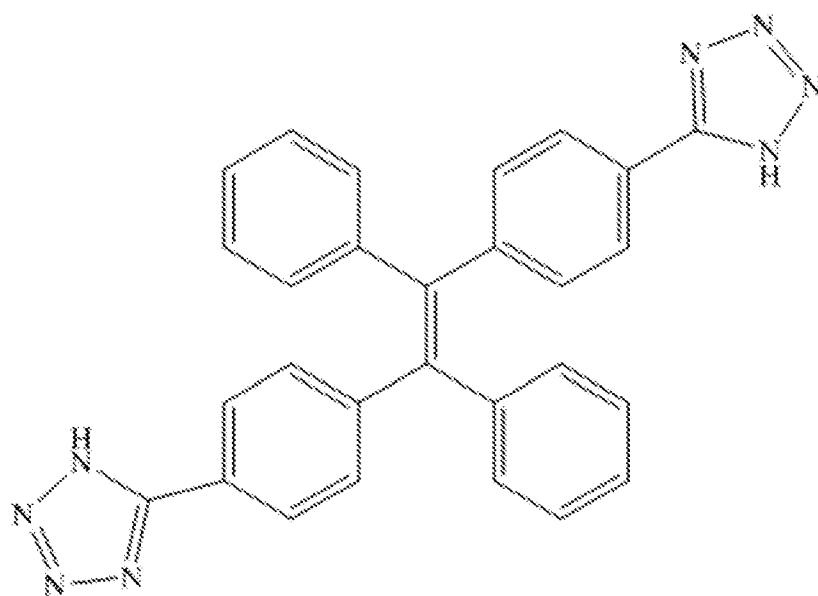
3. The method of claim 1, wherein the compound is a compound selected from the group consisting of:



TPE-4TA,

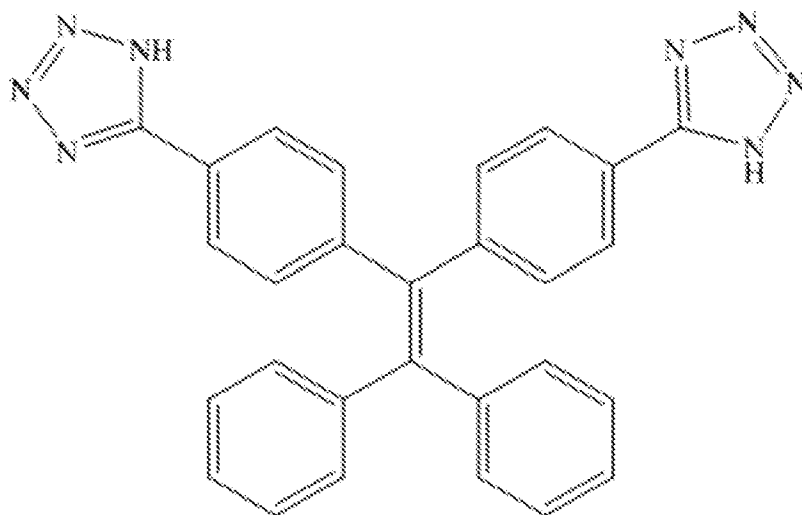


TPE-2TA-a,



TPE-2TA-b,

and



TPE-2TA-c.

4. The method of claim 1, wherein the compound binds with the human serum albumin and the binding of the compound with the human serum albumin produces the emission.
5. The compound of claim 4, wherein the biological fluid is human urine.
6. A method of determining human serum albumin concentration in a biological fluid test sample, comprising:
 - detecting for presence of human serum albumin in the biological fluid test sample according to claim 1,
 - measuring fluorescence intensity of the emission detected; and
 - determining the concentration of human serum albumin in the biological fluid based on a predetermined correlation between a known concentration of human serum albumin in a biological fluid reference sample and a fluorescence intensity produced when the fluorescent compound is added to the biological fluid reference sample.
7. The method of claim 6, wherein the biological fluid test sample is human urine and the biological fluid reference sample is a separate sample of human urine.
8. The method of claim 7, wherein the predetermined correlation is determined by:
 - obtaining a plurality of human urine reference samples from a population;
 - determining the human serum albumin concentration in each of the human urine reference samples;
 - separating each urine reference sample into a first portion and a second portion;

adding the fluorescent compound to the first portion of each reference sample to provide a plurality of target samples;

adding phosphate buffer to the second portion of each reference sample to provide a plurality of blank samples;

obtaining fluorescence intensity of the target sample and the blank sample for each reference urine sample at a maximum wavelength emission;

subtracting the fluorescence intensity of the blank sample from the fluorescence intensity of the respective target sample for each human urine reference sample to provide a reference fluorescence intensity for each reference human urine sample;

plotting a linear curve of known human serum albumin concentration of each human urine reference sample against the corresponding reference intensity to provide a calibration curve, the calibration curve providing the predetermined correlation.

9. The method claim 8, wherein a volume ratio of urine to fluorescent compound in each of the target samples ranges from about 1:6 to about 1:12.

10. A device for detecting human serum albumin concentration in a biological fluid, comprising:

a hollow housing defined by a top wall, a bottom wall, and a plurality of side walls connecting the top wall and the bottom wall;

a sample inlet window defined within the top wall of the hollow housing;

first and second detection windows defined within the top wall and spaced from the sample inlet window;

a sample pad disposed within the housing below the sample inlet window;

a conjugate pad disposed within the housing adjacent the sample pad, the conjugate pad including at least one fluorescent compound, the at least one fluorescent compound being capable of aggregation-induced emission;

an incubation pad disposed within the housing, adjacent the conjugate pad on a side opposite the sample pad;

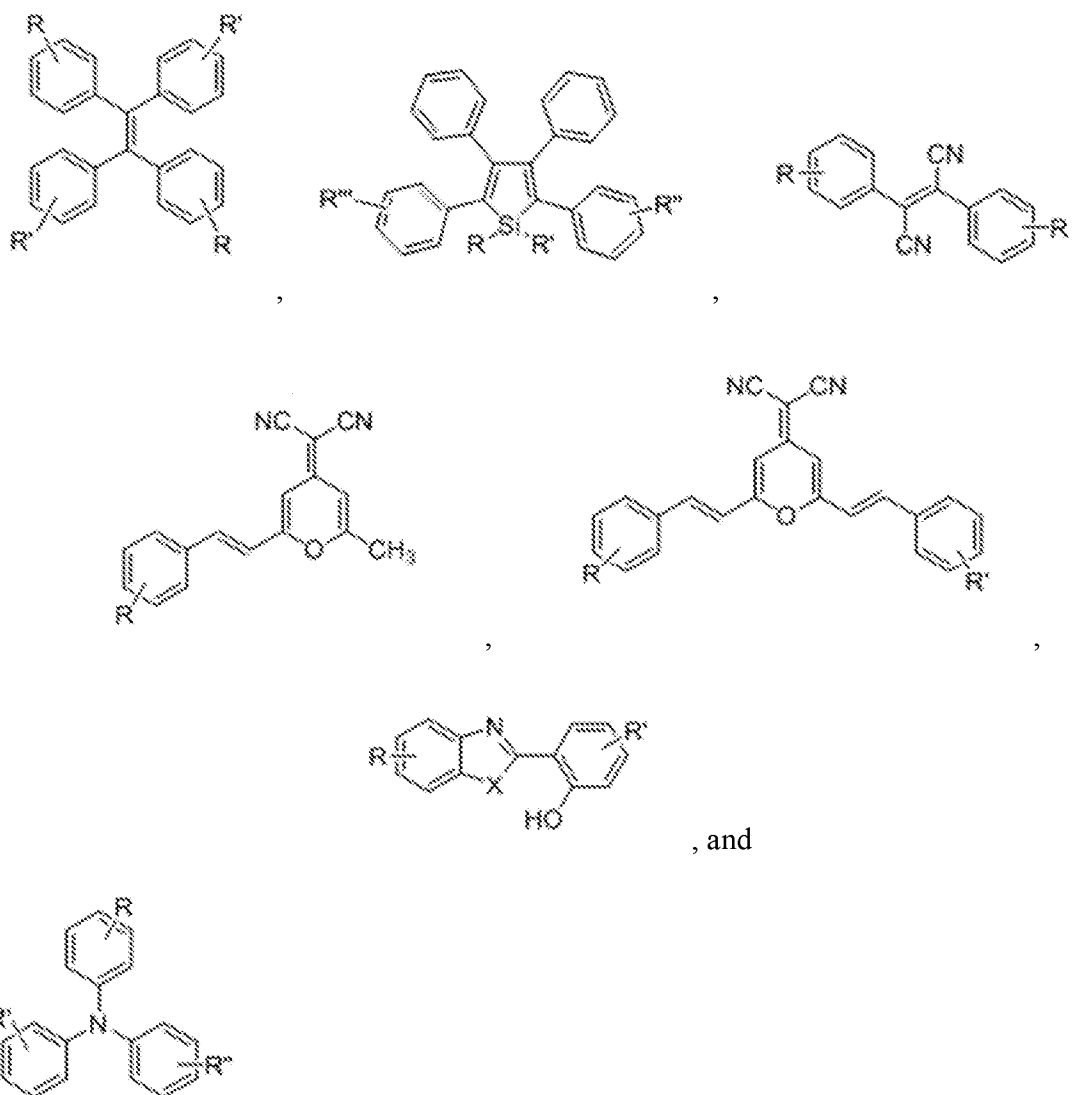
a first detection pad disposed within the housing, adjacent the incubation pad and below the first detection window, the first detection pad including a test line comprising albumin-specific antibodies;

a second detection pad disposed within the housing, spaced from the first detection pad and below the second detection window, the second detection pad including a control line comprising human serum albumin; and

a portable fluorescent reader coupled to the housing.

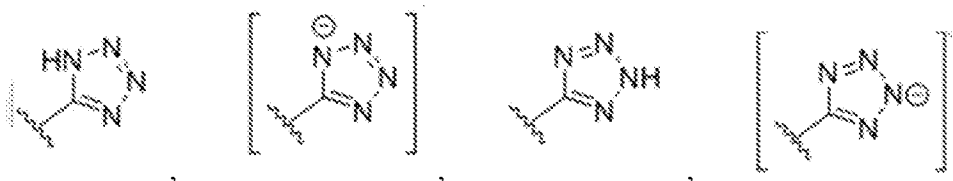
11. The device of claim 10, further comprising an absorbent pad adjacent the second detection pad.

12. The device of claim 10, wherein the fluorescent compound has a backbone structural formula selected from the group consisting of :



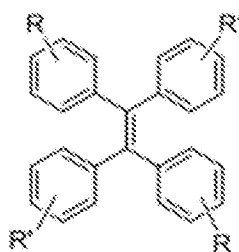
wherein X is O or S;

wherein at least one of R, R', R'' or R''' is independently selected from the group consisting of

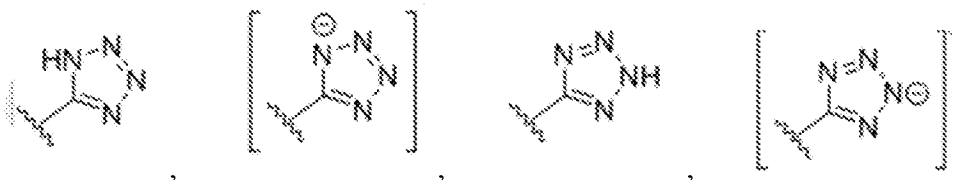


and wherein all other of the R, R', R'', and, R''' groups are selected from the group consisting of H, heteroatom, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

13. The device of claim 12, wherein the fluorescent compound has the following backbone structural formula:

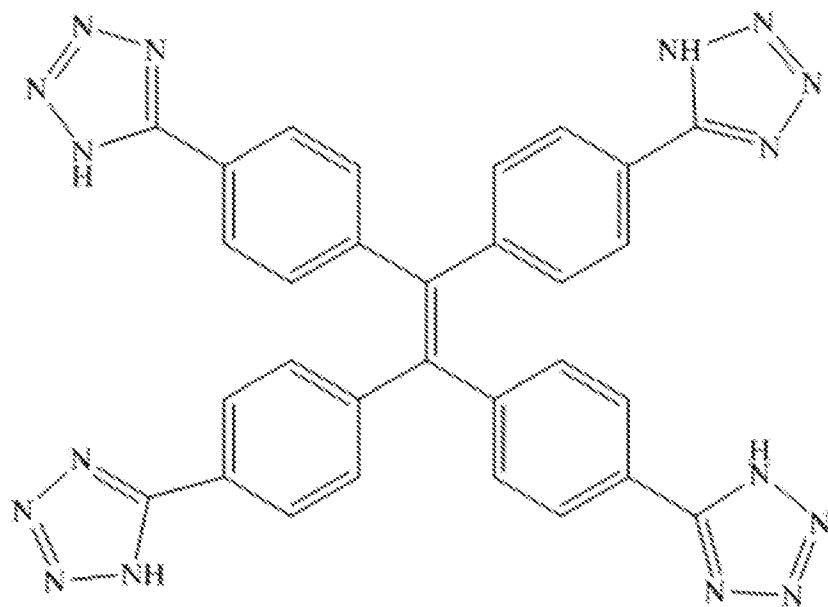


wherein at least two of the R and R' groups are independently selected from the group consisting of

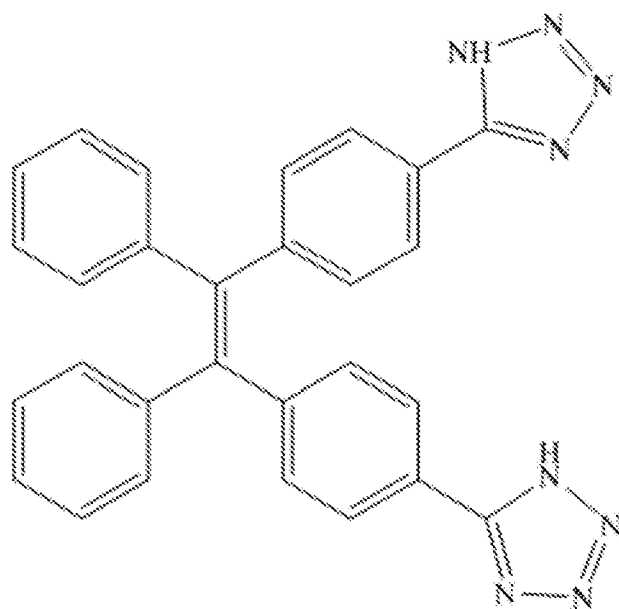


and wherein all other of the R and R' groups are selected from the group consisting of H, heteroatom, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

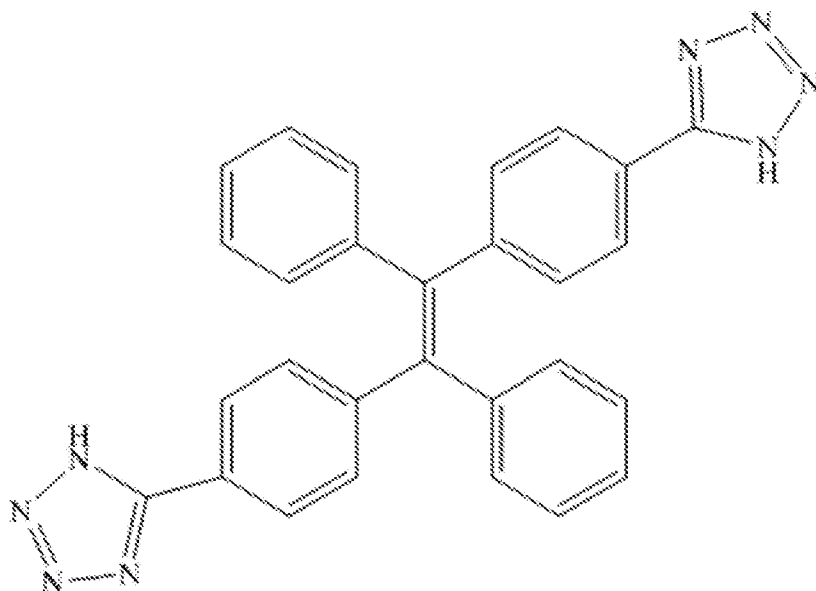
14. The device of claim 12, wherein the compound is a compound selected from the group consisting of:



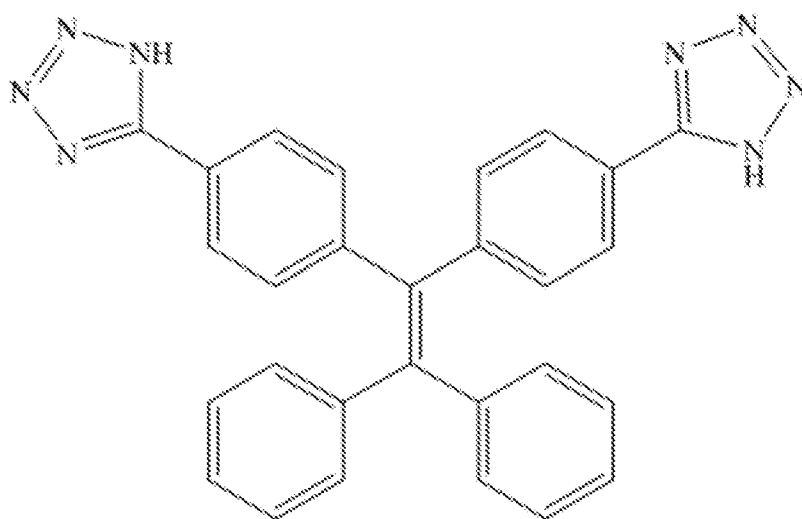
TPE-4TA,



TPE-2TA-a,



TPE-2TA-b, and



TPE-2TA-c.

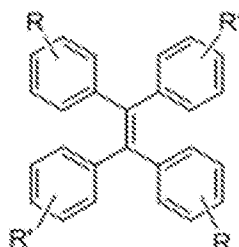
15. A method for detecting for presence of human serum albumin in a biological fluid test sample, comprising:

contacting the biological fluid test sample with a fluorescent compound to provide a mixture;

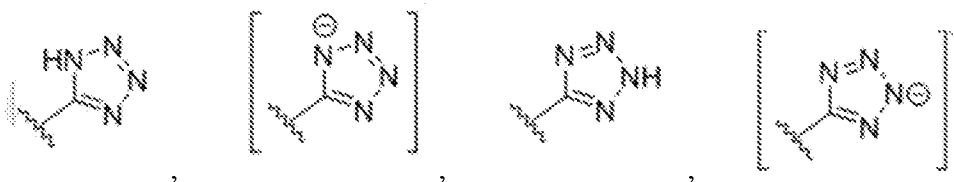
irradiating the mixture with ultraviolet light; and

determining a presence of human serum albumin when an observable emission is detected, wherein

the fluorescent compound has a backbone structural formula selected from the group consisting of:

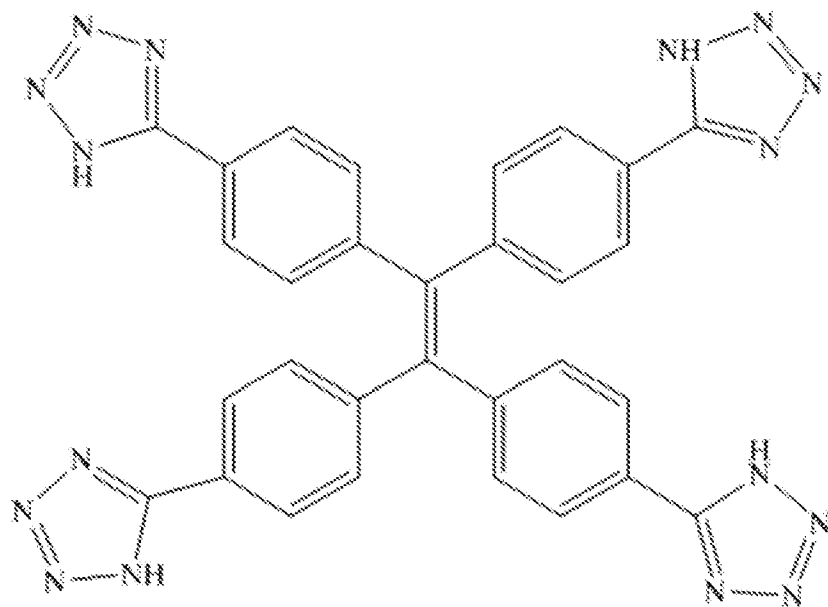


wherein at least two of the R and R' groups are independently selected from the group consisting of

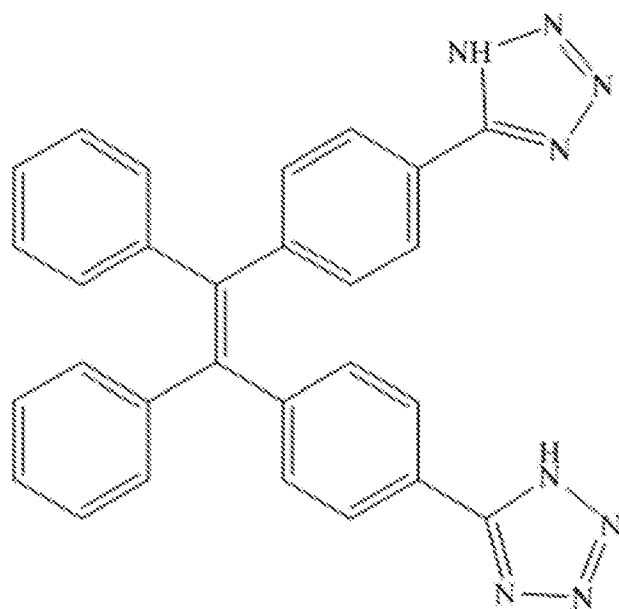


and wherein all other of the R and R' groups are selected from the group consisting of H, heteroatom, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl.

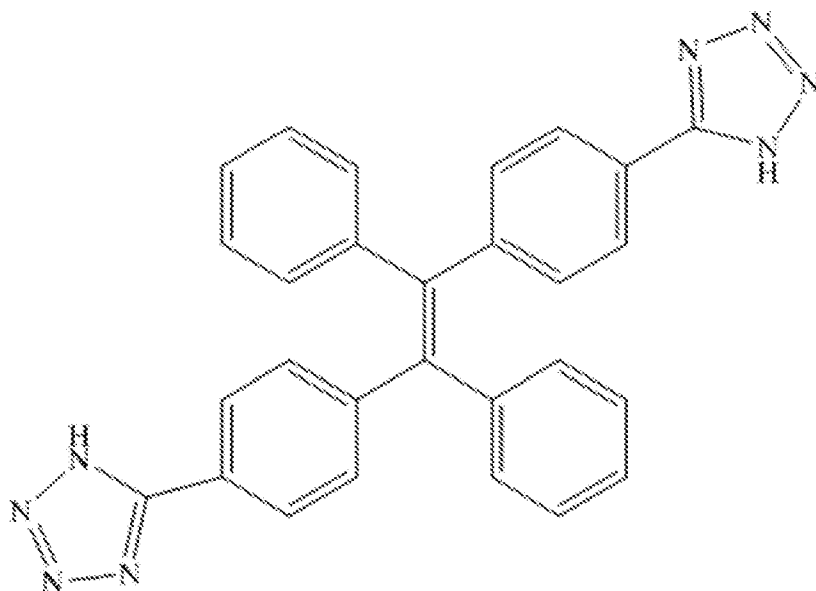
16. The method of claim 15, wherein the compound is a compound selected from the group consisting of:



TPE-4TA,

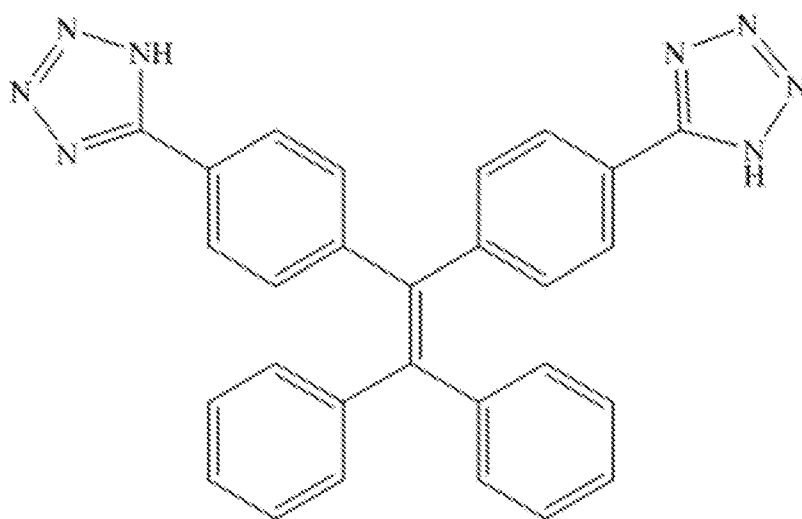


TPE-2TA-a,



TPE-2TA-b,

and



TPE-2TA-c.

17. The method of claim 15, wherein the compound binds with human serum albumin and the binding of the compound with human serum albumin produces the emission.

18. A method of determining human serum albumin concentration in a biological fluid test sample, comprising:

detecting for presence of human serum albumin in the biological fluid test sample according to claim 15,

measuring fluorescence intensity of the emission detected; and

determining the concentration of human serum albumin in the biological fluid based on a predetermined correlation between a known concentration of human serum albumin in a biological fluid reference sample and a fluorescence intensity produced when the fluorescent compound is added to the biological fluid reference sample.

19. The method of claim 18, wherein the biological fluid test sample is human urine and the biological fluid reference sample is human urine.

20. The method of claim 19, wherein the predetermined correlation is determined by:

obtaining a plurality of human urine reference samples from a population;

determining the human serum albumin concentration in each of the human urine reference samples;

separating each urine reference sample into a first portion and a second portion;

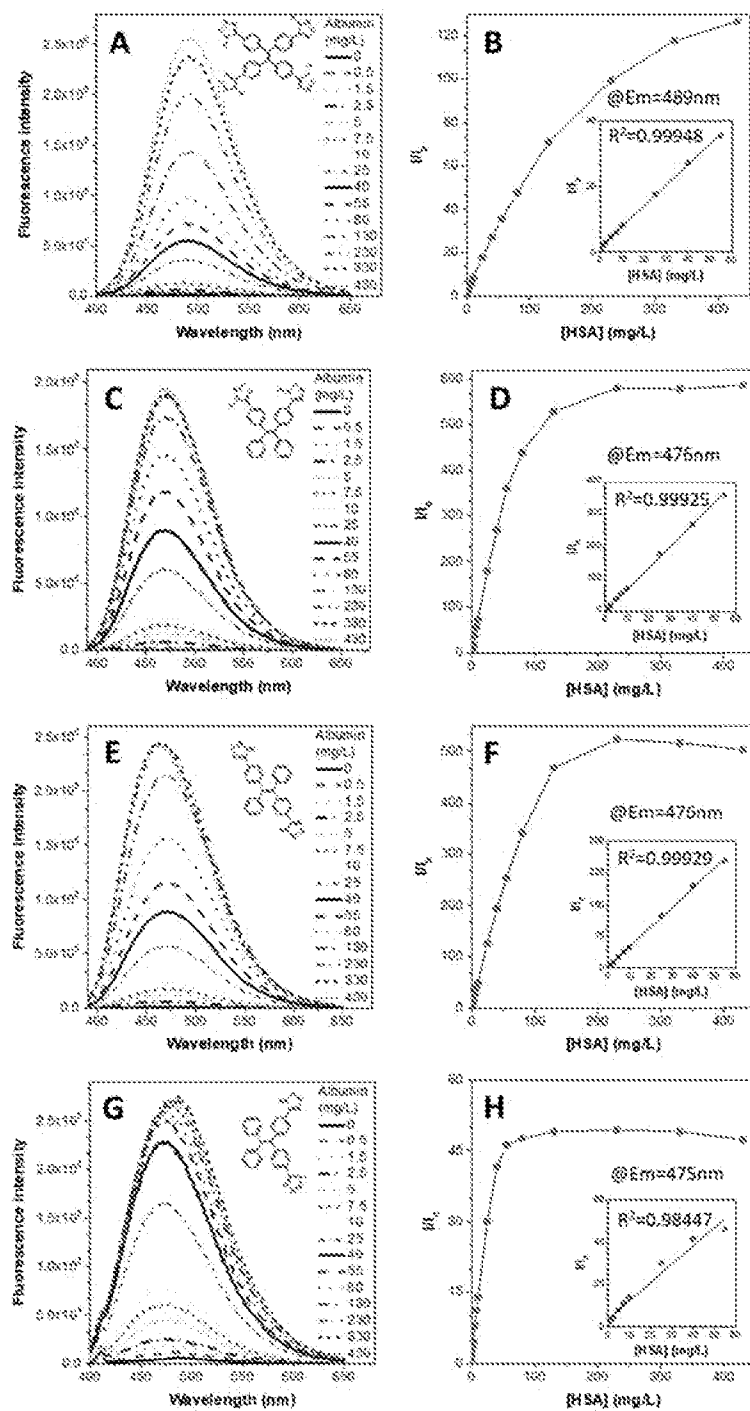
adding the fluorescent compound to the first portion of each reference sample to provide a plurality of target samples;

adding phosphate buffer to the second portion of each reference sample to provide a plurality of blank samples;

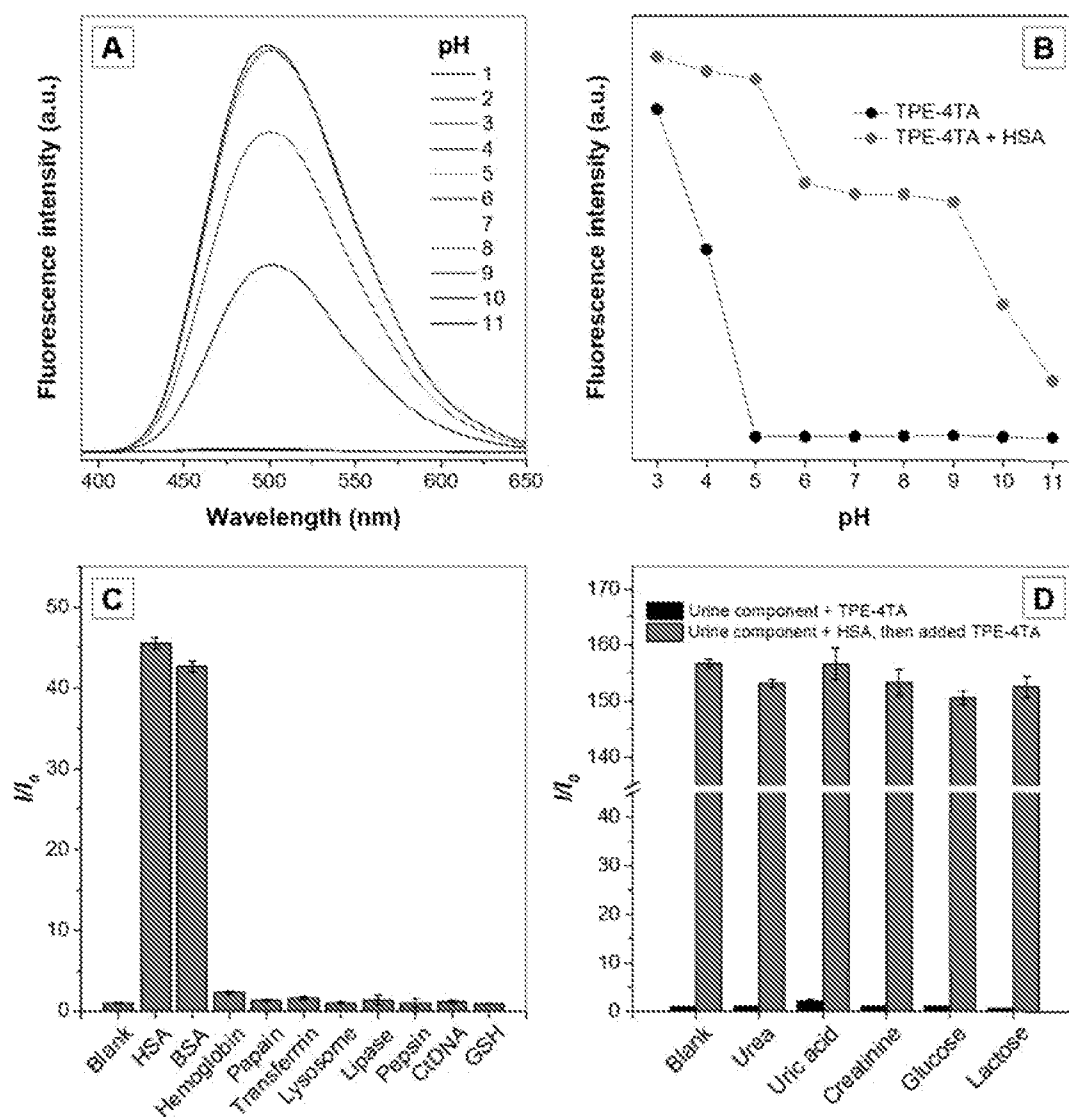
obtaining fluorescence intensity of the target sample and the blank sample for each reference urine sample at a maximum wavelength emission;

subtracting the fluorescence intensity of the blank sample from the fluorescence intensity of the respective target sample for each human urine reference sample to provide a reference fluorescence intensity for each reference human urine sample;

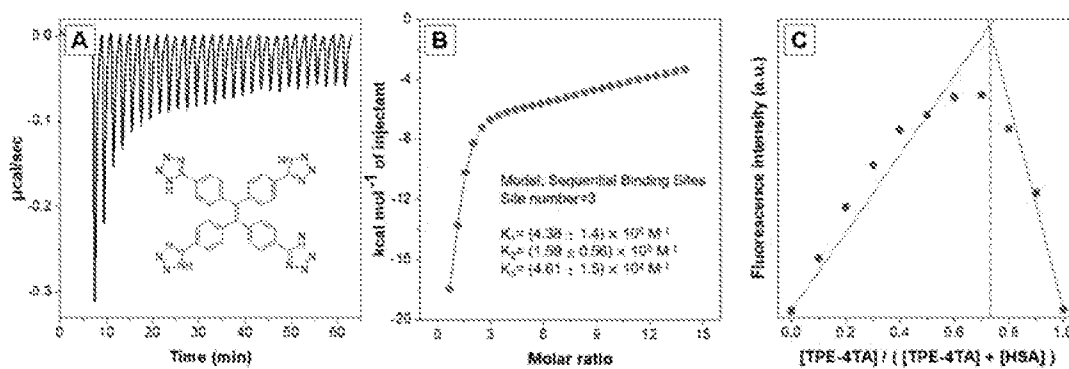
plotting a linear curve of known human serum albumin concentration of each human urine reference sample against the corresponding reference intensity to provide a calibration curve, the calibration curve providing the predetermined correlation.



Figs. 1A-1H



Figs. 2A-2D



Figs. 3A-3C

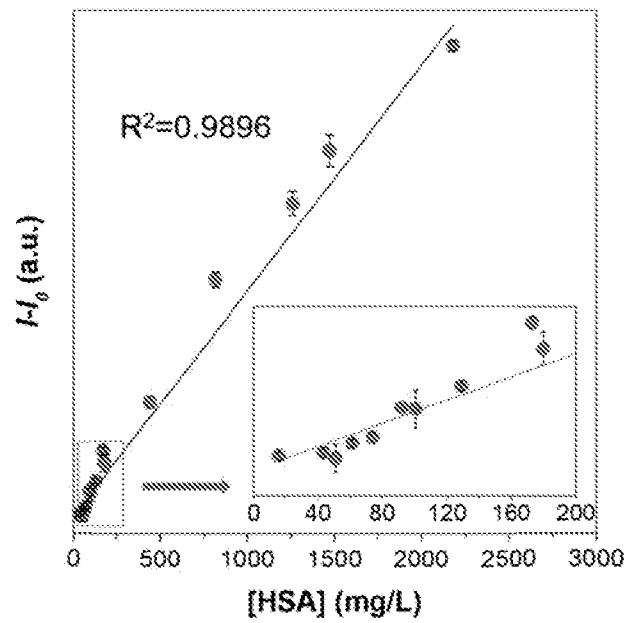


Fig. 4

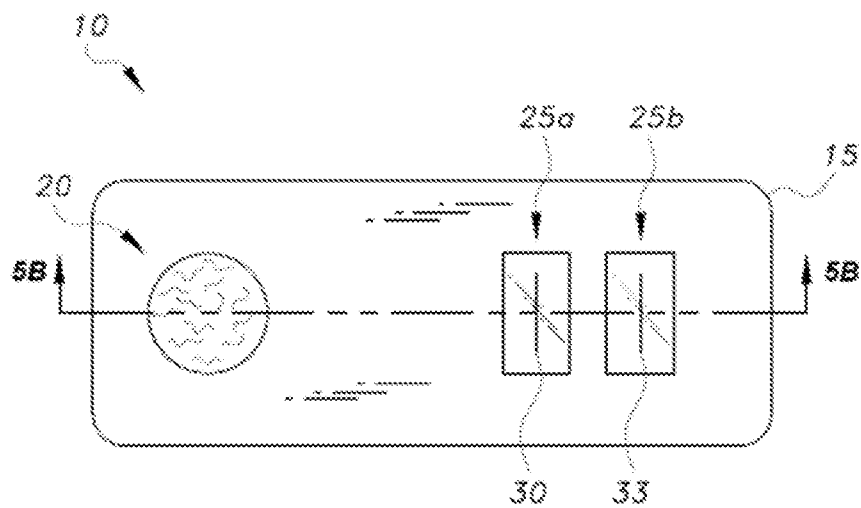


Fig. 5A

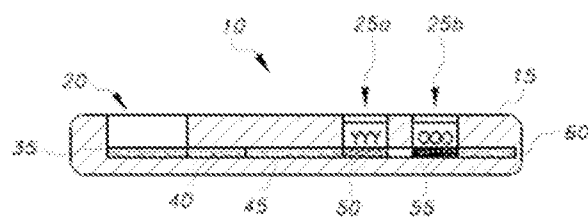


Fig. 5B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2019/102792

A. CLASSIFICATION OF SUBJECT MATTER

G01N 21/64(2006.01)i; C07D 257/04(2006.01)i; C09K 11/06(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N; C07D; C09K

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

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

DWPI; SIPOABS; CNKI; CNABS; GOOGLE; STNext; Web of Science: albumin, fluorescent, ultraviolet, tetrazolate, fluid, AIE, aggregation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	TU Y. et al. "Specific and Quantitative Detection of Albumin in Biological Fluids by Tetrazolate-Functionalized Water-Soluble AIEgens." <i>ACS Appl. Mater. Interfaces</i> , Vol. 11, 25 July 2019 (2019-07-25), pages 29619-29629	1-20
PA	WO 2019091389 A1 (UNIV. HONG KONG SCIENCE & TECHNOLOGY) 16 May 2019 (2019-05-16) the abstract, claims 1-20	1-20
A	WO 2015164784 A1 (UNIV. RUTGERS) 29 October 2015 (2015-10-29) the abstract, claims 1-21	1-20
A	CN 102706839 A (UNIV. HONG KONG SCIENCE & TECHNOLOGY) 03 October 2012 (2012-10-03) the abstract, claims 1-45	1-20
PA	WO 2018192521 A1 (UNIV. HONG KONG SCIENCE & TECHNOLOGY) 25 October 2018 (2018-10-25) the abstract, claims 1-20	1-20



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

11 November 2019

Date of mailing of the international search report

28 November 2019

Name and mailing address of the ISA/CN

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2019/102792

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YAN N.et al. "Real-time monitoring of the dissolution kinetics of silver nanoparticles and nanowires in aquatic environments using an aggregation-induced emission fluorogen." <i>Chem. Commun.</i> , Vol. 54, 09 April 2018 (2018-04-09), pages 4585-4588	1-20
A	XIE S.et al. "Fluorogenic Ag ⁺ -Tetrazolate Aggregation Enables Efficient Fluorescent Biological Silver Staining." <i>Angew. Chem. Int. Ed.</i> , Vol. 57, 14 April 2018 (2018-04-14), pages 5750-5753	1-20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2019/102792

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **1-9,15-20**
because they relate to subject matter not required to be searched by this Authority, namely:
 - [1] Claims 1-9 and 15-20 direct to a method for detecting for presence of human serum albumin in a biological fluid test sample, and do not meet the criteria set out in PCT Rules 39.1(iv). The search has been carried out and based on the use of the fluorescent compound for the manufacturing of a medicament for detecting for presence of human serum albumin in a biological fluid test sample.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2019/102792

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2019091389	A1	16 May 2019	None			
WO	2015164784	A1	29 October 2015	US	2017044428	A1	16 February 2017
				EP	3134416	A1	01 March 2017
				JP	2017519721	A	20 July 2017
				EP	3134416	A4	04 July 2018
				CN	106459095	A	22 February 2017
CN	102706839	A	03 October 2012	CN	102706839	B	12 August 2015
				HK	1174687	A1	22 April 2016
WO	2018192521	A1	25 October 2018	CN	110234735	A	13 September 2019