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- (54) **Title:** AIE BIOPROBES EMITTING RED OR YELLOW FLUORESCENCE

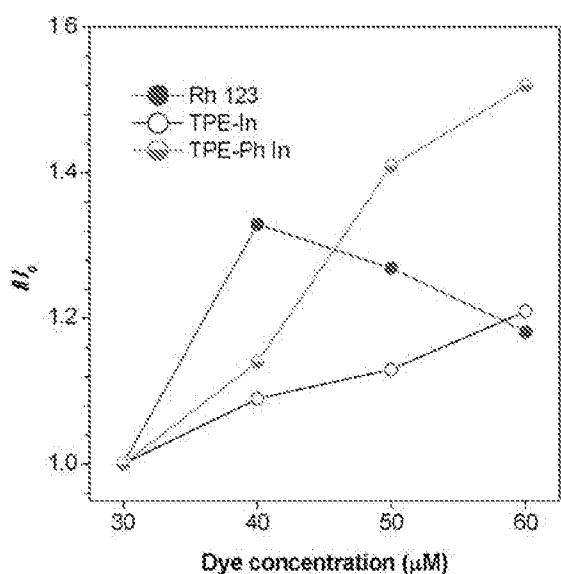


FIG. 2

- (57) **Abstract:** The present subject matter relates to red emitting mitochondria-targeted aggregation induced emission (AIE) probes as an indicator for membrane potential and mouse sperm activity. The present subject matter relates to AIE-active fluorescent probes for reactive oxygen species (ROS) detection and related biological applications, such as inflammation imaging and glucose assay, as well as the preparation and application of red fluorescent AIEgens.



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TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,
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AIE BIOPROBES EMITTING RED OR YELLOW FLUORESCENCE

RELATED APPLICATIONS

[1] The present patent application claims priority to provisional U.S. Patent Application No. 62/285,826 filed November 10, 2015 and PCT application No. PCT/CN2016/089911, both of which are incorporated by reference herein in their entirety.

TECHNICAL FIELD

[2] The present subject matter relates to red emitting mitochondria-targeted aggregation induced emission (AIE) probes as an indicator for membrane potential and mouse sperm activity. The present subject matter relates to AIE-active fluorescent probes for reactive oxygen species (ROS) detection and related biological applications, such as inflammation imaging and glucose assay, as well as the preparation and application of red fluorescent AIEgens.

BACKGROUND

[3] Fluorescent dyes have been used widely in modern biological studies and have facilitated the development of fluorescent microscopes. Fluorescent imaging is a powerful tool to look beyond tissue and observe single cells and, nowadays, has an important role in the progression and noninvasive study on gene expression, protein function, protein-protein interactions, and many other cellular processes. Furthermore, fluorescent imaging has proved to be a powerful tool in examining the microscopic structures of polymer blends. Particularly, far-red to near-infrared (FR/NIR) fluorescent dyes are beneficial for *in vivo* imaging, as the effects of optical absorption and intrinsic auto-fluorescence may be minimized. Higher degrees of tissue penetration can be achieved due to a longer fluorescent wavelength. For the same reasons, the

imaging of microscopic polymer blend structures utilizing FR/NIR fluorescent dyes allows for obtaining insight not only of the surface pattern, but also of deeper layers.

[4] Regarding the molecular design of fluorogens, the aggregation-caused quenching (ACQ) effect has always given rise to photo-bleaching and attenuation in fluorescence intensity upon aggregation, thus resulting in restrictions of long-time monitoring of organelles and lower performance.

[5] In 2001, molecules exhibiting AIE characteristics were discovered. Luminogens with AIE properties are almost non-fluorescent when molecularly dissolved, but become highly emissive when aggregates are formed. Restriction of intramolecular rotation (RIR) is deemed to be the major mechanism for the AIE effect. Thus, AIE active molecules are highly emissive in the aggregated and/or crystalline state due to restriction of intra-molecular motions (RIM), allowing applications in various areas, such as in OLEDs and bioprobes for cell imaging and tracking. AIE active molecules have already been applied successfully as bioprobes, proving high photostability and high bio-compatibility.

[6] Mitochondria are dynamic organelles that exist in almost all eukaryotic cells. The mitochondrial morphology is regulated by a set of proteins. The mutations of these proteins are reported to be associated with diseases, including neurodegenerative and cardiovascular diseases. The major function of mitochondria is to generate energy and approximately 95% of the primary source of energy used in eukaryotic cells and ATP is produced by mitochondria.

[7] In order to synthesize ATP, mitochondria continuously oxidize substrates and maintain a proton gradient across the lipid bilayer in the respiratory electron transport chain with a large membrane potential ($\Delta\Psi_m$). The $\Delta\Psi_m$ is a vital parameter reflecting the mitochondrial functional status, and thus is closely related to cell health, injury and function. Thus, the

maintenance of mitochondrial function is crucial. The mitochondria $\Delta\Psi_m$ is an essential indicator for assessing the physiology, viability, and fertilization potential of sperm, the male germ cell. As the mitochondria provide energy for sperm movement, abnormal $\Delta\Psi_m$ in sperm mitochondria may lead to mitochondria dysfunction and result in male infertility. Consequently, development of efficient methods for monitoring mitochondrial morphology, as well as $\Delta\Psi_m$, is of great importance for both biomedical research and early diagnosis of related diseases.

[8] Various cationic fluorescent dyes have been developed to target mitochondria, and some of the dyes, such as Rhodamine 123 (Rh 123), TMRE/TMRM, and JC-1, can be utilized to monitor mitochondria $\Delta\Psi_m$. However, the photostability of these dyes leaves much to be desired due to their detrimental concentration-quenching emission effect. Such effect only allows the use of diluted solutions of the probes for imaging (generally $< 1\text{--}30\text{ nM}$), which easily leads to fast photobleaching of the probes when a harsh laser beam is used as the excitation light source.

[9] The situation is even more complicated when the concentration-quenching dyes are used to measure the $\Delta\Psi_m$. For example, the elevation of the $\Delta\Psi_m$ resulting in the increase of dye amounts in the mitochondria may lead to either the enhancement or the decrease of the fluorescent signal, depending on whether the dye concentration is within the non-quenching range. JC-1 is the most widely used fluorescent indicator for $\Delta\Psi_m$. However, JC-1 is highly sensitive to dye loading concentration and time. Many references have reported the complexities and false results of using JC-1 for measuring $\Delta\Psi_m$. Therefore, development of a non-self-quenching, photostable mitochondrial probe to reveal the $\Delta\Psi_m$ in living cells is in high demand.

[10] Through molecular endeavors, mitochondria-targeting AIE probes have been successfully developed. However, most of these probes emit at a short wavelength region and are unresponsive to the $\Delta\Psi_m$ changes. On the other hand, probes emitting at a longer wavelength

region offer various advantages such as minimum photo-damage to biological samples, deep tissue penetration, and little interference from auto-fluorescence. Efficient red emitting probes with excellent photostability and functionality are thus highly desirable.

[11] ROS are chemically reactive molecules containing oxygen. Hydrogen peroxide (H_2O_2) is one of the most well-known ROS and is widely used in industry and daily life for rinsing, bleaching, and disinfecting. The level of residual H_2O_2 in waste water is an important parameter for state standards of waste discharge, as high concentration of H_2O_2 may cause oxidative damage to the environment. Furthermore, hydrogen peroxide is becoming a popular molecule in living organisms, since scientists have disclosed more biological processes in which H_2O_2 participates and plays different roles. For example, H_2O_2 serves as a common indicator of oxidative stress, induces antioxidant defenses in many tissues, and is a biological product in many enzyme-catalyzed metabolic reactions. In particular, glucose can be converted to gluconolactone under the catalysis of glucose oxidase (GOx), accompanied by the generation of H_2O_2 . By analyzing the amount of enzymatically-produced H_2O_2 , biological molecules of concern, such as specific enzymes and important substrates (e.g., glucose), may be quantified indirectly.

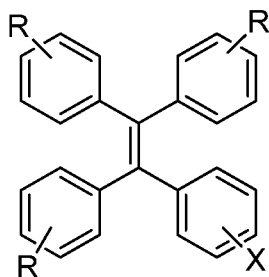
[12] Another important ROS is peroxynitrite (ONOO^-), which has attracted increasing attention from researchers due to its multiple implications in biological systems. Specifically, an elevated level of ONOO^- generation is a key fact of inflammation *in vivo*. As a protective immune response generated by organisms against pathogens or harmful stimuli, inflammation is becoming an increasingly popular topic, as it has portentous implications of various major diseases such as cancer, cardiopathy, diabetes, and Alzheimer's disease. Inflammation is a cardinal characteristic of ischemic heart disease and is a crucial mechanism in coronary artery disease progression, which takes place in pathologically vulnerable regions of the brains of

Alzheimer's patients as well. More importantly, it has been established that 15-20% of all cancers are preceded and induced by chronic inflammation. Consequently, detection and imaging of inflammation *in vivo* would be undoubtedly beneficial to the early diagnosis and prevention of carcinoma before metastasis and diffusion.

[13] Many fluorescent probes for ROS detection have been reported in the prior art, examples of which have been reported by HeeChol Kang (US 20130287689 A1), Jianghong Rao (US 20140004049 A1), and Allan Korsgaard Poulsen (WO 2008044138 A1). Generally, most prior art references face several problems, such as inferior sensitivity, complicated syntheses, susceptibility to environment, and limited working conditions in solutions.

SUMMARY

[14] In an embodiment, the present subject matter is directed to a long wavelength probe having aggregation induced emission characteristics comprising at least one fluorophore comprising a backbone structure having the formula:

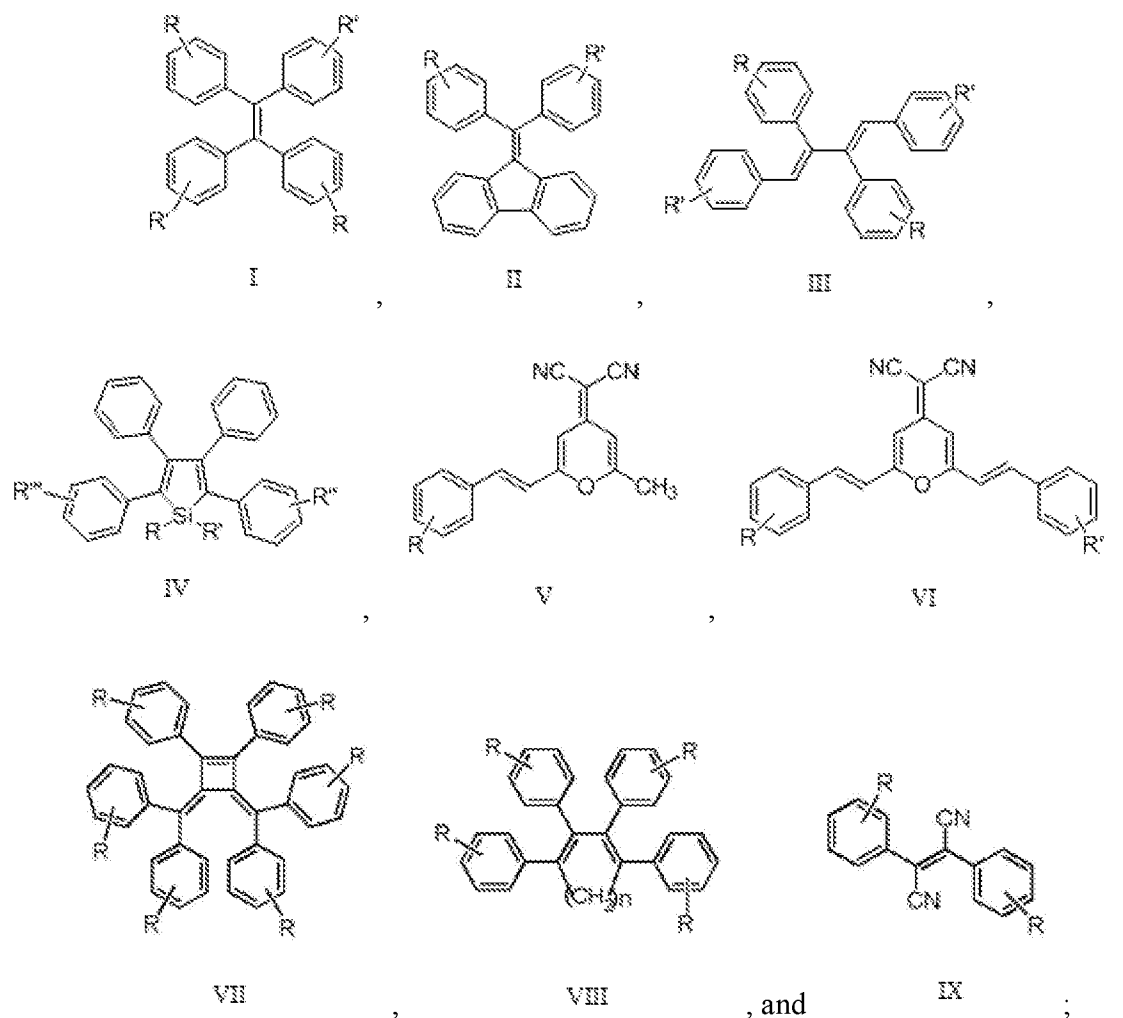


wherein

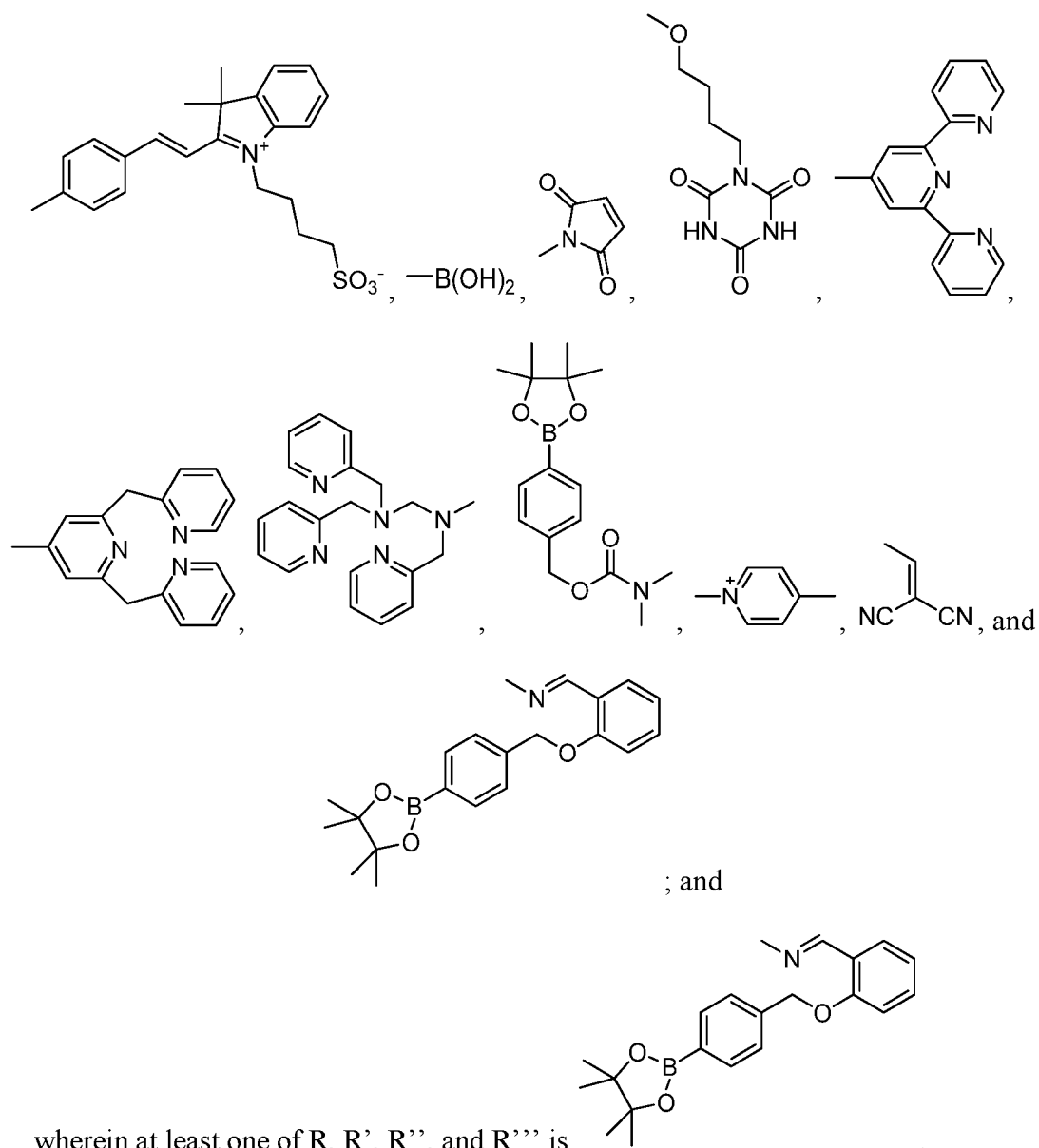
each R is independently selected from the group consisting of H, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl; and

X is at least one chromophore which can conjugate with at least one fluorophore.

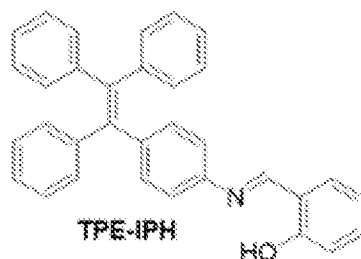
[15] In an embodiment, the present subject matter is directed to a highly sensitive and selective probe for H_2O_2 and ONOO^- detection comprising AIE luminogens comprising a backbone structure selected from the group consisting of:



wherein each R, R', R'', and R''' are independently selected from the group consisting of

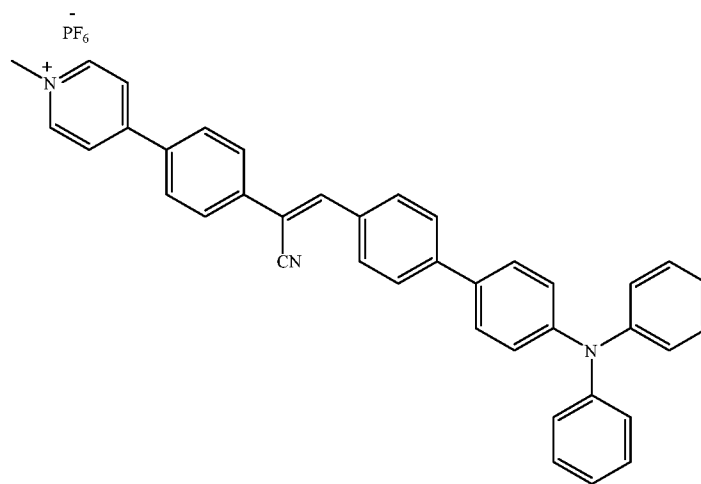


[16] In an embodiment, the present subject matter is directed to a highly sensitive and selective probe for H_2O_2 and ONOO^- detection comprising AIE luminogens comprising, as a backbone structure:



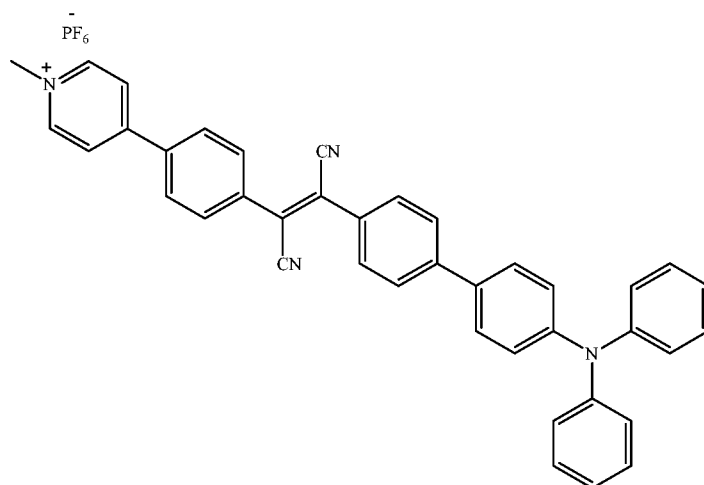
[17] In an embodiment, the present subject matter is directed to a method of preparing the probe of the present subject matter comprising fabricating nanoparticles of the AIE luminogens in a PEG matrix.

[18] In an embodiment, the present subject matter is directed to a probe for generating and/or tracking reactive oxygen species (ROS) under UV irradiation comprising red fluorescent AIEgens having the structure:



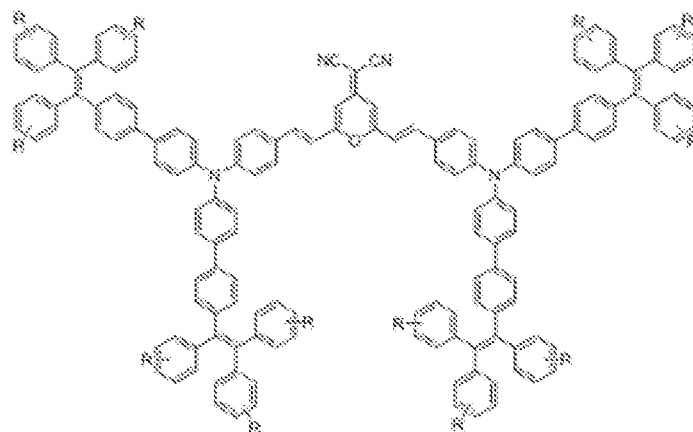
Compound 6c.

[19] In an embodiment, the present subject matter is directed to a probe for monitoring long-term morphology changes of a plasma membrane comprising red fluorescent AIEgens having the structure:



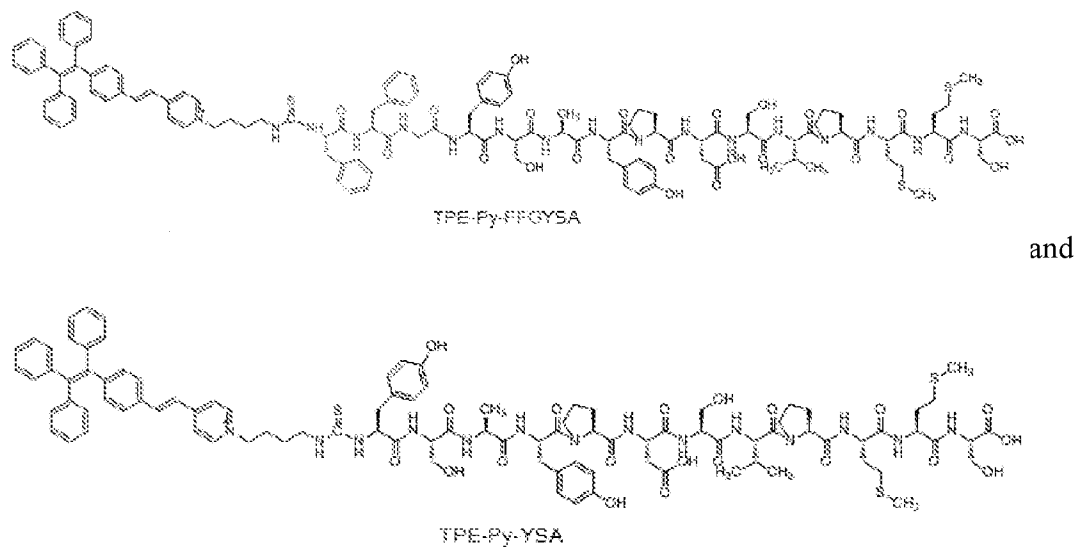
Compound 12.

[20] In an embodiment, the present subject matter is directed to a probe comprising near-infrared AIE luminogens comprising the structure:



TPE-TETRAD.

[21] In an embodiment, the present subject matter is directed to a probe for organelle targeting comprising red fluorescent AIEgens selected from the group consisting of



BRIEF DESCRIPTION OF DRAWINGS

[22] FIG. 1A-B show emission spectra of (A) TPE-In and (B) TPE-Ph-In in DMSO and DMSO/water mixtures with 99% water fractions (f_w). Dye concentration: 10 μ M; excitation wavelength: 450 nm. Inset: photographs of (A) TPE-In and (B) TPE-Ph-In in DMSO/water mixtures with f_w values of 0 and 99 vol% under 365 nm UV irradiation.

[23] FIG. 2 shows a plot of the relationship between fluorescent intensity and fluorophore concentration of Rh123, TPE-In and TPE-Ph-In in aqueous solution (1% DMSO).

[24] FIG. 3A-B shows plots of cytotoxicity of luminogens TPE-In and TPE-Ph-In evaluated on HeLa cells by MTT assay.

[25] FIG. 4 shows the signal loss (%) of fluorescent intensity of TPE-Ph-In and MT with increasing number of scans.

[26] FIG. 5A-B shows (A) the changes of emission intensity of HeLa cells stained with TPE-Ph-In (5 μ M) upon treated with 10 μ g/mL oligomycin and then 20 μ M CCCP. Excitation wavelength: 488 nm. Inset: snapshots of the cells in different period of time during the treatment

of stimulants. Scale bar: 20 μm . (B) The fluorescent intensity of the unstained blank HeLa cells, untreated TPE-Ph-In stained HeLa cells, oligomycin treated TPE-Ph-In stained HeLa cells and CCCP treated TPE-Ph-In stained HeLa cells analyzed by flow cytometry.

[27] **FIG. 6A-D** shows the flow cytometry analysis of HeLa cells (A), stained with 4 μM TPE-Ph-In for 30 min (B), treated stained cells with 10 $\mu\text{g/mL}$ oligomycin for 25 min (C) or 20 μM CCCP for 25 min (D). Excitation wavelength: 488 nm; emission wavelength: 585 ± 11.5 nm.

[28] **FIG. 7A-B** shows (A) ^1H NMR and (B) ^{13}C NMR spectra of TPE-IPB in CD_2Cl_2 .

[29] **FIG. 8A-B** shows (A) ^1H NMR and (B) ^{13}C NMR spectrum of TPE-IPH in CDCl_3 .

[30] **FIG. 9A-D** shows (A) UV spectra of TPE-IPH and TPE-IPB in acetonitrile. (B) PL spectra of TPE-IPB in acetonitrile/water mixtures with different fractions (f_w). Concentration: 20 μM . (C) PL spectra of TPE-IPH in acetonitrile/water mixtures with different fractions (f_w). Concentration: 20 μM . (D) a plot of relative PL intensity (I/I_0) at 549 nm versus the composition of the acetonitrile/water mixture of TPE-IPB.

[31] **FIG. 10A-E** shows (A) a time-dependent PL spectra of TPE-IPB in acetonitrile/buffer mixture (1:9, v/v; pH 10.0) in the presence of H_2O_2 (150 μM) at 37°C . (B) a plot of PL intensity of TPE-IPB in the presence and absence of H_2O_2 versus incubation time. Inset: photographs illumination of the solutions (a) before and (b) after incubation with H_2O_2 taken under 365 nm UV illumination. (C) PL spectra of TPE-IPB in acetonitrile/buffer mixture (1:9, v/v; pH 10.0) incubated with different concentrations of H_2O_2 for 40 min at 37°C . (D) a plot of PL intensity at 540 nm versus H_2O_2 concentration. Solution concentration: 40 μM ; excitation wavelength: 373 nm. (E) fluorescent photographs of TPE-IPB deposited on filter paper after immersed into buffer solutions (10 mM, pH = 10) with different concentrations of H_2O_2 .

[32] **FIG. 11** shows PL response of TPE-IPB in buffer solution (10 mM, pH = 10.0) buffer solution to different reactive oxygen species. $[\text{ClO}^-] = 100 \mu\text{M}$, $[\text{TBHP}] = 100 \mu\text{M}$, $[\text{O}_2^{\cdot-}] = 100 \mu\text{M}$, $\cdot\text{OH}$: 20 mg FeSO_4 in 0.1 M H_2O_2 , $[\text{ROO}\cdot] = 100 \mu\text{M}$, $[\text{}^1\text{O}_2] = 100 \mu\text{M}$, $[\text{ONOO}^-] = 100 \mu\text{M}$, $[\text{H}_2\text{O}_2] = 100 \mu\text{M}$.

[33] **FIG. 12A-B** shows (A) a plot of relative PL intensity (I/I_0) of TPE-IPB in acetonitrile/buffer mixture (1:9, v/v; pH 10.0) incubated with GOx (2 U/mL) and glucose versus concentrations of adscititious glucose; blue line: in acetonitrile/buffer mixture (1:9, v/v; pH 10.0); red line: in acetonitrile/buffer mixture with 1% FBS. Inset: linear calibration curve of glucose assay in the same condition. (B) PL response of TPE-IPB in acetonitrile/buffer mixture to different saccharides. Solution concentration: 40 μM ; incubation time: 1 h; incubation temperature: 37°C; $[\text{GOx}]$: 2 U/mL; $[\text{Gal}]$: 1 mM; $[\text{Man}]$: 1 mM; $[\text{Fru}]$: 1 mM; $[\text{Lac}]$: 0.5 mM; $[\text{Suc}]$: 0.5 mM; $[\text{Dex}]$: 0.18 mg/mL; $[\text{Glu}]$: 1 mM. Inset: photographs of solutions incubated with different saccharides taken under 365 nm UV illumination.

[34] **FIG. 13** shows PL intensity change of TPE-IPB (40 μM) at 536 nm upon incubation with 200 μM of ONOO^- (red bar) and H_2O_2 (green bar) at different pH values.

[35] **FIG. 14A-D** shows (A) a schematic illustration of TPE-IPB nanoprobe and their “turn-on” sensing of ONOO^- . (B) a photograph of TPE-IPB aggregates and TPE-IPB nanoprobe in PBS buffer (pH 7.4) taken under daylight. (C) size distribution of TPE-IPB nanoprobe and TPE-IPB aggregates in PBS buffer measured by DLS. (D) a TEM image of TPE-IPB nanoprobe.

[36] **FIG. 15A-D** shows (A) a plot of I/I_0 versus various RONS in PBS buffer (pH 7.4). I_0 and I are the PL intensities of TPE-IPB nanoprobe solution at the RONS concentrations of 0 and 400 μM , respectively. (B) PL spectra of TPE-IPB nanoprobe treated with different concentrations of ONOO^- . (C) time-dependent PL relative intensity (I/I_0) of TPE-IPB nanoprobe

and TPE-IPB aggregates upon addition of ONOO^- , respectively. I_0 and I are the PL intensities of nanoprobe/aggregate solution in the absence of RONS and at different time points after treatment with 400 μM of ONOO^- , respectively. (D) photostability comparisons among TPE-IPH nanoprobes, QD565, and FITC in MCF-7 cells under continuous irradiation for 10 min. I_0 and I are the initial PL intensity and the PL intensity of each sample at different time points.

[37] **FIG. 16A-B** shows (A) blood test parameters of the mice with (diagonal) and without (solid) TPE-IPB nanoprobe administration ($n = 4$ per group). No statistically significant differences were found between the treated and untreated groups. (B) typical images of H&E-stained liver, spleen and kidney slices from TPE-IPB nanoprobe-injected healthy mice.

[38] **FIG. 17A-E** shows (A) a schematic illustration of the AIE light-up nanoprobes applied for specific *in vivo* inflammation imaging. (B) *in vivo* non-invasive fluorescent images of *MRSA* infection-induced inflammation-bearing nude mice before and after intravenous injection of TPE-IPB nanoprobes for designated time intervals. The white circle indicates the inflammatory region. (C) *ex vivo* fluorescent images of various tissues of inflammation-bearing mice after treatment with TPE-IPB nanoprobes for 3 h. (D) semiquantitative analysis of fluorescence intensity in various tissues. * represents statistical significance ($P < 0.05$) versus other tissues. Data are presented as mean $\pm$ standard deviation ($n = 5$). (E) representative CLSM images of slices of infected and uninfected skin tissues from the TPE-IPB nanoprobe-treated mice. The blood vessels were immunostained by platelet/endothelial cell adhesion molecule 1 (PECAM-1, red fluorescence).

[39] **FIG. 18A-F** shows (A, B) *In vivo* non-invasive fluorescent images of both *MRSA* and *E. coli*-infected mice before and after (A) vancomycin and (B) penicillin treatment for designated time intervals. The TPE-IPB nanoprobes were intravenously injected into the mice prior to

antibiotic treatment and after treatment for 7 and 14 days, respectively. (C, D) Typical images of H&E-stained slices of infection regions from mice treated with (C) vancomycin and (D) penicillin for 14 days. (E, F) Typical CLSM images of slices of infection regions from mice treated with (E) vancomycin and (F) penicillin for 14 days. The TPE-IPB nanoprobe was injected on day 14 and the blood vessels were immunostained by PECAM-1.

[40] **FIG. 19** shows absorption spectra of Compound **6a** in DMSO solution.

[41] **FIG. 20** shows absorption spectra of Compound **6b** in DMSO solution.

[42] **FIG. 21** shows absorption spectra of Compound **6c** in DMSO solution.

[43] **FIG. 22** shows absorption spectra of Compound **12** in DMSO solution.

[44] **FIG. 23A-B** shows (A) PL spectra of Compound **6a** in different toluene fraction (f_t) in toluene/ DMSO mixture. (B) Plot of I/I_0 versus f_t . I = PL intensity of **6a** in pure DMSO solution at 650 nm; Concentration: 10 μ M; Ex.: 460 nm.

[45] **FIG. 24A-B** shows (A) PL spectra of Compound **6b** in different toluene fraction (f_t) in toluene/ DMSO mixture. (B) a plot of I/I_0 versus f_t . I = PL intensity of **6b** in pure DMSO solution at 650 nm; Concentration: 10 μ M; Ex.: 460 nm.

[46] **FIG. 25A-B** shows (A) PL spectra of Compound **6c** in different toluene fraction (f_t) in toluene/ DMSO mixture. (B) a plot of I/I_0 versus f_t . I = PL intensity of **6c** in pure DMSO solution at 620 nm; Concentration: 10 μ M; Ex.: 440 nm.

[47] **FIG. 26A-B** shows (A) PL spectra of Compound **12** in different toluene fraction (f_t) in toluene/ DMSO mixture. (B) a plot of I/I_0 versus f_t . I = PL intensity of **12** in pure DMSO solution at 720 nm; Concentration: 10 μ M; Ex.: 460 nm.

[48] **FIG. 27** shows absorption spectra of Compound **16** in THF solution.

[49] **FIG. 28** shows absorption spectra of Compound **17** in THF solution.

[50] **FIG. 29A-B** shows (A) PL spectra of Compound **16** in THF/water mixture with different water fractions (f_w). (B) a plot of relative PL intensities versus f_w . I_0 are the PL intensities at 580 nm of the dyes in THF; Dye concentration: 10 μ M; excitation wavelength: 410 nm.

[51] **FIG. 30A-B** shows (A) PL spectra of Compound **17** in THF/water mixture with different water fractions (f_w). (B) a plot of relative PL intensities versus f_w . I_0 are the PL intensities at 680 nm of the dyes in THF; Dye concentration: 10 μ M; excitation wavelength: 525 nm.

[52] **FIG. 31A-B** shows (A) UV spectra and (B) PL spectra of Compound **6a** mixed with different phospholipid, DNA and RNA in 1% DMSO in HEPES pH 7.4 buffer. Concentration: 10 μ M; Ex.: 460 nm.

[53] **FIG. 32A-B** shows PL spectra of Compound **6a** with DNA and treated with (A) DNase and (B) RNase in HEPES pH 7.4 buffer. Concentration: 10 μ M; Ex.: 460 nm.

[54] **FIG. 33A-E** shows confocal images of HeLa cell stained with (A and C) Compound **6a** and (B and D) SYTO RNAselect taken under continuous excitation. (E) FL signal loss of HeLa cell stained with ASCP or SYTO RNAselect with increasing no. of scan. **6a**: Ex.: 560 nm, Em: 650-750 nm; SYTO RNAselect : Ex.: 488, Em: 500-600 nm.

[55] **FIG. 34** shows MTT assay viability of a HeLa cell stained with different concentration of Compound **6a** for 8 h. Data are expressed as mean value of five separate trials.

[56] **FIG. 35** shows PL spectra in confocal images, where $\circ$: PL signals in lipid droplets; Δ : PL signals outside lipid droplets.

[57] **FIG. 36** shows nanoparticle platforms investigated and proposed applications.

[58] **FIG. 37A-B** shows (A) the molecular rotation of phenyl groups on TPE-TETRAD. (B) TETRAD solutions in THF/water mixtures containing different volume fractions of water. The photographs were taken under the illumination of a UV lamp.

[59] **FIG. 38A-B** shows (A) PL spectra of TPE-TETRAD in THF/water mixture with different water fractions (fw). (B) a plot of relative PL intensities (I/I_0) versus fw. I_0 are the PL intensities at 668 nm of the dyes in THF solutions; Dye concentration: 10 μ M; excitation wavelength: 488 nm. Inset: photographs of (A) TPE-TETRAD water fraction and (B) TPE-TETRAD thin film.

[60] **FIG. 39A-B** shows (A) ROS generation capabilities of TPE-TETRAD and (B) a MTT assay evaluating the ROS cytotoxicity of TPE-TETRAD PEG nanoparticles in ON/OFF white light conditions..

[61] **FIG. 40** shows a schematic diagram of MSN@AIE synthesis. Inset pictures reveal the particles fluorescence in solution and morphology using TEM.

[62] **FIG. 41A-B** shows (A) a dynamic light scattering hydrodynamic diameter comparison of TPE-TETRAD nanoaggregates and their MSN encapsulated counterparts, and (B) a photoluminescence emission spectrum of TPE-TETRAD nanoaggregates and the MSN encapsulated TPE-TETRAD nanoparticles.

[63] **FIG. 42** shows a schematic diagram of TPE-TETRAD/AuNP PEG nanoparticle conjugates. Inset pictures reveal the AuNPs morphology using TEM.

[64] **FIG. 43A-G** shows (A-C) Co-staining with Mito-tracker. (D-F) CLSM images show the intracellular ROS levels of A549 cancer cells received different treatments by using DCFH as the ROS indicator. (D) Probe +, Light -; (E) Probe +, Light +; (F) Probe +, Light +, NAC+. (G) Cell viabilities of A549 cells after various treatments indicated.

[65] **FIG. 44A-D** shows (A) a clonogenic formation upon different treatments. (B) the quantitative data for clonogenic assay of (A). ** represents $P < 0.01$. (C) a clonogenic formation after treatment with different popularly used radiosensitizer. (D) the quantitative data for clonogenic assay of (C). ** represents $P < 0.01$.

[66] **FIG. 45A-C** shows a Western blot analysis of (A) p-ERK, ERK, p-Akt and Akt as well as (B) Bcl-XL, Bcl-2, BAD, and Caspase-3 from A549 cells with various treatments indicated. (C) a schematic illustration of the pathway that indicates how Compound **6b** serves as an effective radiosensitizer to irradiation. **FIG. 45D** shows a Western blot analysis of p-ERK, p-Akt, Bcl-2, Bax and BAD with various treatments indicated.

[67] **FIG. 46A-B** shows overlay confocal images of HeLa cells stained with Compound **12** (3.5 μM) for 5 min before (pseudored color) and after (pseudogreen color) being incubated with (A) Hg^{2+} (100 μM) and (B) control for 40 min. Conditions: $\lambda_{\text{ex}} = 488 \text{ nm}$ and $\lambda_{\text{em}} = 600\text{-}750 \text{ nm}$

[68] **FIG. 47** shows the morphology changes of HeLa cells after trypsin treatment.

[69] **FIG. 48A-H** shows confocal images merged with bright field of HeLa cells stained with Compound **12** (3.5 μM) for 5 min, and without (A) and with (B-H) trypsin treatment in different times. Inset images: enlarged images without bright field. Conditions: λ_{ex} : 488 nm and λ_{em} : 600-750 nm.

[70] **FIG. 49** shows confocal images of HeLa cells co-stained with Compound **6c** (4 μM) and H2DCFDA (10 μM) under different irradiation times with 405 nm. Conditions: AIE-Mem-ROS: λ_{ex} : 405 nm, λ_{em} : 500-600 nm; PI: λ_{ex} : 488 nm, λ_{em} : 490-650 nm.

[71] **FIG. 50** shows confocal images of HeLa cells co-stained with Compound **6c** (4 μM) and PI (3 μM) under different irradiation times with 405 nm. Conditions: **6c**: λ_{ex} : 405 nm, λ_{em} : 500-600 nm; PI: λ_{ex} : 560 nm, λ_{em} : 580-740 nm.

[72] **FIG. 51** shows the change in fluorescent intensity at 650 nm in confocal images.

[73] **FIG. 52** shows cell viability of HeLa cells incubated with Compound **6c** in dark (Black) and ASCP-TPA pretreated with white light irradiation for 2 min and followed by in dark (grey).

[74] **FIG. 53A-C** shows (A) PL spectra of TPE-Py-NCS (10 μ M) in THF/hexane mixtures with different hexane fractions (f_H). (B) a plot of peak intensities *versus* f_H . (Inset) Photographs of TPE-Py-NCS in THF/hexane mixtures with different f_H taken under hand-held UV lamp with 365 nm illumination. (C) PL spectra of TPE-Py-FFGYSA (1 μ M) and TPE-Py-YSA (1 μ M) in PBS buffer with and without addition of PC-3 cell lysate. Excitation at 405 nm for (A-C).

[75] **FIG. 54A-D** shows CLSM images of (A) PC-3 cancer cells and (B) smooth muscle cells after staining with monoclonal anti-EphA2 antibody/Alexa Fluor 633-conjugated secondary antibody. (C) and (D) are the corresponding fluorescence/transmission overlay images of (A) and (B), respectively.

[76] **FIG. 55A-I** shows CLSM images of (A) TPE-Py-FFGYSA and (B) anti-EphA2 antibody/Alexa Fluor 633-conjugated secondary antibody co-stained PC-3 cancer cell. The cells were treated with TPE-Py-FFGYSA at 37°C for 90 min. (C) is the overlay image of (A) and (B). CLSM images of PC-3 cancer cells after incubation with TPE-Py-FFGYSA (D) at 0°C for 1 h, followed by further incubation of the cells at 37°C for (E) another 10 and (F) 60 min, respectively. (G-I) are the corresponding fluorescence/transmission overlay images of (D-F), respectively. [TPE-Py-FFGYSA] = 1 μ M for (A-I).

[77] **FIG. 56A-B** shows (A) a CLSM image of free YSA peptides (500 μ M) pre-treated PC-3 cancer cells after incubation with TPE-Py-FFGYSA (1 μ M) at 37°C for 90 min. (B) is the corresponding fluorescence/transmission overlay image of (A).

[78] **FIG. 57A-H** shows CLSM images of (A) smooth muscle cells and (B) PC-3 cancer cells after incubation with TPE-Py-FFGYSA at 37°C for 90 min. CLSM images of (C) PC-3 cancer cells and (D) smooth muscle cells after incubation with TPE-Py-YSA at 37°C for 90 min. (E-H) are the corresponding fluorescence/transmission overlay images of (A-D), respectively. [TPE-Py-FFGYSA] = [TPE-Py-YSA] = 1 μ M.

[79] **FIG. 58A-C** shows (A) the fluorescence intensity (FI) of DCF at 530 nm and (B) the relative absorbance of DPBF at 418 nm as functions of light irradiation time of TPE-Py-FFGYSA (1 μ M) in aqueous solution with and without addition of vitamin C (VC). (C) CLSM images show the intracellular ROS levels of PC-3 cells received different treatments by using DCF-DA as the ROS indicator. The cells were incubated with TPE-Py-FFGYSA (1 μ M) at 37°C for 90 min. Light irradiation (0.1 W cm⁻²) was performed for 2 min. [NAC] = 1 mM.

[80] **FIG. 59A-C** shows (A) cell viabilities of PC-3 cancer cells and smooth muscle cells received different treatments of TPE-Py-FFGYSA (1 μ M)/light irradiation for 48 h, respectively. (B) cell viabilities of TPE-Py-FFGYSA (1 μ M)-incubated PC-3 cancer cells after addition of 32 nM of Ptx for 24 h. Single light irradiation (0.1 W cm⁻², 2 min) were performed at 0, 3, 6, 9, or 12 h post Ptx addition. (C) cell viabilities of PC-3 cancer cells after addition of various concentrations of Ptx for 48 h. The PC-3 cells were received different treatments of TPE-Py-FFGYSA (1 μ M)/light irradiation. For (A) and (C), light irradiations (0.1 W cm⁻², 2 min) were performed three times at 12, 24, and 36 h post addition of Ptx (Ptx is 0 nM for (A)), respectively. Data are presented as mean $\pm$ s.d. for (A-C). ** in (B) and (C) represents $P < 0.01$ versus the Ptx alone group (Probe $\square$; light $\square$), respectively.

[81] **FIG. 60A-C** shows (A) a western blot analysis of various protein expressions in PC-3 cancer cells received different treatments. (B) a western blot analysis in the absence and presence

of NAC. (C) a schematic illustration of the proposed synergistic mechanism based on the western blot data.

DETAILED DESCRIPTION

Definitions

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

[83] 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.

[84] “Aggregation-induced emission” means the fluorescence/phosphorescence is turned on upon aggregation formation or in the solid state. When molecularly dissolved, the material is nonemissive. However, the emission is turned on when the intramolecular rotation is restricted.

[85] “Emission intensity” means the magnitude of fluorescence/phosphorescence normally obtained from a fluorescence spectrometer or fluorescence microscopy measurement.

[86] “Fluorophore” means a molecule which exhibits fluorescence.

[87] “Luminogen” means a molecule which exhibits luminescence.

[88] “AIEgen” means a molecule exhibiting AIE characteristics.

[89] 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.

[90] 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.

[91] 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.”

[92] 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.

Abbreviations

AEE	aggregation enhanced emission
AIE	aggregation-induced emission
ACQ	aggregation-caused quenching
ASCP	(Z)-4-(4-(1-cyano-2-(4-(dimethylamino)phenyl)vinyl)phenyl)-1-methylpyridin-1-ium hexafluorophosphate(V)
AuNP	gold nanoparticles
CAMs	cell adhesion molecules
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
CLSM	confocal laser scanning microscopy

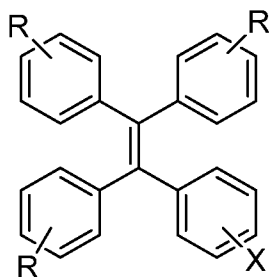
DCF	dichlorofluorescein
DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPBF	1,3-diphenylisobenzofuran
DPPE	1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine
<i>E. coli</i>	<i>Escherichia coli</i>
EPR	enhanced permeability and retention
FBS	Fetal Bovine Serum
FITC	fluorescein isothiocyanate
FR/NIR	far-red to near-infrared
GNP	gold nanoparticles
GOx	glucose oxidase
H&E	hematoxylin and eosin
H ₂ O ₂	hydrogen peroxide
HRMS	high-resolution mass spectroscopy
I/I_0	relative peak intensity
LC	liquid chromatography
LOD	detection limit
LPS	lipopolysaccharide
MADLI-TOF	matrix assisted laser desorption ionization time-of-flight
Mito-GFP	CellLight® Mitochondria-GFP
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MT	MitoTracker red FM
MTR	MitoTracker® Red CMXRos
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
NIR	near infrared
NMR	nuclear magnetic resonance
OLED	organic light-emitting diodes
ONOO ⁻	peroxynitrite
PBS	phosphate buffer saline
PEG	polyethylene glycol
PET	photo-induced electron transfer
PL	photoluminescence
PMA	phorbol 12-myristate 13-acetate
Ptx	paclitaxel
RAW264.7	macrophage cells
RIM	restriction of intramolecular motions
RIR	restriction of intramolecular rotations
RNA	ribonucleic acid
RNase	ribonuclease
RONS	reactive oxygen nitrogen species
ROS	reactive oxygen species

TBHP	tert-Butyl hydroperoxide
TEM	transmission electron microscope
TICT	Twisted Internal Charge Transfer
TOCL	Tetraoleoylcardiolipin
TPE	tetraphenylethene
TPE-IPH	2-(((4-(1,2,2-triphenylvinyl)phenyl)imino)methyl)phenol
TPE-TPP	tetraphenylethene-triphenylphosphonium
UV	ultraviolet
$\Delta\Psi_m$	membrane potential

Red Emitting Mitochondria-Targeted AIE Probe as an Indicator for Membrane Potential and Mouse Sperm Activity

[93] In an embodiment, the present subject matter relates to a red-emitting long wavelength luminogen and its use for staining mitochondria and monitoring the change of mitochondrial membrane potential and mouse sperm activity. In particular, the present subject matter relates to luminogens comprising TPE derivatives having AIE and AEE characteristics and their use in tracing the change of intracellular mitochondrial membrane potential and evaluating the sperm vitality. The present subject matter relates to cationic light-emitting materials comprising heterocycle-functionalized luminogens prepared via attachment of the heterocycle unit to the AIE unit through vinyl functionality. These cationic light-emitting materials exhibit long wavelength emission, as well as aggregation-induced emission.

[94] In one embodiment in this regard, the present subject matter is directed to a long wavelength probe having aggregation induced emission characteristics comprising at least one fluorophore comprising a backbone structure having the formula:

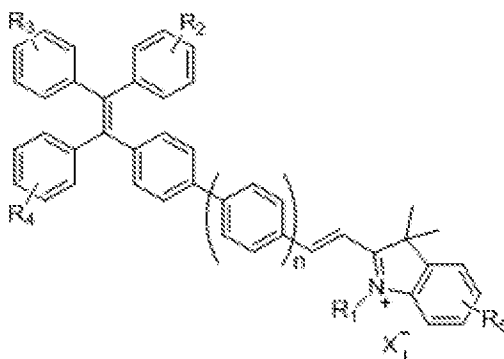


wherein

each R is independently selected from the group consisting of H, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl; and

X is at least one chromophore which can conjugate with at least one fluorophore.

[95] In an embodiment, the fluorogen of the present subject matter has a backbone structure of:

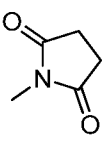


wherein

R_1 , R_2 , R_3 , R_4 , and R_5 are independently selected from the group consisting of C_nH_{2n+1} , $C_{10}H_7$, $C_{12}H_9$, OC_6H_5 , $OC_{10}H_7$, $OC_{12}H_9$, $C_nH_{2n}COOH$, $C_nH_{2n}NCS$, $C_nH_{2n}N_3$, $C_nH_{2n}NH_2$, $C_nH_{2n}Cl$,

$C_nH_{2n}Br$, $C_nH_{2n}I$, and $(CH_2)_nCOOR'$;

R' is independently selected from the group consisting of $C_nH_{2n}NCS$, $C_nH_{2n}N_3$, $C_nH_{2n}NH_2$,

$C_nH_{2n}Cl$, $C_nH_{2n}Br$, $C_nH_{2n}I$ and ;

X_1 is independently selected from the group consisting of I, Cl, Br, PF_6 , ClO_4 , BF_4 , BPh_4 , and CH_3PhSO_3 ; and

$n = 0$ to 20.

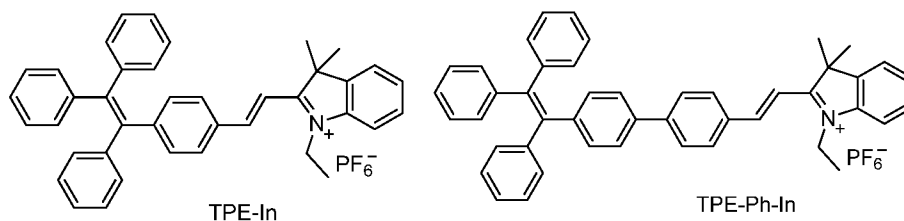
[96] In an embodiment, the probe of the present subject matter is used to label

mitochondria in living cells. In an embodiment, the probe of the present subject matter is used to indicate a change in mitochondrial membrane potential. In an embodiment, the probe of the present subject matter is used *in situ* to monitor a change of $\Delta\Psi_m$ in living cells. In an embodiment, the probe of the present subject matter is used to evaluate sperm vitality by monitoring membrane potential differences in mouse sperm cells and sperm activity.

[97] In an embodiment, the specific luminogen TPE-In emits weakly at 694 nm in DMSO. When the water fraction reached 99% in the solvent mixture, stronger red fluorescence was observed. The AIE effect is even more obvious for TPE-Ph-In (FIG. 1). The emission of TPE-Ph-In is enhanced about 70 times upon aggregates formation. Both TPE-In and TPE-Ph-In are AIE active and are therefore free of the self-quenching problem encountered by most conventional mitochondria probes, such as Rh 123 (FIG. 2).

[98] In an embodiment, the cytotoxicity of the two luminogens on HeLa cells was assessed using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. As illustrated in FIG. 3, the cell viability is significantly reduced when 5.0 μM TPE-In is added to the HeLa cells. In contrast, TPE-Ph-In shows almost no cytotoxicity. Even when the concentration of TPE-Ph-In in the culture medium is as high as 8.0 μM , the cell growth is not obviously affected. The planarity of TPE-In may facilitate the molecules to interact with DNA and thus lead to higher cytotoxicity.

[99] TPE-In exhibits better planarity: one of the phenyl rings on the TPE unit is coplanar with the indolium unit, whereas TPE-Ph-In adopts more of a twisted configuration. The chemical structures of TPE-In and TPE-Ph-In are as follows:



[100] Due to its configuration, TPE-Ph-In was chosen for a cell staining test. Pre-experiments showed the dye to be cell-permeable and able to stain mitochondria specifically. Compared with TPE-TPP (tetraphenylethene-triphenylphosphonium), a UV-excited AIE mitochondrial probe with blue-emission, TPE-Ph-In can be excited with a 488 nm laser and therefore is more compatible with confocal microscopy and manifests higher signal-to-noise ratio. To test the mitochondrial selectivity of TPE-Ph-In, a co-localization experiment was performed with commercial CellLight® Mitochondria-GFP (Mito-GFP), a green fluorescent protein targeted to mitochondria. The stained cells give out red fluorescence from TPE-Ph-In and green fluorescence from Mito-GFP, respectively. Merged imaging shows that the distribution of TPE-Ph-In in cells is totally consistent with that of Mito-GFP, indicating the high selectivity of TPE-Ph-In towards mitochondria.

[101] Furthermore, an advantage of utilizing a small organic dye over a fluorescent protein for cell staining was revealed. In the co-staining experiment, it was observed that all the cells are uniformly stained with the red emitting TPE-Ph-In, while in the green channel of Mito-GFP, several cells emit obviously weaker than others in some image fields. This is attributed to the inhomogeneous transfection rate and the different expression levels of Mito-GFP in cells, which is also mentioned in the Mito-GFP user manual. To investigate the photostability of TPE-Ph-In, the TPE-Ph-In stained cells were continuously scanned by a confocal microscope. After 40 scans, the signal loss of TPE-Ph-In was less than 10% of the original intensity, and no significant

difference was observed between the first and the 40th fluorescent images. However, when MitoTracker red FM (MT), a commercially red emission mitochondria probe, was tested under the same excitation power (FIG. 4), the fluorescence signal loss of MT reached up to 90% of the initial value and almost no fluorescence remained after only 8 scans. Undoubtedly, TPE-Ph-In possesses much higher photostability than commercial MT, implying its capability in long-term mitochondrial imaging and morphological analysis.

[102] The $\Delta\Psi_m$ is the major driving force for cationic lipophilic dyes to enter and stain the mitochondria, leading to the possibility that the mitochondrial function could be evaluated by a $\Delta\Psi_m$ sensitive probe. To examine the response of TPE-Ph-In towards $\Delta\Psi_m$ changes, membrane-potential stimulants, oligomycin and carbonyl cyanide 3-chlorophenylhydrazone (CCCP), were applied to increase or decrease the $\Delta\Psi_m$ prior to the staining process, respectively. When the cells were treated with oligomycin, significant enhancement of the red fluorescent signals was observed. Once the cells were treated with CCCP, TPE-Ph-In could no longer effectively accumulate in the mitochondria, resulting in the decrease of the fluorescence signals. Results demonstrate that the accumulation of TPE-Ph-In in the mitochondria depends on the $\Delta\Psi_m$. More importantly, the fluorescence signals of TPE-Ph-In can directly represent the $\Delta\Psi_m$ based on the positive correlation between the fluorescent intensity and the local dye concentration in mitochondria, which is difficult to achieve for traditional dyes suffering from the concentration quenching effect.

[103] To further examine the feasibility of using TPE-Ph-In to follow the real-time *in situ* change of $\Delta\Psi_m$ in living cells, different stimulants were added sequentially and the fluorescent intensity and imaging was observed by confocal microscope. HeLa cells were first stained with 5 μ M TPE-Ph-In for 30 min (FIG. 5A). The red emission from mitochondria was observed. The

fluorescence signal was increased by 1.5-fold when the cells were treated with 10 $\mu\text{g/mL}$ oligomycin. The enhanced emission from mitochondria can be retained for a period of time. Further addition of 20 μM CCCP into the medium immediately lead to a drastic decrease of emission intensity. As mentioned above, the treatment of oligomycin and CCCP can lead to the increase and decrease of $\Delta\Psi_{\text{m}}$, respectively, which is reflected on the change of the fluorescence signal of TPE-Ph-In. Because of its high signal-to-noise ratio and low background, no washing step is required during the entire process, thus providing a convenient method for tracing the micro-environment changes in living cells.

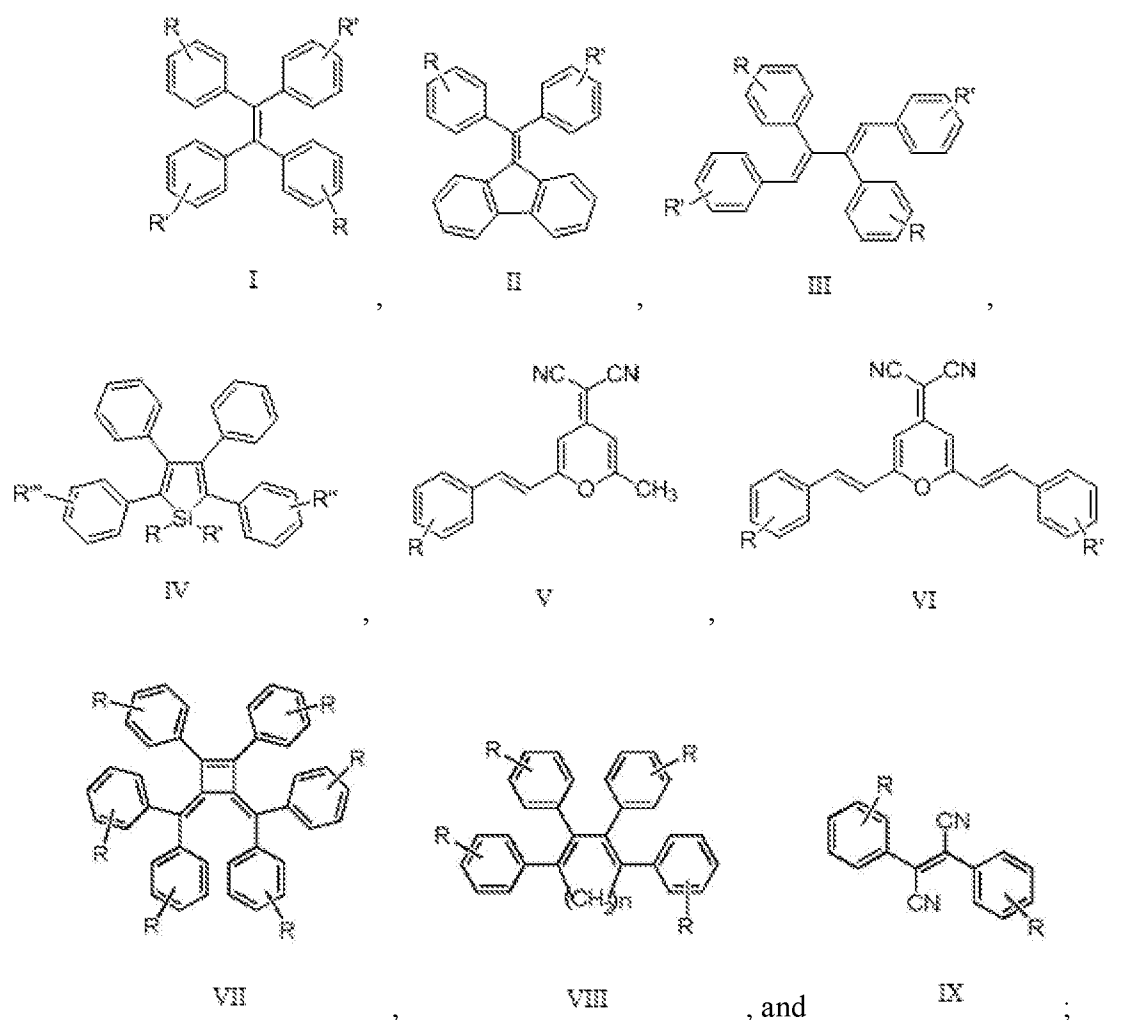
[104] In order to explore the application of TPE-Ph-In for high-throughput analysis, flow cytometry was added to the study. HeLa cells stained with 4 μM TPE-Ph-In for 30 min (FIG. 6B) exhibited a mean fluorescent intensity of about 2866, which can be easily differentiated from the unstained cells (FIG. 6A). In accordance with the results of the confocal images, a slight increase in the mean fluorescent intensity was detected when the cells were treated with oligomycin for 25 min (FIG. 6C). In sharp contrast, after incubation of TPE-Ph-In stained cells and CCCP for 25 min, the mean fluorescent intensity was decreased 5.4-fold (FIG. 6D), which is also consistent with the results collected by confocal microscopy. The mean intensity is plotted in FIG. 5B. The use of flow cytometry thus offers a high-throughput and quantitative analysis manner for TPE-Ph-In to probe cells in different environments.

[105] The good biocompatibility and the membrane potential-dependent fashion of TPE-Ph-In inspired exploration of its feasibility to evaluate sperm vitality. Mouse sperm cells were stained with 5 μM TPE-Ph-In for 1 h. Under the fluorescent microscope, the midpieces of sperms presented various degrees of fluorescence intensity. To gain further insight into this phenomenon, the dynamic motion of sperms stained with TPE-Ph-In was tracked and recorded. Results reveal

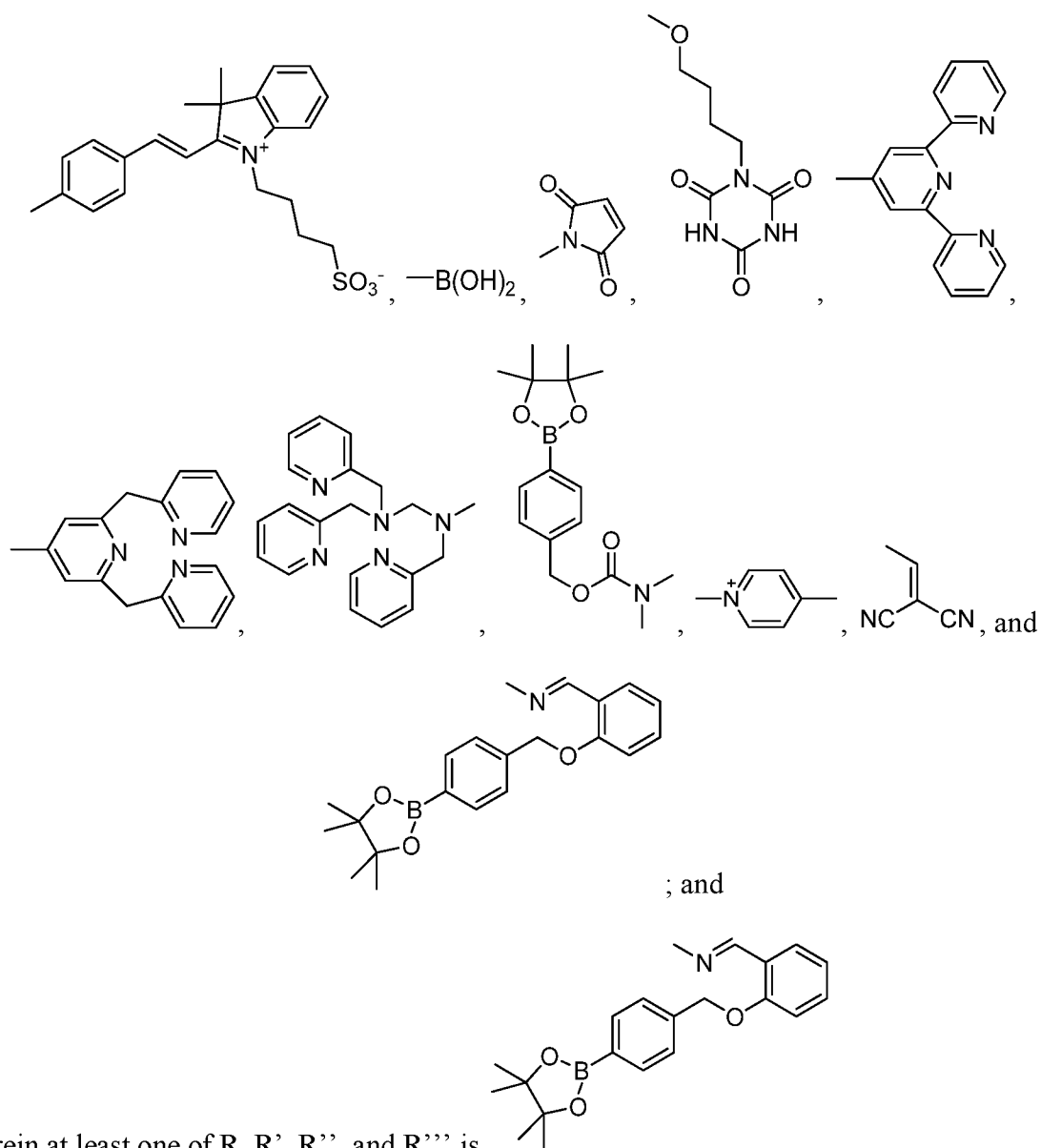
the bright red fluorescence comes from the energetic sperm, while the non-vital sperm only gave faint red fluorescence and even non-fluorescence. The fluorescence intensity in the TPE-Ph-In stained mitochondria reflects the mitochondrial mobility, suggesting TPE-Ph-In is a promising fluorescent probe for monitoring the function of sperm.

AIE-Active Fluorescent Probes for ROS Detection and Related Biological Applications

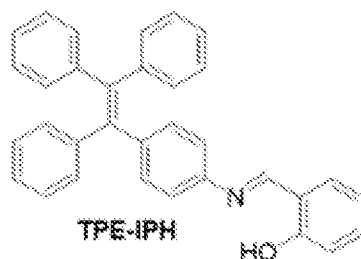
[106] In a further embodiment, the present subject matter is directed to a highly sensitive and selective probe for H_2O_2 and ONOO^- detection comprising AIE luminogens comprising a backbone structure selected from the group consisting of:



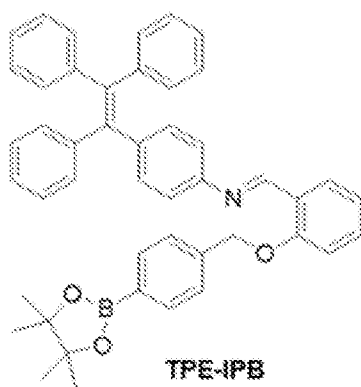
wherein each R, R', R'', and R''' are independently selected from the group consisting of



[107] In an embodiment, the present subject matter is directed to a highly sensitive and selective probe for H_2O_2 and ONOO^- detection comprising AIE luminogens comprising, as a backbone structure:



[108] In another embodiment, the present subject matter is directed to a highly sensitive and selective probe for H_2O_2 and ONOO^- detection comprising AIE luminogens comprising, as a backbone structure:



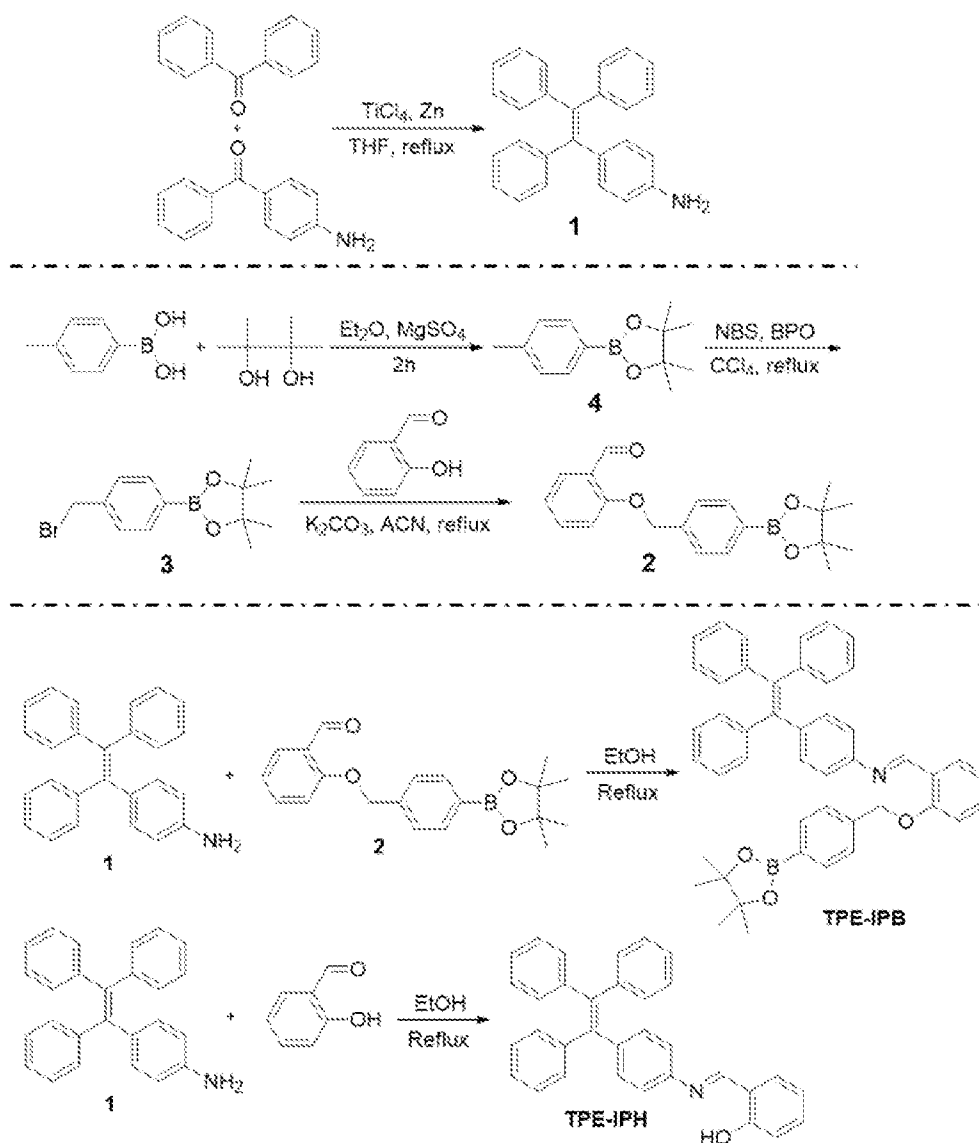
[109] In an embodiment, the probe of the present subject matter is used for sensing glucose in buffer solutions and serum samples. In an embodiment, the probe of the present subject matter may be in an aggregated state or solid state. In an embodiment, the AIE luminogens are used as imaging agents for inflammation *in vivo*.

[110] In an embodiment, the present subject matter is directed to a method of preparing the probe of the present subject matter comprising fabricating nanoparticles of the AIE luminogens in a PEG matrix.

[111] Accordingly, the present subject matter further relates to a model compound demonstrating feasibility and advantages of AIE probes for ROS detection, particularly the probes TPE-IPB and TPE-IPH. TPE-IPB consists of three parts: TPE as a fluorophore, imine as

an emission mediator, and phenyl boronic ester as the ROS recognition site. The probe is non-emissive in both the solution state and aggregation state, but emits strong yellow fluorescence in the presence of H_2O_2 or ONOO^- . Since H_2O_2 can be generated by the oxidation reaction of D-glucose catalyzed by GOx, the probe is capable of sensing glucose concentration indirectly through quantifying enzymatically-produced H_2O_2 . TPE-IPB nanoprobe can serve as safe probes for imaging inflammation *in vivo* in a selective and high-contrast manner, which also shows a unique merit in visualizing *in vivo* treatment efficacy of anti-inflammatory agents.

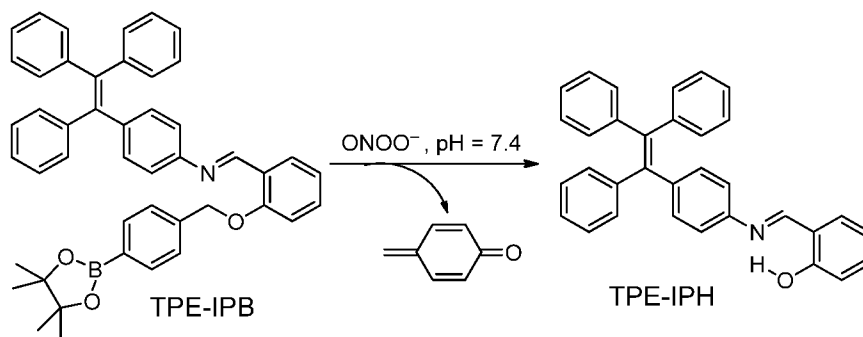
[112] The synthetic route to TPE-IPB and TPE-IPH are as follows:



[113] TPE-IPB was synthesized *via* condensation reaction between TPE- NH_2 (**1**) and phenyl boronic ester modified benzaldehyde (**2**) in mild conditions. The chemical structure of TPE-IPB was confirmed by standard spectroscopic techniques including NMR and HRMS (FIG. 7A-B) with exact mass of 667.3258.

[114] While TPE is a well-known molecule for its facile synthesis, high quantum efficiency and good photostability in the aggregate/solid state, how to regulate the emission of TPE, especially at the molecular level, remains challenging. As shown herein, the imine group ($\text{C}=\text{N}$)

is able to quench the TPE emission. Two theories would explain the quenching mechanism. One is the process of photo-induced electron transfer (PET) which retards radiative decay through electron transferring from donor orbital to acceptor orbital. The other is the *cis-trans* isomerization process of C=N which serves as a channel for non-radiative decay from excited states to ground states. Consequently, TPE-IPB is deactivated in the aggregate state due to the existence of imine. Moreover, the moiety of phenylboronicpinacol ester, acting as a cleavable group to respond to ROS, is also incorporated into TPE-IPB. In the presence of ROS, particularly H_2O_2 and ONOO^- , the phenylboronicpinacol ester will be cleaved through oxidative reaction followed by the release of a small molecule. At neutral conditions, the residue after reaction of TPE-IPB with ONOO^- or H_2O_2 will be protonated and converted to 2-(((4-(1,2,2-triphenylvinyl)phenyl)imino)methyl)phenol (TPE-IPH). The design rational of the TPE-IPB for H_2O_2 and ONOO^- detection is as follows:



[115] Regarding TPE-IPH, it is hypothesized that intramolecular hydrogen bonding between the proton of the hydroxyl group and the lone pair of nitrogen electrons on the imine will be formed. On the basis of the above-mentioned two theories, the formation of intramolecular hydrogen bonding will enable the electron lone pair of nitrogen atom to be both localized and anchored, while fixing the imine conformation, leading to the significant inhibition of the imine quenching effect. Furthermore, it is also assumed that TPE-IPH will possess an AIE

signature; that is, despite of the formation of intramolecular hydrogen bonding, TPE-IPH remains non-fluorescent in dilute solution due to the dynamic rotations of the phenyl rings but emits intensely in the aggregate state by a RIM mechanism.

[116] To verify the hypotheses, the TPE-IPH was chemically synthesized according to the synthetic route, as depicted above. The purity and identity of the synthesized TPE-IPH were confirmed by NMR and HRMS (FIG. 8) with an exact mass of 451.1936. TPE-IPH is the expected product of TPE-IPB after reaction with H_2O_2 , the optical properties of both TPE-IPH and TPE-IPB were first investigated.

[117] As shown in FIG. 9A, the absorption spectra of TPE-IPH and TPE-IPB in acetonitrile display their absorption maximums at 373 nm and 347 nm, respectively. The bathochromic absorption of TPE-IPB is induced by intramolecular hydrogen bonding, which fixes the conformation of the C=N structure and makes the two phenyl rings connected with imine more planar, leading to better conjugation of the whole molecule. Since both TPE-IPH and TPE-IPB contain the TPE moiety, their AIE features were further examined in acetonitrile/water mixtures. Regarding TPE-IPB, when adding water into its acetonitrile solution, the photoluminescence (PL) intensity was maintained at a low level and the spectra showed no obvious peak (FIG. 9B). This should be ascribed to a PET effect or a *cis/trans* isomerization process of the unanchored imine group, which quenches the emission of TPE even in the aggregation state. With increasing the water content in acetonitrile/water mixture from 0 to 60%, the PL intensity of TPE-IPH remained quite weak. However, further addition of water into the mixture resulted in a sharp and significant enhancement of PL intensity. At 90 vol% water content, the yellow fluorescence was more than 30-fold stronger than that in the pure acetonitrile solution (FIG. 9C and 9D), which clearly indicates TPE-IPH is AIE-active.

[118] Fluorescence detection of H₂O₂ and glucose

[119] First the incubation time for the reaction-based cleavage process from TPE-IPB to TPE-IPH was optimized. The PL spectra of TPE-IPB were recorded every 2 min upon addition of 150 μ M of H₂O₂. As presented in FIG. 10A and 10B, the fluorescence at 540 nm underwent a gradual increase with incubation time while the control group without addition of H₂O₂ remained almost non-emissive. The PL intensity reached the plateau when incubated with H₂O₂ for 40 min, demonstrating the cleavage reaction approached to a saturated state.

[120] Since TPE-IPB can be stoichiometrically converted to TPE-IPH by H₂O₂, this opened the possibility of establishing a working curve for the quantitative detection of H₂O₂. Accordingly, experiments were conducted with different concentrations of H₂O₂. As shown in FIG. 10C, the PL intensity at 540 nm increased along with concentration of H₂O₂ from 0 to 200 μ M. The corresponding peak intensities versus the concentration of H₂O₂ are plotted in FIG. 10D, which displays a linear relationship in the range from 0 to 100 μ M with the square of correlation coefficient equal to 0.999. Meanwhile, the detection limit (LOD) of H₂O₂ is estimated to be 100 nM (S/N = 3 and n = 11). As selectivity is another important parameter for sensing technic, PL responses of TPE-IPB to other ROS were further examined. FIG. 11 illustrates that H₂O₂ induced significant enhancement of PL intensity while ONOO⁻ had some interference on the detection system because phenyl boronicpinacol ester also responds to ONOO⁻. Other ROS including hypochlorite, tert-Butyl hydroperoxide (TBHP), singlet oxygen and hydroxyl radical etc. hardly increase the PL intensity of TPE-IPB at the same conditions.

[121] In consideration of the good performance of TPE-IPB in suspending aggregation for H₂O₂ detection, the possibility of H₂O₂ sensing in the solid state was also considered. Filter paper deposited by TPE-IPB was immersed into buffer solutions (pH 10.0) with different

concentration of H_2O_2 and taken out after incubation for 40 min. It was obviously noticed that higher concentrations of H_2O_2 rendered the filter paper more emissive (FIG. 10E), indicating that test paper deposited by TPE-IPB could potentially act as a H_2O_2 reporter. This phenomenon demonstrated the broad adaptability of TPE-IPB to working conditions due to the AIE property of TPE-IPH.

[122] Since the quantitative detection of H_2O_2 was realized successfully, a cascade of applications based on TPE-IPB is possible. For example, it is well known that D-glucose will be oxidized to gluconolactone in the presence of GOx accompanied by the generation of H_2O_2 . Moreover, the quantity of H_2O_2 produced in the process is stoichiometrically proportional to the amount of glucose. Thus TPE-IPB could indirectly sense D-glucose when GOx coexist in the probe solution through quantifying the concentration of H_2O_2 . As expected, the PL intensity of TPE-IPB enhances when the concentration of glucose increases and the saturated intensity is more than 10-fold than the initial level. The relative peak intensity (I/I_0) is plotted in FIG. 12A (white dots), which shows a good linear relationship with glucose concentration in the range from 0 to 200 μM .

[123] Another possible application is diagnostics for diabetes mellitus. Normally, the concentration of fasting blood-glucose in human serums is fluctuant from 3.6 mM to 6.1 mM. The postprandial glucose level may go up but is usually less than 10 mM. If the glucose levels exceed 11 mM, this quite possibly indicates a diabetes patient. To avoid limits in detecting the glucose concentration of serum samples, 1% Fetal Bovine Serum (FBS) was added into the buffer solution to mimic the real serum environment and to dilute the glucose concentration as well. The result is depicted in FIG. 12A (solid dots). Compared to the white dot curve (in buffer), the solid dot curve (1% FBS) shows almost the same assay range (0 to 200 μM) and similar slope

(~0.04). The only difference is the larger intercept of the solid dot curve, which means FBS contains glucose. The result demonstrates TPE-IPB owns excellent anti-interference ability and performs well in a serum environment. It also proves the reliability of this assay method. Besides, the selectivity of TPE-IPB toward glucose was examined among different saccharides. As shown in FIG. 12B, about 15 fold PL enhancement was observed in the probe solution with glucose while other saccharides such as galactose, mannose, fructose, lactose, sucrose and dextran only induced tiny enhancement, which illustrates the superior selectivity of TPE-IPB to glucose.

[124] Fluorescence detection of ONOO⁻

[125] Inspired by the selectivity test to different ROS, TPE-IPB may also respond to ONOO⁻ as it induces considerable enhancement on the fluorescence of TPE-IPB. Thus the pH effect on the PL responses of TPE-IPB to H₂O₂ and ONOO⁻ was investigated. As shown in FIG. 13, ONOO⁻ can turn on the fluorescence of TPE-IPB at physiological conditions (pH 7.4) and buffer basicity can promote the cleavage reaction leading to higher enhancement, while H₂O₂ can only activate TPE-IPB at strong basic conditions (pH 9.0-10.0). This result indicates ONOO⁻ shows higher reactivity with TPE-IPB. Based on this finding, TPE-IPB could potentially also be applied for ONOO⁻ detection. Accordingly, TPE-IPB was formulated utilizing biocompatible lipid-PEG₂₀₀₀ as the encapsulation matrix, affording TPE-IPB-loaded lipid-PEG₂₀₀₀nanoprobes (TPE-IPB nanoprobes). During the nanoprobe formation, the hydrophobic lipid and TPE-IPB molecules entangled with each other, and the resultant aggregates became the core of the nanoprobes.

[126] On the other hand, the hydrophilic PEG chains stretched into the aqueous phase and thus acted as an outer layer, stabilizing the nanoprobes (FIG. 14A). Using bare TPE-IPB aggregates without any matrix encapsulation as a control, FIG. 14B shows the photograph of

TPE-IPB aggregates and TPE-IPB nanoprobe in PBS buffer at the same fluorogen concentration (40 μM). The PBS solution of TPE-IPB aggregates appeared blue-white and a bit turbid. In comparison, the PBS solution of TPE-IPB nanoprobe was clearer, revealing that the lipid-PEG matrix can help TPE-IPB better dissolve in an aqueous solution. The dynamic light scattering (DLS) results revealed that TPE-IPB aggregates have a wide size distribution with an average size of ~ 377 nm in PBS buffer, while the TPE-IPB nanoprobe has much smaller hydrodynamic diameters of ~ 34 nm and a narrower distribution (FIG. 14C). The morphology of TPE-IPB nanoprobe was studied by transmission electron microscopy (TEM). As shown in FIG. 14D, the TPE-IPB nanoprobe was uniform and spherical in shape with a diameter of ~ 30 nm. In addition, unlike TPE-IPB aggregates that are prone to forming precipitation in PBS buffer, the TPE-IPB nanoprobe exhibited outstanding colloidal stability, as evidenced by their stable size distribution, even after 2 weeks in PBS buffer.

[127] The response of TPE-IPB nanoprobe to ROS was subsequently investigated. A variety of ROS including TBHP, ClO^- , $\cdot\text{OH}$, $\text{ROO}^\cdot$, $\text{O}_2^{\cdot-}$, H_2O_2 and ONOO^- were co-incubated with TPE-IPB nanoprobe in PBS buffer at pH 7.4, respectively. Of note, only ONOO^- can largely switch on the nanoprobe fluorescence, whereas other reactive oxygen nitrogen species (RONS) induce negligible changes on the emission intensity (FIG. 15A). This result demonstrates that TPE-IPB nanoprobe is highly selective for ONOO^- detection at physiological pH. FIG. 15B exhibits the PL spectra of TPE-IPB nanoprobe in PBS buffer at pH 7.4 upon addition of various amounts of ONOO^- . The PL intensity of the nanoprobe at 538 nm almost linearly enhances with the increase of ONOO^- concentration from 100 to 400 μM . Compared with the emission of nanoprobe itself in PBS buffer, a 20-fold PL enhancement is observed after treatment of TPE-IPB nanoprobe with 400 μM of ONOO^- . This result suggests

that ONOO^- is able to turn on the TPE-IPB nanoprobe fluorescence with satisfactory signal-to-background ratio, which results in highly emissive TPE-IPH nanoprobes.

[128] The kinetic studies were also performed by incubation of TPE-IPB nanoprobes or TPE-IPB aggregates with ONOO^- (400 μM) in PBS buffer at 37°C. FIG. 15C displays the time-dependent variations in the maximum PL intensity. Upon addition of ONOO^- , the TPE-IPB nanoprobe fluorescence dramatically switched on, which reached a plateau in 20 min with around 20-fold PL enhancement. In contrast, the PL intensity of TPE-IPB aggregates only elevated 5 times, for more than 40 min. This result reveals that TPE-IPB nanoprobes possess much faster ONOO^- response speed and larger fluorescent “turn-on” ratio than the aggregates, which should be attributed to their smaller particle size and higher surface area. This also highlights the necessity and importance of employing the lipid-PEG matrix. The photostability of the resultant emissive TPE-IPH nanoprobes was assessed with commercially available QD565 and fluorescein isothiocyanate (FITC) as the references. The fluorescence changes of the nanoprobe, QD565 or FITC-treated MCF-7 cancer cells were monitored under continuous laser scanning for 10 min. As shown in FIG. 15D, the TPE-IPH nanoprobes only bear ~7% PL intensity loss after continuous irradiation for 10 min, which is comparable to that of QD565 (~5% loss) and much better than the performance of FITC (~53% loss). As QDs are well known for their strong photobleaching resistance, this result demonstrates the high photostability of the fluorescent nanoprobes. On the basis of the above results together, it is reasonable to conclude that the TPE-IPB nanoprobes can indeed serve as an AIE light-up nanoprobe for specific ONOO^- detection.

[129] **Specific *in vivo* imaging of inflammation**

[130] It is known that immune cells such as macrophages would migrate to the inflammatory region and activate to release a large amount of RONS including ONOO^- . Hence, the TPE-IPB nanoprobe was utilized for detecting endogenously generated ONOO^- in macrophage cells (RAW264.7) before *in vivo* applications. To induce the elevated generation of RONS such as ONOO^- and ClO^- , the macrophages were successively treated with bacterial cell wall lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA). The macrophages with and without LPS/PMA stimulation were subsequently incubated with TPE-IPB nanoprobe, respectively, followed by imaging with confocal laser scanning microscopy (CLSM). The unstimulated macrophages exhibited similar fluorescence intensity to the cell background, while the LPS/PMA-treated macrophages contained intense fluorescence signals, indicating that the fluorescence of TPE-IPB nanoprobe can dramatically light up in macrophages under conditions related to inflammation. Furthermore, *N*-acetylcysteine (NAC) was employed to block the produced ROS in LPS/PMA-treated macrophages. After RONS scavenging by NAC, the fluorescence signals in the nanoprobe-incubated cells were significantly reduced, verifying that the fluorescence "turn-on" roots in the specific interaction between TPE-IPB nanoprobe and the endogenously generated ROS.

[131] Furthermore, the *in vivo* toxicity of TPE-IPB nanoprobe was evaluated. The nanoprobe was intravenously administrated into healthy mice with the concentration 5 times higher than that used for the following *in vivo* imaging experiment. The data including mouse body weight changes, the blood chemistry tests as well as the histological analyses of important organs (liver, spleen and kidney) reveal that the TPE-IPB nanoprobe is quite safe for *in vivo* applications (FIG. 16).

[132] The application of the above described AIE light-up nanoprobe for *in vivo* inflammation imaging was next examined. As the blood vessels are permeable and the activated immune cells produce substantial amounts of ONOO^- in the inflammation area, the TPE-IPB nanoprobe will preferentially accumulate to the inflammatory region by EPR effect, where the elevated generation of ONOO^- will significantly light up the nanoprobe fluorescence, resulting in imaging of the inflammation *in vivo* in a selective and high-contrast manner (FIG. 17A).

[133] To validate these nanoprobe can indeed provide a new opportunity for specific *in vivo* inflammation imaging, bacterial infection-induced inflammation-bearing nude mice were used as model animals, which were established by subcutaneous inoculation of methicillin-resistant *Staphylococcus aureus* (MRSA) into the left back of mice. The activation of an inflammatory response localized to the infection region was verified by the histological analysis. After intravenous injection of TPE-IPB nanoprobe, the inflammation-bearing mice were imaged by a Maestro EX *in vivo* imaging system with removal of mouse autofluorescence via spectral unmixing. As shown in FIG. 17B, no detectable fluorescence signal was observed before nanoprobe injection, whereas explicit fluorescent delineation of inflammatory region with negligible fluorescence signals in any other tissues was achieved at 0.5 h post nanoprobe administration. The fluorescence signal at the inflammatory site also increased over time and became extremely intense after intravenous injection of TPE-IPB nanoprobe for 3 h. It is encouraging that there has never been any detectable fluorescence signal observed in other mouse tissues post nanoprobe administration.

[134] The excellent selectivity of the AIE light-up nanoprobe in inflammation imaging were further confirmed by the *ex vivo* imaging of different tissues (FIG. 17C). The semi-quantitative study of the harvested tissues revealed that the average fluorescence intensity in the

bacterial infected skin is ~12-fold higher as compared to that in other tissues (FIG. 17D). It is well known that nanomaterials are prone to uptake by reticuloendothelial system (RES) organs such as liver and spleen. The negligible fluorescence signals in RES organs of TPE-IPB nanoprobe-treated mice revealed that the AIE light-up nanoprobe fluorescence would not turn on in normal tissues. This result manifests that TPE-IPB nanoprobe are highly specific for visualizing an *in vivo* inflammatory region with elevated production of ONOO⁻. Furthermore, the infected and uninfected skins were also sliced for blood vessel staining and CLSM imaging. As shown in FIG. 17E, emissive yellow fluorescence dots were clearly observed around the blood vessels in the bacterial infection region. In contrast, nearly no nanoprobe fluorescence signals were seen in the slice of normal skins. This result at single-cell resolution confirms the fluorescence light-up of our nanoprobe in the inflammatory region.

[135] Visualization of *in vivo* treatment efficacy of anti-inflammatory agents

[136] The discovery of new probes that is conducive to see the *in vivo* treatment efficacy of anti-inflammatory agents will be clinically valuable for selection of efficient and infallible drugs. To estimate the capacity of the present AIE light-up nanoprobe in this application, two kinds of bacteria, *MRSA* and *Escherichia coli* (*E. coli*) were subcutaneously inoculated at the left and right back of nude mice, respectively, to establish the inflammation-bearing mouse model. The mice were then divided into two groups and treated with different antibiotics, respectively, for 2 weeks. The mice in the first group received systemic administration of vancomycin by intraperitoneal injection every day. Vancomycin is a significant antibiotic for the treatment of infections caused by Gram-positive bacteria such as *MRSA*. However, *E. coli* is a typical Gram-negative bacterium, which is resistant to vancomycin. On the other hand, the mice in the second

group were treated with another antibiotic, penicillin, which is potent and sensitive to *E. coli* strain, but is ineffective against *MRSA* bacterial infection.

[137] Upon inflammation formation, the TPE-IPB nanoprobe was intravenously administered into the mice in both groups before the antibiotic treatments. As shown in FIG. 18A and 18B, strong fluorescence signals can be clearly observed at both sides of back (only in the infection regions) from mice in both two groups prior to treatments. Furthermore, after antibiotic treatments for 7 and 14 days, respectively, the AIE light-up nanoprobe was also injected into the mice of both groups. It was observed that both the fluorescence signals from *MRSA*-infected foci in the vancomycin-treated mice (FIG. 18A) and *E. coli*-infected foci in the penicillin-treated mice (FIG. 18B) significantly decrease over time and almost vanished on day 14. In comparison, the fluorescence intensity from the inflammatory region induced by the bacteria resistant to the corresponding injected antibiotic was still maintained, even after 14-day treatments.

[138] On day 14, the mice in two antibiotic-treated cohorts were sacrificed and all of the bacterial infected skin tissues were resected and sliced for further analyses. The histological examinations reveal the severe immune cell infiltrate in the hematoxylin and eosin (H&E)-stained slices from vancomycin-treated *E. coli*-infected foci (FIG. 18C) and penicillin-treated *MRSA*-infected foci (FIG. 18D), respectively, indicating typical inflammation. In contrast, greatly alleviated inflammation along with nearly no evidence of bacteria was observed from both the slices of vancomycin-treated *MRSA*-infected foci (FIG. 18C) and penicillin-treated *E. coli*-infected foci (FIG. 18D). In addition, it was also found that there were large number of emissive nanoprobe around the blood vessels in the inflammatory regions, whereas significantly less nanoprobe fluorescence were detected in the areas with effective antibiotic therapy (FIG.

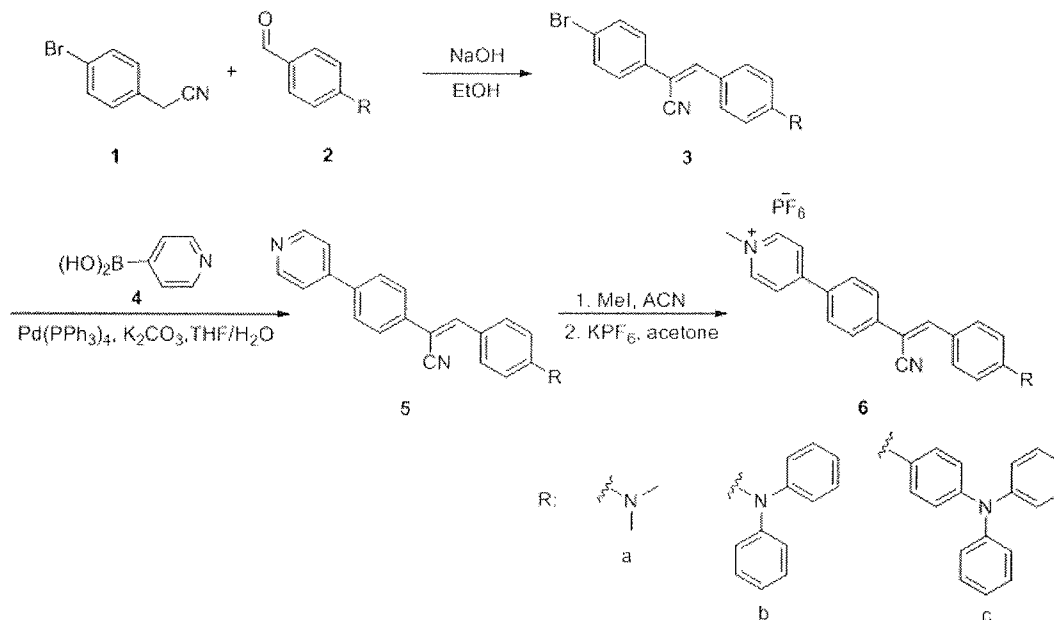
18E and 18F). This result agreed well with the non-invasive data in FIG. 18A and 18B. The results together not only verify the good selectivity and high treatment efficacy of the antibiotics used in this study, but also demonstrate that the present AIE light-up nanoprobe can offer non-invasive and real-time information, reporting the *in vivo* treatment efficacy of anti-inflammatory agents.

Red Fluorescent AIEgens for Organelle Targeting

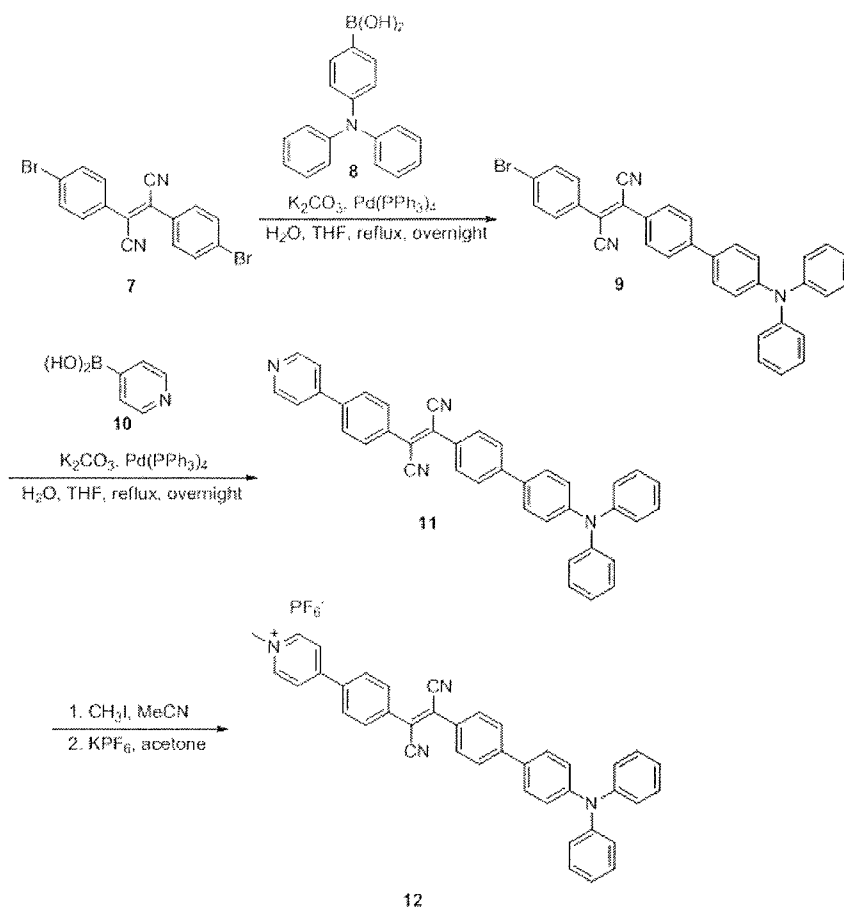
[139] The present subject matter relates to several groups of red-emitting AIEgens and their biological applications. Furthermore, targeting moieties are attached to these molecules for organelle-specific imaging.

[140] Synthesis and general properties

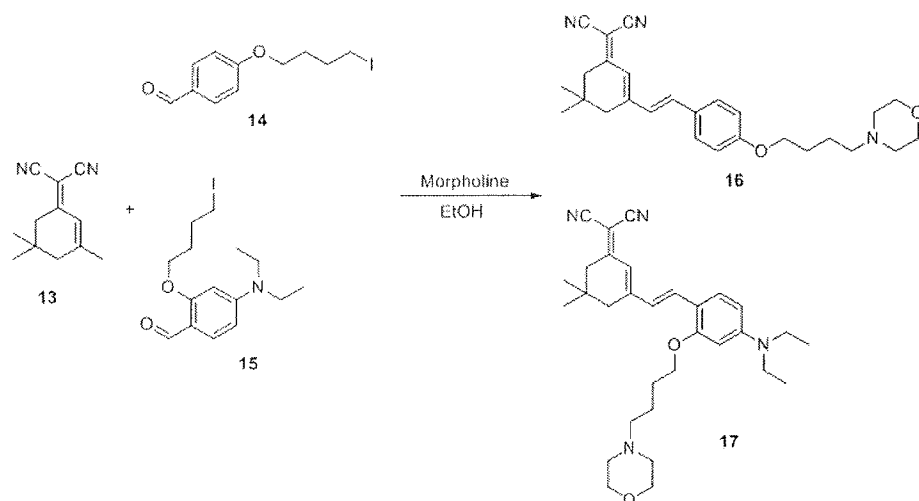
[141] Compounds **6(a-c)** were synthesized according to the following scheme:



[142] Compound **12** was synthesized according to the following scheme:



[143] Compounds **16** and **17** were synthesized according to the following scheme:



[144] The synthetic routes were based on Knoevenagel condensation and Suzuki coupling. The product was characterized by NMR and mass spectroscopy and both gave satisfactory analysis data corresponding to the molecular structures.

[145] The absorption region of compounds **6(a-c)** and **12** in different solvents was around 450 nm and the fluorescence in different solutions was varied from 600 nm to 650 nm (FIG. 19-22). It was a typical Twisted Internal Charge Transfer (TICT) characteristic, that the non-polar solvents increased and blue-shifted the fluorescent signals, while the polar solvents decreased and red-shifted the fluorescent signals. A DMSO and toluene solvent mixture was used for study of AIE characteristics, where DMSO played as a good solvent and toluene played as a poor solvent. There was no fluorescence in pure DMSO solvent, but enhancement and a blue-shift in fluorescence were observed with increasing toluene fraction content in DMSO. Below 80% toluene content in toluene/DMSO mixture, the enhancement was due to TICT effect by the non-polar toluene. But above that, the dramatic enhancement was due to the AIE characteristic. Once the aggregates were formed, the intra-molecular motions were restricted, in which the energy was relaxed in fluorescence rather than non-radiative relaxation. For compounds **16** and **17**, the absorptions were 410 and 525 nm, respectively (FIG. 27-28). Compound **16** was AEE active and the fluorescence around 600 nm was enhanced and red shifted due to an increasing water fraction (FIG. 29). In compound **17**, the fluorescence around 650 nm increased, but decreased with a 50% water fraction (FIG. 30), which may be due to the distance of molecules being closed, through pi-pi interaction.

[146] **Application of 6a**

[147] Cell localization properties

[148] Since the absorption region was in the visible light region, fluorescence was sensitive

to polarity and the mitochondria targeting group has a high potential to be a cell imaging probe. HeLa cells were incubated with compound **6a** (5 μ M) for 30 minutes. Surprisingly, there were two colors in fluorescent images, orange in the mitochondria and an unexpected red in the nucleolus. First, the mitochondria-specificity was confirmed. Confocal imaging of HeLa cells co-stained with compound **6a** and MitoTracker Green demonstrated the localization of their fluorescent signals overlapping. In order to separate two organelles' signals, confocal images were performed and the emission signals were collected from 500 to 750 nm. Two different wavelengths were used for excitation, 405 and 560 nm. Upon 405 nm excitation, signals can only be found in the mitochondria; in 560 nm excitation, signals can also be found in the nucleolus. Based on the observations that a single probe can show dual-color in cells imaging and different selectivity under different excitations, there were likely two different interactions in nucleolus and mitochondria, resulting two different fluorescent colors.

[149] The major and the most abundant components were phospholipids in the mitochondrial membrane, while DNAs and RNAs were the most abundant components in the nucleolus. To mimic the cell environment, different types of phospholipid, DNA, and RNA solutions were prepared and mixed with compound **6a** solution.

[150] The absorption maxima of the mixture of compound **6a** and phospholipid were at 430 nm, but there was 30 nm red-shifted in addition of DNA or RNA (FIG. 31A). The emission also showed red-shift from 600 to 680 nm in phospholipids and DNA or RNA mixture (FIG. 31B). The results suggested two different colors in cell imaging were due to different interactions, but also suggested that mitochondria specificity in 405 nm excitation and nucleolus specificity in 560 nm excitation happened because the absorption in phospholipid and DNA or RNA was different. A 405 nm excitation can be absorbed more in phospholipid solutions than in DNA or

RNA solution. At the same time, a 560 nm excitation can be absorbed more in DNA or RNA solution than in phospholipid solutions.

[151] The interaction between compound **6a** and mitochondrial phospholipid should be electrostatic attraction. There was no shift in absorption in phospholipid solutions and the strongest emission was from TOCL solution which carried two negative charges. It was proposed that the mechanism of the interactions between compound **6a** and RNA or DNA was electrostatic attraction and intercalation. DNA and RNA were also carrying negative charge due to the phosphate groups. Compound **6a** carries a positive charge and can be attracted to DNA or RNA. But after approaching, compound **6a** may diffuse and slide into the hydrophobic environment in DNA or RNA. This is explained by the red-shifted of the absorptions with DNA or RNA, which implied that the structure of compound **6a** became more co-planar. The fluorescence was also red-shifted in a more co-planar structure. Moreover, other possibilities include the intercalation site may still be a polar environment because the base pairs in the backbone were hydrogen bonding, in which the emission became more red-shifted due to TICT effect; and the red-shifted emission may be caused by D-A complex formation in the intercalation site.

[152] Mechanism of nucleolus specificity

[153] In the nucleolus, there were abundant proteins and RNAs, such as ribosomal proteins and rRNA. To further understand the interaction of compound **6a** in the nucleolus, a digest test of ribonuclease (RNase) and deoxyribonuclease (DNase) was performed in fixed cells and SYTORNASElect was used as control. It was clear that the signals in nucleolus were disappeared after RNase treatment, but remained unchanged in DNase treatment. This was direct evidence to prove that the majority of signals in nucleolus were from the interaction between ASCP and RNA.

[154] To mimic the digest test, the change in PL spectra was investigated (FIG. 32). Interestingly, fluorescence signals were only decreased significantly in RNase treatment due to the difference in digestion of DNase and RNase. DNase I and RNase I were used, which digests single- and double-stranded DNA to oligodeoxyribonucleotides containing a 5' phosphate; which degrades all RNA dinucleotide bonds leaving a 5' hydroxyl and 2', 3' cyclic monophosphate. Although there was DNase treatment, DNA can only be cut in small pieces in where ASCP can still interact.

[155] In FIG. 48A, there was fluorescence from interaction with DNA, but the red color in cell imaging was not related to DNA but due to the difference in the DNA purchased for experimentation and DNA in cells were different. Cellular DNA was packaged and ordered by histone, but there was no histone in *in vitro* DNA. Histone carried partial positive charge to interact with DNA. The positive charge in DNA was likely neutralized, and then the attraction to compound **6a** was decreased.

[156] Signals analysis, Photostability and cytotoxicity

[157] The fluorescent signals from mitochondria and nucleolus had overlapped partially, even in 560 nm excitation, and attempts were made to minimize the signals from mitochondria and emphasize a distinct fluorescent nucleolus by collecting the range of signals from 650 to 750 nm. The approach was successful to have a higher contrast fluorescent nucleolus (FIG. 33A). At the same time, compound **6a** was compared with SYTO RNASelect in confocal imaging. Photostability was important for monitoring the mitochondria and nucleolus morphological changes and studying these processes and relationship. Under continuous scanning with 560 nm excitation, the signals of compound **6a** were almost kept above 98% in the 50th scan, but signals of SYTO RNASelect were almost around 0% in the 15th scan (FIG. 33B). The morphologies of

nucleolus were still clear and distinct, but the fluorescent signals of SYTORNASelect disappeared.

[158] Cytotoxicity of compound **6a** was tested in HeLa cells by MTT assay (FIG. 34). The results showed that the cell viability was above 90% in different dye concentrations and proved the biocompatibility of compound **6a**. Since compound **6a** was only incubated to cells in 30 minutes, it was considered as non-toxic. To consider the study on changing of nucleolus and mitochondria, compound **6a** fulfills the requirement of high photostability and biocompatibility and high specificity. Importantly, the signals can be separated for study and can be observed in different fluorescent colors. Compound **6a** has a high potential for monitoring the changing and relationship of nucleolus and mitochondria.

[159] **Application of 6b, 6c and 12**

[160] Compound **6b** was utilized as a mitochondrial targeting dye and compounds **6c** and **12** were used for cell membrane targeting. Compounds **6c** and **12** were carrying positive charge and much longer hydrophobic part than compound **6b**. They can be tracked in the double layer lipid of cell membrane. However, compounds **6b** and **6c** were found that they can be used for two photon imaging, but also generate ROS under UV irradiation. They can also be utilized for image-guided photodynamic therapy. They may help study on cell apoptosis and necrosis.

[161] Radiosensitization effect

[162] To study the oxidized effect of **6b**, A549 cancer cells were incubated with ASCP-2P for 2 h. Then a ROS detector, DCFH, was added into the medium, followed by the expose to white light (25 mW cm^{-2}) for 1 min. After that, the cells were immediately observed under CLSM. As shown in FIG. 48D-F, the green fluorescence represented the level of intracellular ROS. The brighter the green fluorescence was, the higher the level of intracellular ROS was. It is

noted that **6b** could greatly increase the level of intracellular ROS as represented by brighter fluorescence, which indicated that light was an effective trigger to the induction of ROS by **6b** (FIG. 43E). Moreover, co-treatment of an antioxidant agent, N-acetylcysteine (NAC), with **6b** substantially reversed the induction of ROS, demonstrating that the ROS-inducing effect of **6b** by light could be abrogated by antioxidant NAC (FIG. 43F).

[163] XTT assay was employed to evaluate the anticancer effect of **6b**. As shown in FIG. 43G, **6b** without light exposure was almost non-toxic to A549 cells. There were nearly 90% cells alive even at the highest concentration (80 μ M). However, with the exposure to white light for 1 min, **6b** led to dose-dependent cell death. The IC₅₀ value was about 33 μ M. In addition, co-treatment of NAC significantly attenuated the cytotoxic effect of **6b** with light. For instance, 80 μ M of **6b** led to more than 90% cell death while more than 75% cells were alive upon NAC co-treatment.

[164] Clonogenic assay was performed to evaluate the radiosensitization effect of **6b**. Prior to irradiation, A549 cancer cells were incubated with **6b** (5 μ M) for 2 h to ensure the targeting delivery of **6b** to mitochondria. After that, irradiation was given at a series of doses (2, 4, 6 Gy). Cells were then immediately seeded into 6 well plates to study the colony forming ability. As shown in FIG. 44A and 44B, **6b** without light showed no radiosensitization effect when compared to irradiation alone. However, the exposure of **6b**-treated cells to light significantly sensitized cancer cells to radiation. The calculated SER10 was 1.62.

[165] Recent studies showed that certain drugs have radiosensitization effect, such as paclitaxel, cisplatin, etc. Clinical studies also demonstrated that paclitaxel has been recommended as a standard therapy for synchronized chemo/radiotherapy. In addition, several studies focused on potential nanomaterials that may possess radiosensitization effect. Gold

nanoparticles (GNP) were one of the most promising radiosensitization agents in the field of nanotechnology, and therefore the radiosensitization effects of **6b** with paclitaxel and GNP were compared. FIG. 44C and 44D showed that **6b** with light was the most effective agent that could sensitize lung cancer cells to irradiation. There was a significant difference between the colony forming ability in cells treated with **6b** and Paclitaxel or GNP. As calculated from the curve, SER10 of paclitaxel was 1.32 while that of GNP was 1.19. Both were significantly lower than SER10 of **6b**, which reached 1.62, the highest among the three agents.

[166] To investigate the underlying mechanism of radiosensitization effect, a lower dose of **6b** (5 μ M) was chosen to combine with irradiation. 5 μ M of **6b** with light induced very little apoptosis, which can be considered as almost non-toxic. As shown in FIG. 45A, irradiation alone inhibited the phosphorylation of both Akt and ERK, whereas **6b** with light barely influenced the expression of p-Akt and p-ERK. More importantly, combination of **6b** with light and irradiation significantly blocked the phosphorylation process, indicating the synergistic effect of the inhibition of p-Akt and p-ERK. Furthermore, the down-stream apoptotic pathways were also evaluated by western blotting (FIG. 45B). **6b** with light induced little apoptosis, while irradiation inhibited the expression of anti-apoptotic proteins (Bcl-2, Bcl-XL) and promoted the expression of pro-apoptotic proteins (Bax, BAD). One of the most important apoptotic marker, caspase-3, underwent significant decrease of pro-caspase-3 and increase of cleaved caspase-3. Moreover, the combination of irradiation and **6b** with light was much more effective in inducing apoptosis than irradiation alone or **6b** with light.

[167] In addition, an antioxidant agent NAC was used as a ROS scavenger to investigate if the radiosensitization effect of **6b** was mainly dependent on the induction of intracellular ROS. It is obvious that NAC significantly attenuated the inhibitory effect of **6b** on the expression of p-

Akt and p-ERK, which reversed the induction of down-stream apoptotic pathway. For example, co-treatment of NAC substantially decreased the expression of anti-apoptotic Bcl-2 and strengthened the expression of the pro-apoptotic Bax and BAD after the exposure to **6b** with light, which clearly demonstrated that the radiosensitization effect of **6b** was closely related to the induction of intracellular ROS by light. It is known that radiation resistance in cancer cells had very close relationship to the modulation of PI3k/Akt and MAPK pathways. Meanwhile, several studies demonstrated that constitutive expression of PI3k/Akt, which protect the cells from apoptosis, play a vital role in chemosensitization of cancer cells. The efficient ROS-inducing effect of **6b** was triggered by light exposure and it can act as an effective radiosensitizer to irradiation through the inhibition of p-Akt and p-ERK and the following induction of apoptosis (FIG. 45C and D).

[168] Morphology changes in plasma membrane

[169] Programmed cell death is called apoptosis and the death caused by external factor is called necrosis. The morphology of membrane is changed during either apoptosis or necrosis. Morphology changed of plasma membrane is related to the health of cells. However, the dynamic changes are rarely recorded. Since the dye can target plasma membrane selectively and show highly photostability and high biocompatibility, it could be utilized for long-term tracking in plasma membrane.

[170] Mercury is one of the most toxic elements to human and animal. Hg^{2+} can cause dysfunction of cells and induce cell death. Hua et al. have reported that Hg^{2+} ions change the morphology of membrane and induce bleb formation which is a common sign of cell death. **12** is used to monitor the dynamic changes of HeLa cells under Hg^{2+} treatment. In FIG. 43, pseudored color and pseudogreen color represent before and after Hg^{2+} treatment. It was found that there are

changes in plasma membrane. A bleb is formed under 40 min of the treatment. Bleb formation implies Hg^{2+} interacts with cytoskeleton (FIG. 46A), resulting actin filament disruption. When the actin filament is damaged, the hydrostatic pressure in the disrupted sits is increased and forces the bilayer membrane out. To confirm the bleb formation is not caused by the dye, a control experiment is performed and it is found that the morphology of plasma membrane do not change a lot. The possibility of monitoring morphology changes of plasma membrane under toxic conditions by **12** is demonstrated.

[171] Cell adhesion is essential and widely used in biological experiment. It is a process of interaction and attachment of cell to a surface, substrate or another cell. The interaction is driving by the action of transmembrane glycoproteins, called cell adhesion molecules (CAMs). CAMs are the proteins on the cell surface and bind to extracellular matrix. Selectins, integrins, syndecans and cadherins are the examples of CAMs. In the process of adhesion on coverslips, the morphology of cells is changed from sphere to be flattened on the surface of coverslips. The process is well studied and the key events in adhesion are hypothesized. The adherent cells can be detached by trypsin which is a protease to cleave peptide bonds. When CAMs is digested by trypsin, the adherent cells are going to leave the surface and the flattened shape is returned to be spherical (FIG. 47). **12** may be used to monitor the process of the detachment of adherent cells.

[172] First, adherent cells are imaged by using a confocal microscope (FIG. 48A). After addition of trypsin, the images are recorded in different times (FIG. 48B-H). The appearances of the cells are changed to a smaller sphere, meaning that the cells are leaving from the coverslip. But more interestingly, some small spheres surrounding the cells are observed after 7 min. It is firstly observed by using fluorescent technique. It is proposed that there is disassociation of the bilayer membrane during detachment. When trypsin is added and start to digest the CAMs, the

cells start to leave in lack of enough binding points on surfaces. It is meant that some CAMs may still not be digested even though the cells are leaving. At binding points, the plasma membrane is enforced to leave, but CAMs keep the membrane on the coverslip. As a result, some membrane may be cleaved from cells in this pulling. Since the membrane is bilayer phospholipid, the cleaved membranes tend to form micelle. The present dye is tracked in the bilayer. The process of detachment can be monitored by observation of the shape of cells. On the other hand, some micro-events like the micelle formation can also be monitored in detailed. The results suggest that **12** is a potential candidate to monitor morphology changes of plasma membrane in long-term.

[173] ROS-induced changes in plasma membrane

[174] After it was confirmed that **6c** can generate ROS in vitro, attempts were made to confirm that the bleb formation was related to ROS. **6c** and H2DCFDA were co-stained in HeLa cells and their corresponding signals were recorded (FIG. 49). After 405 nm excitation as irradiation for 4 minutes, the signals from the ROS sensor were turned on gradually and became highly emissive, meaning that there was ROS generated under the irradiation. Meanwhile, it was found that the signals of **6c** were going into the cells from the plasma membrane. It may imply that the rigidity of the membrane was changed during the irradiation. The dye cannot be tracked tightly, then diffused inside.

[175] Considering the rigidity during light irradiation, attempts were made to use PI to study the permeability after disruption of the rigidity in plasma membrane. PI cannot enter living cells, but can enter dead cells because of the permeability of the plasma membrane. When it entered cells, it interacted with DNA and turned on as red emission. After HeLa cells stained with **6c**, PI was introduced to the cell culture. Before irradiation, no red fluorescent signals from

PI were found, but the signals were from the present dye in the membrane (FIG. 50). The fluorescence was turned on and enhanced gradually under irradiation, meaning that PI was entering cells and interacting with DNA. The increased signals were collected for a real-time monitoring of turn-on process of PI (FIG. 51). Importantly, this result implies that the permeability of plasma membrane was weakened or gone after irradiation. ROS was not disrupted F-actin cortex, but also phospholipid of the plasma membrane.

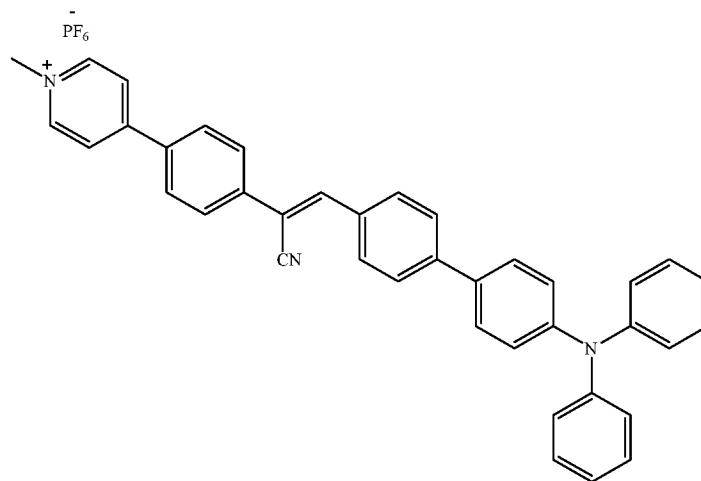
[176] Biocompatibility

[177] After the properties of **6c** were investigated, it was suggested that it has potential to be a photosensitizer for PDT. MTT assay was used to study the possibility of this idea (FIG 52). PDT requires a non-toxic agent and that the agent can generate toxins like ROS for cells killing. The biocompatibility of **6c** in dark or without light irradiation was good in terms of the high percentage of cell viability, and the percentages were dramatically decreased upon light irradiation. This light-controlled process combined with the fluorescence of **6c** can be further developed as image-guided PDT.

[178] **Application of 16 and 17**

[179] Compound **16** was utilized for lysosome targeting. The selectivity was confirmed by a commercial dye, lyso-tracker red. It can also be used for two photon imaging in order to give a higher resolution and high signal-to-noise ratio. Compound **17** was used for lipid droplet imaging. In confocal images, the signals come from the whole cells. However, when the range for collection of emission was changed from 520 to 630 nm, the signals only come from lipid droplets because the environment of lipid droplets was non-polar, which will shift the emission of compound **17** into more blue regions (FIG. 35).

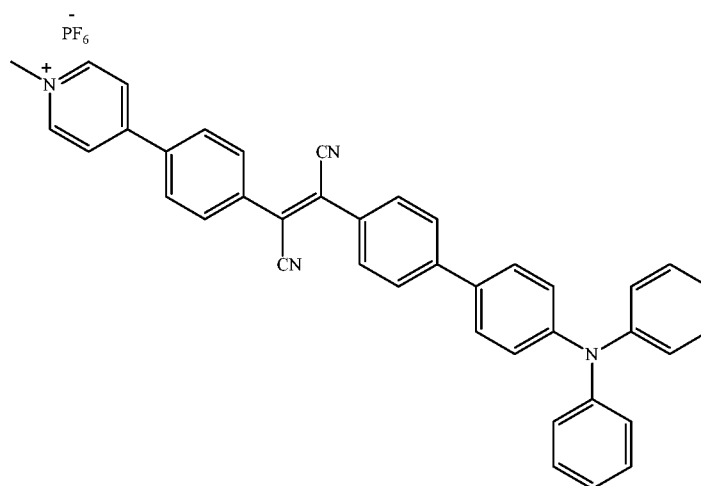
[180] In an embodiment, the present subject matter is directed to a probe for generating and/or tracking reactive oxygen species (ROS) under UV irradiation comprising red fluorescent AIEgens having the structure:



Compound 6c.

[181] In an embodiment, the probe of the present subject matter is a dye.

[182] In an embodiment, the present subject matter is directed to a probe for monitoring long-term morphology changes of a plasma membrane comprising red fluorescent AIEgens having the structure:



Compound 12.

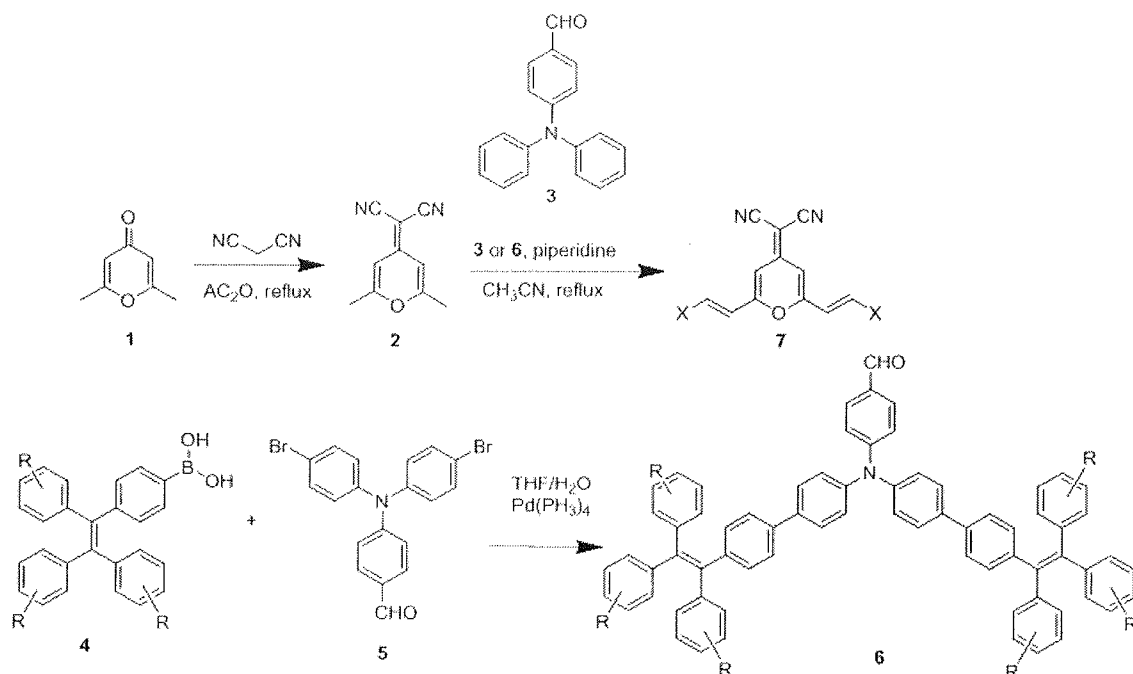
[183] In an embodiment, the probe of the present subject matter provides cell membrane staining.

Near-Infrared AIE Luminogens for Biological Applications

[184] Synthesis and General Properties

[185] The featured class of compounds is based on the TPE-TETRAD scaffold which exhibited near-infrared emission, a large Stokes shift, low cytotoxicity, and high photostability. The favorable AIE and TICT properties made it possible to utilize this dye synergistically within various multifunctional nanoparticle platforms. Encapsulating the NIR AIE nanoaggregates emitters using PEG polymers, mesoporous silica, and biomolecular matrix yields uniformly sized NPs with high brightness and low cytotoxicity. Proposed applications relating to bioimaging, long-term cell tracing and as organic light-emitting diodes (OLED) are considered.

[186] The synthetic strategy used to synthesize this unique class of NIR emitters was based on the well-established Knoevenagel condensation and Suzuki coupling reactions. The detailed synthetic route to TPE-TETRAD (compound **6**) is shown in the following scheme:



[187] Optical Properties

[188] Following an established structural design principle in which AIE luminogen components are covalently bonded to conventional fluorophores to endow them with AIE properties, four TPE units were bonded to a molecular framework consisting of TPA-DCM. The overall structure was shown to have AIE and TICT characteristics. TPE-TETRAD exhibits an emission maximum at 668 nm in THF, which is 53 nm red-shifted from that of TPA-DCM.

[189] By gradually increasing the water fraction, the emission of TPE-TETRAD is dramatically weakened and the emission color is bathochromically shifted, due to the increase in the solvent polarity and the transformation to the TICT state. The fluorophores emission is restored at $f_w \approx 50$ vol % and is intensified with a further increase in f_w . Meanwhile, the emission maximum is gradually red-shifted to ~ 675 nm when f_w reaches 90 vol %. These data verify the anticipation that TPE-TETRAD is a luminogen with both TICT and AIE characteristics. The maximum emission absorption was at 509 nm yielding a relatively large stokes shift of 160 nm.

[190] A full listing of the various quantum yields and lifetimes achieved using TPE-TETRAD in various states from solid to nanoparticle morphologies is outlined in Table 1.

Table 1: Quantum Yield and Lifetime of Various TETRAD Species

	TETRAD momomolecules (in THF)	TETRAD nanoaggregates (in H ₂ O)	TETRAD PEG Nanoparticles (in H ₂ O)	TETRAD solid state (thin film)
Quantum yeild	20.94%	20.78%	12.52 %	23.41%
Lifetime	3.97 ns	1.55 ns	4.12 ns	3.55 ns

[191] Most notably, the solid state emission was as high as 23.41% with a lifetime of 3.55 ns. The two-photon cross section was 313 GM at 830nm making this dye extremely useful for deep-tissue imaging and biological applications. This can be seen in the two photon excited emission spectra of TPE-TETRAD under 800 nm where there is a significant overlap with the biological window.

[192] Nanoparticle Fabrication and Bioapplication

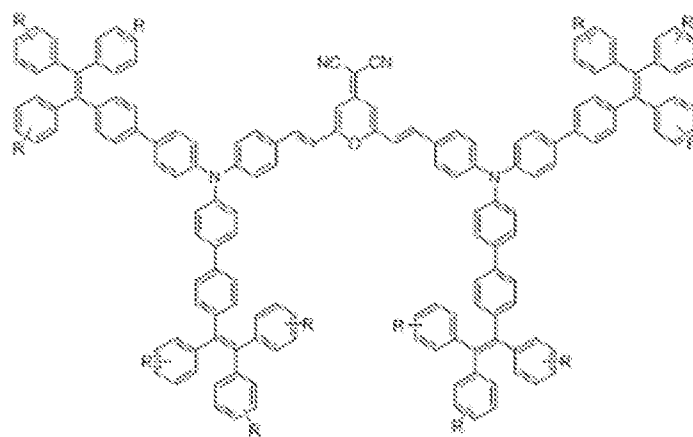
[193] To further explore the biological applications of TPE-TETRAD, three different nanoparticle platforms were investigated. The first and most basic approach involved the encapsulating the TPE-TETRAD dye using DSPE-PEG₂₀₀₀. This was achieved by dispersing TPE-TETRAD in THF and slowly adding it to an aqueous solution of DSPE-PEG₂₀₀₀. Subsequently, the TPE-TETRAD molecules aggregate and entangle with the hydrophobic domains of the DSPE-PEG₂₀₀₀. Stable nanoparticles formed instantly upon sonication. The THF was then removed and purified by filtration through a 0.45 μ m microfilter. The negative Zeta potential of the purified NPs suggests that the NPs are stabilized by outer layers of ionized carboxylic groups. The NPs were 230 nm as confirmed by dynamic light scattering.

[194] The NPs had a very low cytotoxicity as revealed in the MTT assay preformed.

Additionally, it has been shown that highly conjugated molecules exhibit ROS capabilities. This is highly unfavorable for long term cell tracking studies, due to unintentional cytotoxicity effects from longer term confocal microscope laser exposure. The TPE-TETRAD dye and nanoparticles do not generate a significant amount of ROS. However, the TPE-TETRAD NPs were able to be internalized by HeLa cells and showed very bright fluorescence.

[195] The next nanoparticle system that was investigated involved coating the outside of the TPE-TETRAD nanoaggregates with a mesoporous silica coating to endow the particle with superior long term biostability and multifunctional drug delivery capabilities. The cytotoxicity was observed to be very low as shown by a MTT assay performed where the working concentration lead to greater than 95% cell survival. The third and final nanoparticle platform that was investigated involved incarcerating gold nanoparticles (AuNP) into the TPE-TETRAD and PEG matrix described earlier.

[196] In an embodiment, the present subject matter is directed to a probe comprising near-infrared AIE luminogens comprising the structure:



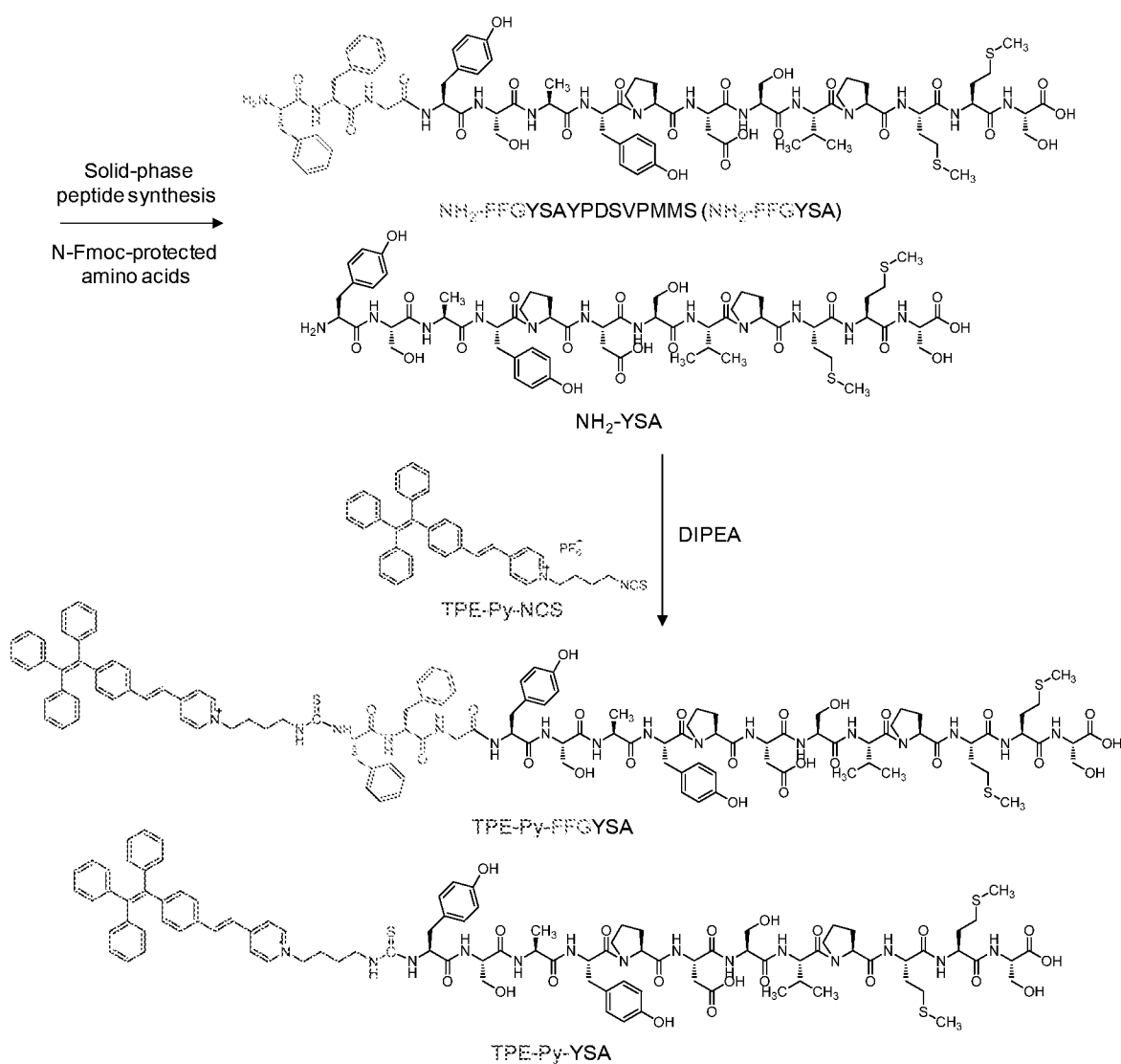
TPE-TETRAD.

[197] In an embodiment, the probe of the present subject matter is used for deep tissue imaging. In an embodiment, the probe of the present subject matter is used for drug delivery. In

an embodiment, the probe of the present subject matter is internalized by HeLa cells.

Synthesis and Characterization of TPE-Py-FFGYSA and TPE-Py-YSA

[198] An isothiocyanate-functionalized AIEgen, namely TPE-Py-NCS, was synthesized and characterized with standard spectroscopic techniques. The synthetic route toward TPE-Py-FFGYSA and TPE-Py-YSA is shown below:



[199] The peptide of NH₂-FFGYSA was synthesized through standard solid-phase peptide

synthesis, which was then characterized by liquid chromatography(LC), ^1H NMR, and HRMS. The reaction between the isothiocyanate group on TPE-Py-NCS and the amine group of NH_2 -FFGYSA yielded TPE-Py-FFGYSA in 70% yield. The purity and chemical structure of the final product were also confirmed by LC, ^1H NMR, and HR-MS. As a control, TPE-Py-YSA without FFG sequence was synthesized and characterized as well following the same procedures as that for TPE-Py-FFGYSA.

[200] Optical Properties

[201] The AIE characteristic of TPE-Py-NCS was demonstrated by measuring its photoluminescence (PL) spectra in tetrahydrofuran (THF)/hexane solvent mixtures. As shown in FIG. 53A and FIG. 53B, TPE-Py-NCS shows relatively weak emission peaked at ~ 626 nm in pure THF solution. With the increase of hexane content in THF/hexane mixtures from 0 to 70%, the PL intensity slightly enhances with evident blue-shift of the emission wavelength. This phenomenon should be ascribed to the typical TICT effect with decreased polarity of solvent mixtures when hexane fraction is elevated. Further increase of hexane fraction in the mixture leads to a dramatic PL enhancement with a constant peak at ~ 595 nm, which illustrates the pure AIE effect of T.

[202] PE-Py-NCS. The emission spectra of TPE-Py-FFGYSA and TPE-Py-YSA in phosphate buffered saline (PBS) buffer are depicted in FIG. 53C, respectively. Both TPE-Py-FFGYSA and TPE-Py-YSA are weakly fluorescent in PBS buffer, although the emission of TPE-Py-FFGYSA is ~ 2.2 -fold higher than that of TPE-Py-YSA. It is noted that the PL spectra of TPE-Py-FFGYSA and TPE-Py-YSA are nearly unchanged when they are incubated in pure water, PBS buffer, Dulbecco's Modified Eagle Medium (DMEM) and DMEM containing fetal bovine serum, respectively. This suggests that TPE-Py-FFGYSA and TPE-Py-YSA are

capable of serving as fluorescence turn-on probes applicable for complex biological environments.

[203] Specific Imaging of PC-3 Cancer Cells

[204] It has been reported that EphA2 proteins are highly overexpressed in human prostate PC-3 cancer cells, which is also confirmed by the staining experiment of PC-3 cells with commercial monoclonal anti-EphA2 antibody and subsequent fluorescent secondary antibody (FIG. 54). Therefore, PC-3 cell lysates were used to treat with TPE-Py-FFGYSA and TPE-Py-YSA, respectively, which was followed by PL measurement. As depicted in FIG. 53C upon addition of PC-3 cell lysates, the emission of both TPE-Py-FFGYSA and TPE-Py-YSA peaked at ~575 nm are greatly enhanced. Noteworthy, after treatment with PC-3 cell lysates, the fluorescence intensity of TPE-Py-FFGYSA is ~3.7 times higher than that of TPE-Py-YSA, indicating the larger fluorescence signal output of TPE-Py-FFGYSA.

[205] It was investigated whether the theranostic agent can image EphA2 proteins that are overexpressed in the cancer cells in a selective and high-contrast manner. In this experiment, PC-3 cancer cells and human smooth muscle cells were utilized as EphA2-positive and negative cells, respectively. Through antibody staining experiment, it is verified that smooth muscle cells express very few EphA2 proteins (FIG. 54), revealing that this normal cell line can act as a good EphA2-negative control. It is important to note that most of EphA2 receptors exist as dimers on the cancer cell membrane; nevertheless, after interaction with the specific ligands (*i.e.*, anti-EphA2 antibody or YSA peptide), the ligand-bound EphA2 dimers are prone to assemble into larger clusters on the membrane, followed by internalization into cytoplasm.

[206] TPE-Py-FFGYSA (1 μ M) was then applied to incubate with PC-3 cancer cells. Upon incubation at 37°C for 90 min, PC-3 cancer cells were imaged by confocal laser scanning microscopy (CLSM). As shown in FIG. 55A, distinct dots with bright yellow fluorescence are

explicitly observed around the nucleus of PC-3 cells, indicating that the TPE-Py-FFGYSA fluorescence can be significantly switched on in the cancer cells. To validate that what TPE-Py-FFGYSA visualized were indeed EphA2 clusters, the PC-3 cells were also co-stained with monoclonal anti-EphA2 antibody and fluorescent secondary antibody. It is found that the yellow fluorescence from TPE-Py-FFGYSA (FIG. 55A) and red fluorescence from antibodies (FIG. 55B) are colocalized pretty well in the cell (FIG. 55C). As the anti-EphA2 antibody is known to specifically bind to EphA2, the aforementioned result reasonably verifies that TPE-Py-FFGYSA is able to target and light up EphA2 clusters in PC-3 cancer cells. Additionally, the PC-3 cells were pretreated with free YSA peptides and subsequently incubated with TPE-Py-FFGYSA at 37°C for 90 min. The CLSM image as displayed in FIG. 56 reveals that the fluorescence signal in the PC-3 cells is significantly reduced upon blocking of EphA2 receptors. This result demonstrates that the fluorescence turn-on of TPE-Py-FFGYSA results from its specific binding with EphA2 receptors.

[207] To test the feasibility of TPE-Py-FFGYSA in tracking the intracellular movement of EphA2, the PC-3 cancer cells were first incubated with TPE-Py-FFGYSA at 0°C, as the protein internalization is energy-dependent. After incubation at 0°C for 1 h, intense fluorescence signals from TPE-Py-FFGYSA are observed on the membranes of PC-3 cancer cells (FIG. 55D), indicating that the EphA2 receptors are originally distributed on the cell membrane. Alternatively, after treatment with TPE-Py-FFGYSA at 0°C for 1 h, the PC-3 cells were washed and incubated in culture medium for another 10 and 60 min, respectively, followed by imaging of the live cells with CLSM. Upon further incubation of the cells at 37°C for 10 min, it is obvious that yellow fluorescent signals are located in both the cell membrane and cytoplasm (FIG. 55E), suggesting that the internalization of EphA2 receptors occurs when the cells are

rejuvenated at 37°C. Dramatically, a vast majority of the fluorescent patches are observed in the cytoplasm post further cell incubation at 37°C for 60 min (FIG. 55F), suggesting the nearly complete internalization of EphA2 clusters into the PC-3 cancer cells. This result reveals that TPE-Py-FFGYSA can monitor the intracellular movement of EphA2 in live PC-3 cancer cells.

[208] Furthermore, the targeting capability and specific fluorescence turn-on signature of TPE-Py-FFGYSA toward EphA2 were estimated using EphA2-negative smooth muscle cells as the control. As shown in FIG. 57A, there are very few fluorescence signals detected in the smooth muscle cells upon incubation with TPE-Py-FFGYSA (1 μ M) at 37°C for 90 min, indicating that TPE-Py-FFGYSA is highly specific for lighting up EphA2 that are overexpressed in cancer cells. Moreover, TPE-Py-YSA without FFG sequence was also utilized as a control probe. FIG. 57B and FIG. 57C show the CLSM images of PC-3 cancer cells after incubation with TPE-Py-FFGYSA (1 μ M) and TPE-Py-YSA (1 μ M), respectively, at 37°C for 90 min. Compared with TPE-Py-FFGYSA-treated cells, less staining areas with weaker fluorescence is observed for TPE-Py-YSA-treated cells. Quantitative analysis with Image Pro Plus software suggests that the average fluorescence intensity from TPE-Py-FFGYSA-treated cells is ~4.0-fold higher than that from TPE-Py-YSA-treated PC-3 cells, which agrees well with the cell lysate titration data (FIG. 53C). This comparative experiment manifests that as compared to TPE-Py-YSA, TPE-Py-FFGYSA is capable of visualizing EphA2 proteins in cancer cells in a more sensitive and higher-contrast manner. In addition, CLSM image of smooth muscle cells post incubation with TPE-Py-YSA at 37°C for 90 min displays very few fluorescence signals in the normal cells (FIG. 57D), which shows nearly no difference to TPE-Py-FFGYSA-treated smooth muscle cells (FIG. 57A) in terms of the number as well as fluorescence intensity of the fluorescence patches in the cells.

[209] The larger fluorescence signal throughput of TPE-Py-FFGYSA than TPE-Py-YSA for EphA2 imaging in PC-3 cancer cells should be attributed to the FFG sequence between the AIEgen and YSA. As EphA2 receptors form clusters in cancer cells, a considerable number of probes will be significantly enriched in the EphA2 clusters due to the specific binding of the protein and YSA. At the surface of protein clusters, it is reasonable to envision that as compared to TPE-Py-YSA, more and tighter TPE-Py-containing assemblies or aggregates will form for TPE-Py-FFGYSA under the action of FF by virtue of its excellent self-assembly property when capped with an aromatic group, which will thus restrict the intramolecular rotations of phenyl rings of TPE-Py more effectively, leading to higher fluorescence signal output. As a consequence, TPE-Py-FFGYSA can image EphA2 clusters in cancer cells in a more sensitive and higher-contrast manner, by the simple incorporation of three amino acids FFG.

[210] Adjuvant Amplification of Antitumor Efficacy of Paclitaxel

[211] Next, the ability of TPE-Py-FFGYSA to generate ROS under light irradiation, which is a prerequisite to be an AIE adjuvant, was studied. In this experiment, 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) was used as a ROS indicator, which is non-emissive but can change to fluorescent dichlorofluorescein (DCF) through rapid oxidation reaction in the presence of ROS. As shown in FIG. 58A, upon continuous exposure of the aqueous solution of TPE-Py-FFGYSA to white light irradiation, efficient ROS production is found, as evidenced by the significant increase in the fluorescence intensity of DCF peaked at 530 nm. Such fluorescence enhancement of indicator could be effectively suppressed when vitamin C was added to scavenge the generated ROS. The capacity of TPE-Py-FFGYSA in ROS generation was further confirmed by another ROS indicator, 1,3-diphenylisobenzofuran (DPBF), *via* monitoring the decrease in DPBF absorbance at 418 nm (FIG. 58B).

[212] The ROS production of TPE-Py-FFGYSA in PC-3 cancer cells was also assessed using DCF-DA as the indicator. After incubation with TPE-Py-FFGYSA (1 μ M) at 37°C for 90 min, PC-3 cells were exposed to light irradiation for 2 min. As shown in FIG. 58C, intense green emission from DCF can be distinctly seen inside the TPE-Py-FFGYSA-treated cells upon light irradiation. Nevertheless, after the cells were pretreated with *N*-acetylcysteine (NAC), a ROS scavenger, the fluorescence intensity of DCF in the TPE-Py-FFGYSA-treated cells after light irradiation is greatly reduced. These results indicate efficient ROS generation from TPE-Py-FFGYSA that targets EphA2 clusters in the PC-3 cancer cells. In comparison, the TPE-Py-FFGYSA-treated cells without exposure to light exhibit similarly weak green fluorescence to the control cells (FIG. 58C), suggesting that light irradiation is necessary for TPE-Py-FFGYSA to produce ROS.

[213] The application of TPE-Py-FFGYSA as an AIE adjuvant to enhance the cytotoxicity of Ptx was studied by MTT assay. The exogenous ROS generated by TPE-Py-FFGYSA will not kill cancer cells, but provides an intracellular oxidation environment to amplify the antitumor efficacy of Ptx. It is demonstrated that the 48 h viabilities of PC-3 cancer cells and smooth muscle cells after treatments with TPE-Py-FFGYSA itself (1 μ M), “TPE-Py-FFGYSA (1 μ M) + light irradiation” or pure light irradiation are all above 95% (FIG. 59A), indicating that by optimizing the experimental condition, TPE-Py-FFGYSA is non-toxic to both cancer and normal cells even exposure to light. This result reveals that TPE-Py-FFGYSA is promising to serve as an adjuvant with very low cytotoxicity. When the light irradiation performed would lead to synergistic antitumor effect of TPE-Py-FFGYSA and Ptx was next studied.

[214] After incubation with TPE-Py-FFGYSA at 37°C for 90 min, PC-3 cancer cells were washed and exposed to 32 nM of Ptx. Subsequently, single irradiation with white light (0.1 W

cm⁻², 2 min) were carried out at 0, 3, 6, 9, or 12 h post addition of Ptx, which was followed by MTT assays at 24 h. As shown in FIG. 59B, upon light irradiation at 0, 3, or 6 h post Ptx addition, the PC-3 cell viabilities show no obvious difference to that without light irradiation (Probe +; Light □). Encouragingly, when light irradiation is performed at 12 h post addition of Ptx, significantly enhanced cytotoxicity of Ptx is found. This result implies that after interaction of PC-3 cells with Ptx for 12 h, an intramolecular oxidation environment is important for the drug to perform better.

[215] Furthermore, after TPE-Py-FFGYSA-treated PC-3 cells were incubated with a series of doses of Ptx, light irradiations (0.1 W cm⁻²) were performed three times at 12, 24, and 36 h post Ptx addition, respectively. Each irradiation lasted for 2 min. The MTT assays at 48 h as depicted in Figure FIG. 59C reveal that the treatments of TPE-Py-FFGYSA without light irradiation (Probe +; Light □) and pure light irradiation without adding TPE-Py-FFGYSA (Probe □; Light +) have negligible interference on the cytotoxicity of Ptx. It is noteworthy that the antitumor efficacy of Ptx is dramatically amplified by the treatment of “TPE-Py-FFGYSA + light irradiation” (Probe +; Light +).

[216] As calculated from the cytotoxicity curves in FIG. 59C, the IC₅₀ value of Ptx alone (Probe □; Light □) is 75.9 nM; when Ptx is combined with “TPE-Py-FFGYSA + light irradiation”, the IC₅₀ value decreases to a significantly lower value of 7.8 nM, which is only 10.3% of the original IC₅₀ value. Previous study reported that amifostine as a chemosensitizer could lower the IC₅₀ value to ~14% of the value of Ptx alone, which has been well accepted as a superb performance in enhancing the antitumor efficacy of Ptx. It is important to emphasize that “TPE-Py-FFGYSA + light” cannot lead to cell death (FIG. 59A). Hence, it is reasonable to conclude that with the help of light irradiation, TPE-Py-FFGYSA can serve as an extremely effective

adjuvant for synergistic antitumor therapy with Ptx by virtue of the effect of “0+1 > 1”.

[217] Mechanistic Study

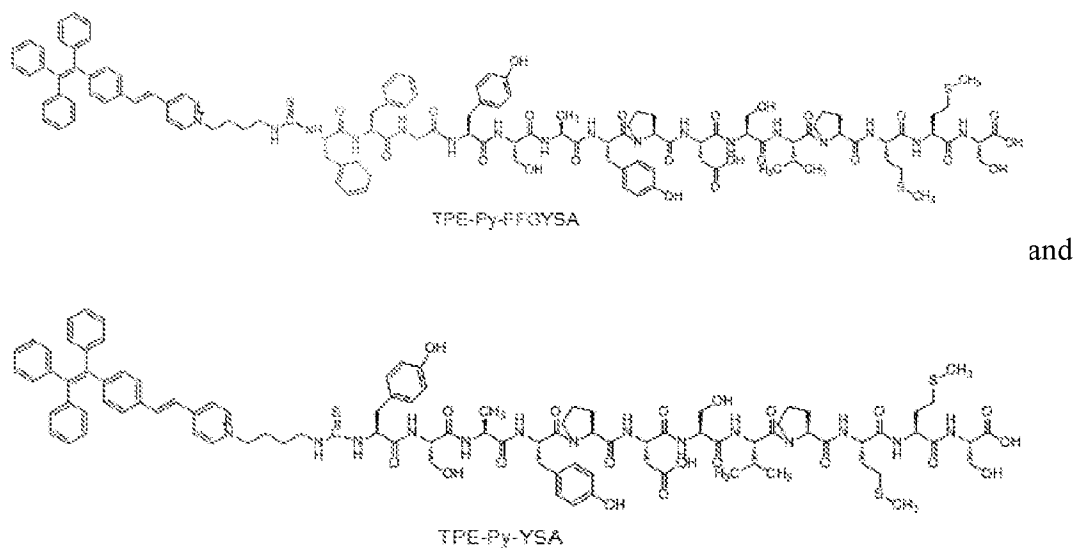
[218] The expression of related proteins was examined by western blot to study the possible mechanism of such synergistic antitumor effect between Ptx and “TPE-Py-FFGYSA + light irradiation”. As shown in FIG. 60A, in the absence of Ptx, “TPE-Py-FFGYSA + light irradiation” (Ptx $\square$; Probe +; Light +) has nearly no impact on the expression of proteins in PC-3 cancer cells, compared with the untreated cells (Ptx $\square$; Probe $\square$; Light $\square$). This result verifies that “TPE-Py-FFGYSA + light irradiation” does indeed not lead to death of PC-3 cells. Moreover, there is also no significant difference between the protein expression of PC-3 cells treated with Ptx alone (Ptx +; Probe $\square$; Light $\square$) and “Ptx + TPE-Py-FFGYSA without light irradiation” (Ptx +; Probe +; Light $\square$), which further confirms that exposure to light is the key factor to initiate the synergistic effect.

[219] It is noteworthy that the expression of phosphorylatedAkt (p-Akt) is inhibited more substantially by the combination of Ptx and “TPE-Py-FFGYSA + light irradiation” (Ptx +; Probe +; Light +), when compared with Ptx alone (FIG. 60A). Since p-Akt is a very important survival signal in cancer cells, earlier studies have demonstrated that constitutive expression of p-Akt undermines the sensitivity of cancer cells toward Ptx. It is thus proved in the present study that inhibition of the phosphorylation of Akt proteins plays a pivotal role in the chemosensitization effect of “TPE-Py-FFGYSA + light irradiation” on Ptx.

[220] Furthermore, the down-stream apoptotic pathway was also evaluated by western blot. It is obvious that the combination of Ptx and “TPE-Py-FFGYSA + light irradiation” is much more effective on inducing mitochondria-originated apoptosis by increasing the cytoplasm expression of cytochrome c and decreasing the expression of anti-apoptotic protein Bcl-2,

compared with other four control treatments. Moreover, the expression of one of the most important apoptotic markers, pro-caspase-3, undergoes the most attenuated under combinational treatment (FIG. 60A). Furthermore, the presence of NAC as an antioxidant is able to significantly abolish the synergistic antitumor efficacy (FIG. 60B). Therefore, these results together elucidate the underlying synergistic mechanism, that is, the elevated intracellular ROS level resulting from “TPE-Py-FFGYSA + light irradiation” amplifies the action of Ptx by enhancing the inhibition of p-Akt and thus inducing mitochondria-originated apoptosis more efficiently (FIG. 60C).

[221] In an embodiment, the present subject matter is directed to a probe for organelle targeting comprising red fluorescent AIEgens selected from the group consisting of



[222] In an embodiment, the probe of the present subject matter is used for imaging PC-3 cancer cells. In an embodiment, the probe of the present subject matter is used for ROS generation. In an embodiment, the probe of the present subject matter is used as an adjuvant for antitumor therapy with Paclitaxel.

EXAMPLES**[223] Synthesis of compound 3**

[224] In a 100 mL round bottom flask, 4-bromophenylacetonitrile (0.69 g, 3.50 mmol) and compound **2** (3.00 mmol) were dissolved and stirred in 40 mL of ethanol. Sodium hydroxide (0.14 g, 3.50 mmol) in 5 mL of ethanol was added into the mixture dropwise. After 2 hours, precipitates were formed and filtered out. The pale yellow solid was obtained in the yield of 80%.

[225] Compound **3a**: ¹H NMR (400 MHz, CDCl₃, δ(ppm)): 7.86 (d, 2H, J = 8.4 Hz), 7.54-7.48 (m, 4H), 7.38 (s, 1H), 6.73 (d, 2H, J = 8 Hz), 3.07 (s, 6H). HRMS (MALDI-TOF) m/z 326.0417 (M⁺, calcd. 326.0419).

[226] Compound **3b**: ¹H NMR (400 MHz, CDCl₃, δ(ppm)): 7.97 (d, 1H, J = 8.4 Hz), 7.69 (d, 1H, J = 8.0 Hz), 7.58-7.47 (m, 6H), 7.43 (s, 1H), 7.38-7.27 (m, 4H), 7.22 (d, 1H, J = 8.0 Hz), 7.16-7.12 (m, 4H), 7.07 (d, 2H, J = 4.4 Hz). HRMS (MALDI-TOF) m/z 450.0778 (M⁺, calcd. 450.0732).

[227] Compound **3c**: ¹H NMR (400 MHz, CDCl₃, δ(ppm)): 7.78 (d, 2H, J = 8.8 Hz), 7.56-7.49 (m, 5H), 7.40 (s, 1H), 7.34-7.29 (m, 6H), 7.13-6.98 (m, 8H), 6.82 (d, 2H, J = 8.8 Hz). HRMS (MALDI-TOF) m/z 526.1036 (M⁺, calcd. 526.1045).

[228] Synthesis of compound 5 and compound 11

[229] Into a 100 mL two-necked round bottom flask equipped a condenser, **3** or **9** (0.31 mmol), (4-hydroxyphenyl)boronic acid (0.045 g, 0.39 mmol), potassium carbonate (0.42 g, 3.06 mmol), and Pd(PPh₃)₄ (0.011 g, 0.0092 mmol) were dissolved in 20 mL distilled THF and 3 mL water under nitrogen. The mixture was heated to reflux overnight. After being cooled to room temperature, the mixture was extracted with dichloromethane three times. The organic phase was combined and washed with water and dried over anhydrous sodium sulfate. After the evaporation

of solvents, the crude product was purified by silica gel column chromatography using DCM/ethyl acetate in the volume ratio of 99:1 as eluent. The orange solid was obtained in a yield of 74%.

[230] Compound **5a**: ^1H NMR (400 MHz, CDCl_3 , $\delta(\text{ppm})$): 8.68 (d, 2H, $J = 4.4$ Hz), 8.68 (d, 2H, $J = 8.4$ Hz), 7.76-7.68 (m, 4H), 7.55 (d, 2H, $J = 4.4$ Hz), 7.26 (s, 1H), 6.74 (d, 2H, $J = 8.4$ Hz), 3.08 (s, 6H). ^{13}C NMR (100 MHz, CDCl_3 , $\delta(\text{ppm})$): 150.7, 149.4, 149.3, 144.6, 142.4, 137.4, 137.5, 131.2, 130.9, 129.2, 127.0, 126.8, 125.4, 120.9, 120.9, 120.7, 111.0, 110.6, 106.0, 39.4, 39.3. HRMS (MALDI-TOF) m/z 325.1575 (M^+ , calcd. 325.1579).

[231] Compound **5b**: ^1H NMR (400 MHz, CDCl_3 , $\delta(\text{ppm})$): 8.68 (d, 2H, $J = 4.4$ Hz), 7.82-7.76 (m, 1H), 7.72 (d, 1H, $J = 8.0$ Hz), 7.66 (d, 2H, $J = 7.6$ Hz), 7.58 (d, 2H, $J = 8.0$ Hz), 7.52 (d, 2H, $J = 4.8$ Hz), 7.35-7.27 (m, 4H), 7.18 (d, 2H, $J = 8.4$ Hz), 7.11-7.06 (m, 5H), 6.83 (d, 2H, $J = 8.4$ Hz). HRMS (MALDI-TOF) m/z 449.1916 (M^+ , calcd. 449.1892).

[232] Compound **5c**: ^1H NMR (400 MHz, CDCl_3 , $\delta(\text{ppm})$): 8.70 (d, 2H, $J = 5.6$ Hz), 8.00 (d, 2H, $J = 8.4$ Hz), 7.83 (d, 2H, $J = 8.4$ Hz), 7.74-7.68 (m, 4H), 7.63 (s, 1H), 7.55-7.52 (m, 4H), 7.31-7.27 (m, 4H), 7.16-7.14 (m, 6H), 7.09-7.05 (m, 2H). HRMS (MALDI-TOF) m/z 525.2238 (M^+ , calcd. 525.2205).

[233] Compound **11**: ^1H NMR (400 MHz, CDCl_3 , $\delta(\text{ppm})$): 8.75 (d, 2H, $J = 4.0$ Hz), 7.99-7.95 (m, 4H), 7.83 (d, 2H, $J = 8.4$ Hz), 7.76 (d, 2H, $J = 8.4$ Hz), 7.58 (d, 2H, $J = 5.6$ Hz), 7.54 (d, 2H, $J = 8.4$ Hz), 7.32-7.28 (m, 4H), 7.17 (d, 2H, $J = 8.4$ Hz), 7.08 (t, 2H, $J = 7.2$ Hz). HRMS (MALDI-TOF) m/z 550.4575 (M^+ , calcd. 550.2157).

[234] Synthesis of compound **6** and **12**

[235] Into a 100 mL two-necked round bottom flask equipped with a condenser, **5** or **11** (0.154 mmol) was dissolved in 5 mL of acetonitrile. 0.1 mL of iodomethane was added and

refluxed with 8 hours. After cooling to room temperature, the mixture was added in diethyl ether dropwise. The precipitates were formed and filtered out. The dark red solid was obtained in a yield of 95%. The obtained solid was dissolved in acetone and 5 mL of saturated KPF₆ solution was added. The mixture was stirred for 1 hour. The solvent was removed by compressed air and precipitates were formed. The precipitates was filtered out and washed by water. The dark red solid was obtained in 95%.

[236] Compound **6a**: ¹H NMR (400 MHz, DMSO-d₆, δ(ppm)): 8.98 (d, 2H, J = 6.8 Hz), 8.53 (d, 2H, J = 6.8 Hz), 8.18 (d, 2H, J = 8.4 Hz), 8.03 (s, 1H), 7.93 -7.40 (m, 4H), 6.83 (d, 2H, J = 8.8 Hz), 4.29 (s, 3H), 3.03 (s, 6H). ¹³C NMR (100 MHz, DMSO-d₆, δ(ppm)): 153.0, 152.0, 145.3, 144.4, 138.3, 132.0, 131.6, 128.5, 125.6, 123.5, 120.3, 118.9, 111.4, 100.3, 46.8. HRMS (MALDI-TOF) m/z 340.1826 (M⁺, calcd. 340.1814).

[237] Compound **6b**: ¹H NMR (400 MHz, DMSO-d₆, δ(ppm)): 8.95 (d, 2H, J = 6.8 Hz), 8.50 (d, 2H, J = 6.8 Hz), 8.17 (d, 2H, J = 8.4 Hz), 8.07 (s, 1H), 7.95 (d, 2H, J = 8.4 Hz), 7.89 (d, 2H, J = 8.8 Hz), 7.39 (t, 4H, J = 8.0 Hz), 7.20-7.13 (m, 6H), 6.93 (d, 2H, J = 8.8 Hz), 4.30 (s, 3H). HRMS (MALDI-TOF) m/z 464.2133 (M⁺, calcd. 464.2127).

[238] Compound **6c**: ¹H NMR (400 MHz, DMSO-d₆, δ(ppm)): 8.97 (d, 2H, J = 6.8 Hz), 8.51 (d, 2H, J = 6.8 Hz), 8.23-8.19 (m, 3H), 8.06-7.99 (m, 4H), 7.85 (d, 2H, J = 8.4 Hz), 7.71 (d, 2H, J = 8.4 Hz), 7.33 (t, 4H, J = 7.6 Hz), 7.10-7.03 (m, 8H), 4.30 (s, 3H). HRMS (MALDI-TOF) m/z 540.2458 (M⁺, calcd. 540.2440).

[239] Compound **12**: ¹H NMR (400 MHz, DMSO-d₆, δ(ppm)): 9.01 (d, 2H, J = 6.8 Hz), 8.54 (d, 2H, J = 6.8 Hz), 8.28 (d, 2H, J = 8.4 Hz), 8.10 (d, 2H, J = 8.4 Hz), 7.96-7.89 (m, 4H), 7.73 (d, 2H, J = 8.8 Hz), 7.35 (t, 4H, J = 7.6 Hz), 7.11-7.01 (m, 8H), 4.33 (s, 3H). HRMS (MALDI-TOF) m/z 565.4226 (M⁺, calcd. 565.2392).

[240] Synthesis of compound 9

[241] Into a 100 mL two-necked round bottom flask equipped a condenser, **7** (0.62 mmol), **8** (0.045 g, 0.39 mmol), potassium carbonate (0.42 g, 3.06 mmol) and Pd(PPh₃)₄ (0.011 g, 0.0092 mmol) were dissolved in to 20 mL distilled THF and 3 mL water under nitrogen. The mixture was heated to reflux overnight. After being cooled to room temperature, the mixture was extracted with dichloromethane three times. The organic phase was combined and washed with water and dried over anhydrous sodium sulfate. After the evaporation of solvents, the crude product was purified by silica gel column chromatography using DCM/ethyl acetate in the volume ratio of 99:1 as eluent. The orange solid was obtained in a yield of 56%.

[242] Compound **9**: ¹H NMR (400 MHz, CDCl₃, δ(ppm)): 7.91 (d, 2H, J = 8.8 Hz), 7.74-7.69 (m, 5H), 7.54 (d, 2H, J = 8.8 Hz), 7.34-7.28 (m, 5H), 7.17 (d, 6H, J = 8.4 Hz), 7.08 (d, 2H, J = 8.0 Hz). HRMS (MALDI-TOF) m/z 553.1021 (M⁺, calcd. 553.0977).

[243] Synthesis of compound 16 and 17

[244] Compound **13** (0.5 mmol) and **16** or **17** (0.5 mmol) was added to a 25 mL two necked round-bottom flask equipped with a condenser under nitrogen. 10 mL of dried ethanol was added and the mixture underwent reflux. 2 mL of morpholine was further added and the reaction was for 2 hours. After being cooled to room temperature, the mixture was extracted with dichloromethane for three times. The organic phase was combined and washed with water and dried over anhydrous sodium sulfate. After the evaporation of solvents, the crude product was purified by silica gel column chromatography using hexane/ethyl acetate in the volume ratio of 7:3 as eluent. The orange solid was obtained in a yield of 56%.

[245] Compound **16**: ¹H NMR (400 MHz, CDCl₃, δ(ppm)): 7.46 (d, 2H, J = 8.8 Hz), 7.04 (d, 1H, J = 16.0 Hz), 6.92 (d, 2H, J = 8.8 Hz), 6.85 (d, 1H, J = 6.4 Hz), 6.80 (s, 1H), 4.03 (t, 2H,

J = 6.4 Hz), 3.72 (t, 4H, J = 4.4 Hz), 2.60 (s, 2H), 2.46-2.39 (m, 8H), 1.87-1.81 (m, 2H), 1.72-1.65 (m, 2H), 1.08 (s, 6H). HRMS (MALDI-TOF) m/z 432.2664 (M⁺, calcd. 432.2651).

[246] Compound **17**: ¹H NMR (400 MHz, CDCl₃, δ(ppm)): 7.42-7.40 (m, 2H), 6.90 (d, 1H, J = 16.0 Hz), 6.70 (s, 1H), 6.31 (d, 1H, J = 6.4 Hz), 6.09 (d, 1H, J = 2.4 Hz), 4.06 (t, 2H, J = 6.4 Hz), 3.72 (t, 4H, J = 4.4 Hz), 3.41 (q, 4H, J = 7.2 Hz), 2.56 (s, 2H), 2.48-2.45 (m, 8H), 1.96-1.89 (m, 2H), 1.77-1.70 (m, 2H), 1.21 (t, 6H, J = 6.8 Hz), 1.06 (s, 6H). HRMS (MALDI-TOF) m/z 502.3295 (M⁺, calcd. 502.3308).

[247] Synthesis of compound 6(TPE-TETRAD)

[248] The malononitrile derivative was prepared according to the reported experimental procedures. The fluorophores were prepared as shown in the scheme for the detailed synthetic route to TPE-TETRAD (compound **6**). Pd(PPh₃)₄ (150 mg) was added into a stirred mixture of 833 mg (0.19 mmol) of 2Br-TPA-DCM, 2.17g (5.8 mmol) of the malononitrile derivative **2** and 1.5g of K₃PO₄ (5 mmol) in 50 mL of THF and 8 mL of water under nitrogen. The mixture was heated to 70°C for 36 h to obtain. After filtration and solvent evaporation under reduced pressure, the product was purified by silica-gel column chromatography using hexane/DCM as the eluent. The product CHO-TPA-2TPE was obtained in 50% yield (900 mg).

[249] Synthesis of compound 7

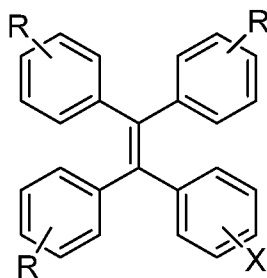
[250] Subsequently, piperidine (0.3 mL) was added into a stirred mixture of 198 mg (0.2 mmol) of CHO-TPA-2TPE and 14.5 mg (0.1mmol) of DCM in 5 mL of acetonitrile. The mixture was heated to 100°C for 72 h. After cooling to room temperature, the solution was extracted with dichloromethane (DCM; 100 mL) twice, washed with water, and dried over Na₂SO₄. After filtration and solvent evaporation under reduced pressure, the product was purified by silica-gel column chromatography using hexane/DCM as the eluent. TPE-TETRAD (compound **7**) was

obtained in 40% yield (200 mg) as red powder. All the compounds were characterized by ^1H NMR, ^{13}C NMR and high resolution mass spectrometry, which confirmed the structures.

[251] With the information contained herein, various departures from precise descriptions of the present subject matter will be readily apparent to those skilled in the art to which the present subject matter pertains, without departing from the spirit and the scope of the below claims. The present subject matter is not considered limited in scope to the procedures, properties, or components defined, since the preferred embodiments and other descriptions are intended only to be illustrative of particular aspects of the presently provided subject matter. Indeed, various modifications of the described modes for carrying out the present subject matter which are obvious to those skilled in chemistry, biochemistry, or related fields are intended to be within the scope of the following claims.

We claim:

1. A long wavelength probe having aggregation induced emission characteristics comprising at least one fluorophore comprising a backbone structure having the formula:

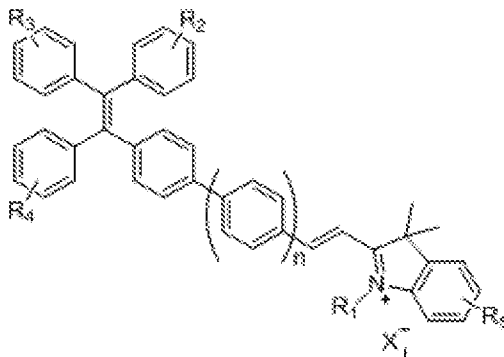


wherein

each R is independently selected from the group consisting of H, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, and heteroaryl; and

X is at least one chromophore which can conjugate with at least one fluorophore.

2. The probe of claim 1, wherein the fluorogen has a backbone structure of:

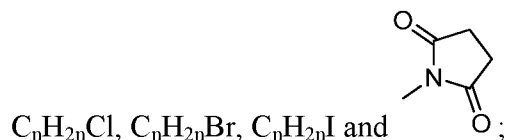


wherein

R_1 , R_2 , R_3 , R_4 , and R_5 are independently selected from the group consisting of C_nH_{2n+1} , $C_{10}H_7$, $C_{12}H_9$, OC_6H_5 , $OC_{10}H_7$, $OC_{12}H_9$, $C_nH_{2n}COOH$, $C_nH_{2n}NCS$, $C_nH_{2n}N_3$, $C_nH_{2n}NH_2$, $C_nH_{2n}Cl$,

$C_nH_{2n}Br$, $C_nH_{2n}I$, and $\left(\text{CH}_2 \right)_n \text{C}(=\text{O})\text{OR}'$;

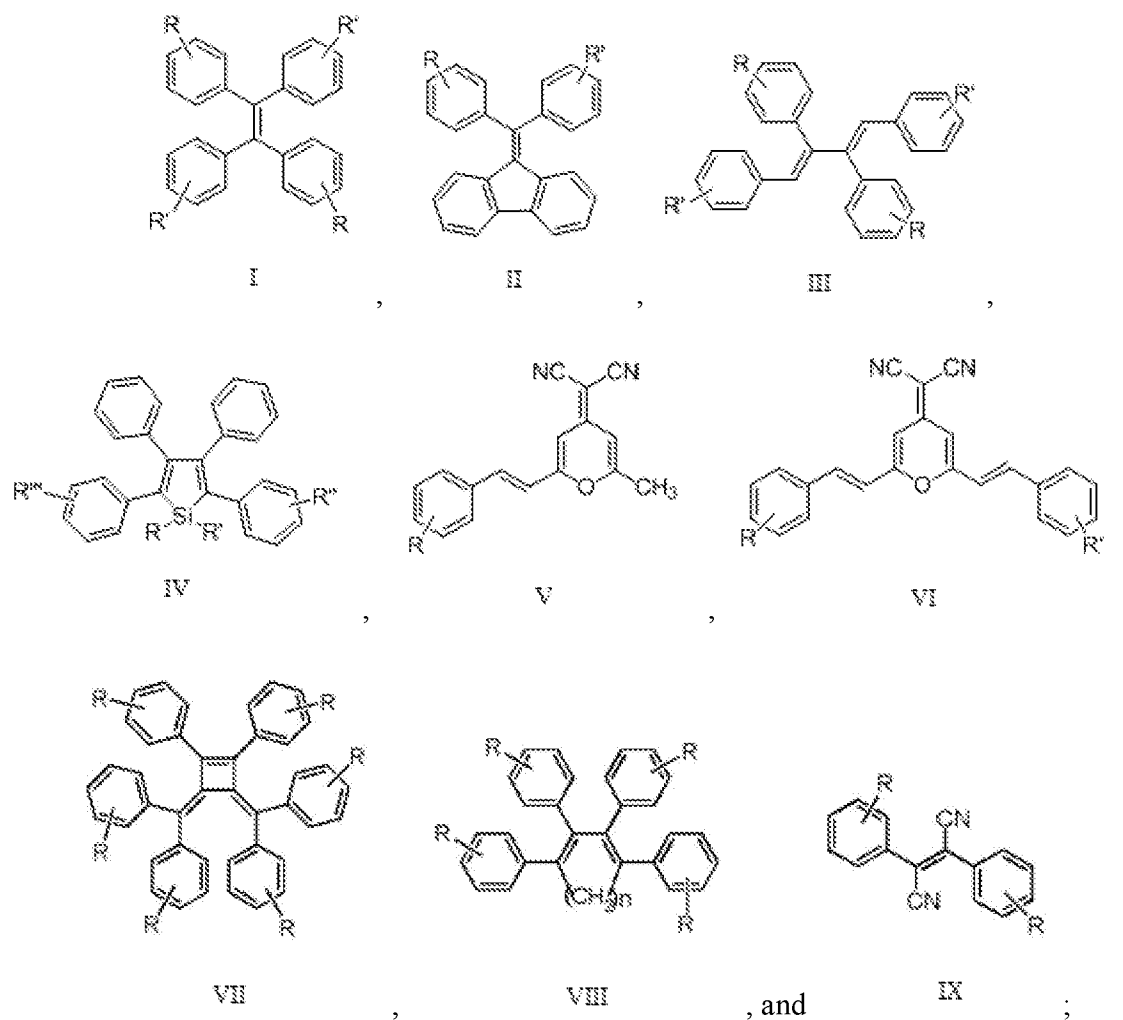
R' is independently selected from the group consisting of $C_nH_{2n}NCS$, $C_nH_{2n}N_3$, $C_nH_{2n}NH_2$,



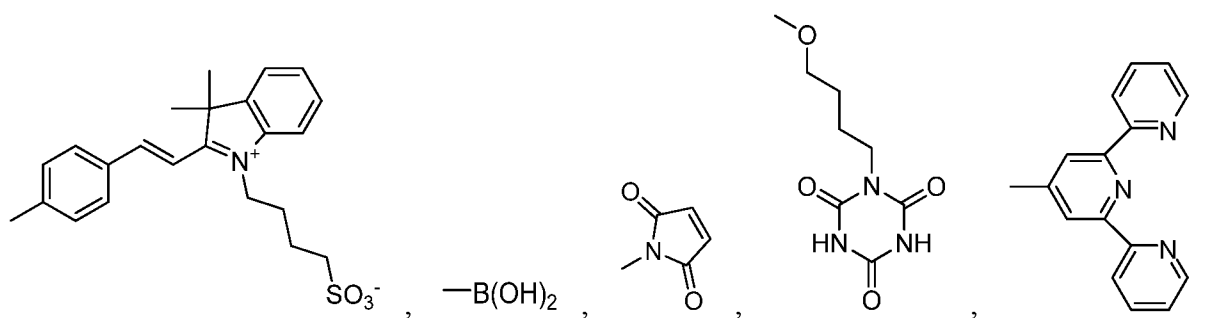
X_1 is independently selected from the group consisting of I, Cl, Br, PF_6 , ClO_4 , BF_4 , BPh_4 , and CH_3PhSO_3 ; and

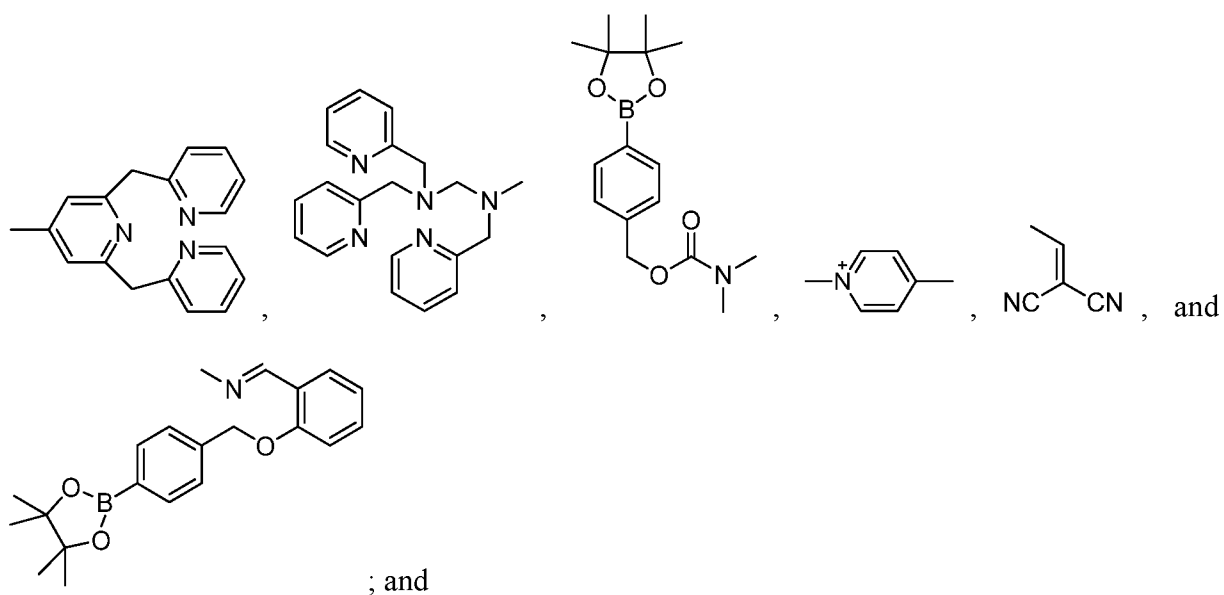
$n = 0$ to 20.

3. The probe of claim 1, wherein the probe is used to label mitochondria in living cells.
4. The probe of claim 1, wherein the probe is used to indicate a change in mitochondrial membrane potential.
5. The probe of claim 1, wherein the probe is used in situ to monitor a change of $\Delta\Psi_m$ in living cells.
6. The probe of claim 1, wherein the probe is used to evaluate sperm vitality by monitoring membrane potential differences in mouse sperm cells and sperm activity.
7. A highly sensitive and selective probe for H_2O_2 and $ONOO^-$ detection comprising AIE luminogens comprising a backbone structure selected from the group consisting of:

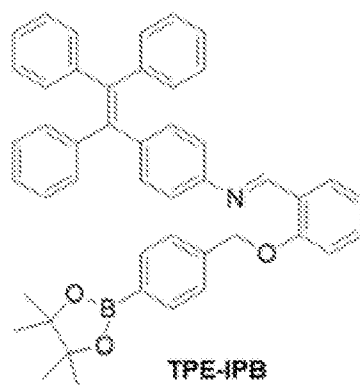


wherein each R, R', R'', and R''' are independently selected from the group consisting of



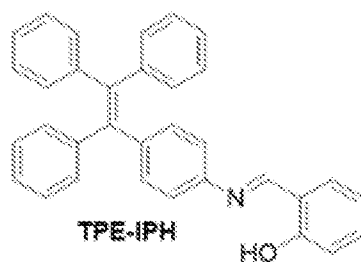


8. The probe of claim 7, wherein the AIE luminogens comprise:

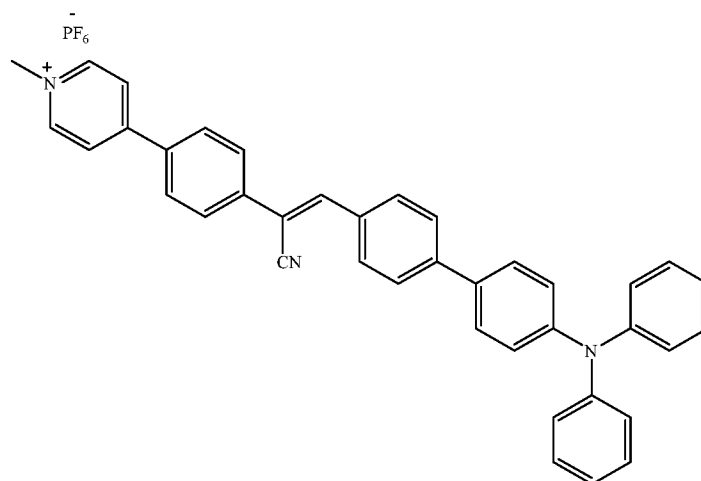


9. The probe of claim 7, wherein the probe is used for sensing glucose in buffer solutions and serum samples.

10. The probe of claim 9, wherein the probe may be in an aggregated state or solid state.
11. The probe of claim 7, wherein AIE luminogens are used as imaging agents for inflammation in vivo.
12. A method of preparing the probe of claim 11 comprising:
 fabricating nanoparticles of the AIE luminogens in a PEG matrix.
13. A highly sensitive and selective probe for H_2O_2 and ONOO^- detection comprising AIE luminogens comprising, as a backbone structure:



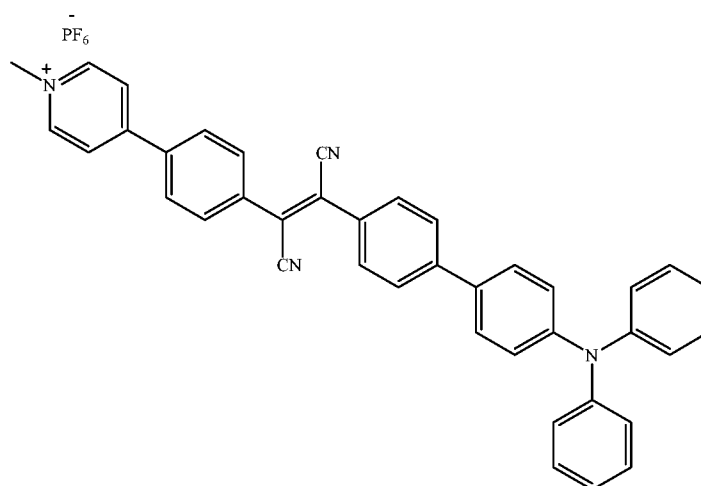
14. A method of preparing the probe of claim 13 comprising:
 fabricating nanoparticles of the AIE luminogens in a PEG matrix.
15. A probe for generating and/or tracking reactive oxygen species (ROS) under UV irradiation comprising fluorescent AIEgens having the structure:



Compound 6c.

16. The probe of claim 15, wherein the probe is a dye.

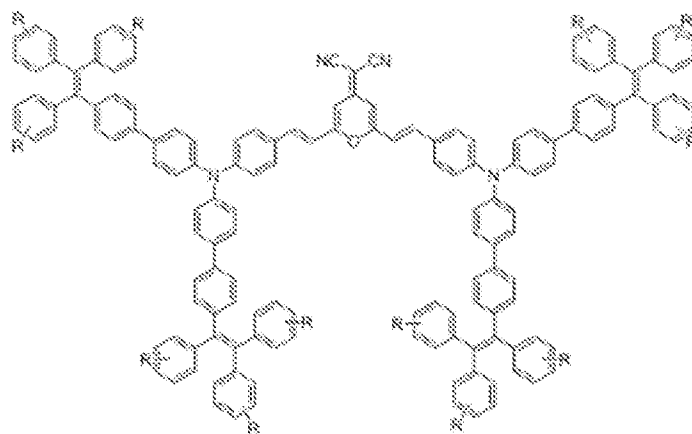
17. A probe for monitoring long-term morphology changes of a plasma membrane comprising red fluorescent AIEgens having the structure:



Compound 12.

18. The probe of claim 17, wherein the probe provides cell membrane staining.

19. A probe comprising near-infrared AIE luminogens comprising the structure:



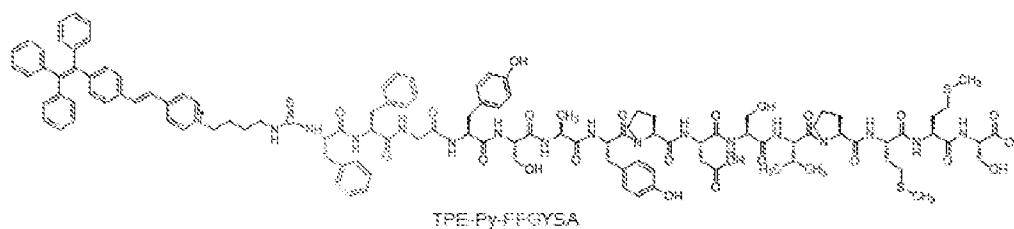
TPE-TETRAD.

20. The probe of claim 19, wherein the probe is used for deep tissue imaging.

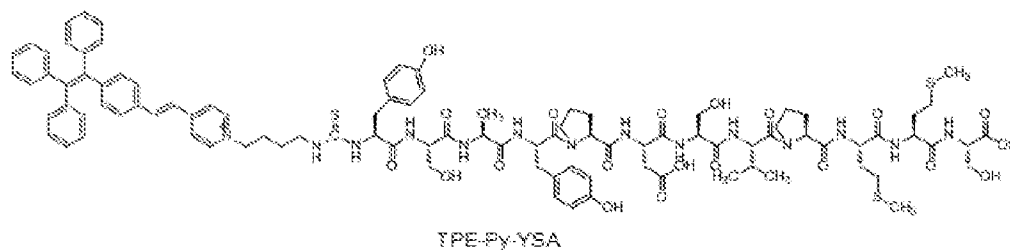
21. The probe of claim 19, wherein the probe is used for drug delivery.

22. The probe of claim 19, wherein the probe is internalized by HeLa cells.

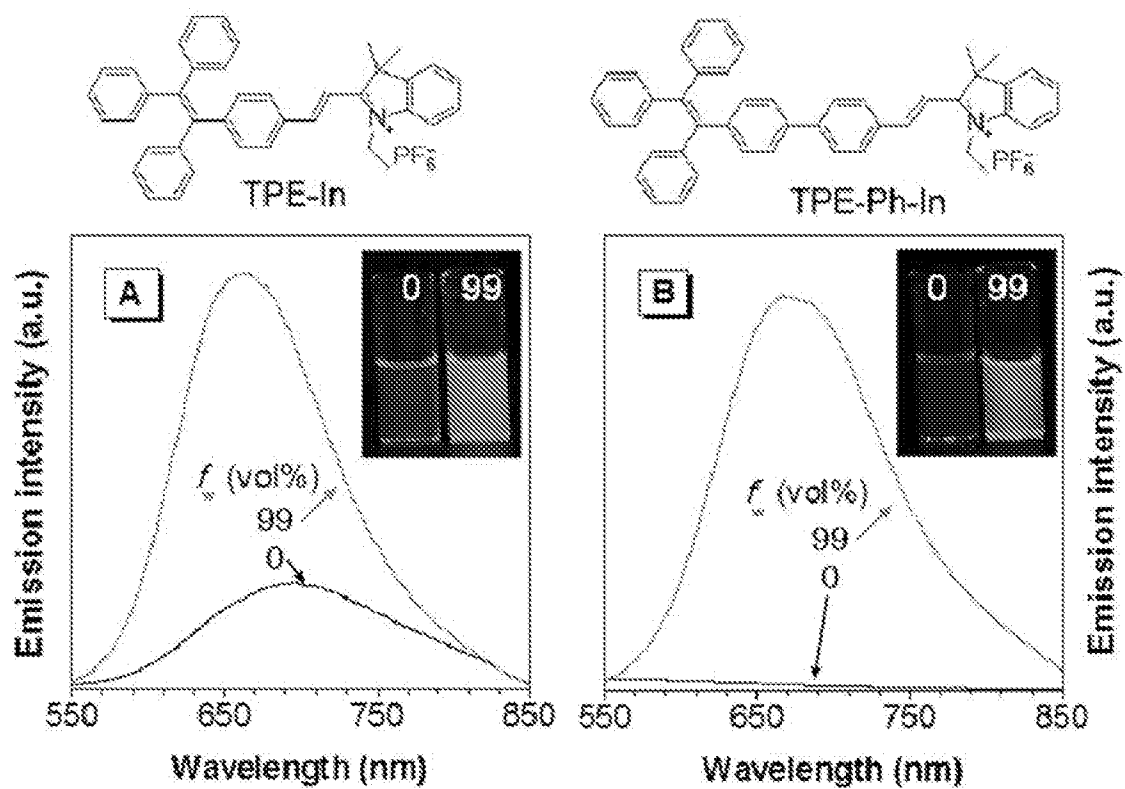
23. A probe for organelle targeting comprising red fluorescent AIEgens selected from the group consisting of



and



24. The probe of claim 23, wherein the probe is used for imaging PC-3 cancer cells.
25. The probe of claim 23, wherein the probe is used for ROS generation.
26. The probe of claim 23, wherein the probe is used as an adjuvant for antitumor therapy with Paclitaxel.



FIGS. 1A-B

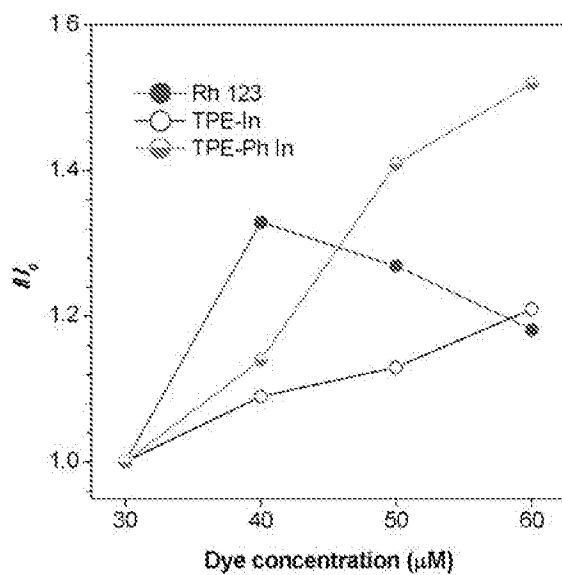
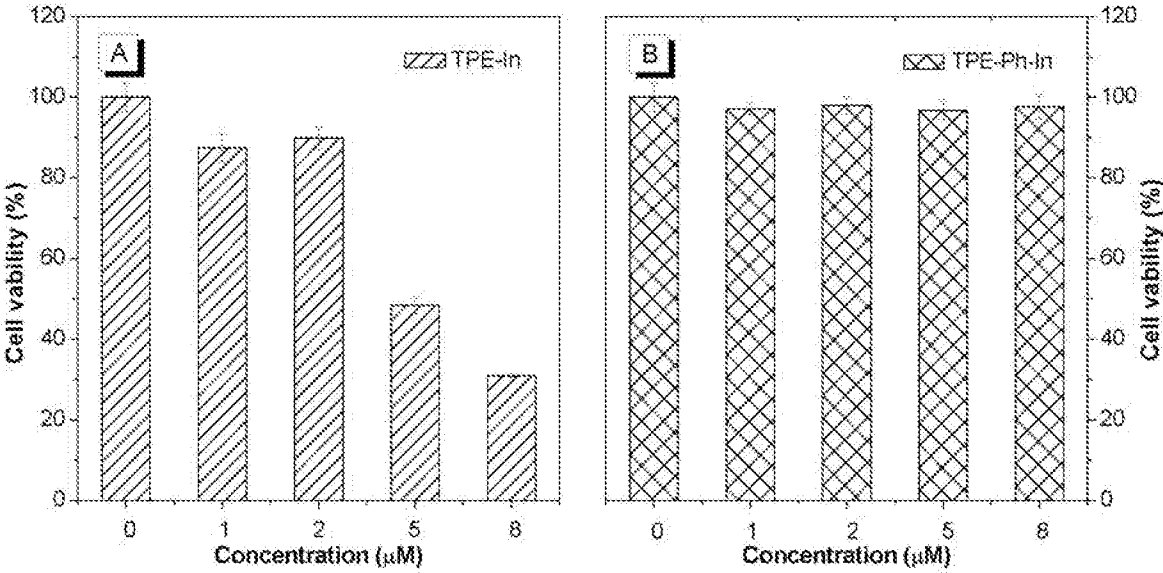


FIG. 2



FIGS. 3A-B

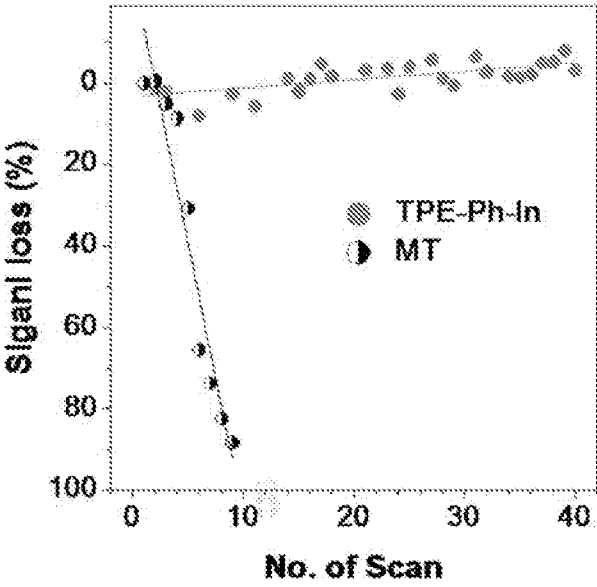
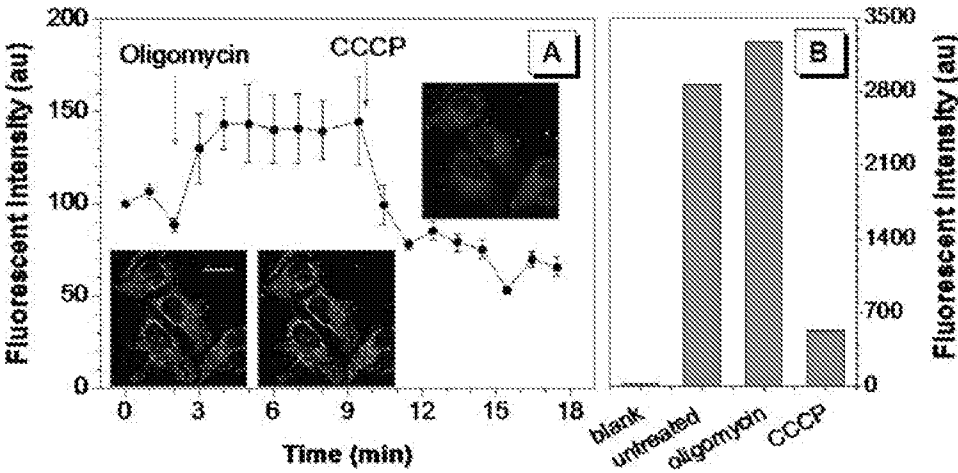
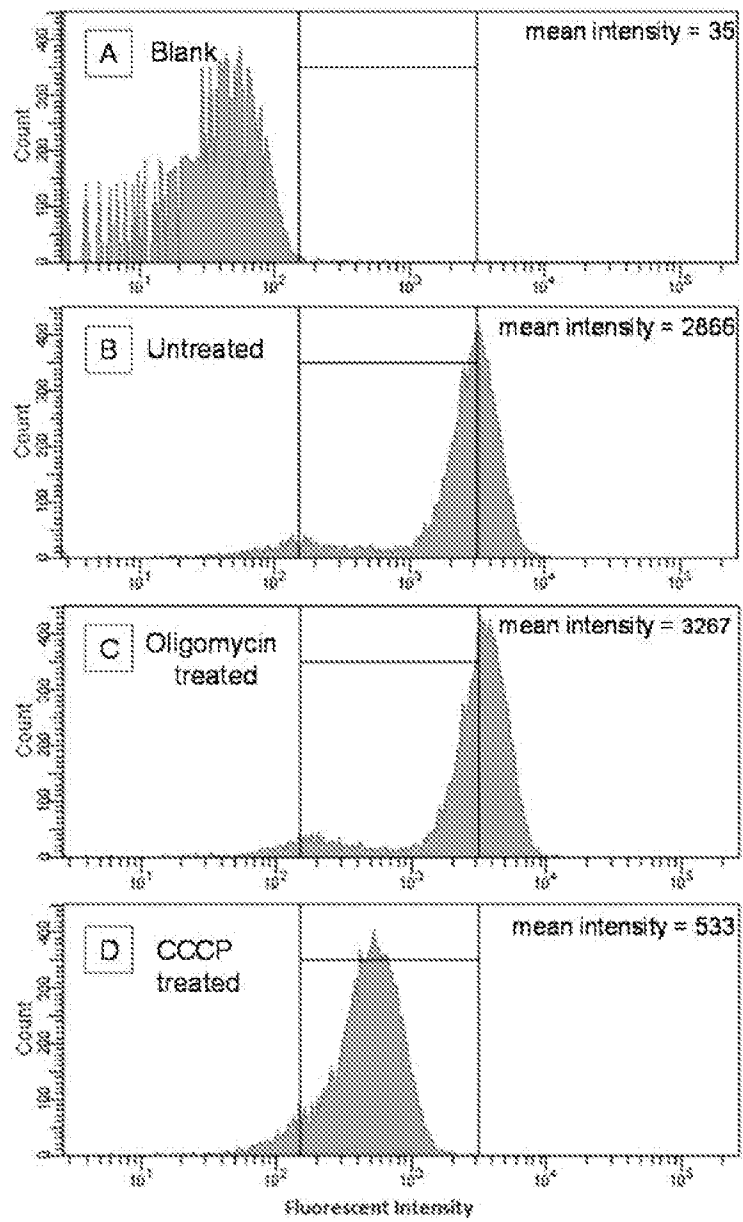


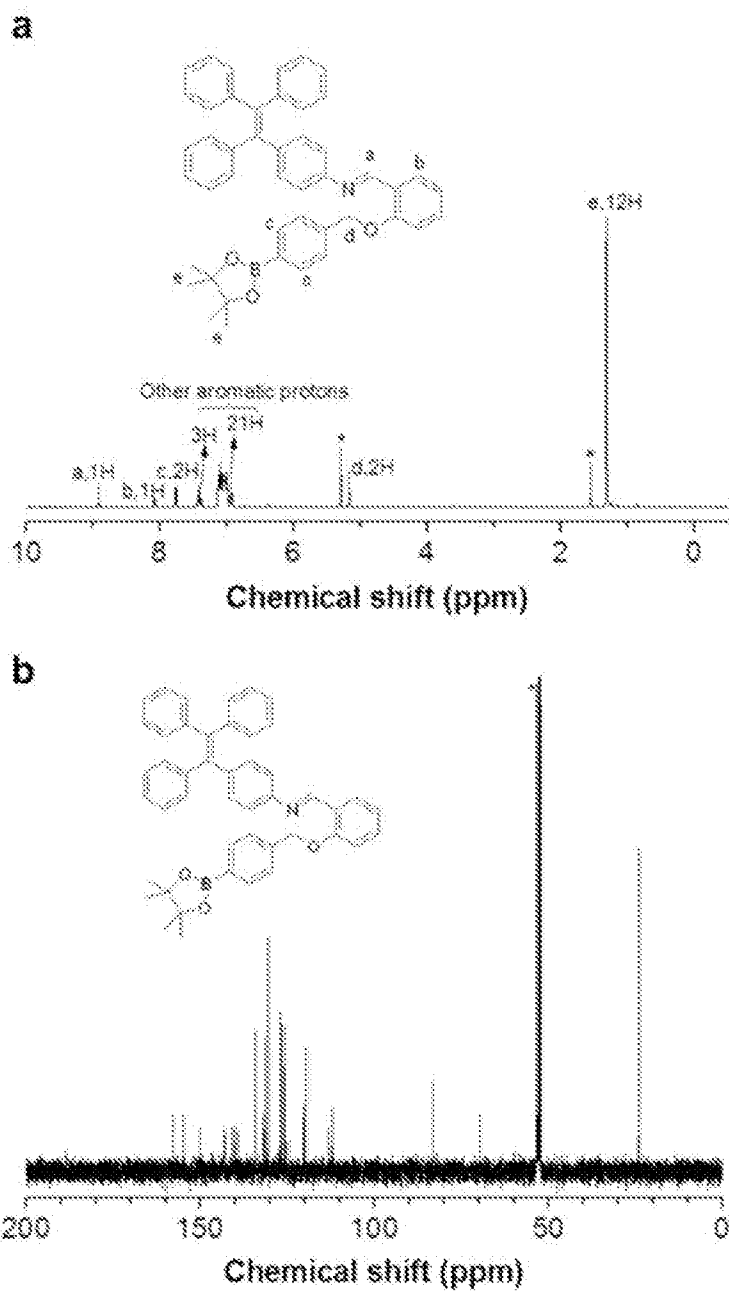
FIG. 4



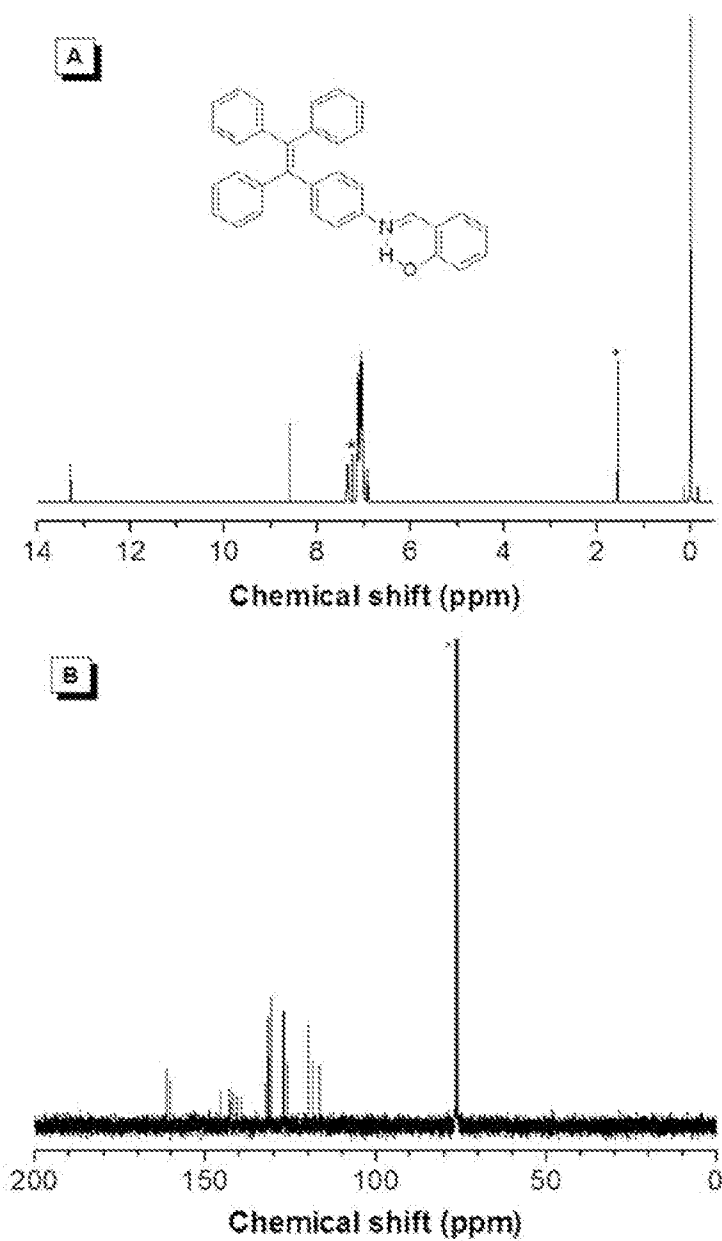
FIGS. 5A-B



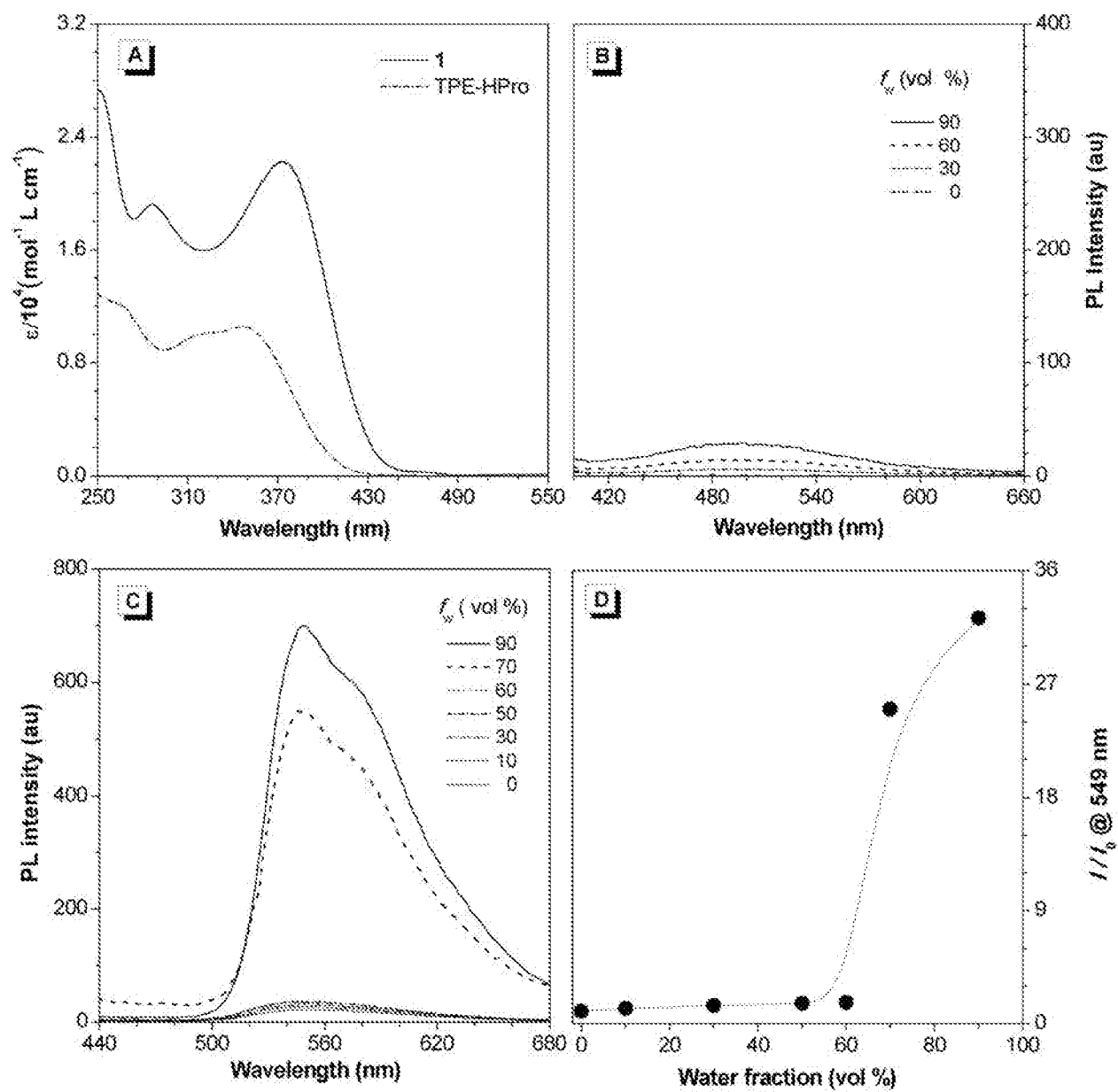
FIGS. 6A-D



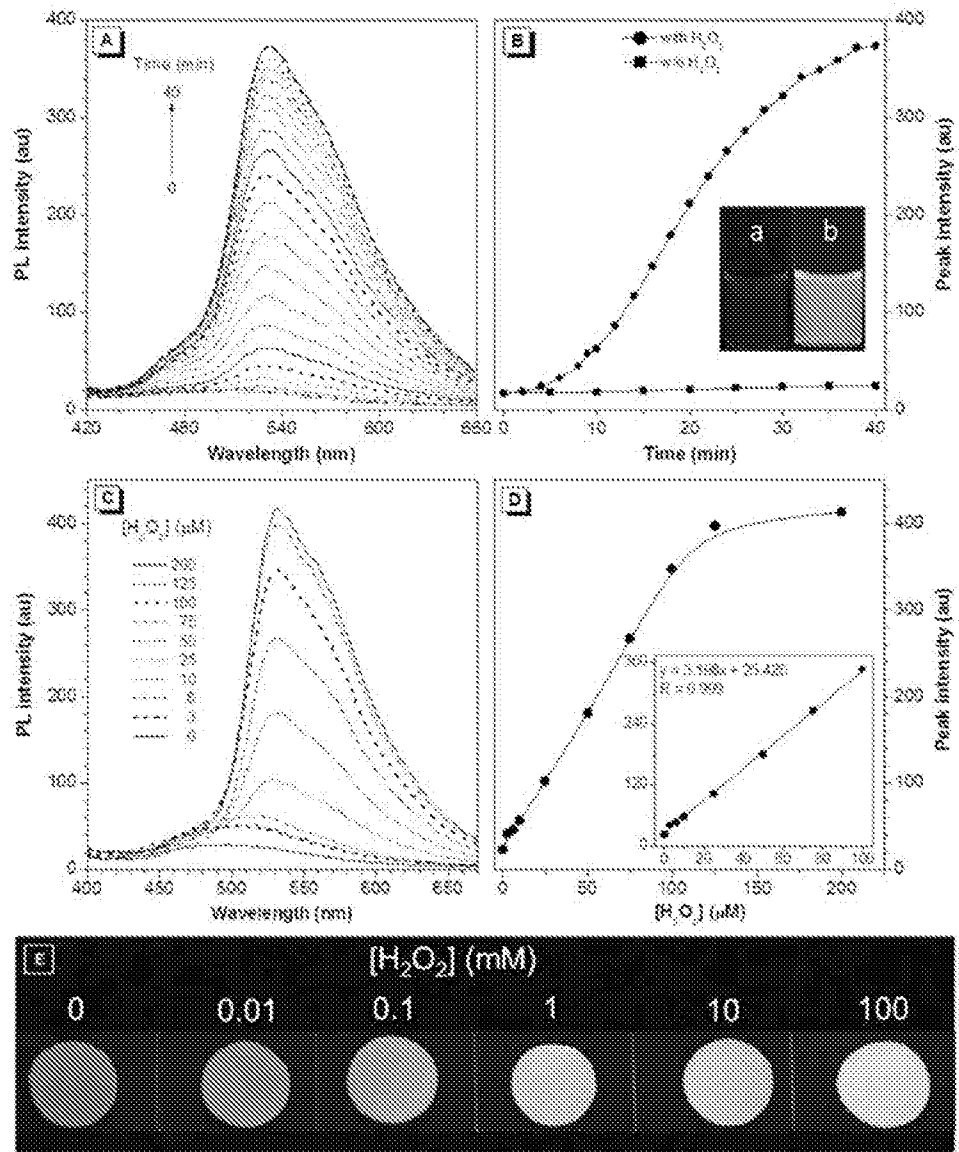
FIGS. 7A-B



FIGS. 8A-B



FIGS. 9A-D



FIGS. 10A-E

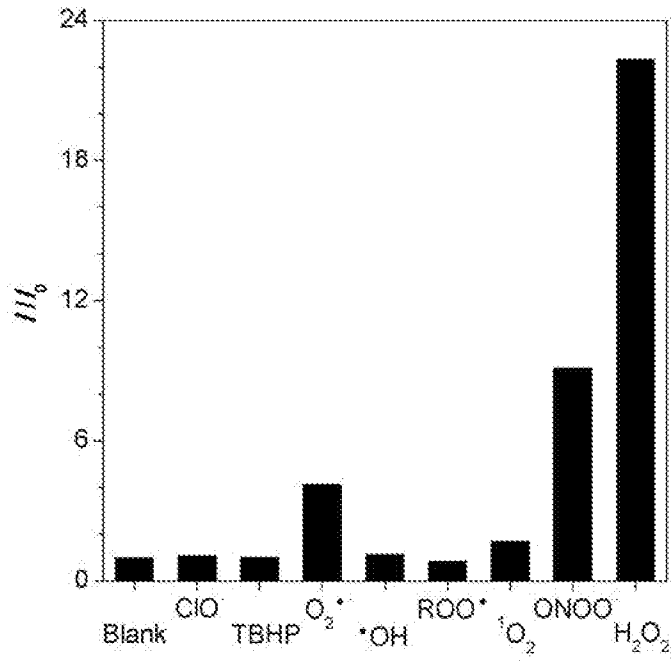
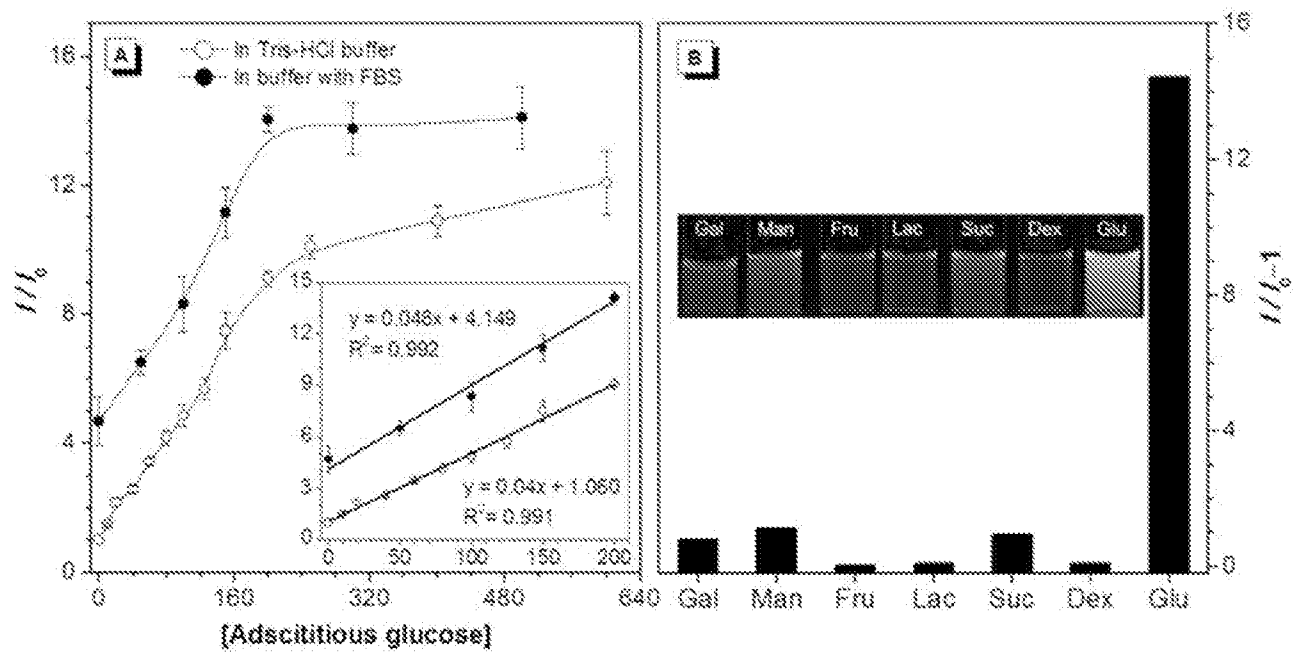


FIG. 11



FIGS. 12A-B

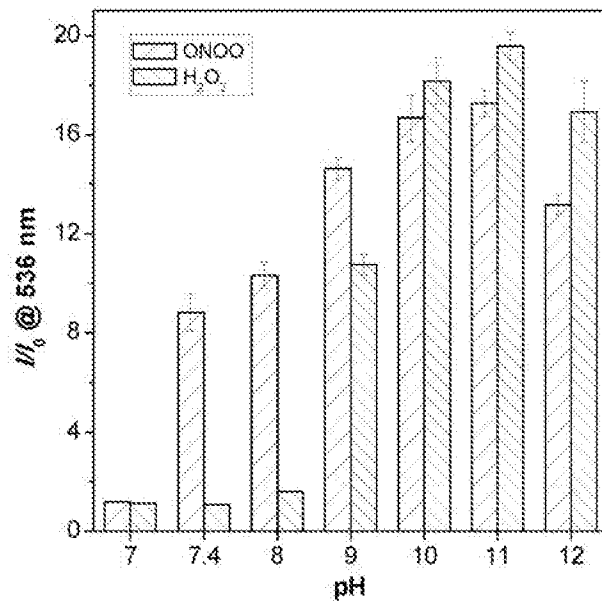
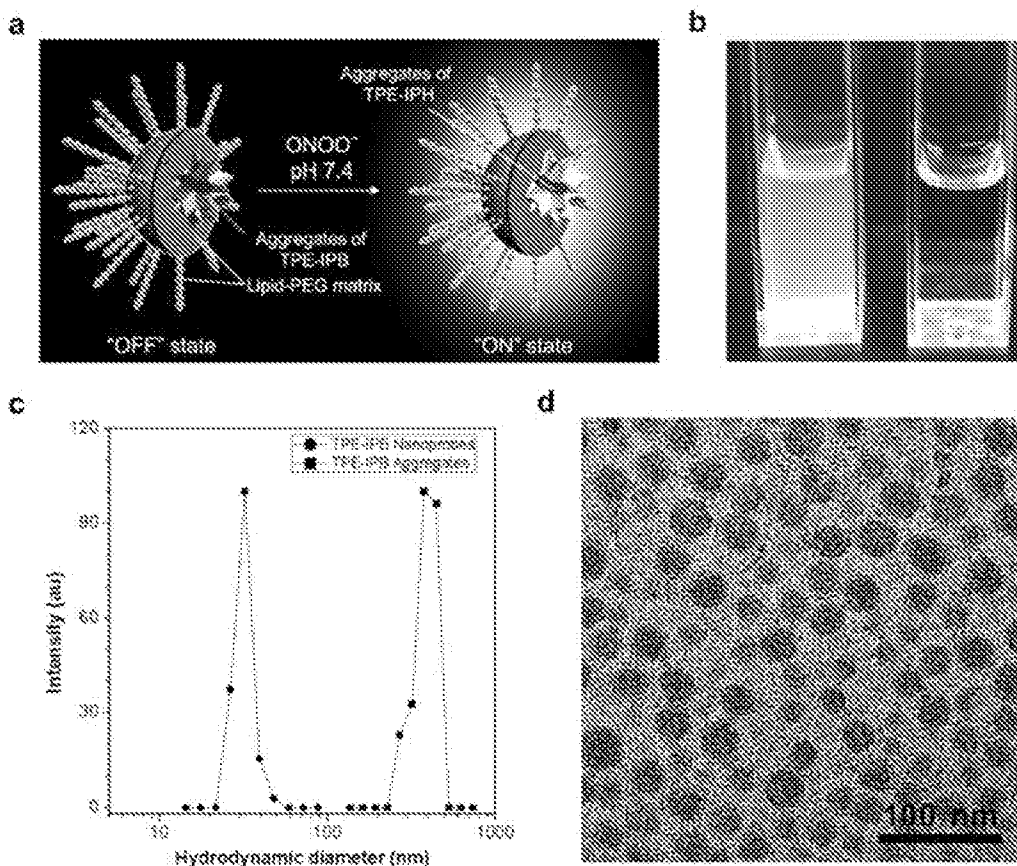


FIG. 13



FIGS. 14A-D

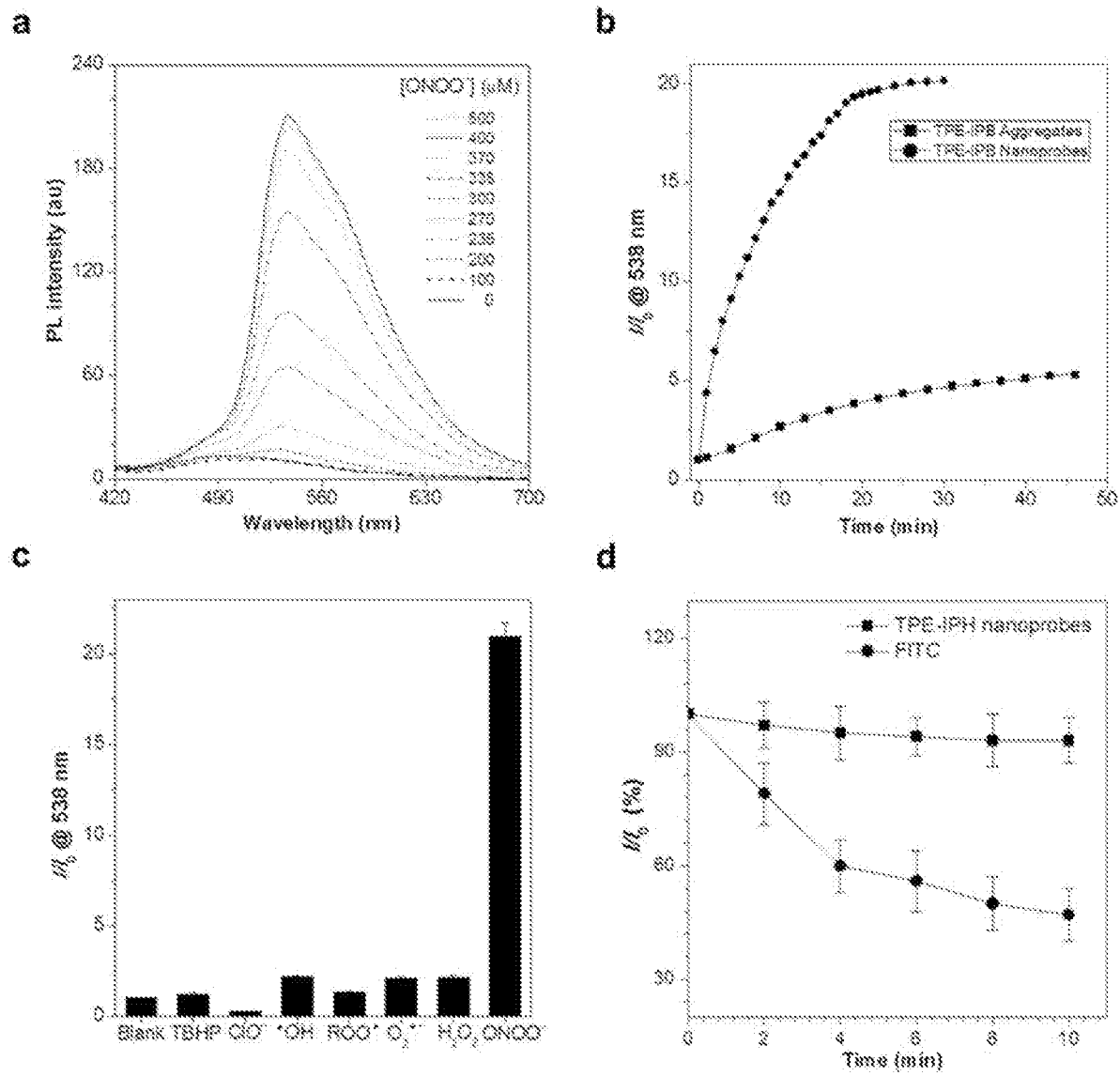


FIG. 15

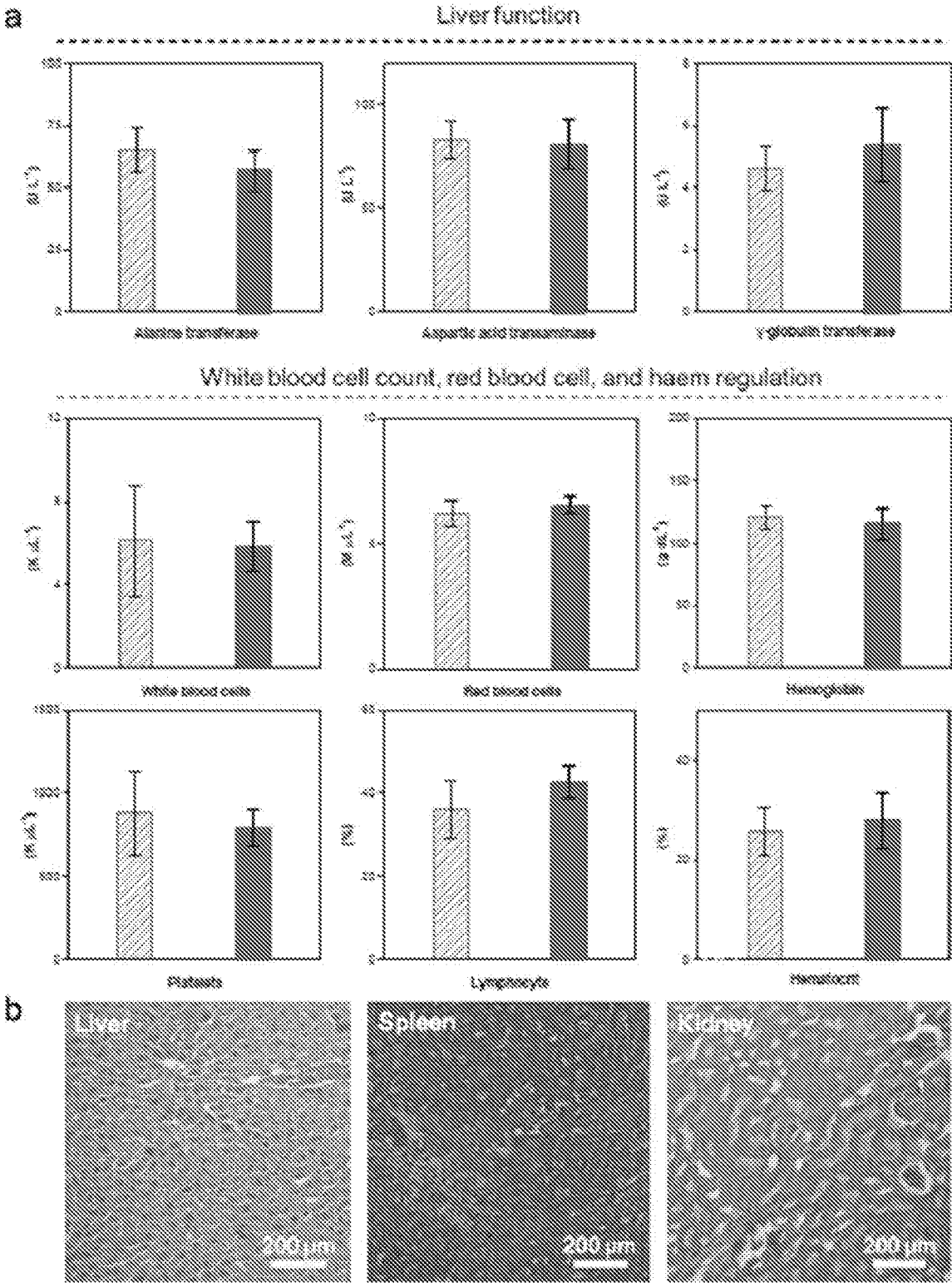
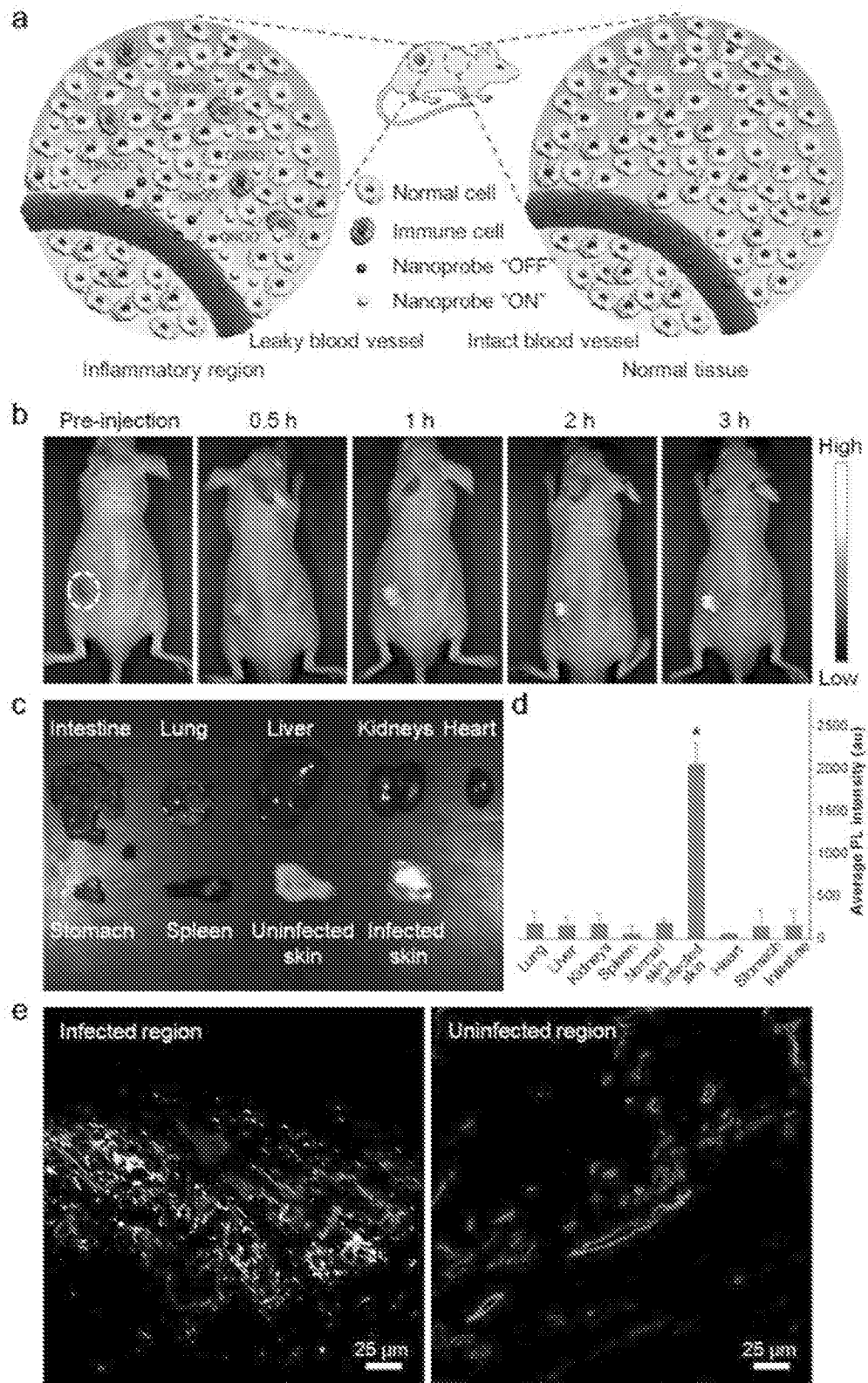
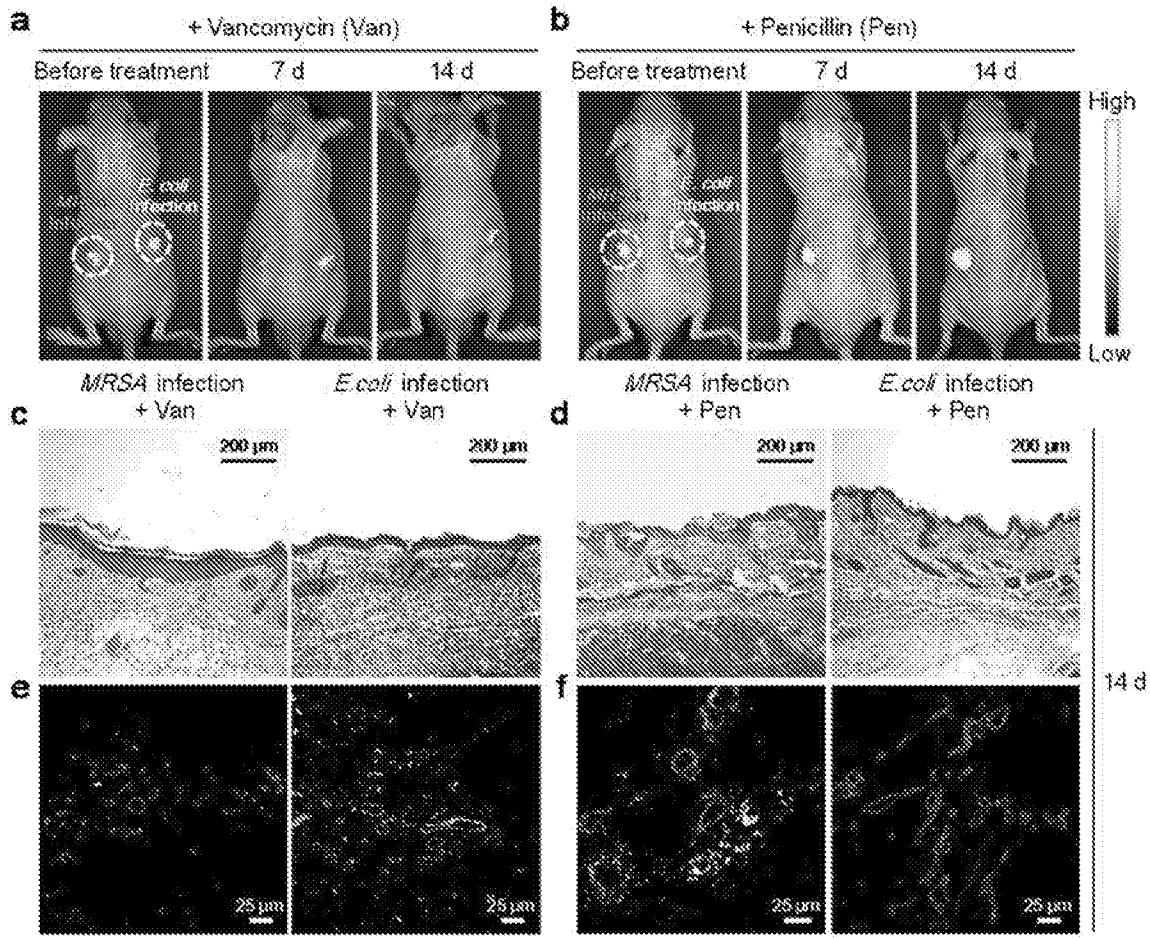


FIG. 16



FIGS. 17A-E



FIGS. 18A-D

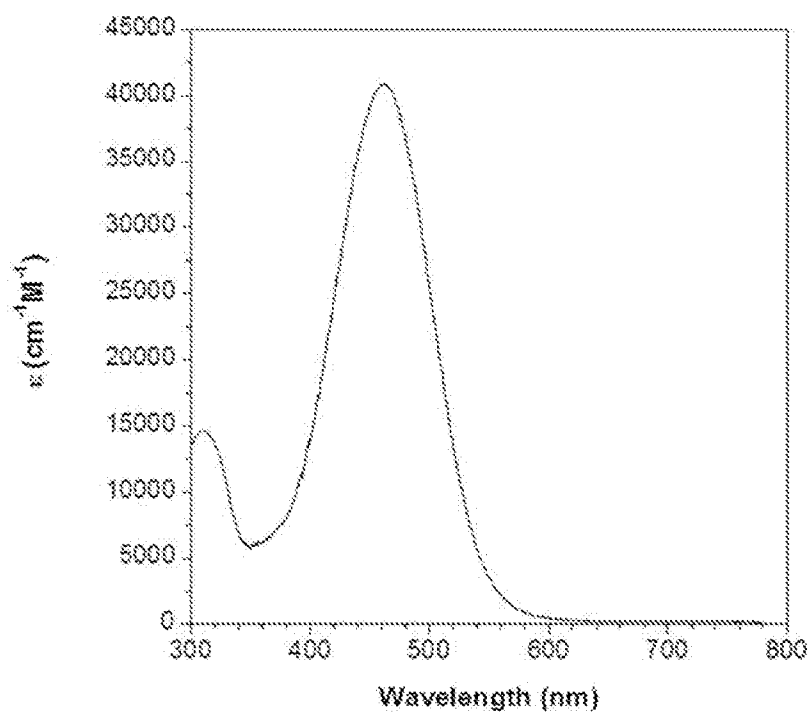


FIG. 19

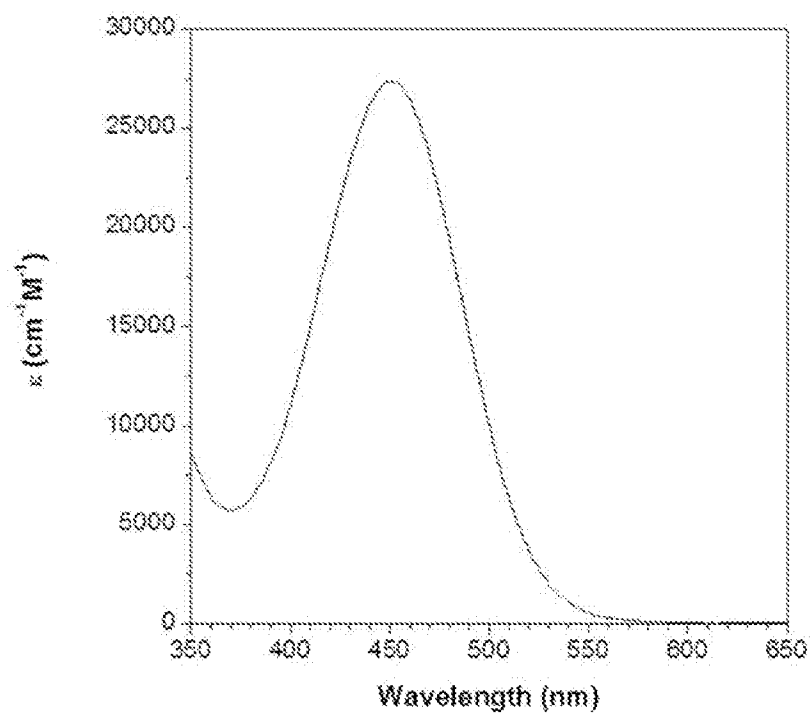


FIG. 20

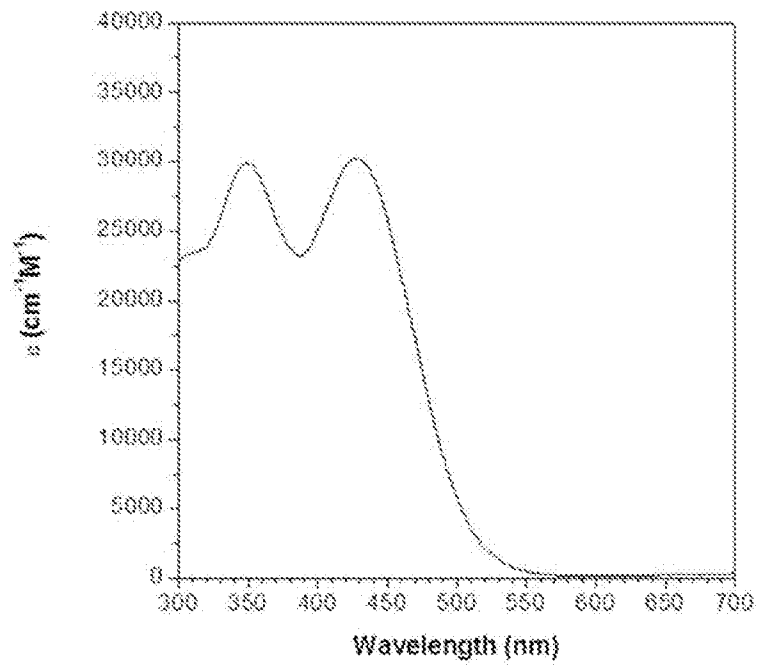


FIG. 21

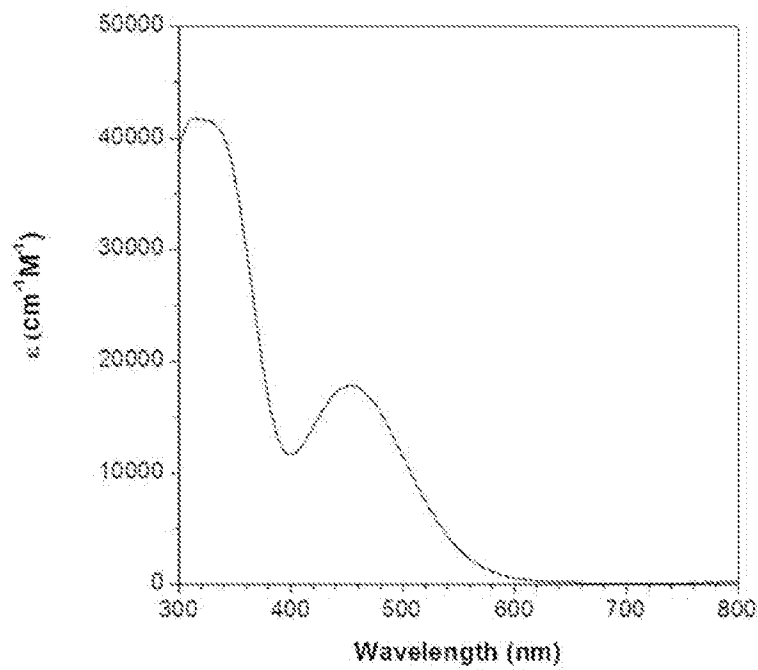


FIG. 22

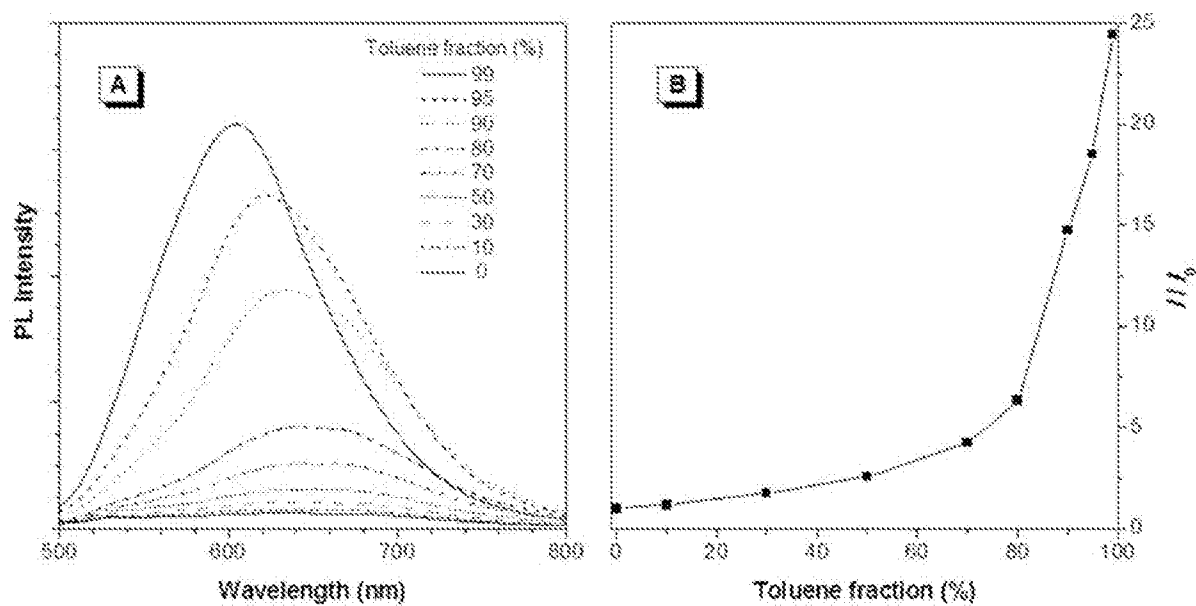


FIG. 23

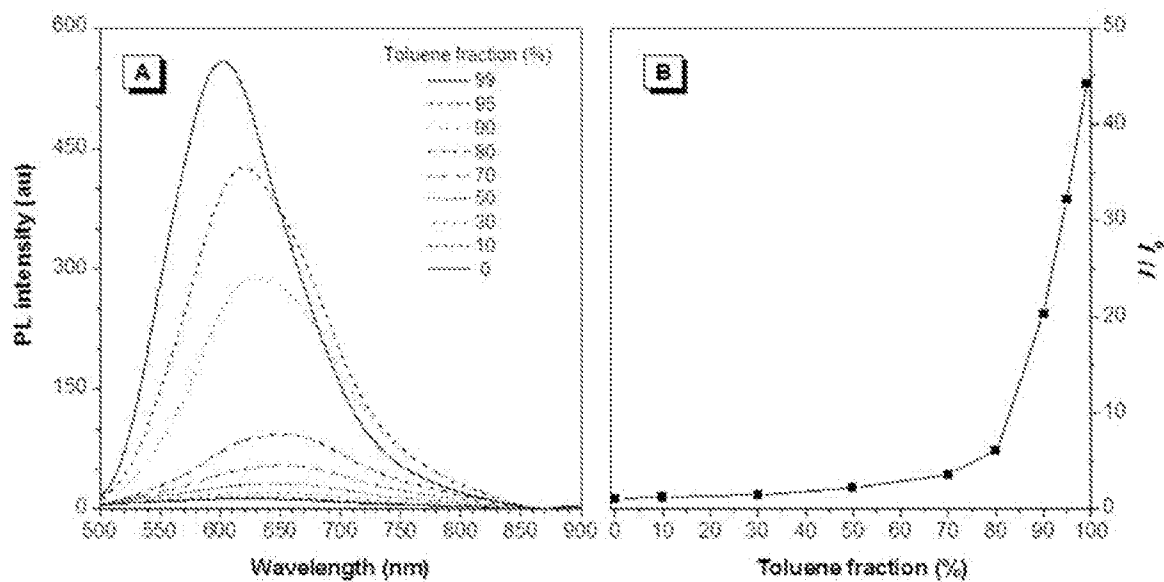


FIG. 24

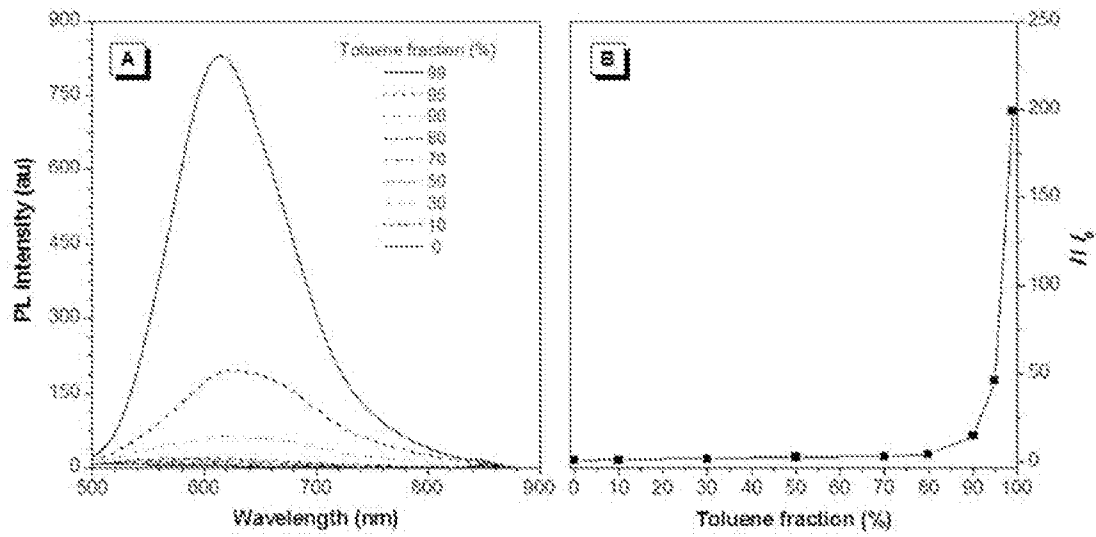


FIG. 25

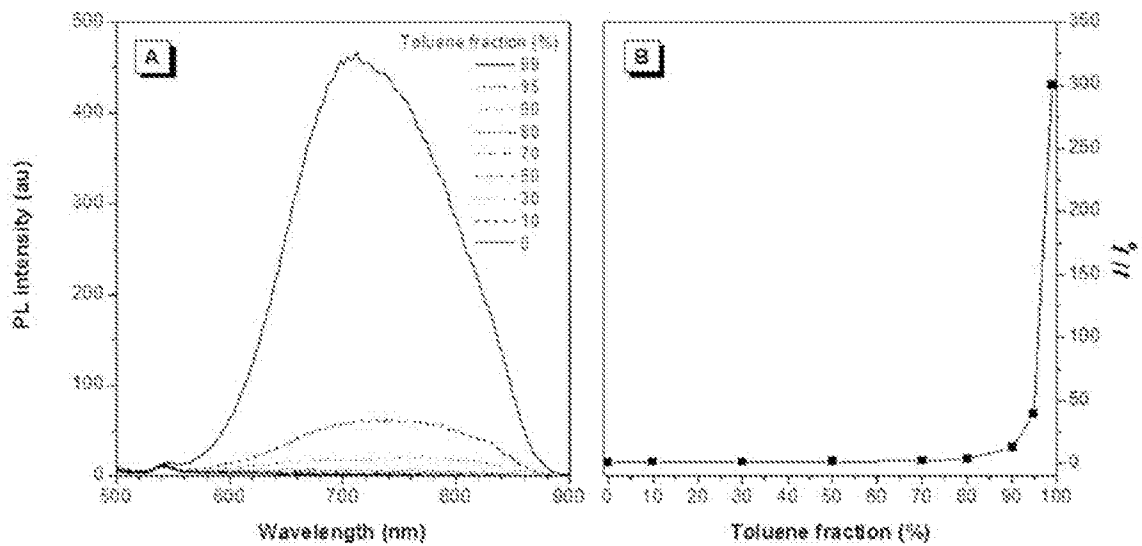


FIG. 26

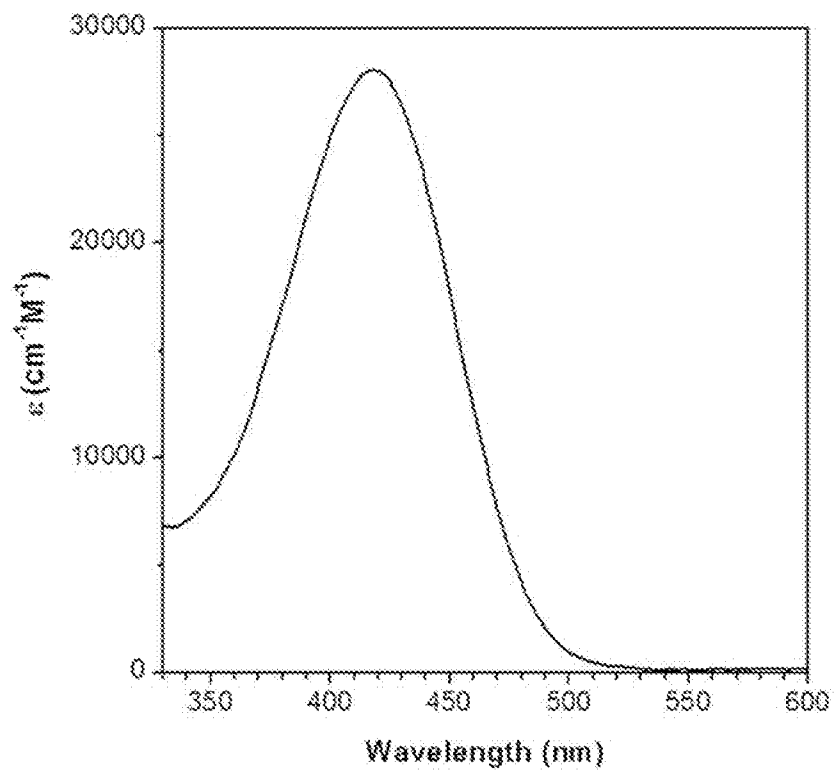


FIG. 27

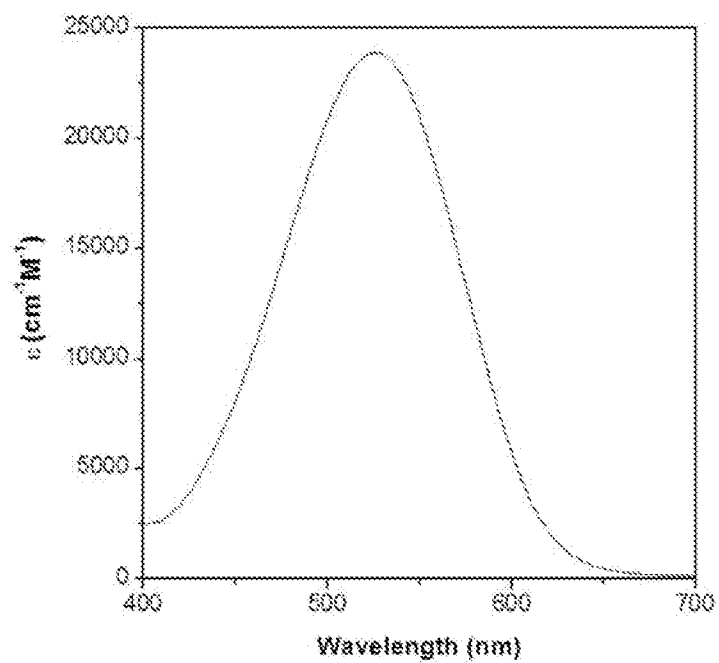


FIG. 28

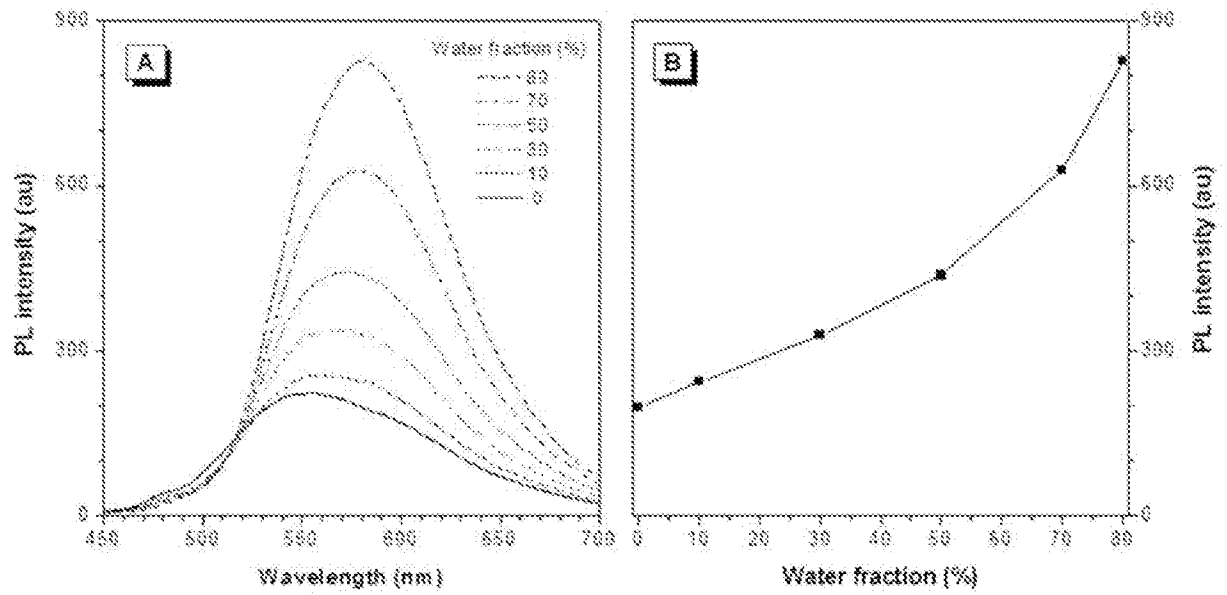


FIG. 29

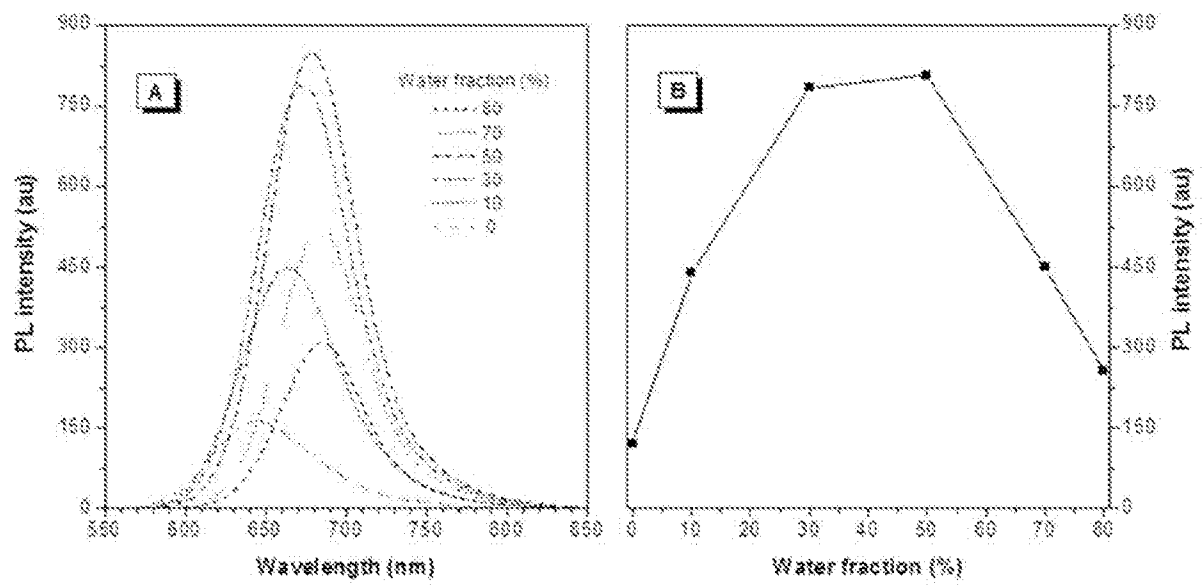


FIG. 30

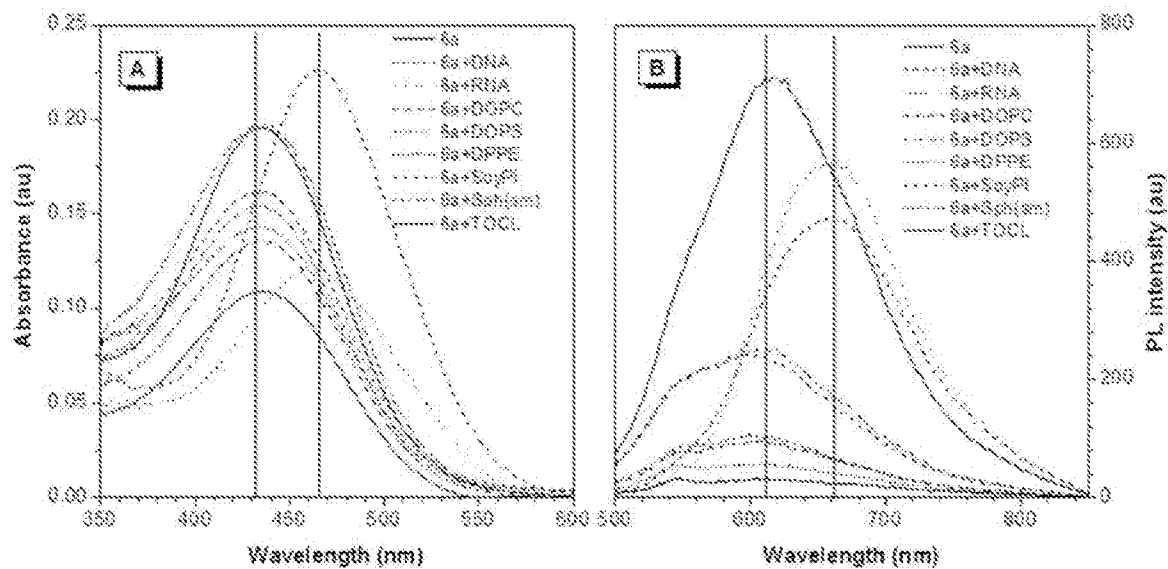


FIG. 31

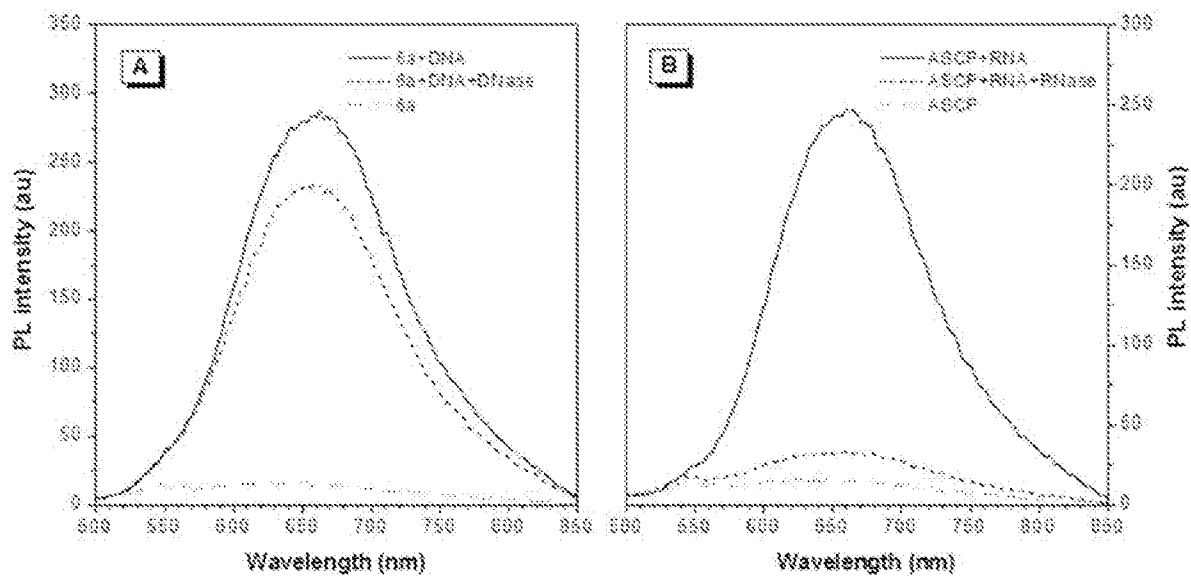


FIG. 32

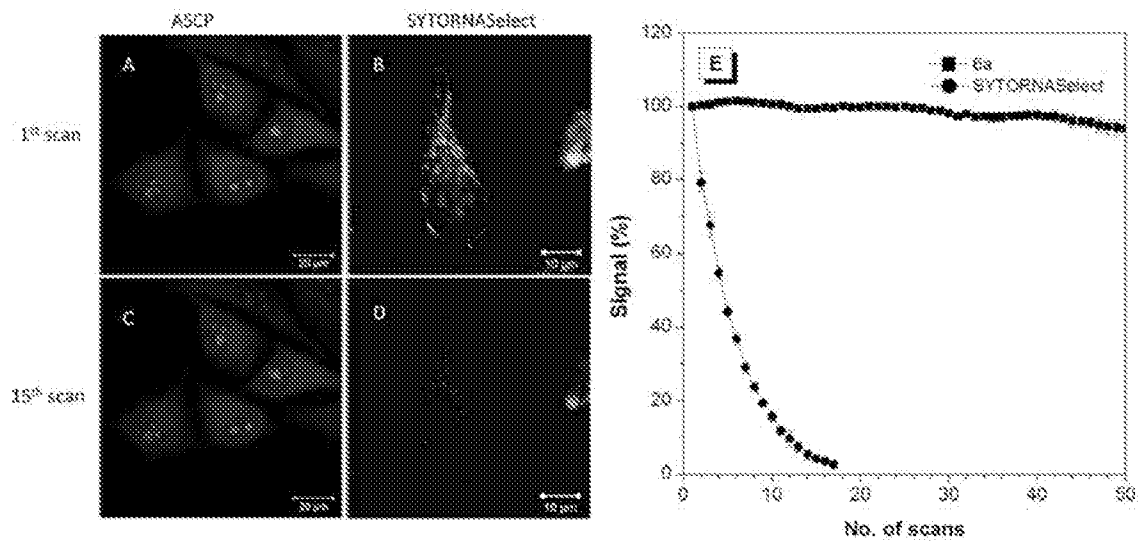


FIG. 33

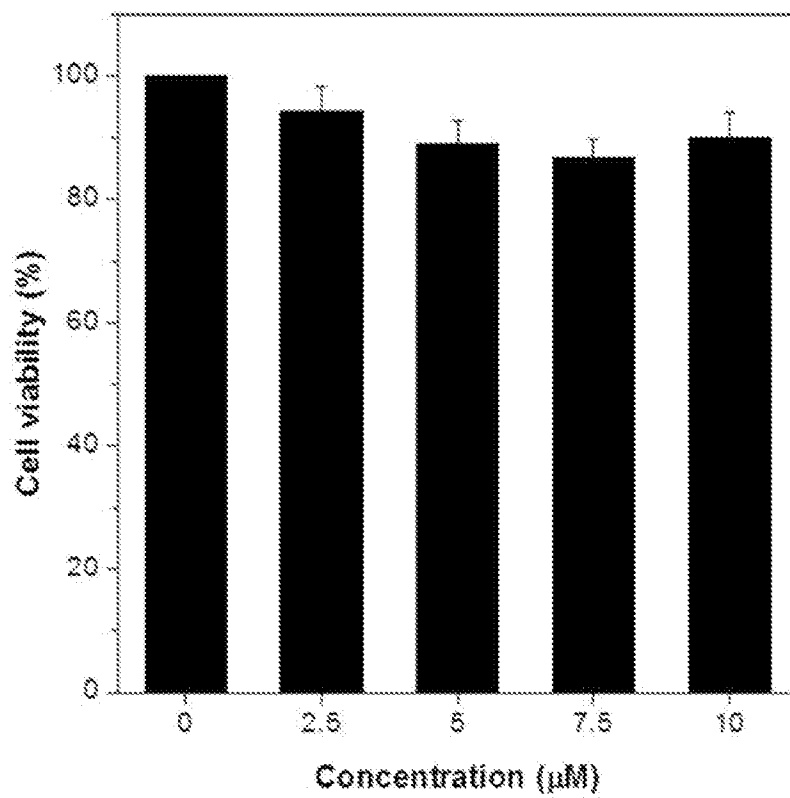


FIG. 34

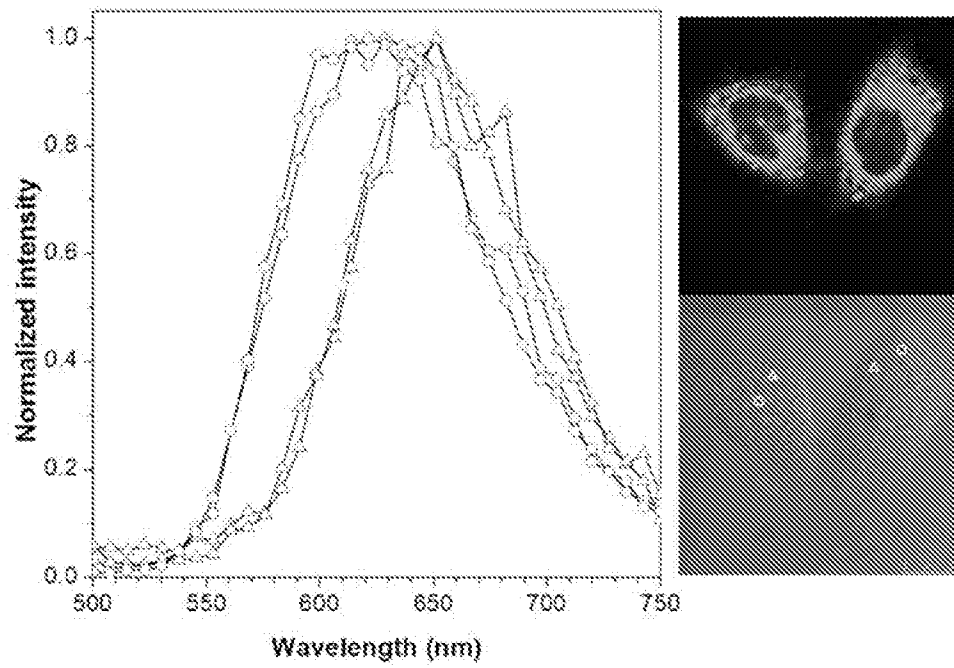


FIG. 35

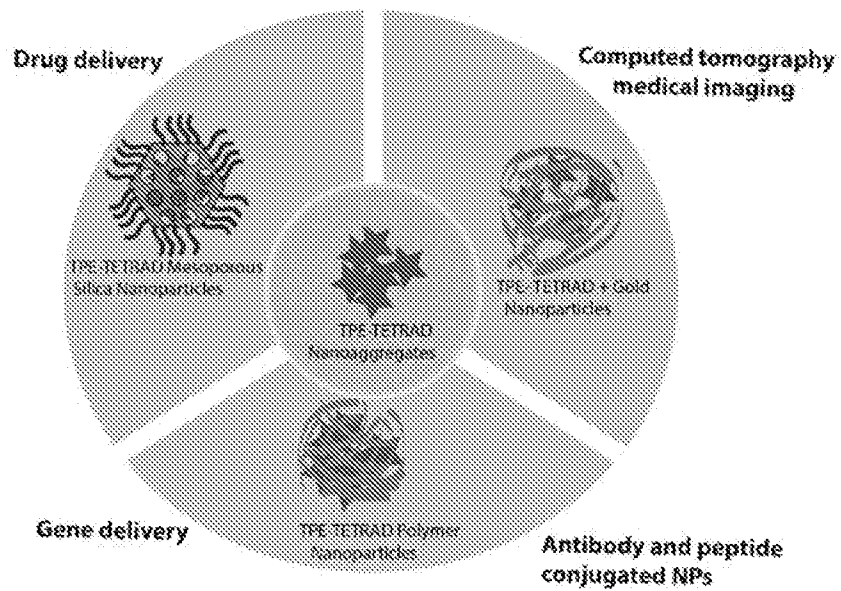


FIG. 36

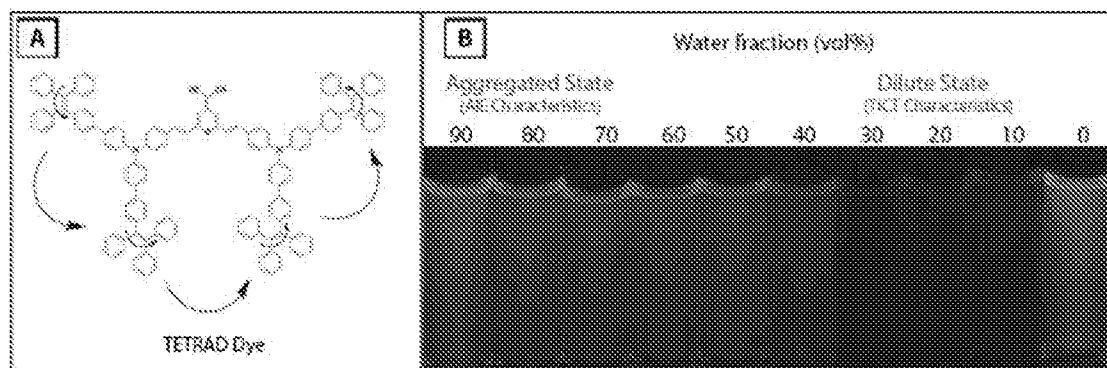


FIG. 37

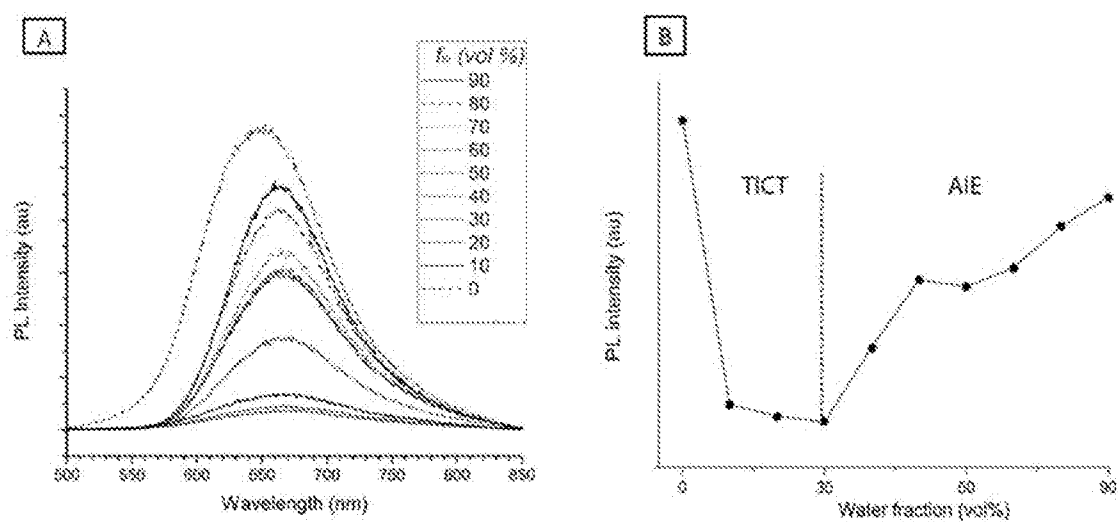


FIG. 38

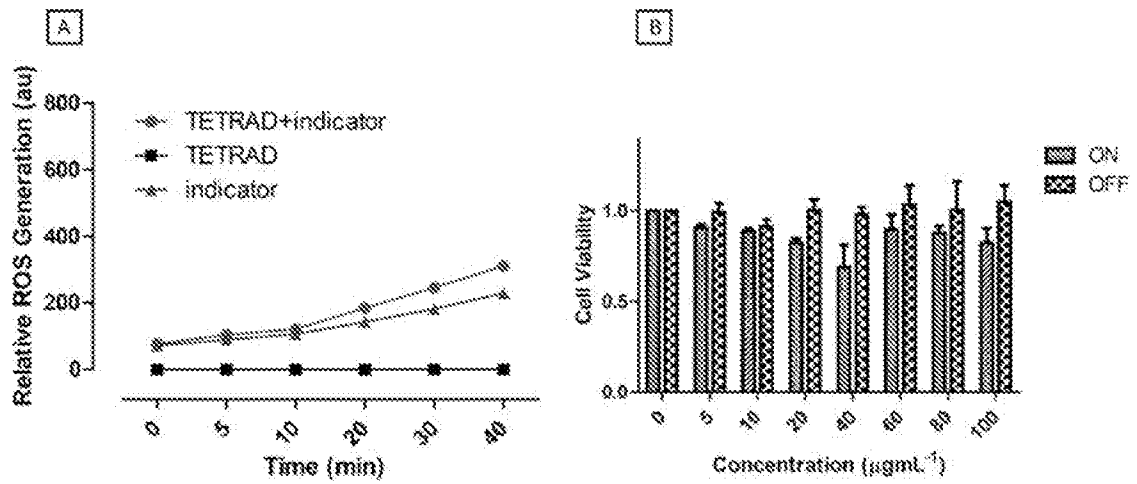


FIG. 39

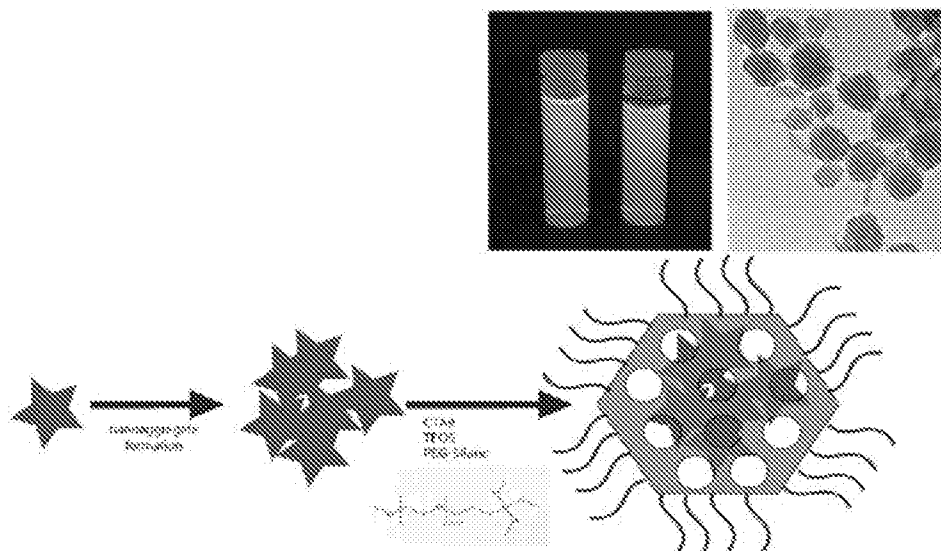


FIG. 40

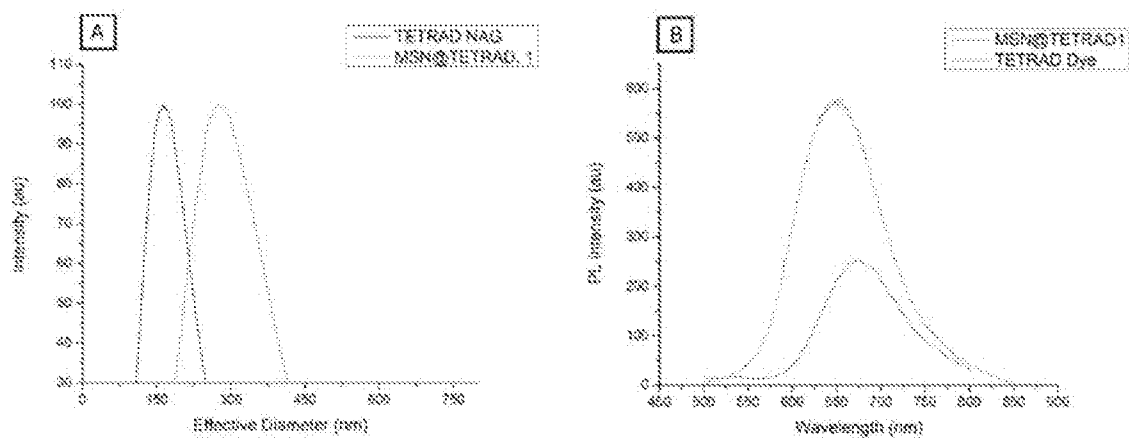


FIG. 41

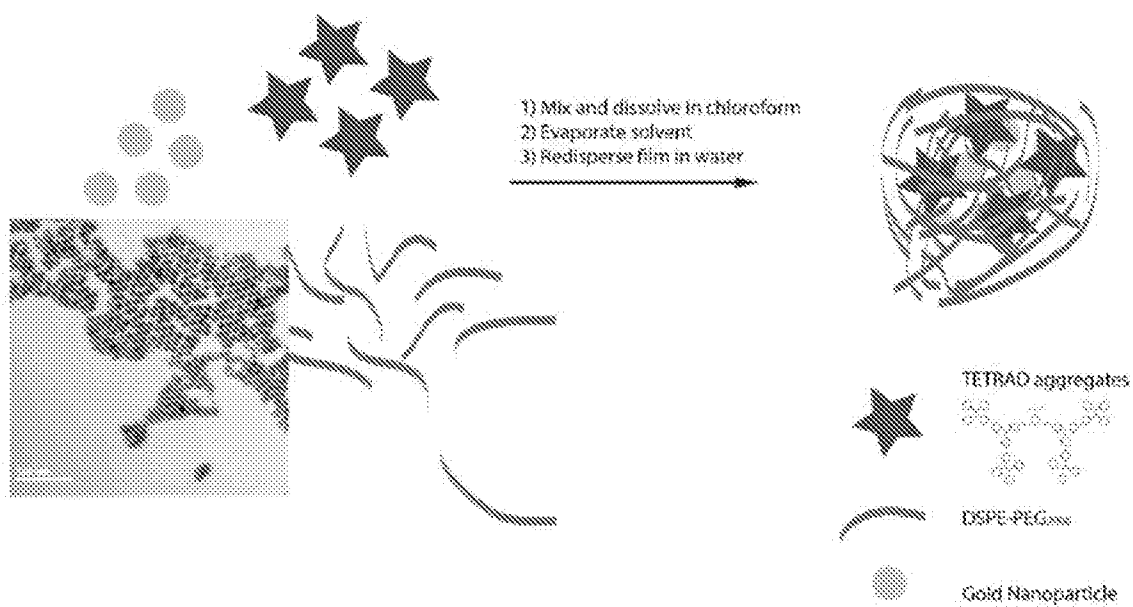


FIG. 42

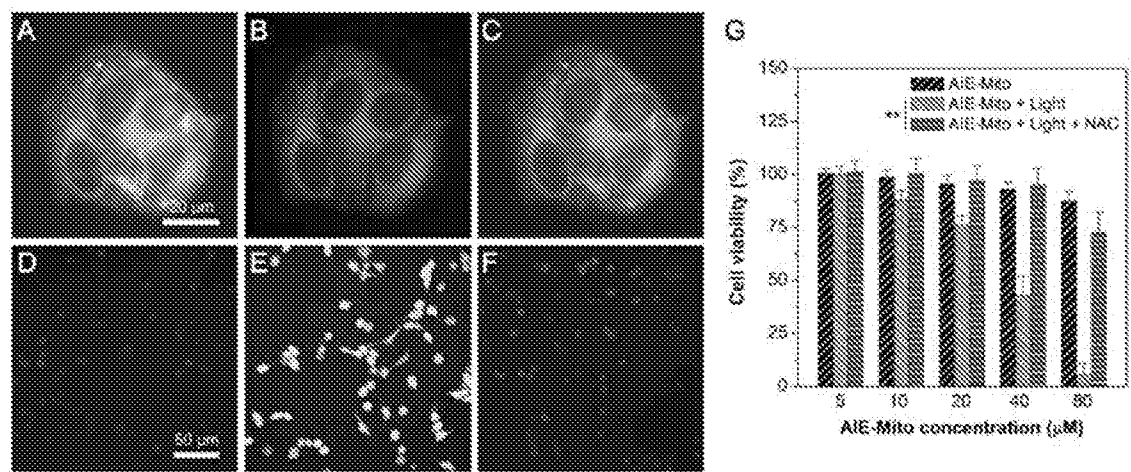


FIG. 43

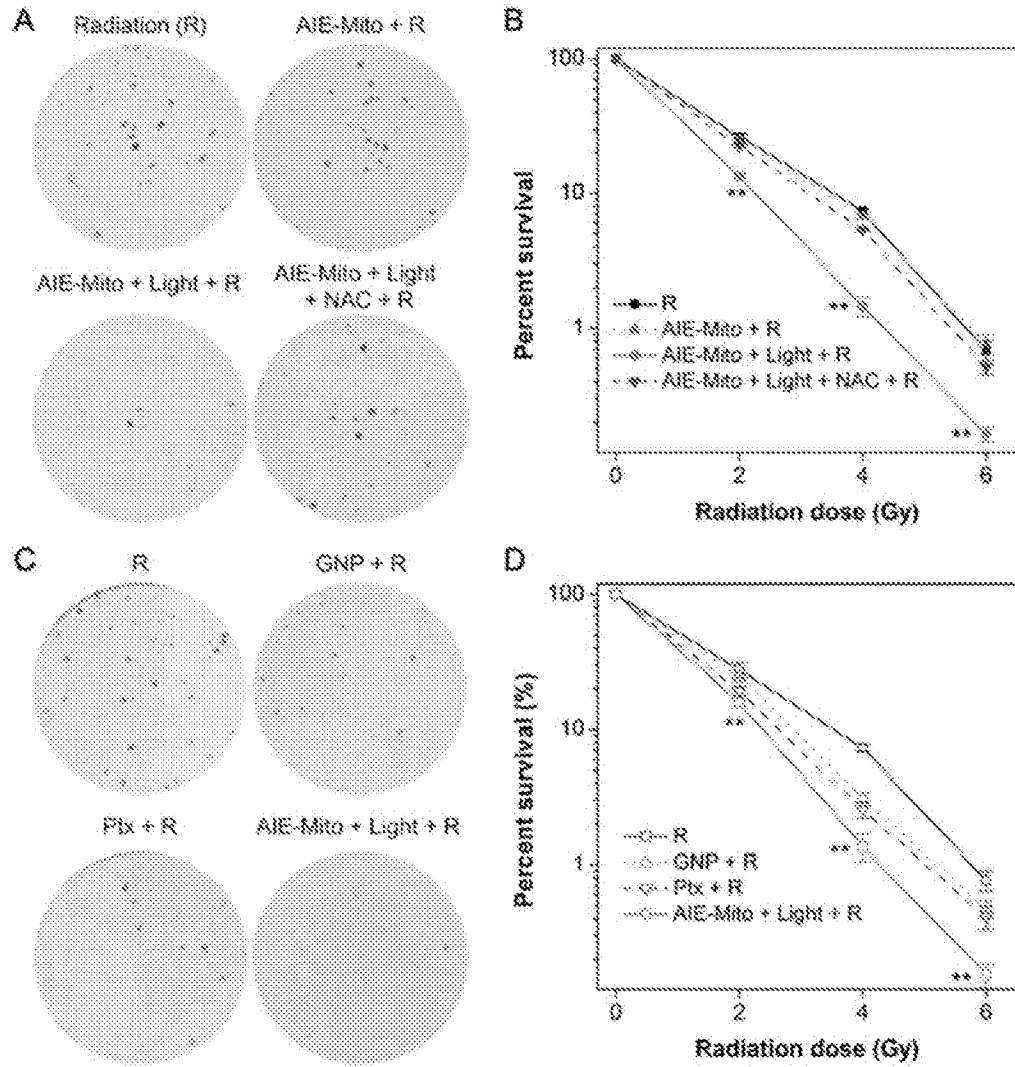


FIG. 44

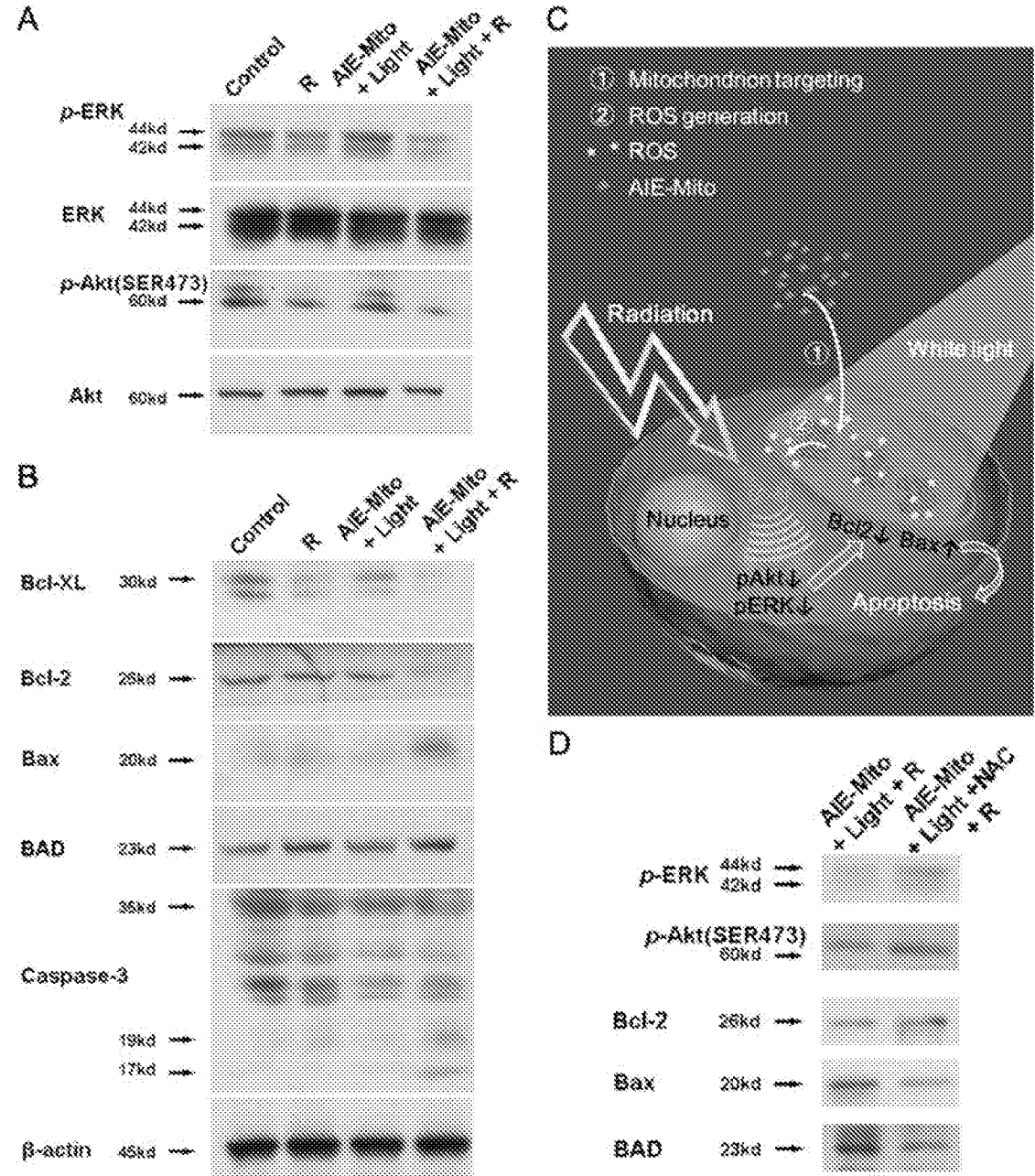


FIG. 45

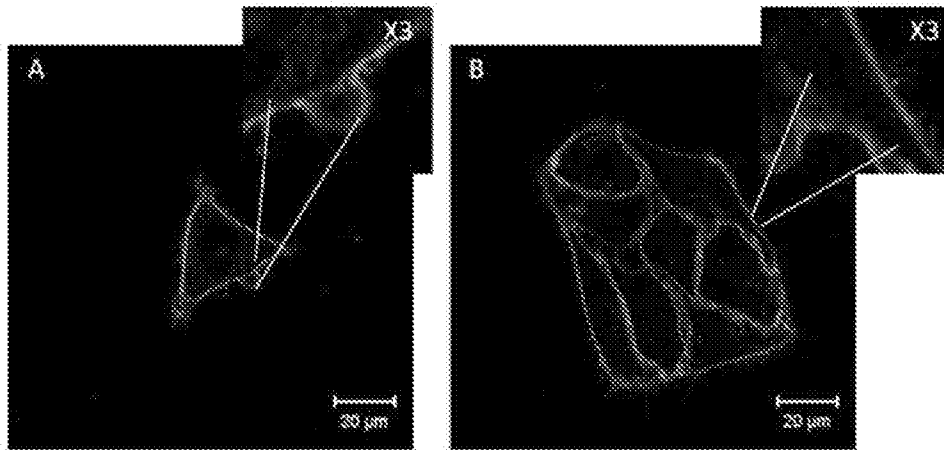


FIG. 46

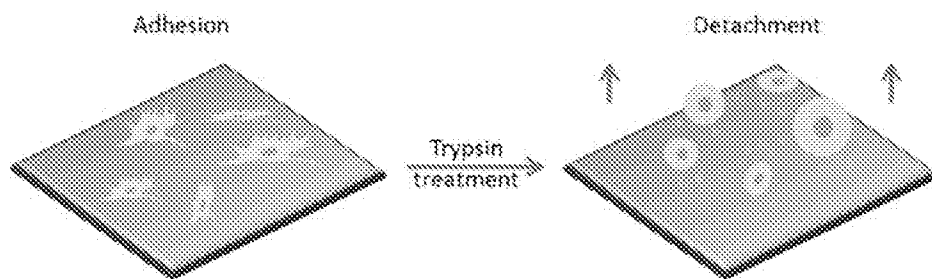


FIG. 47

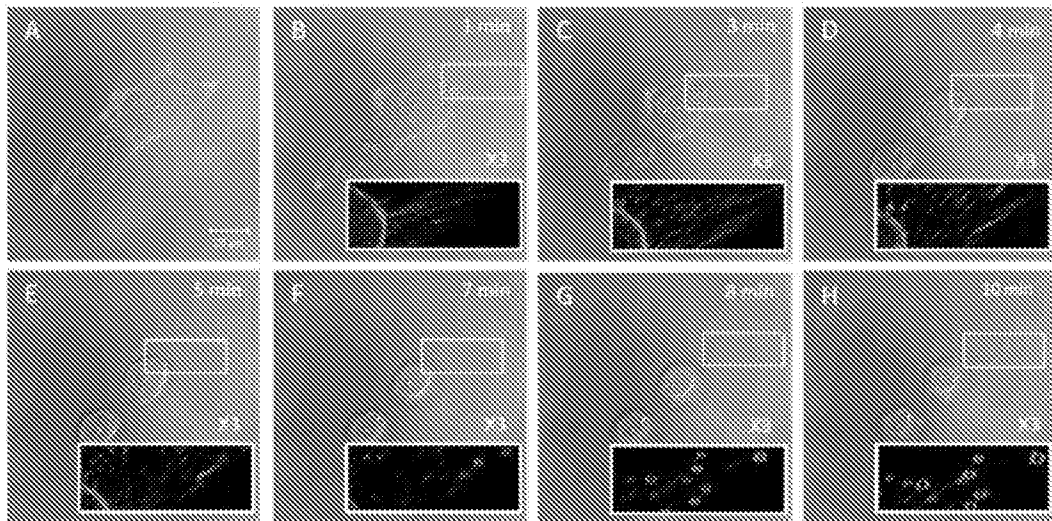


FIG. 48

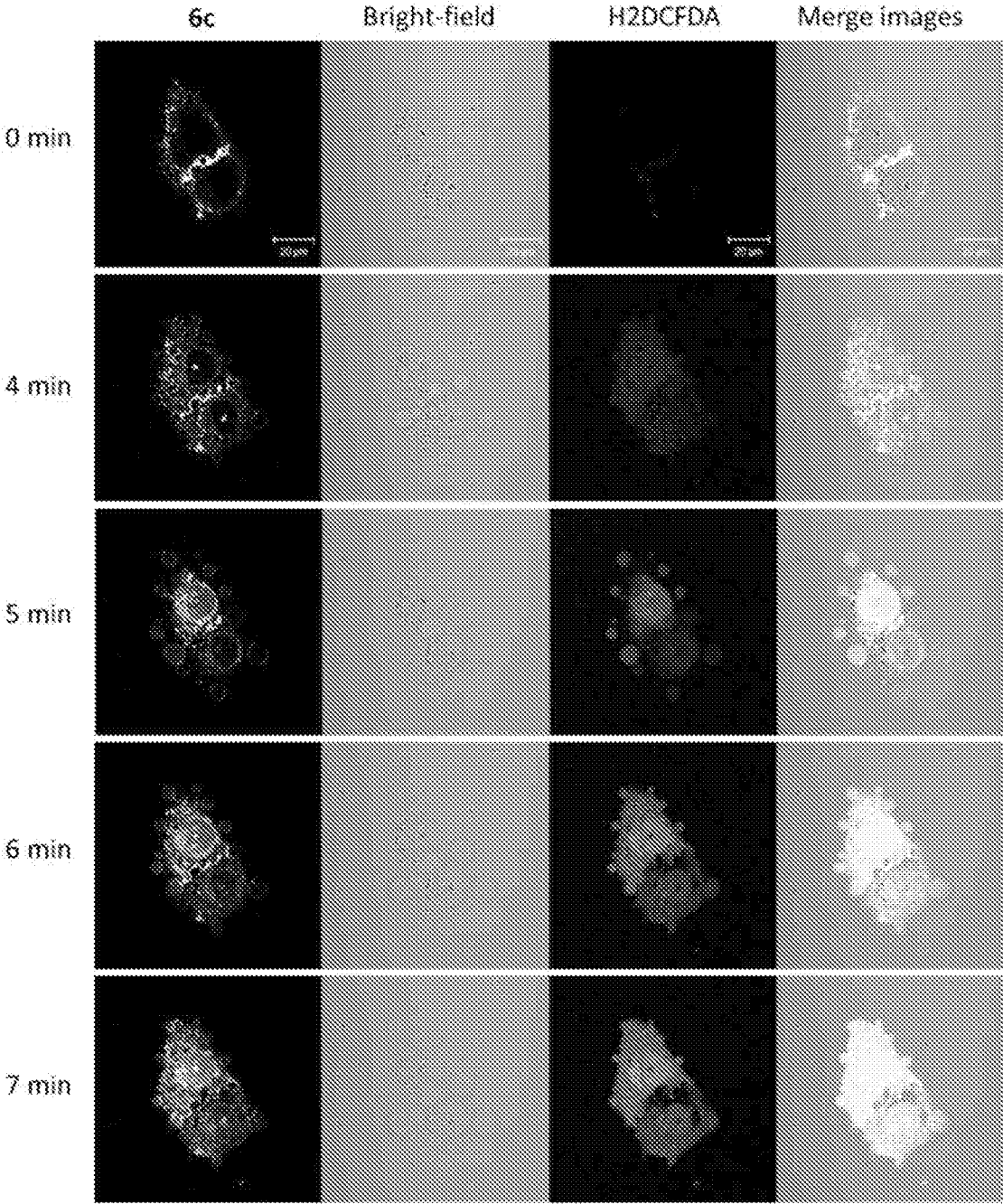


FIG. 49

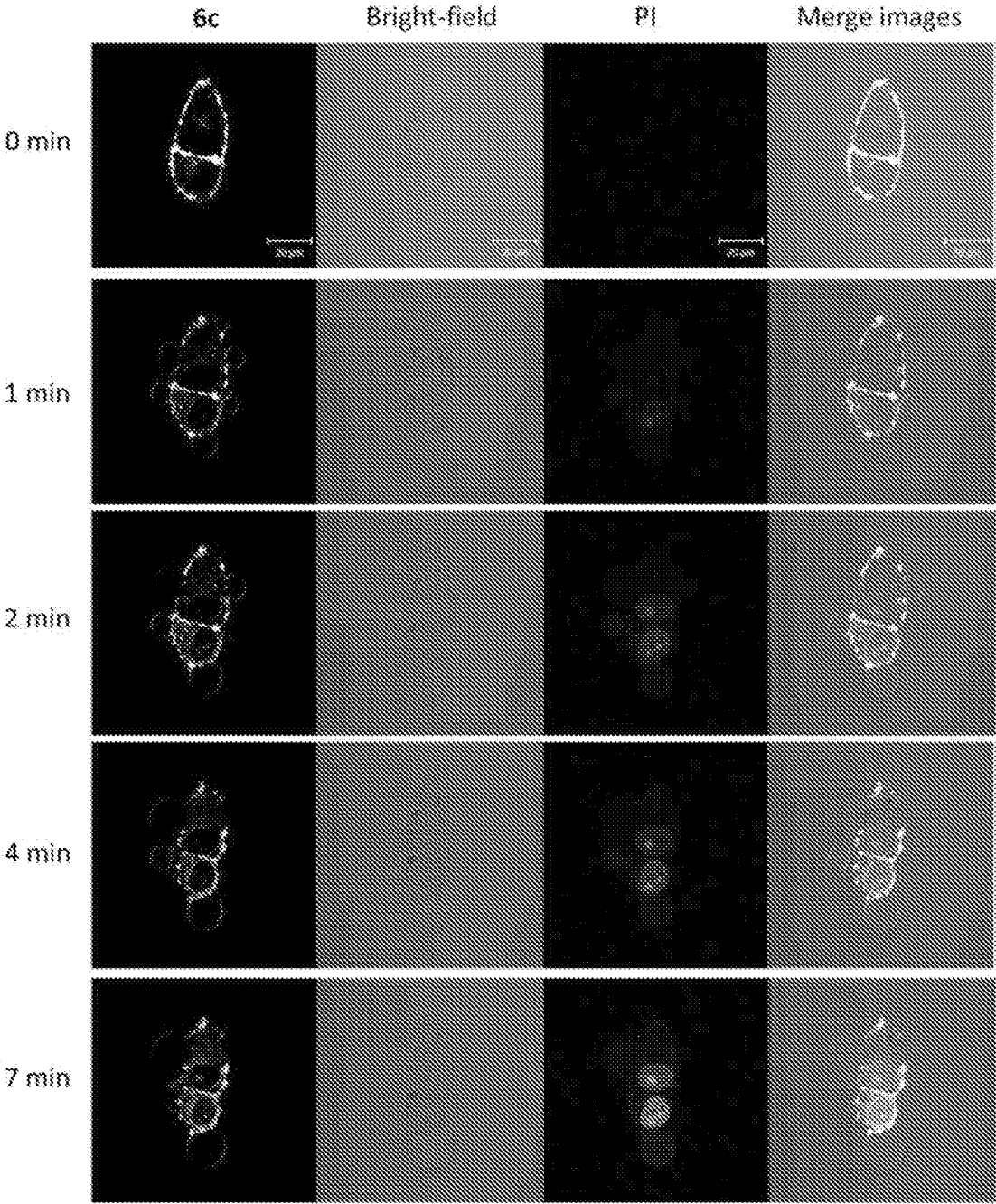


FIG. 50

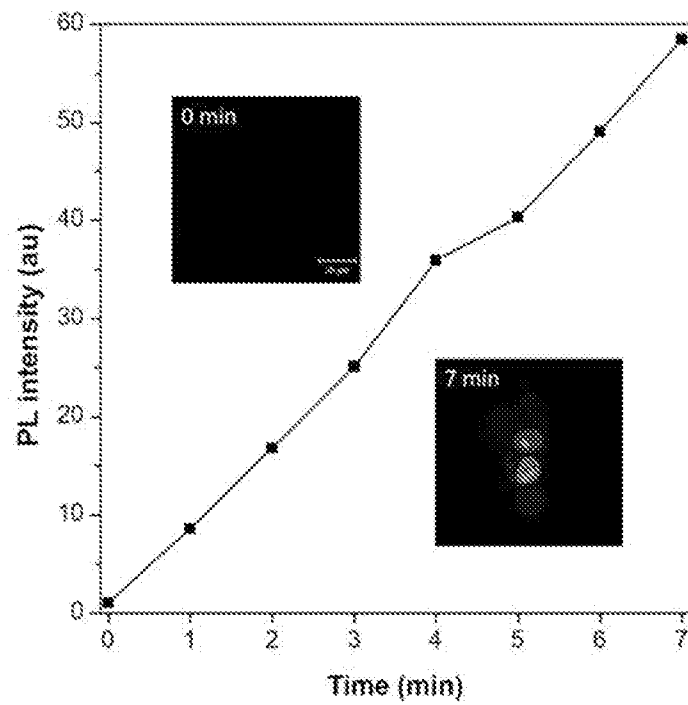


FIG. 51

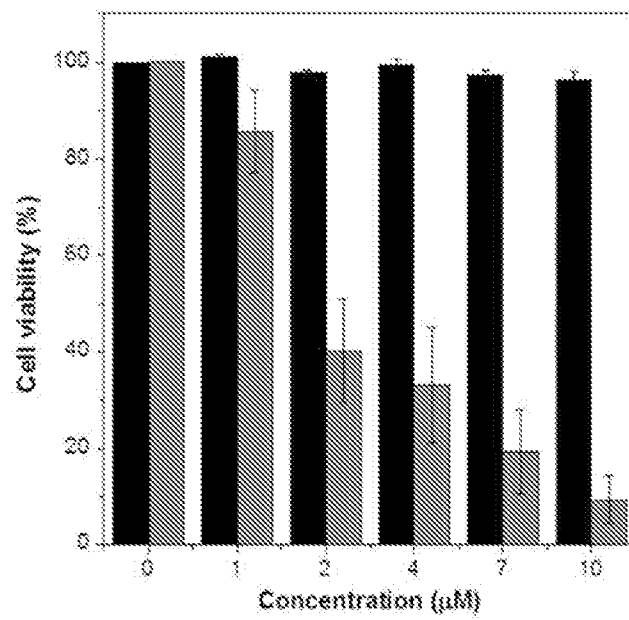


FIG. 52

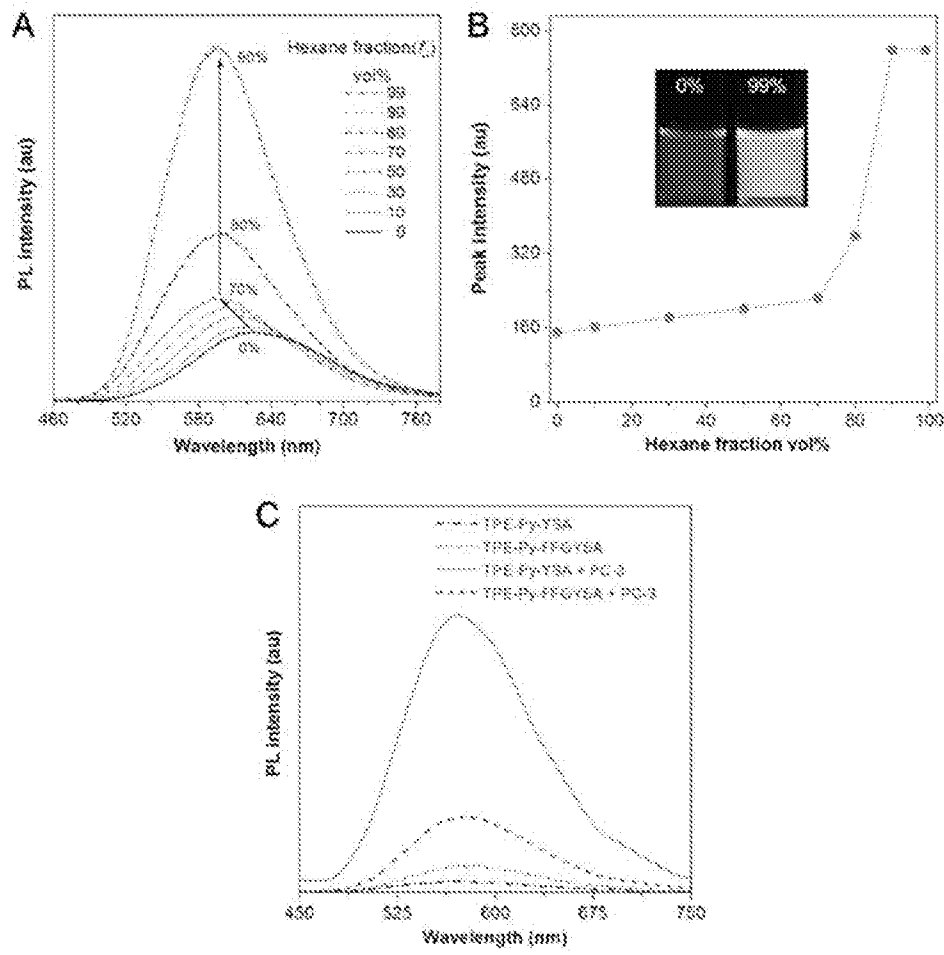


FIG. 53

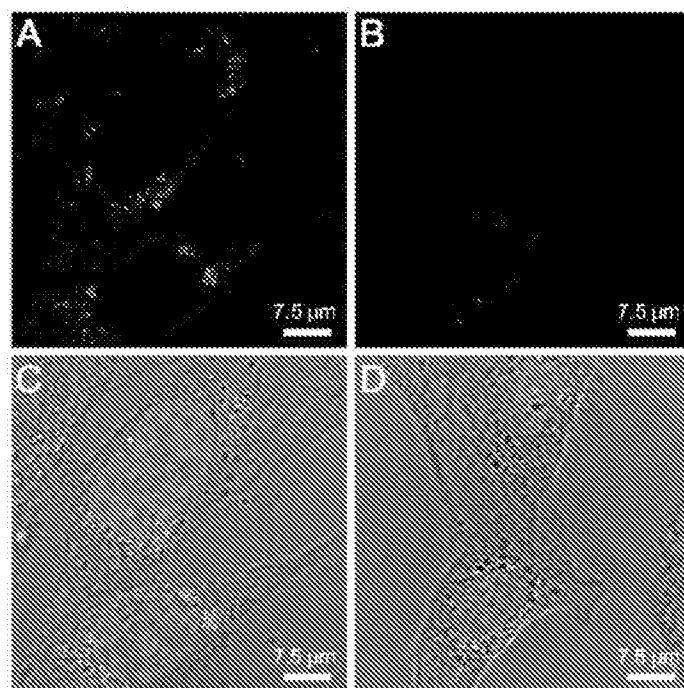


FIG. 54

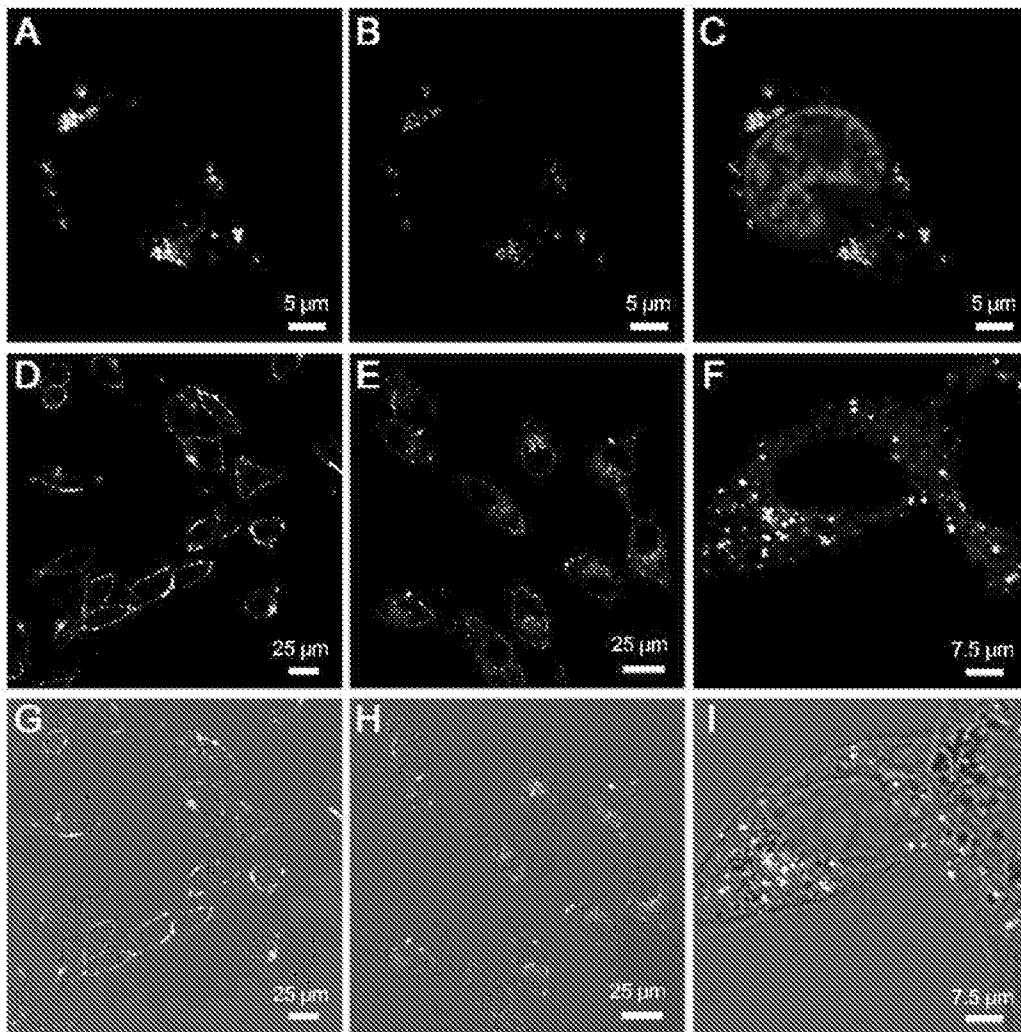


FIG. 55

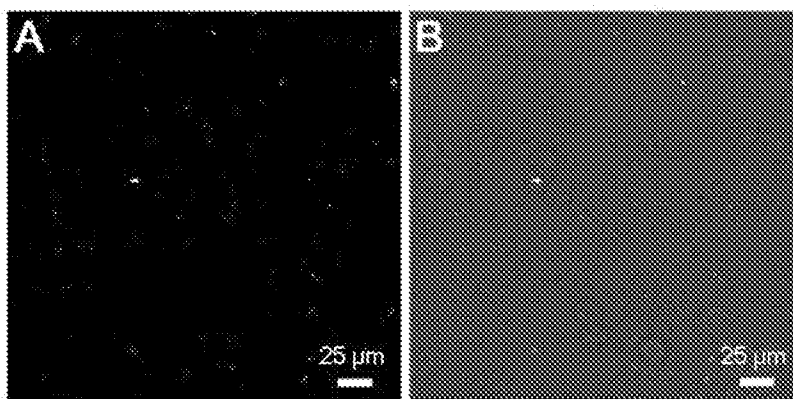


FIG. 56

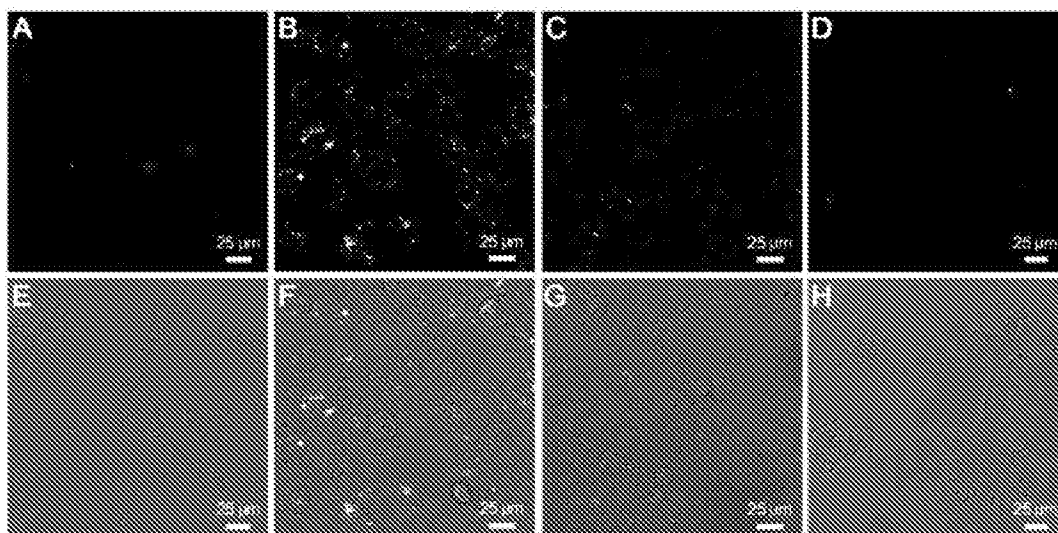


FIG. 57

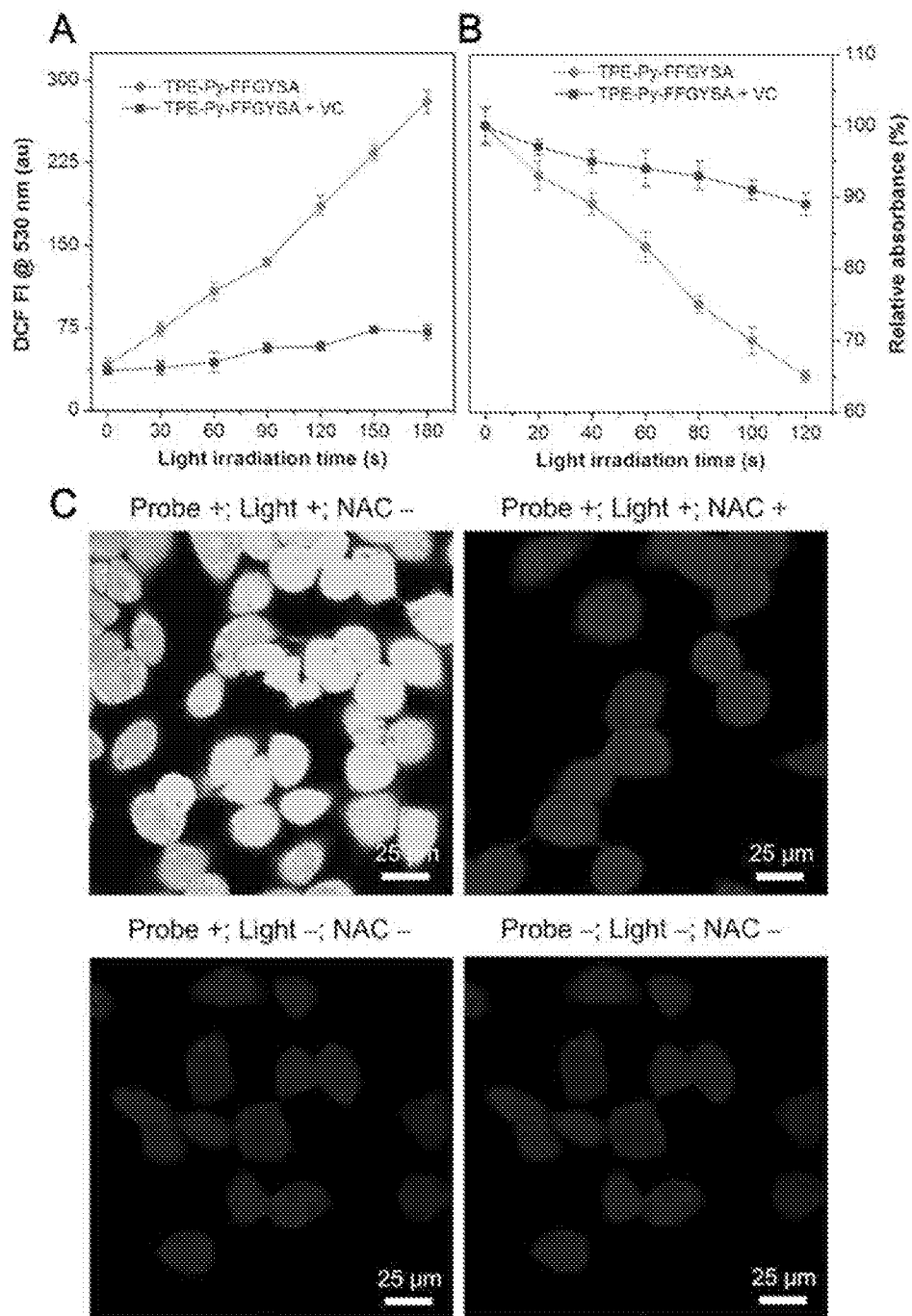


FIG. 58

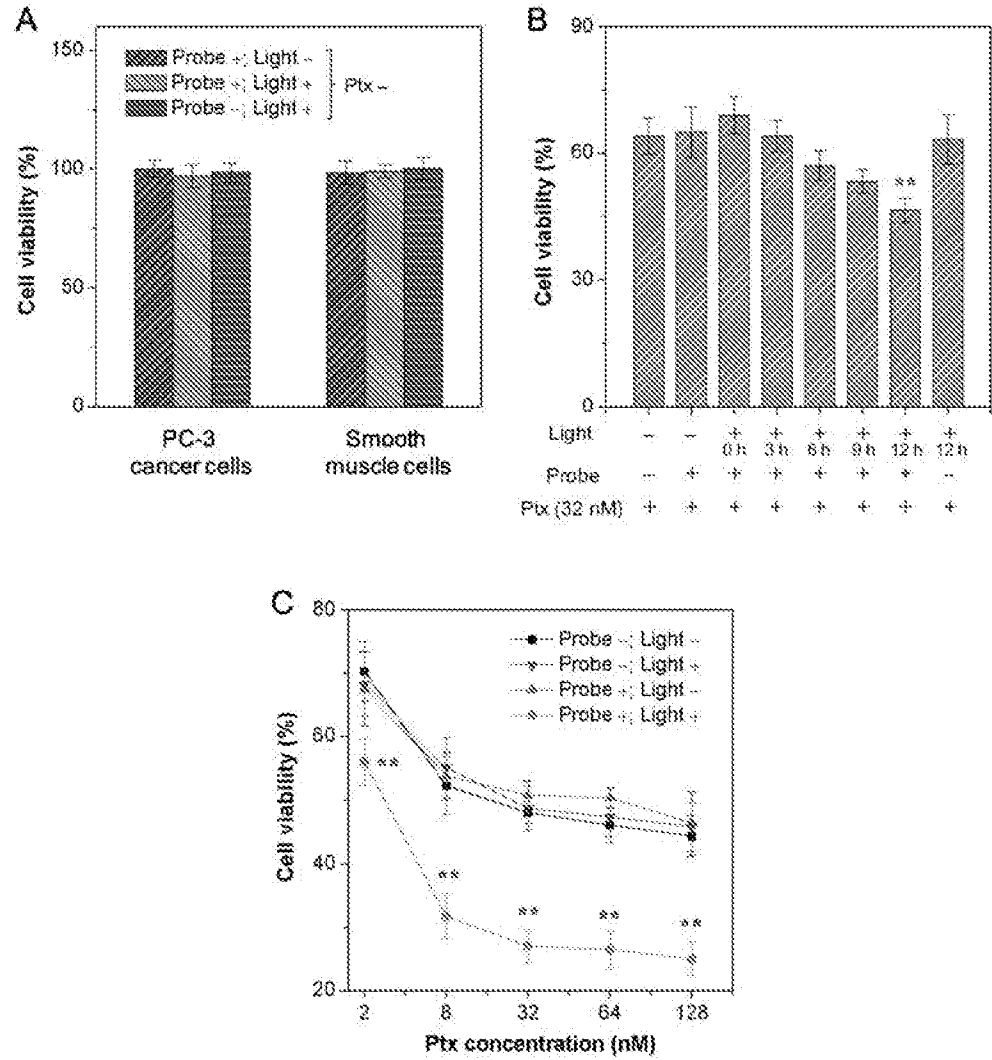


FIG. 59

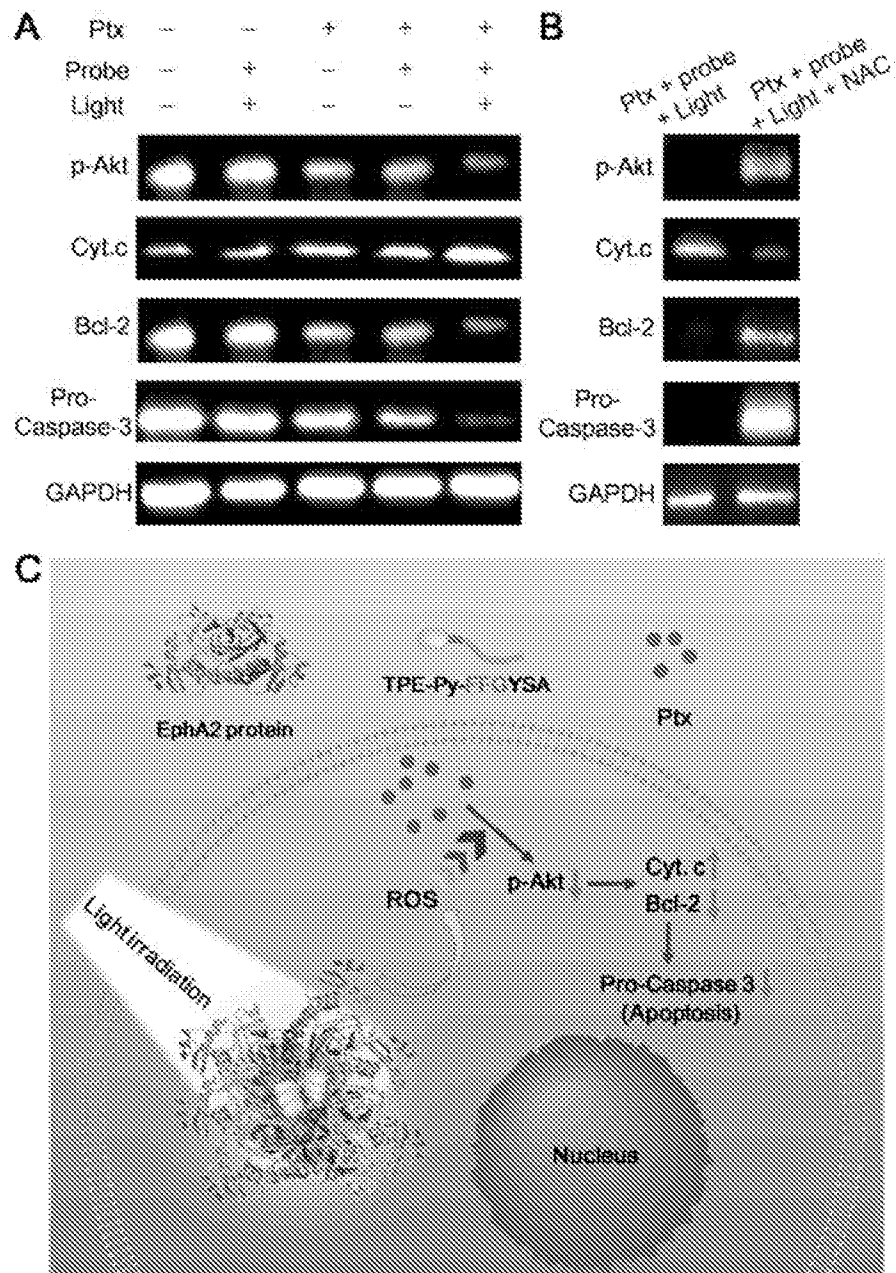


FIG. 60

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2016/104772

A. CLASSIFICATION OF SUBJECT MATTER

G01N 21/64(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 21/-, C09K 11/-

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)

CNKI,DWPI,SIPOABS,STN (CAplus, Registry):aggregation,induce,emission,fluorophore,chromophore,probe

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014212359 A1 (UNIV HONG KONG SCIENCE & TECHN) 31 July 2014 (2014-07-31) claims 1-10	1-7
X	US 2015031607 A1 (UNIV HONG KONG SCIENCE & TECHN) 29 January 2015 (2015-01-29) examples 7-9 and paragraphs [0201]-[0202]	1, 3-7
A	US 2014212359 A1 (UNIV HONG KONG SCIENCE & TECHN) 31 July 2014 (2014-07-31) the whole document	8-26
A	US 2015031607 A1 (UNIV HONG KONG SCIENCE & TECHN) 29 January 2015 (2015-01-29) the whole document	8-26

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

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

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“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

08 January 2017

Date of mailing of the international search report

26 January 2017

Name and mailing address of the ISA/CN

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Facsimile No. (86-10)62019451

Telephone No. (86-10)62086311

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2016/104772

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
US	2014212359	A1	31 July 2014	US	9315465	B2	19 April 2016
US	2015031607	A1	29 January 2015	US	9279806	B2	08 March 2016