



- (51) **International Patent Classification:**  
*C09K 11/06* (2006.01) *C07D 213/38* (2006.01)  
*C07F 7/08* (2006.01)
- (21) **International Application Number:**  
PCT/CN2016/074337
- (22) **International Filing Date:**  
23 February 2016 (23.02.2016)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**  
62/178,512 13 April 2015 (13.04.2015) US
- (71) **Applicant:** THE HONG KONG UNIVERSITY OF SCIENCE AND TECHNOLOGY [CN/CN]; Clear Water Bay, Kowloon, Hong Kong (CN).
- (72) **Inventors:** TANG, Ben Zhong; Department of Chemistry, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong (CN). ZHANG, Weijie; Department of Chemistry, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong (CN). KWOK, Tsz Kin; Department of Chemistry, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong (CN).
- (74) **Agent:** TEE & HOWE INTELLECTUAL PROPERTY ATTORNEYS; Yeping DING, 10th Floor, Tower D, Min-

sheng Financial Center, 28 Jianguomennei Avenue, Dongcheng District, Beijing 100005 (CN).

- (81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Published:**

— with international search report (Art. 21(3))

(54) **Title:** REAL-TIME MONITORING MITOPHAGY PROCESS BY FLUORESCENT PHOTOSTABLE MITOCHONDRIAL SPECIFIC BIOPROBE WITH AIE CHARACTERISTICS

(57) **Abstract:** Provided are a bioprobe comprising AIE luminogens, and a method of imaging cells comprising introducing the bioprobe to a sample containing cells and imaging the cells by monitoring fluorescence emitted from the cell uptake of the bioprobe. Provided further is a method of monitoring a process of mitophagy comprising introducing the bioprobe to a sample containing cells, and monitoring the process of mitophagy by tracking morphological change.



**REAL-TIME MONITORING MITOPHAGY PROCESS BY FLUORESCENT  
PHOTOSTABLE MITOCHONDRIAL SPECIFIC BIOPROBE WITH AIE  
CHARACTERISTICS**

**RELATED APPLICATIONS**

[1] The present patent application claims priority to provisional U.S. Patent Application No. 62/178,512 filed April 13, 2015, which was filed by the inventors hereof and is incorporated by reference herein in its entirety.

**TECHNICAL FIELD**

[2] The present subject matter relates to the development of luminogens with aggregation induced emission (AIE) characteristics, particularly fluorescent bioprobes with AIE characteristics, which may be used to monitor mitochondria and the mitophagy process.

**BACKGROUND**

[3] Autophagy is a process that digests surplus, worn out, and injured organelles, and recovers their nutrients such as amino acid. The term mitophagy has been introduced for the specific process of mitochondrial autophagy, which principally plays an important role to protect organisms against a variety of diseases, including neurodegeneration, heart disease, cancer, infections, and aging. The fluorescence technique was widely used in the research of mitophagy. The fluorescent probe, meanwhile, can selectively illuminate especial cellular organelles and is a powerful tool for monitoring and studying the whole process of mitophagy. Various type of fluorescent probes have been developed to mark mitochondria, such as fluorescent proteins, Quantum dots (QD), and small organic molecules.

[4] Despite fluorescent proteins having good selectivity and sensitivity for staining the mitochondria being known, they may be readily decomposed by proteolytic enzymes due to aggregation-caused quenching (ACQ), as has been shown by for example. Day, R.N. & Davidson, M.W., "The fluorescent protein palette: tools for cellular imaging", *Chemical Society Reviews*, 38, pp. 2887-2921 (2009).

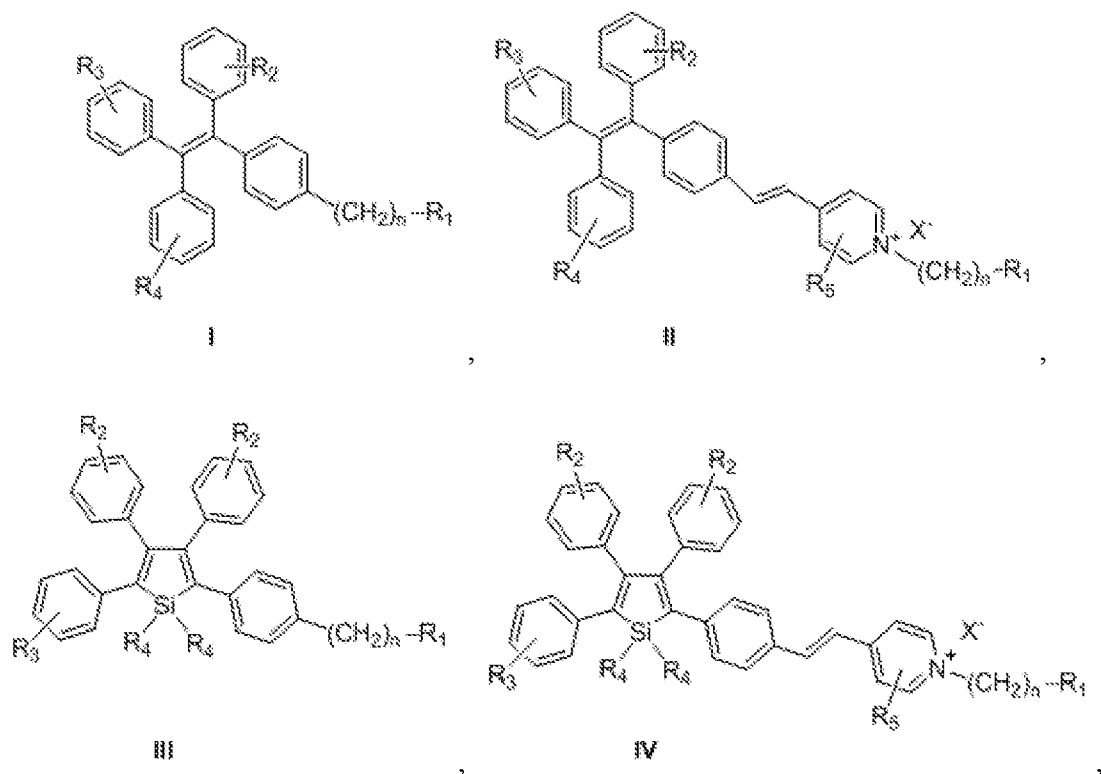
[5] For sensitive detection, fluorescent materials must emit intense visible light upon photoexcitation. ACQ is due to emission quenching caused by the aggregation of fluorophores in the solid state. When dispersed in aqueous media or bound to biomolecules, fluorophore molecules are inclined to aggregate, which usually quenches their fluorescence, and thus greatly limits their effectiveness as bioprobes. The ACQ effect also makes it difficult to assay low-abundance molecular species in biological systems because the fluorescence signals from minimal amounts of fluorophores matching the bioanalyte levels may be too weak to be determined accurately. In addition, at high fluorophore concentrations, the emissions are further weakened, rather than enhanced, due to the ACQ effect.

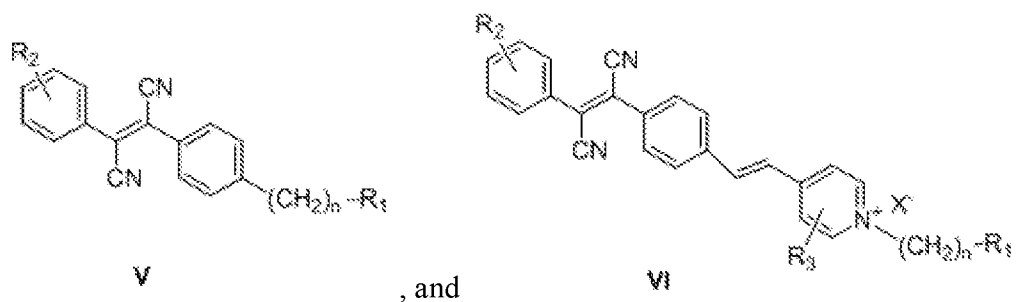
[6] Meanwhile, QD's have heavy metal cores and can be oxidized in complex biological environments, leading to undesired cytotoxicity and unstable fluorescence signals. As compared to the materials mentioned above, organic molecules are richer in variety, free of heavy metals, and are more compatible with living cells. Mitochondria-targeting probes with different functions have been reported in prior art references, examples of which have been reported by Abbotto Alessandro (WO 2007/113321 A1), Zarling David A. (WO 2008/109740 A2), Dario C. Altieri (US 2009/0099080 A1), and Shibnath Ghosal (US 2008/0031862 A1). The photostability of conventional fluorescent dyes for mitochondrial staining, however, leaves much room to be improved.

## SUMMARY

[7] In an embodiment of the present subject matter, a fluorescent bioprobe has been designed and synthesized. The bioprobe can be taken up electrostatically by mitochondria in response to the negative mitochondrial membrane potential. After uptake, it becomes covalently bound to mitochondrial proteins and remains in mitochondria, even if a mitochondrion subsequently depolarizes.

[8] In an embodiment of the present subject matter, the bioprobe comprises AIE luminogens having one or more heterocycle units and a chemical structure selected from the group consisting of:





wherein

$R_1$  is independently selected from  $N_3$  and isothiocyanate;

$R_2$ ,  $R_3$ ,  $R_4$ , and  $R_5$  are independently selected from the group consisting of H, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl,  $C_nH_{2n+1}$ ,  $C_{10}H_7$ ,  $C_{12}H_9$ ,  $OC_6H_5$ ,  $OC_{10}H_7$ ,  $OC_{12}H_9$ ,  $C_nH_{2n}COOH$ ,  $C_nH_{2n}OH$ ,  $C_nH_{2n}CHO$ ,  $C_nH_{2n}COOC_4O_2N$ ,  $C_nH_{2n}NCS$ ,  $C_nH_{2n}N_3$ ,  $C_nH_{2n}NH_2$ ,  $C_nH_{2n}SH$ ,  $C_nH_{2n}Cl$ ,  $C_nH_{2n}Br$ , and  $C_nH_{2n}I$ ;

$n = 0$  to  $20$ ; and

$X$  is a monovalent counterion independently selected from the group consisting of I, Cl, Br,  $PF_6$ ,  $ClO_4$ ,  $BF_4$ ,  $BPh_4$ , and  $CH_3PhSO_3$ .

[9] An embodiment of the present subject matter comprises the bioprobe TPE-Py-NCS, which is constructed from tetraphenylethene-pyridinium (TPE-Py) having AIE features, an isothiocyanate(NCS) functional group, and a positively charged pyridinium unit for specific mitochondrial imaging. The bioprobe, or dye, can specifically localize in the mitochondria, and the alkaline environment inside facilitates chemical reaction of the NCS functionality in the bioprobe with the amino group in the mitochondria. This forms a tight connection, which can resist the organic solvent treatment in bioexperiments.

[10] The bioprobe has high specificity for mitochondria, superior photostability, low cytotoxicity, and high resistance to the loss of mitochondrial membrane potential. Because of these advantages, the bioprobe may be used to monitor the process of mitophagy.

**BRIEF DESCRIPTION OF DRAWINGS**

- [11] **FIGS. 1A-B** show  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra of TPE-Py-NCS in DMSO- $d_6$ .
- [12] **FIG. 2** shows a mass spectrum (MALDI-TOF) of TPE-Py-NCS.
- [13] **FIG. 3** shows UV-vis absorption and photoluminescence (PL) spectra of TPE-Py-NCS in DMSO.
- [14] **FIGS. 4A-B** show PL spectra of TPE-Py-NCS in DMSO/H<sub>2</sub>O mixtures with different water fractions ( $f_w$ ) and a plot of PL intensity versus the composition of the DMSO/H<sub>2</sub>O mixtures of TPE-Py-NCS.
- [15] **FIG. 5** shows particle size analysis of TPE-Py-NCS nanoaggregate in PBS.
- [16] **FIG. 6** shows cytotoxicity of TPE-Py-NCS on HeLa cells determined by MTT assay.
- [17] **FIGS. 7A-C** show fluorescent images of HeLa cells co-stained with TPE-Py-NCS and MitoTracker® Red CMXRos.
- [18] **FIGS. 8A-I** show fluorescent images of HeLa cells stained with (A-C) TPE-Py-NCS (5  $\mu\text{M}$ ), (D-F) TPE-Py (5  $\mu\text{M}$ ), and (J-I) MTR (50 nM) for 15 min. The stained cells (A, D, and G) are without any treatment, (B, E, and H) are fixed with 4% PFA and (C, F, and I) are fixed with 4% PFA followed by washing three times with acetone.
- [19] **FIGS. 9A-B** show (A) confocal images of HeLa cells stained with TPE-Py-NCS and MTR taken under continuous UV excitation at 405 nm (TPE-Py-NCS) and 560 nm (MTR), respectively, as well as (B) a plot of loss in TPE-Py-NCS and MTR emission versus time.
- [20] **FIG. 10** shows confocal images of HeLa cell stained with TPE-Py-NCS and LTR after treatment with rapamycin (50  $\mu\text{g/mL}$ ). Time points (min) were selected to illustrate the onset and completion of mitochondrial digestion by autophagy.

[21] FIGS. 11A-B show confocal images of HeLa cells co-stained with (A) MTR and LTG, and (B) TPE-Py-NCS and LTR, after treatment with rapamycin.

## DETAILED DESCRIPTION

### Definitions

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

[23] 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.

[24] “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.

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

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

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

[28] 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.

[29] 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.

[30] 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.”

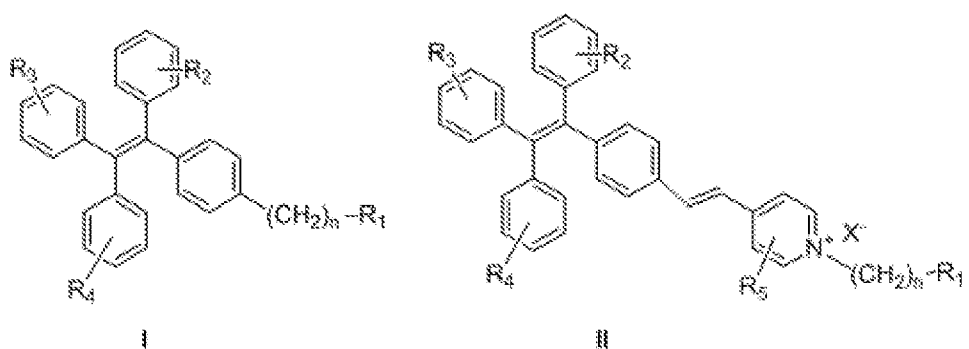
[31] For purposes of better understanding the present teachings and in no way limiting the scope of the teachings, unless otherwise indicated, all numbers expressing quantities, percentages or proportions, and other numerical values used in the specification and claims, are to be understood as being 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.

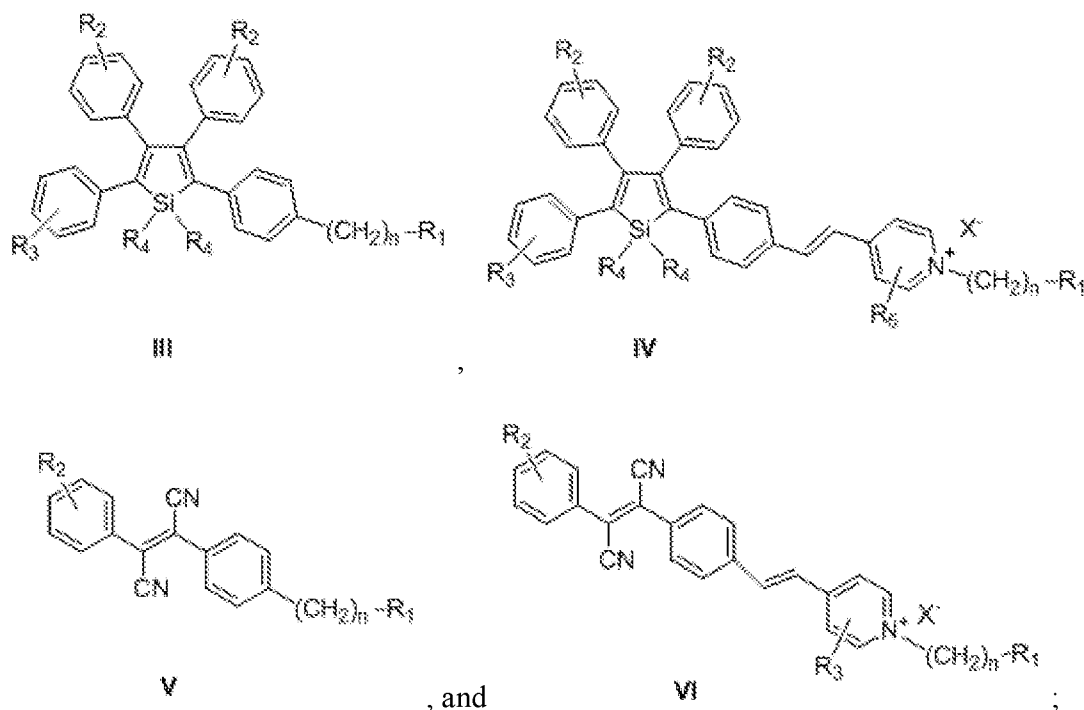
### **Abbreviations**

|         |                              |
|---------|------------------------------|
| ACQ:    | aggregation-caused quenching |
| AIE:    | aggregation-induced emission |
| CMXRos: | chloromethyl-X-rosamine      |
| DLS:    | dynamic light scattering     |
| DMSO:   | dimethylsulfoxide            |
| $f_w$ : | water fraction               |

|             |                                                              |
|-------------|--------------------------------------------------------------|
| HRMS:       | high-resolution mass spectroscopy                            |
| ICT:        | intramolecular charge transfer                               |
| LTG:        | Lysotracker Green                                            |
| LTR:        | Lysotracker Red                                              |
| MADLI-TOF:  | matrix assisted laser desorption ionization time-of-flight   |
| MTR:        | MitoTracker® Red CMXRos                                      |
| MTT:        | 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide |
| NMR:        | nuclear magnetic resonance                                   |
| PBS:        | phosphatebuffer saline                                       |
| PFA:        | paraformaldehyde                                             |
| PL:         | photoluminescence                                            |
| QD:         | quantum dots                                                 |
| TPE:        | tetraphenylethene                                            |
| TPE-Py:     | tetraphenylethene-pyridinium                                 |
| TPE-Py-NCS: | tetraphenylethene-pyridiniumisothiocyanate                   |
| UV:         | ultraviolet                                                  |

[32] In an embodiment, the present subject matter relates to a bioprobe comprising AIE luminogens having one or more heterocycle units and a chemical structure selected from the group consisting of:





wherein

$R_1$  is independently selected from  $N_3$  and isothiocyanate;

R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub>, and R<sub>5</sub> are independently selected from the group consisting of H, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, C<sub>n</sub>H<sub>2n+1</sub>, C<sub>10</sub>H<sub>7</sub>, C<sub>12</sub>H<sub>9</sub>, OC<sub>6</sub>H<sub>5</sub>, OC<sub>10</sub>H<sub>7</sub>, OC<sub>12</sub>H<sub>9</sub>, C<sub>n</sub>H<sub>2n</sub>COOH, C<sub>n</sub>H<sub>2n</sub>OH, C<sub>n</sub>H<sub>2n</sub>CHO, C<sub>n</sub>H<sub>2n</sub>COOC<sub>4</sub>O<sub>2</sub>N, C<sub>n</sub>H<sub>2n</sub>NCS, C<sub>n</sub>H<sub>2n</sub>N<sub>3</sub>, C<sub>n</sub>H<sub>2n</sub>NH<sub>2</sub>, C<sub>n</sub>H<sub>2n</sub>SH, C<sub>n</sub>H<sub>2n</sub>Cl, C<sub>n</sub>H<sub>2n</sub>Br, and C<sub>n</sub>H<sub>2n</sub>I;

n = 0 to 20; and

X is a monovalent counterion independently selected from the group consisting of I, Cl, Br, PF<sub>6</sub>, ClO<sub>4</sub>, BF<sub>4</sub>, BPh<sub>4</sub>, and CH<sub>3</sub>PhSO<sub>3</sub>.

[33] In an embodiment, the AIE luminogens comprise isothiocyanate functional groups. In an embodiment, the AIE luminogens are covalently bonded with proteins on mitochondrial membrane.

[34] In an embodiment, the bioprobe is used for real-time monitoring of a mitophagy

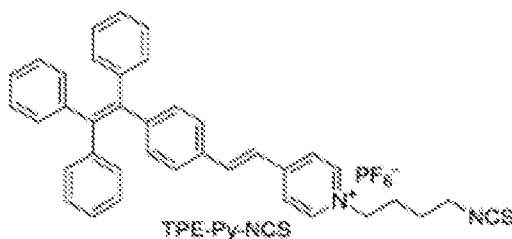
process. In an embodiment, the bioprobe is used for tracking morphological change and monitoring the process of mitophagy, as the AIE luminogens covalently connect with mitochondria. In an embodiment, the bioprobe is used for mitochondria imaging, as the AIE luminogens covalently connect with mitochondria.

[35] In an embodiment, imaging is possible due to fluorescence emitted by mitochondria from cell uptake of the bioprobe. In an embodiment, an imaging sample comprises any kind of cell. The imaging sample may comprise cancer cells such as HeLa cells or MCF-7 cells.

[36] In an embodiment, the present subject matter is directed to a method of imaging cells comprising introducing the bioprobe to a sample containing cells, wherein the AIE luminogens covalently connect with mitochondria; and imaging the cells by monitoring fluorescence emitted from the cell uptake of the bioprobe.

[37] In an embodiment, the present subject matter is directed to a method of monitoring a process of mitophagy comprising introducing the bioprobe to a sample containing cells, wherein the AIE luminogens covalently connect with mitochondria; and monitoring the process of mitophagy by tracking morphological change, wherein fluorescence is emitted from the cell uptake of the bioprobe.

[38] In an embodiment, the bioprobe comprises TPE-Py-NCS as the AIE luminogen:



[39] Taking advantage of the NCS functional group, TPE-Py-NCS is the first AIE-active fluorescent probe covalently connected with mitochondria. TPE-Py-NCS has high specificity for

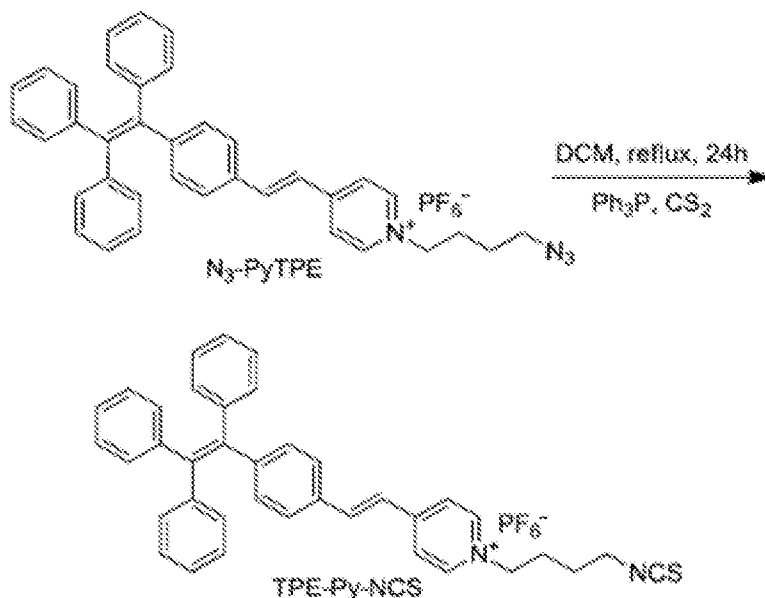
mitochondria, superior photostability, low cytotoxicity, and high resistance to the loss of mitochondrial membrane potential. For example, as shown in Fig. 7, the images stained with TPE-Py-NCS are well overlapped with those labelled with MTR with Pearson's correlation coefficient of 0.97, demonstrating that TPE-Py-NCS can target mitochondria in high specificity. Similarly, as shown in Fig. 9, after 17 scans with a total irradiation time of 10 min, about 20% of the fluorescent signal was lost for TPE-Py-NCS but the mitochondria of HeLa cells can still be observed clearly. In contrast, only 20% initial signal intensity remained in MTR and the mitochondrial image almost disappeared after 17 scans. Evidently, TPE-Py-NCS shows a much higher photo-bleaching resistance and is a better mitochondrial stain than MTR.

[40] Regarding the low cytotoxicity of the bioprobes, as shown in Fig. 6, the cytotoxicity of TPE-Py-NCS on HeLa cells was evaluated using MTT assay. The cell viability exerts little change in the presence of TPE-Py-NCS with a concentration of 7.5  $\mu$ M (working concentration is 5.0  $\mu$ M), suggesting that the fluorogen possesses good biocompatibility. And Fig. 8A-I show that the fluorescence from the mitochondria in fixed cells is mostly retained even after washing with acetone three times, suggesting that except hydrophobic and electrostatic interactions, TPE-Py-NCS form strong and stable chemical bonds with mitochondrial proteins. Accordingly, TPE-Py-NCS can be used for mitochondria imaging and morphological change tracking. It is also well-suited to monitor the process of mitophagy.

[41] In an embodiment, the present subject matter relates to a fluorescent bioprobe with AIE characteristics used for real-time monitoring mitophagy process. In an embodiment, the fluorescence for imaging is emitted by the mitochondrion and results from cell uptake of the bioprobe. In an embodiment, the AIE luminogens comprise isothiocyanate functional groups. In an embodiment, the AIE luminogens are covalently bonded with the proteins on mitochondrial

membrane. In an embodiment, an imaging sample comprises any kind of cell and may be cancer cells such as HeLa cells or MCF-7 cells.

[42] TPE-Py-NCS was synthesized according to the synthetic route shown below:



[43] Reaction of  $\text{N}_3\text{-Py-TPE}$  with carbon disulfide ( $\text{CS}_2$ ) in the presence of triphenylphosphine ( $\text{PPh}_3$ ) furnished the TPE-Py-NCS product. The final product was fully characterized by HRMS,  $^1\text{H}$ , and  $^{13}\text{C}$  NMR spectroscopies, from which results corresponding to structure were obtained (FIGS. 1-2). The optical properties of TPE-Py-NCS were also studied. The UV-vis absorption and photoluminescence (PL) spectra of TPE-Py-NCS in DMSO solution are shown in FIG.3. The absorption and emission maxima of TPE-Py-NCS in DMSO solution are located at 397 nm and 638 nm, respectively. Such a large Stokes shift ( $>240$  nm) of TPE-Py-NCS is due to its extended conjugation as well as intramolecular charge transfer (ICT) effect from the electron-donating TPE moiety to the electron-accepting pyridinium unit. Since bioimaging often uses 405 nm as the excitation source, an excitation light of 405 nm was utilized for the PL measurements. It is known that an AIE molecule will emit weak or no emission in

solution but will fluoresce intensively in either solid or aggregated state.

[44] To verify whether TPE-Py-NCS is AIE-active, PL spectra of DMSO/H<sub>2</sub>O mixtures of TPE-Py-NCS with different water fractions ( $f_w$ ) were recorded. As can be seen from the PL spectra in FIG. 4, the emission in pure DMSO solution is weak and is progressively weakened when up to 70% of water is added to the DMSO solution. However, the PL increased swiftly when the water fractions further increased. At 99% water content, the emission intensity is more than 5-fold higher than that in pure DMSO solution. The PL enhancement is attributed to the formation of nanoaggregates, as confirmed by the dynamic light scattering (DLS) particle size analysis (FIG. 5). Clearly, TPE-Py-NCS is AIE-active. It is noteworthy that the aggregates of TPE-Py-NCS emit bluer color than their isolated species in DMSO solution because of the reduction in the ICT effect and the more twisted conformation in aggregates.

## **Application of TPE-Py-NCS**

### ***1. Mitochondria imaging***

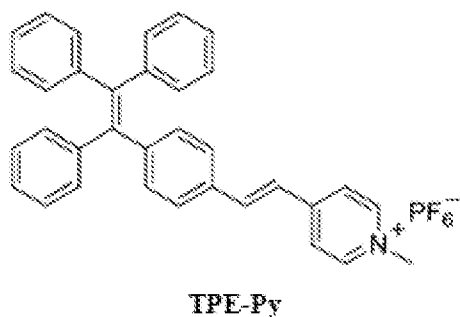
[45] Biocompatibility is one of the important parameters for a fluorogen used in bioimaging applications. The cytotoxicity of TPE-Py-NCS on HeLa cells was evaluated using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. Cell viabilities of more than 85% were observed for the fluorogen concentrations up to 7.5  $\mu$ M (FIG. 6), suggesting that TPE-Py-NCS had good biocompatibility. Next, the application of the TPE-Py-NCS as a fluorescent visualizer for intracellular imaging was explored. After incubation with TPE-Py-NCS (5  $\mu$ M) at 37°C for 15 min, the HeLa cells were washed three times with PBS solution and then imaged. As shown in the fluorescent image in FIG. 7A, an intense yellow fluorescence was observed in the tubular and reticular structures of mitochondria.

[46] To further demonstrate that the TPE-Py-NCS is targeted to mitochondria, MitoTracker® Red CMXRos (MTR), a commercially available mitochondria imaging agent, was used to co-stain the HeLa cells for comparison (FIG. 7B). As shown in FIG. 7C, the position of red fluorescence from MTR was well matched with the yellow fluorescence from TPE-Py-NCS. The Pearson's correlation coefficient between the two fluorescent images was 0.97, indicating that TPE-Py-NCS can stain the mitochondria specifically.

## ***2. Resistance to change of microenvironments***

[47] The mitochondrial membrane contains large number of proteins, where the amino group (NH<sub>2</sub>) and thiol group (SH) are extensively exposed on the surface. Since the NCS group has high reactivity to NH<sub>2</sub> groups, it is hypothesized that the TPE-Py-NCS would chemically react with the NH<sub>2</sub> groups on mitochondria proteins when the AIE dye is taken up by mitochondria in response to the negative membrane potential. To verify this hypothesis, the HeLa cells were first incubated with TPE-Py-NCS and then acetone was used to wash the cells after fixing by PFA. As shown in FIGS. 8A-B, the mitochondria are visualized clearly by TPE-Py-NCS in both live and fixed cells. Interestingly, the fluorescence of mitochondria in fixed cells is mostly preserved after washing with acetone three times (FIG. 8C), suggesting the TPE-Py-NCS can form stable chemical bonds with mitochondrial proteins.

[48] For comparison, the AIE dye TPE-Py was also used to stain HeLa cells under the same conditions. TPE-Py has a similar chemical structure to TPE-Py-NCS, but does not have the NCS group, as shown below:



[49] A bright yellow fluorescence from TPE-Py can be observed in live or fixed cells (FIGS. 8D-E). The fluorescence, however, was lost when the cells were treated with acetone (FIG. 8F), indicating that TPE-Py was dissolved in acetone and then washed away. These results further demonstrated that the fluorescence of TPE-Py-NCS in mitochondria is preserved because of the chemical reaction between the NCS group and NH<sub>2</sub> groups on proteins. On the other hand, the commercial dye MTR containing a thiol-reactive chloromethyl moiety, which can form covalent bonds with mitochondrial proteins, showed results similar to TPE-Py-NCS. Red fluorescence can be observed in the cells with or without treatments (FIG. 8G-I), but the fluorescence was weakened and the fine structure of mitochondria became indistinct after acetone washing. These results may be explained by the amount of NH<sub>2</sub> groups being much more than the thiol groups on proteins. Thus, more TPE-Py-NCS was reacted when compared with the MTR.

### 3. Photostability

[50] To quantitatively investigate the photostability of TPE-Py-NCS and MTR, cells were continuously scanned by confocal microscope (Zeiss laser scanning confocal microscope LSM7 DUO). HeLa cells were stained with 5  $\mu$ M TPE-Py-NCS or 50 nM MTR for 15 min, respectively. 405 nm and 560 nm channels were used to irradiate the TPE-Py-NCS and MTR stained cells, respectively. Excitation power from the two different channels was unified (50  $\mu$ W) with the

help of a power meter. The initial intensity referred to in the first scan of TPE-Py-NCS and MTR stained cells was normalized, and the percentage of fluorescence signal loss was calculated (FIG. 9B). As shown in FIG. 9A, after 10min, the signal loss of TPE-Py-NCS is almost 20%, and there is no significant difference between the first min and 10min. In contrast, 20% fluorescence signal remained in the MTR group. The very weak signal was observed after continuous scanning, about 10min.

#### **4. Mitophagy**

[51] Mitophagy is used to describe the specific organelle autophagy which happens in mitochondria. Mitophagy is key in keeping the cell healthy. In the process, mitochondria are impaired, such as from nutritional deprivation, cell damage, or drug-induced damage. The autophagic is formed, and the injured mitochondria are packed by a sub-cell membrane. Then autophagic is delivered to lysosomes and degraded. By observing the rate of overlap about the mitochondria and lysosome, the emergence of mitophages can be confirmed. This dynamic process is approximately eight minutes. However, the bad photostability of conventional dyes, caused by low working concentrations that present as individual molecules, which will be destroyed with ease by the strong excitation light, restrict monitoring of the process to the fluorescence method. In contrast, due to its AIE feature, TPE-Py-NCS forms nanoaggregates. When exposed to excitation light, the outermost layer of the nanoaggregates may be photobleached. However, the condensed particles can prevent further photobleaching and photo-oxidation by avoiding oxygen diffusion into the particles. Due to this excellent photostability of TPE-Py-NCS, using TPE-Py-NCS as a bioprobe or dye may solve the problem.

[52] To test the performance of TPE-Py-NCS in the process of autophagy, and following the previously reported protocol, the HeLa cell was co-stained with TPE-Py-NCS and LTR. After

TPE-Py-NCS and LTR were loaded, one third of the initial concentration of LTR was kept in the medium for the duration of the experiment to maintain steady state loading. On the confocal microscope stage, the complete growth medium was switched to phosphate buffered saline (PBS) plus 50 $\mu$ g/ml rapamycin. Rapamycin is a well-known drug that can trigger mitophagy. Time series of confocal images were collected from 65 min up to 85 min after autophagic induction. By co-labeling with TPE-Py-NCS and LTR, mitochondrial entrapment inside acidic autolysosomes could be visualized.

[53] As shown in FIG. 10, one region loading by LTR without co-labeling with mitochondria was evident after 72 min in PBS plus rapamycin in the field of view shown. Compared to the appearance and disappearance of autophagosome, the lysosomal is sustained, existing in all images in FIG.10. After 1.5min, the mitochondria at the arrow began to label with LTR. This labeling continues to exist over the next several minutes. After about 7.5min, the LTR-labeled structure disappeared. These images directly show directly the transformation of individual mitochondria into a mitochondrion containing autophagosome/autolysosome.

[54] Random overlap of organelles within the same plane of confocal sections was minimized by opening the pinhole of the confocal microscope for an optical slice thickness of 1 $\mu$ M. Because the diameter of the lysosome is about 0.8  $\mu$ m, and the diameter of the mitochondria is about 1 $\mu$ m, TPE-Py-NCS labeled mitochondria were entrapped consistently and totally within the LTR-labeled autophagosomes/autolysosome. TPE-Py-NCS fluorescence in autophagosomes/autolysosome structures progressively become weaker, as compared to normal mitochondria, which is due to hydrolytic digestion.

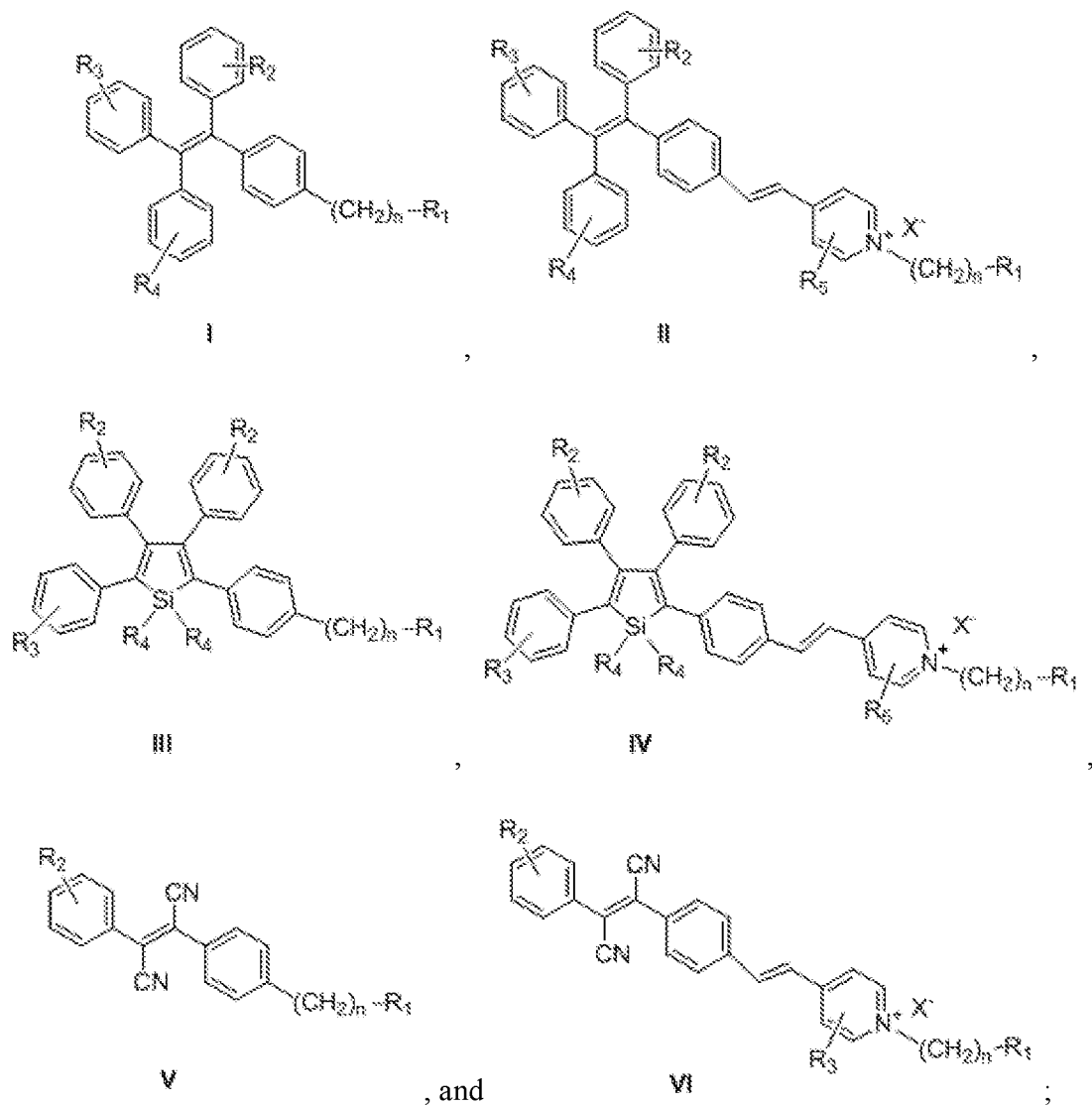
[55] After autophagy beginning, the photostability was investigated again. Following the procedure, before adding the rapamycin, one dish of HeLa was co-stained with TPE-Py-NCS

(5 $\mu$ M) and LTR (150nM) for 30min. The other dish of HeLa, as the control group, was co-stained with MTR (50nM) and LTG (150nM) for 30min. After induction beginning, the continuous scanning image was collected from 70min to 80min after adding rapamycin (50 $\mu$ g/ml) in PBS. As shown in FIG.11A, the signal of the control group became weaker, and in the last image, the overlapped region of MTR and LTG cannot be clearly observed. On the contrary, in FIG.11B, the structure of mitochondria and lysosome are clearly visible, and the overlapped region of mitochondria and lysosome also are displayed with the aid of the yellow and red fluorescence of TPE-Py-NCS and LTR.

[56] 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 bioprobe comprising AIE luminogens having one or more heterocycle units and a chemical structure selected from the group consisting of:



wherein

$R_1$  is independently selected from  $N_3$  and isothiocyanate;

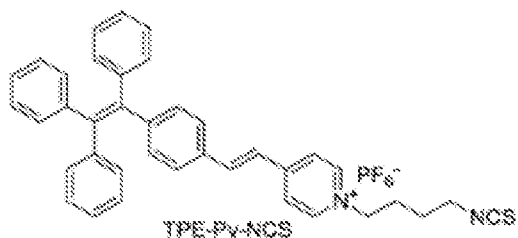
$R_2$ ,  $R_3$ ,  $R_4$ , and  $R_5$  are independently selected from the group consisting of H, alkyl, unsaturated alkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl,  $C_nH_{2n+1}$ ,  $C_{10}H_7$ ,

$C_{12}H_9$ ,  $OC_6H_5$ ,  $OC_{10}H_7$ ,  $OC_{12}H_9$ ,  $C_nH_{2n}COOH$ ,  $C_nH_{2n}OH$ ,  $C_nH_{2n}CHO$ ,  $C_nH_{2n}COOC_4O_2N$ ,  
 $C_nH_{2n}NCS$ ,  $C_nH_{2n}N_3$ ,  $C_nH_{2n}NH_2$ ,  $C_nH_{2n}SH$ ,  $C_nH_{2n}Cl$ ,  $C_nH_{2n}Br$ , and  $C_nH_{2n}I$ ;

$n = 0$  to  $20$ ; and

X is a monovalent counterion independently selected from the group consisting of I, Cl, Br,  $PF_6$ ,  $ClO_4$ ,  $BF_4$ ,  $BPh_4$ , and  $CH_3PhSO_3$ .

2. The bioprobe of claim 1, wherein the bioprobe comprises TPE-Py-NCS as the AIE luminogen:



3. The bioprobe of claim 1, wherein the AIE luminogens comprise isothiocyanate functional groups.

4. The bioprobe of claim 1, wherein the AIE luminogens are covalently bonded with proteins on mitochondrial membrane.

5. The bioprobe of claim 1, wherein the bioprobe is used for real-time monitoring of a mitophagy process.

6. The bioprobe of claim 1, wherein the bioprobe is used for tracking morphological change and monitoring the process of mitophagy, as the AIE luminogens covalently connect with mitochondria.

7. The bioprobe of claim 1, wherein the bioprobe is used for mitochondria imaging, as the AIE luminogens covalently connect with mitochondria.
8. The bioprobe of claim 7, wherein imaging is possible due to fluorescence emitted by mitochondria from cell uptake of the bioprobe.
9. The bioprobe of claim 7, wherein an imaging sample comprises any kind of cell.
10. The bioprobe of claim 9, wherein the imaging sample comprises cancer cells.
11. The bioprobe of claim 10, wherein the cancer cells are HeLa cells or MCF-7 cells.
12. A method of imaging cells comprising:
  - introducing the bioprobe of claim 1 to a sample containing cells, wherein the AIE luminogens covalently connect with mitochondria; and
  - imaging the cells by monitoring fluorescence emitted from the cell uptake of the bioprobe.
13. A method of monitoring a process of mitophagy comprising:
  - introducing the bioprobe of claim 1 to a sample containing cells, wherein the AIE luminogens covalently connect with mitochondria; and
  - monitoring the process of mitophagy by tracking morphological change, wherein fluorescence is emitted from the cell uptake of the bioprobe.

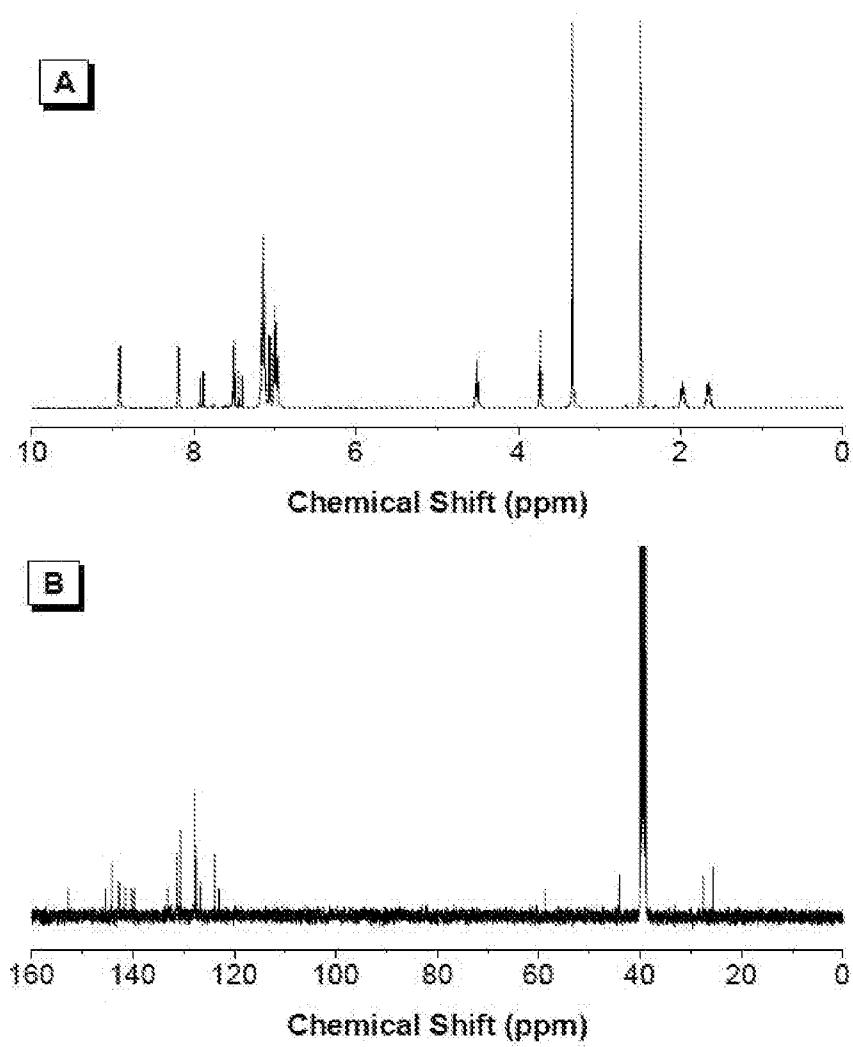


FIG. 1

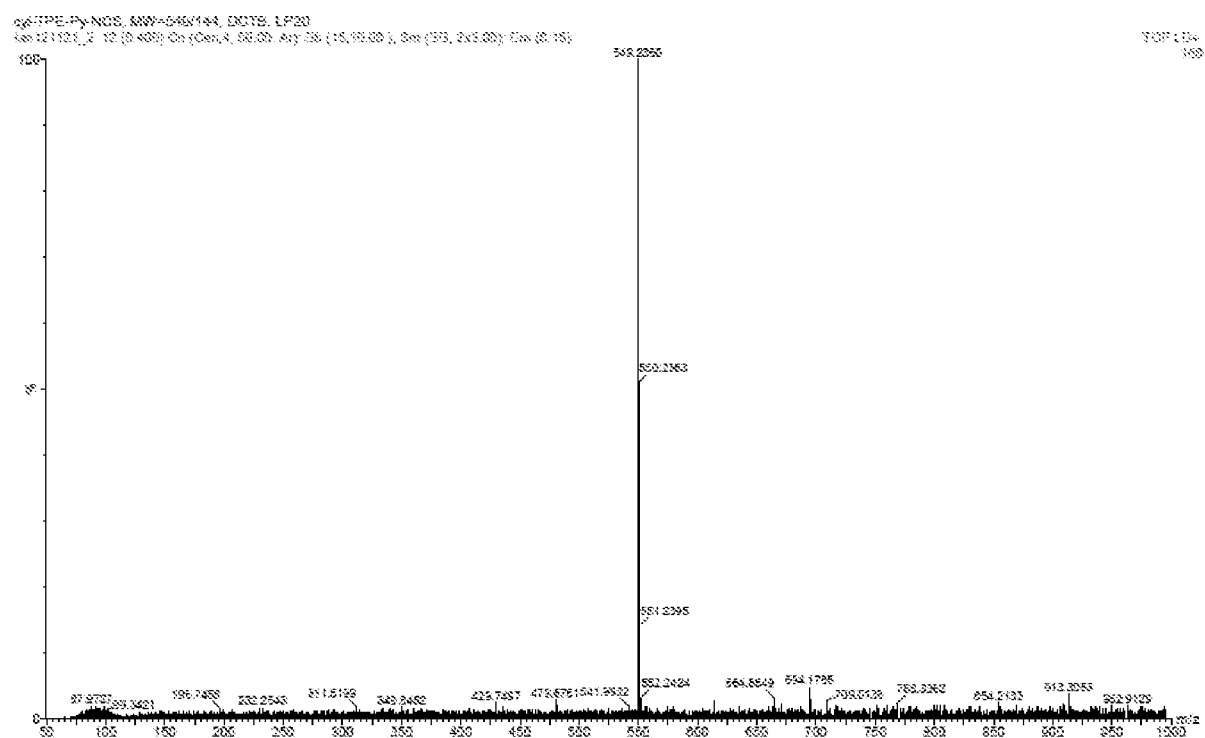


FIG. 2

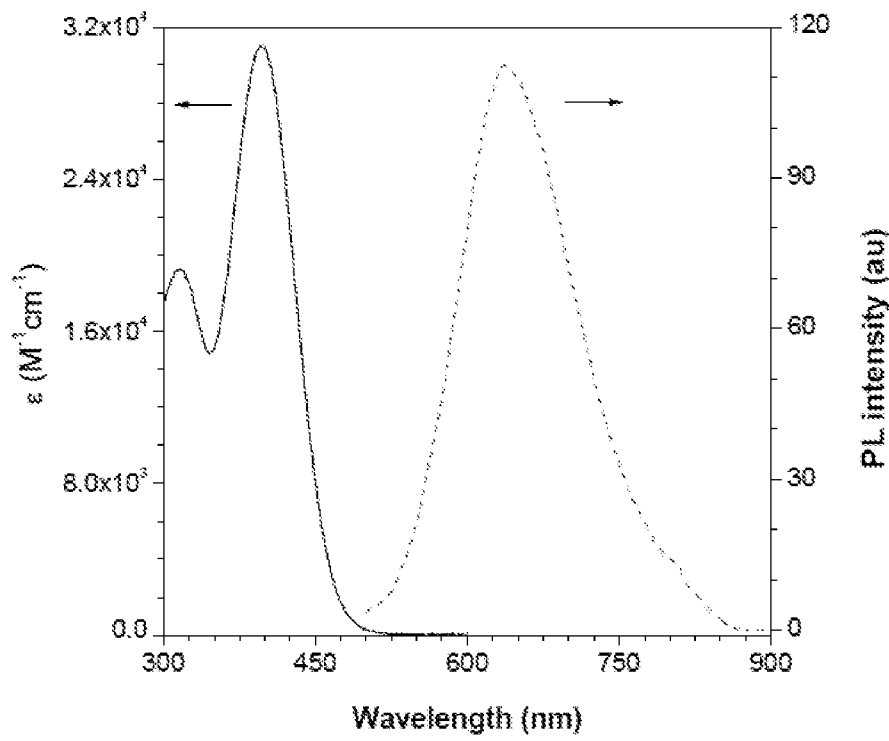


FIG. 3

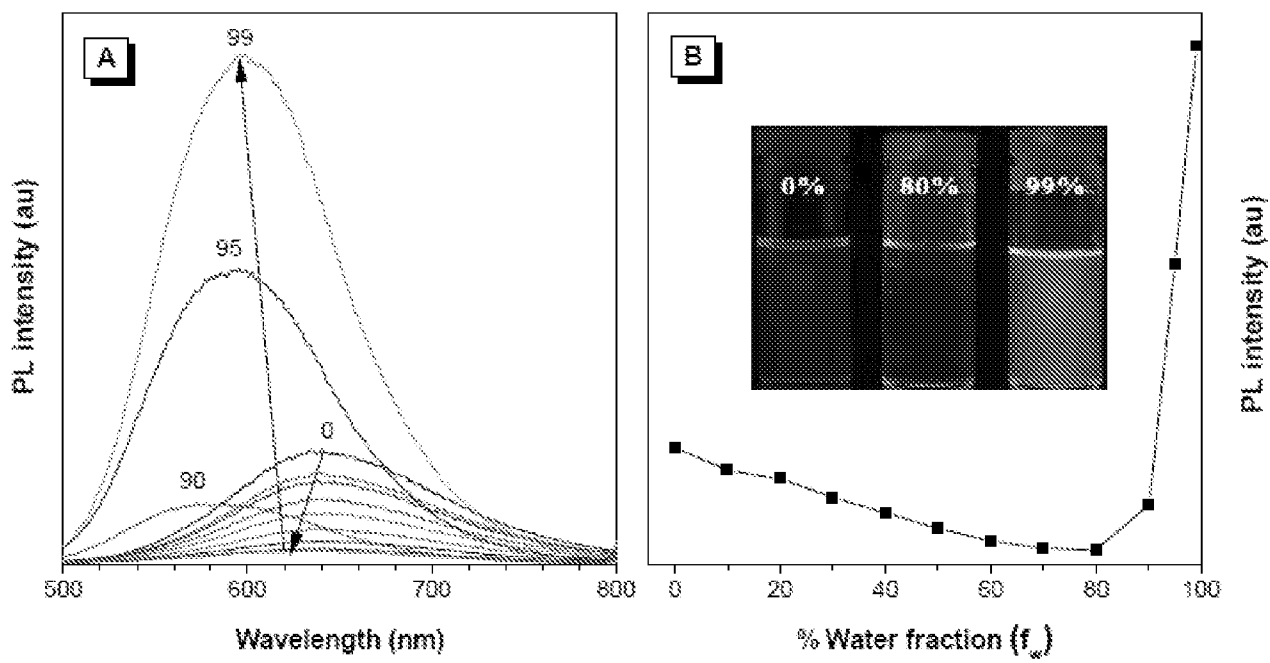


FIG. 4A-B

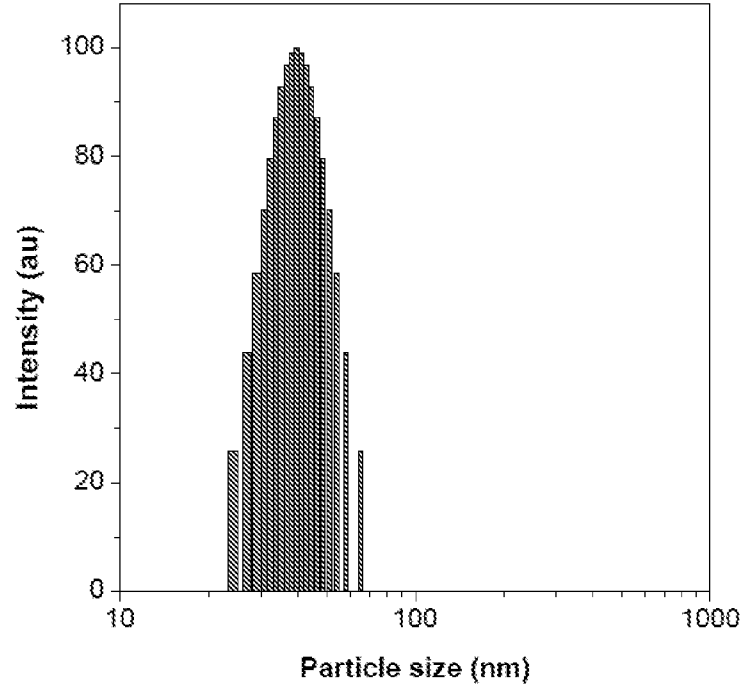


FIG. 5

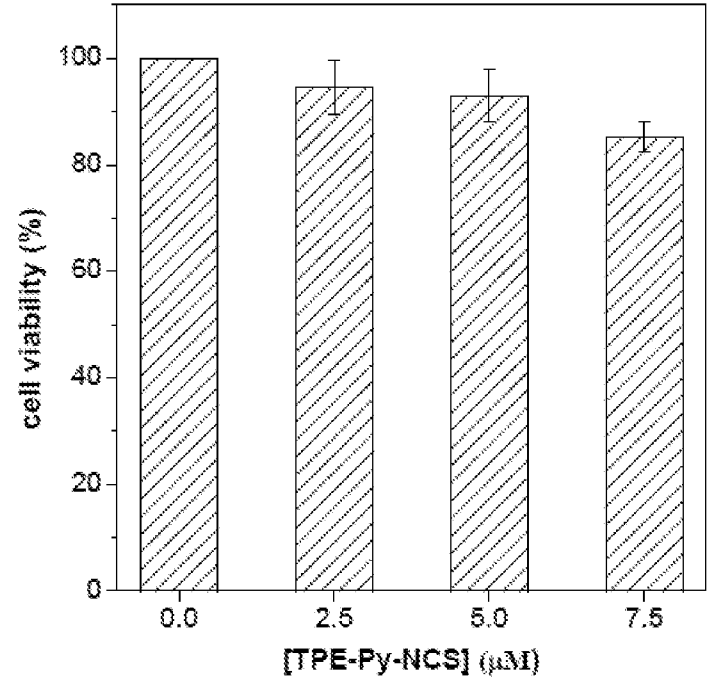


FIG. 6

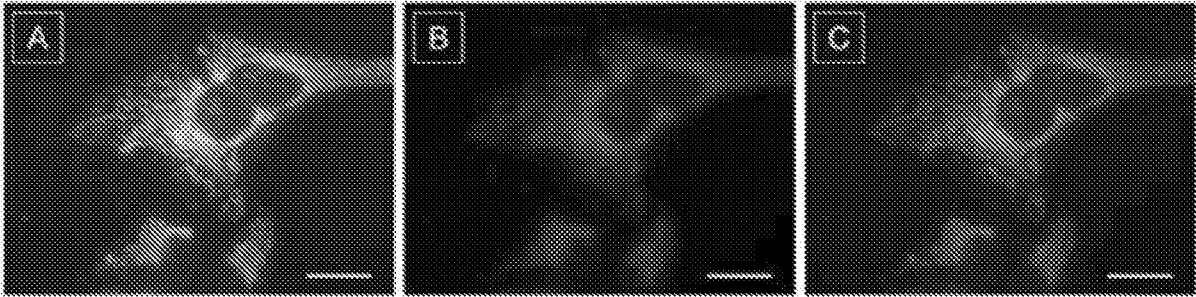


FIG. 7A-C

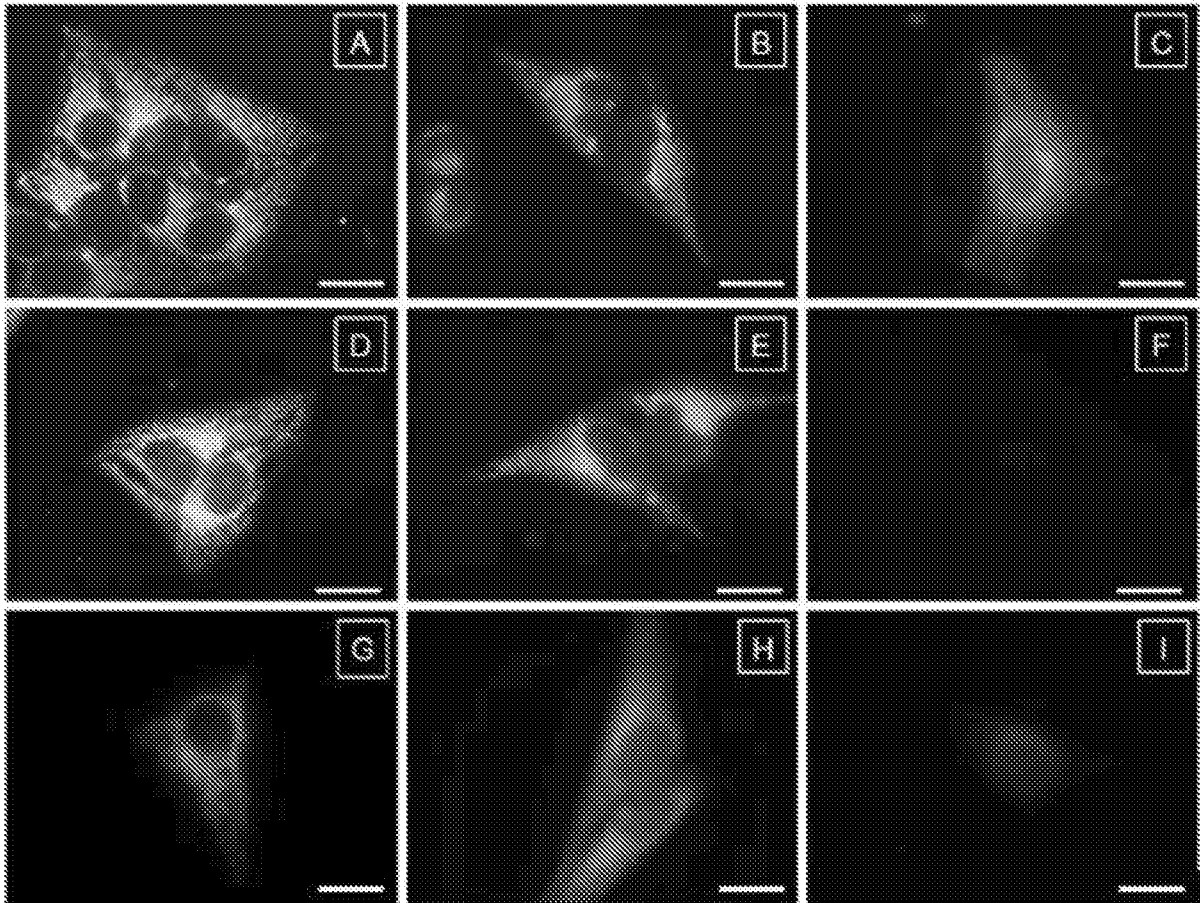


FIG. 8A-I

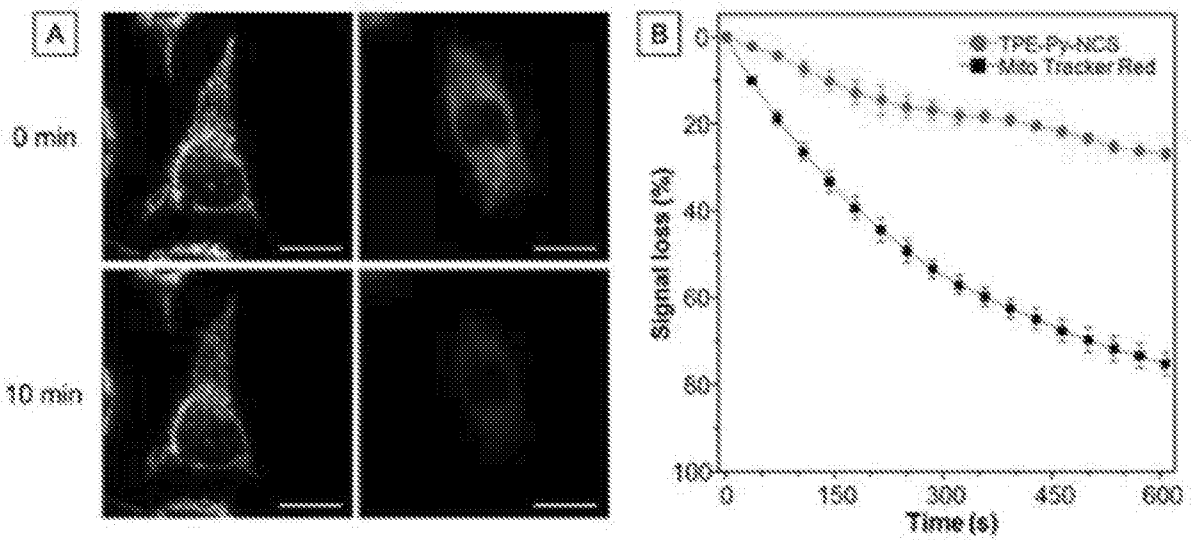


FIG. 9A-B

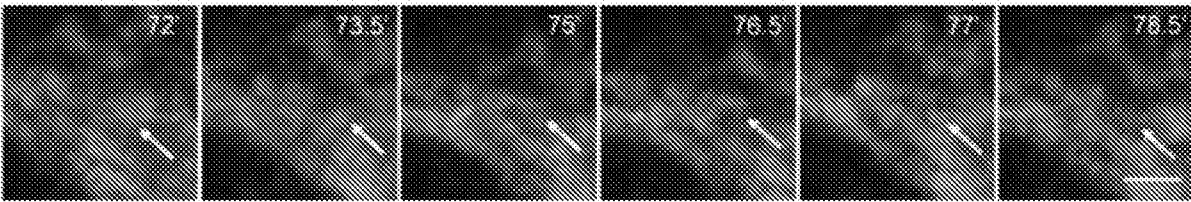


FIG. 10

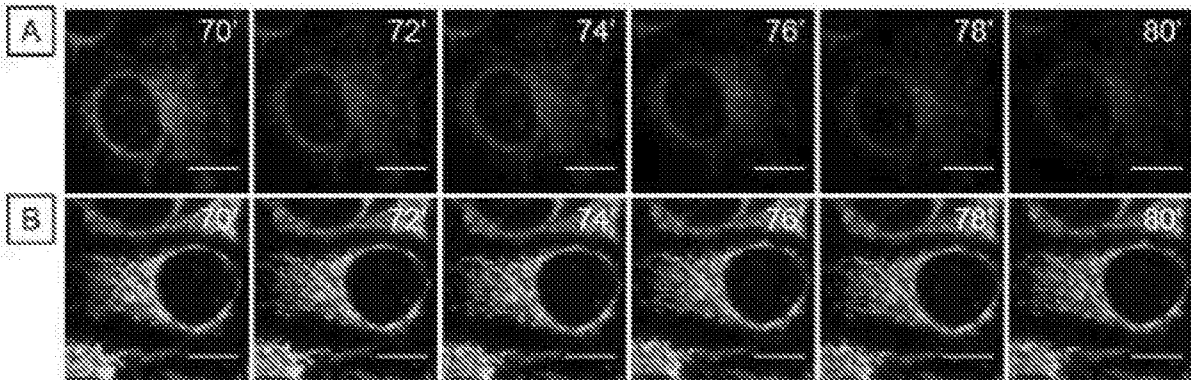


FIG. 11A-B

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2016/074337

**A. CLASSIFICATION OF SUBJECT MATTER**

C09K 11/06(2006.01)i; C07F 7/08(2006.01)i; C07D 213/38(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)

C09K11/-; C07F7/-; C07D213/-

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)

CNPAT,WPI,EPODOC,CASLINK,REG,CAPLUS,MARPAT: AIE, bioprobe, isothiocyanate, ncs, tetraphenylethene, pyridinium, TPE-Py, tetraphenylsilole,diphenylethene

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                 | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | CN 103865522 A (ZHEJIANG UNIVERSITY) 18 June 2014 (2014-06-18)<br>Claim 2                                                                                                                                                                                                                                                          | 1、 4-11               |
| A         | CN 103842472 A (THE HONG KONG UNIVERSITY OF SCIENCE AND TECHNOLOGY) 04 June 2014 (2014-06-04)<br>Claims 1-2                                                                                                                                                                                                                        | 1-13                  |
| A         | CN 102706839 A (THE HONG KONG UNIVERSITY OF SCIENCE AND TECHNOLOGY) 03 October 2012 (2012-10-03)<br>Claims 1-12                                                                                                                                                                                                                    | 1-13                  |
| A         | US 2013210047 A1 (THE HONG KONG UNIVERSITY OF SCIENCE AND TECHNOLOGY) 15 August 2013 (2013-08-15)<br>Claims 1-32                                                                                                                                                                                                                   | 1-13                  |
| X         | Shi Haibin et al. "Fluorescent light-up probe with aggregation-induced emission characteristics for in vivo imaging of cell apoptosis"<br><i>Org. Biomol. Chem.</i> , Vol. 11, 10 September 2013 (2013-09-10),<br>see page 7290, scheme 1 and right column, paragraph 4, page 7292, left column,<br>paragraph 2, page 7293, Fig. 5 | 1-13                  |

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

\* Special categories of cited documents:

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

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

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

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

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

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

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

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

“&” document member of the same patent family

Date of the actual completion of the international search

27 April 2016

Date of mailing of the international search report

20 May 2016

Name and mailing address of the ISA/CN

STATE INTELLECTUAL PROPERTY OFFICE OF THE  
P.R.CHINA  
6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing  
100088, China

Authorized officer

YAN,Hua

Facsimile No. (86-10)62019451

Telephone No. (86-10)82246723

**INTERNATIONAL SEARCH REPORT**  
**Information on patent family members**

International application No.

**PCT/CN2016/074337**

| Patent document<br>cited in search report |            |    | Publication date<br>(day/month/year) | Patent family member(s) |              |    | Publication date<br>(day/month/year) |
|-------------------------------------------|------------|----|--------------------------------------|-------------------------|--------------|----|--------------------------------------|
| CN                                        | 103865522  | A  | 18 June 2014                         | CN                      | 103865522    | B  | 02 December 2015                     |
| CN                                        | 103842472  | A  | 04 June 2014                         | WO                      | 2013029340   | A9 | 11 July 2013                         |
|                                           |            |    |                                      | US                      | 2014328764   | A1 | 06 November 2014                     |
|                                           |            |    |                                      | WO                      | 2013029340   | A1 | 07 March 2013                        |
|                                           |            |    |                                      | SG                      | 11201400944Q | A  | 26 September 2014                    |
| CN                                        | 102706839  | A  | 03 October 2012                      | CN                      | 102706839    | B  | 12 August 2015                       |
| US                                        | 2013210047 | A1 | 15 August 2013                       | None                    |              |    |                                      |