Ratiometric fluorescent probe with AIE property for monitoring endogenous hydrogen peroxide in macrophages and cancer cells

Yong Liu1, Jing Nie2, Jie Niu1, Fangfang Meng1 & Weiying Lin1

Hydrogen peroxide (H2O2) plays a key role in the progression of human illnesses, such as autoimmune and auto-inflammatory diseases, infectious diseases, diabetes, and cancer, etc. In this work, we have described a novel probe, TPE-TLE, which remarkably displayed AIE property and ratiometric fluorescence emission profiles in the presence of H2O2. This ratiometric fluorescent probe with AIE property exhibits outstanding features such as the well-resolved emission peaks, high sensitivity, high selectivity, low cytotoxicity, and good cell-membrane permeability. These excellent attributes enable us to demonstrate the ratiometric imaging of endogenously produced H2O2 in macrophages and cancer cells based on the novel ratiometric probe with AIE property for the first time. By comparing two kinds of cells, it is firstly found that cancer cells should contain much more endogenous H2O2 than macrophages. We expect that TPE-TLE will be useful fluorescent platform for the development of a variety of ratiometric fluorescent probes with AIE property to achieve unique biological applications.

In 2001, the novel phenomenon of aggregation-induced emission (AIE) was first found by Tang’s group1. AIE materials show very weakly fluorescence features in solution state and became intense in the aggregated state2, 3. The above unique finding has become a new method to tackle the aggregation-caused quenching (ACQ) of conventional chromophores and has shown significant academic value and promising applications in cell imaging4, fluorescent sensors and bio-probe materials5, 6. Herein, we describe the development of a novel ratiometric fluorescent probe with AIE property for ratiometric monitoring bioactive small molecule in the living system.

Reactive oxygen species (ROS) are formed as a natural by-product of the normal metabolism of oxygen and play a key role in cell signaling and homeostasis7–9. Generation of excessive ROS is involved in the pathogenesis of various diseases such as cardiovascular disease, cancer and neurological disorders10–12. Hydrogen peroxide (H2O2), a major ROS, exhibits relatively mild reactivity and has attracted intense interest13–17. Because it appears to be involved in signal transduction by reversible oxidation of proteins18. It has been known for many years that H2O2 plays a major role in the progression of human illnesses, such as autoimmune and auto-inflammatory diseases, infectious diseases, diabetes, mutagenesis and, perhaps most importantly, cancer19–23. Consequently, the search for a method that can be used for monitoring H2O2 has always been attractive and challenging.

Owing to the transient nature of H2O2, fluorescent probes, which generally display high sensitivity and can be used to determine spatial and temporal distributions in live specimens, are particularly appealing tools for the detection of ROS and related metabolites4. Most of these probes are intensity-based type, and tend to be interfered by the variations in excitation intensity, probe concentration, etc. To alleviate these problems, a number of ratiometric fluorescent H2O2 probes have been developed25, 26. However, up to present, there have been no reports on an AIE material for ratiometric sensing and imaging endogenous H2O2 in living cells (Table S1). Thus, developing ratiometric fluorescent probe with AIE property is very important due to significant academic value and biological applications of AIE materials. Thus, the goal of our work is to design a ratiometric fluorescent probe with AIE property for ratiometric detecting H2O2 in different cell lines.

1Institute of Fluorescent Probes for Biological Imaging, School of Chemistry and Chemical Engineering, School of Materials Science and Engineering, University of Jinan, Jinan, Shandong, 250022, P.R. China. 2School of Chemical Engineering & Technology, China University of Mining and Technology, Xuzhou, Jiangsu, 221116, P.R. China. Yong Liu and Jing Nie contributed equally to this work. Correspondence and requests for materials should be addressed to W.L. (email: weiiyinglein2013@163.com)
In this work, we described a novel ratiometric H$_2$O$_2$ fluorescent probe with AIE property for the first time. This ratiometric fluorescent probe with AIE property exhibits outstanding features such as the well-resolved emission peaks, high sensitivity, high selectivity, low cytotoxicity, and good cell-membrane permeability. These features were promising this novel AIE material can be successfully applied for ratiometric imaging endogenous H$_2$O$_2$ in living RAW 264.7 macrophages and cancer cells HepG2. Compared to RAW 264.7 macrophages, we firstly found that cancer cells HepG2 should contain much more endogenous H$_2$O$_2$.

**Results and Discussion**

**Preparation of probe.** As we all know, tetraphenylethene (TPE) is an archetypal AIE luminogen$^{27}$. On the basis of our interest on AIE material$^{28}$, we further exploited the unique application of this class of dyes by rational structural modifications. As shown in Fig. 1, we introduced a thiazole group on the TPE-core to afford the novel AIE material TPE-TLE-O. On the other hand, modification of a H$_2$O$_2$ site such as a borate moiety on the TPE-TLE-O gave the new compound TPE-TLE, which exhibited AIE character distinguishing from TPE-TLE-O. We envision that both materials exhibit distinct aggregation fluorescence signals.

For the AIE material TPE-TLE, in the presence of H$_2$O$_2$, the oxidation reaction of H$_2$O$_2$ to the borate moiety will provide TPE-TLE-O, which will decrease aggregation fluorescence signal of TPE-TLE to induce aggregation fluorescence signal of TPE-TLE-O. Therefore, we envisioned that AIE material TPE-TLE may be suitable for ratiometric imaging endogenous H$_2$O$_2$ in living systems.

**Optical properties in various solvents.** Optical properties of probes were basic properties of compounds. These properties provided clues for speculating its optical applications. Thus, with TPE-TLE in hand, we first
The results indicated that this probe had larger Stokes shift in aqueous solutions than organic solvents. The reaction product TPE-TLE-O was confirmed using 1H NMR (Fig. S1). The AIE material was observed at 550 nm. This result suggests that a new compound was formed during the titration process. The probe exhibited highly sensitive to H₂O₂ by an aggregation emission method. We expect that TPE-TLE-O should be an AIE material distinguishing TPE-TLE.

In preparation of probe, we have envisioned that the oxidation reaction of H₂O₂ to the borate moiety will provide TPE-TLE-O in the presence of H₂O₂, which will decrease aggregation fluorescence signal of TPE-TLE to induce aggregation fluorescence signal of TPE-TLE-O. The TPE-TLE-O should be an AIE material distinguishing from TPE-TLE. To prove the above assumption, we investigated optical properties of TPE-TLE-O in the distinct polar environments. As shown in Fig. 5, photographs and fluorescence characteristics of TPE-TLE-O in water/DMF mixtures indicated that the compound TPE-TLE-O was an AIE material distinguishing from TPE-TLE.

The AIE material TPE-TLE should be suitable for ratiometric detecting H₂O₂ based on an aggregation emission method.

Ratiometric responses of probe TPE-TLE to H₂O₂. To examine whether probe TPE-TLE could ratiometric detect H₂O₂, TPE-TLE (5 μM) was treated with H₂O₂ in PBS buffer solution (containing 50% DMF), and further studying the progress of the reaction by fluorescence titration experiment. As shown in Fig. 6a, in the absence of H₂O₂, the probe TPE-TLE exhibited no visible variations in the ratios of emission intensities at 435 nm, suggesting that TPE-TLE was stable in the assay conditions. When increasing concentrations of H₂O₂ were introduced, the fluorescence spectra of probe TPE-TLE exhibited significant changes. The intensity of the emission maximum at 435 nm was gradually decreased with the simultaneous appearance of a new red-shifted emission band centered at 550 nm. The red-shift in the excitation spectra of the TPE with the increase H₂O₂ is due to intramolecular charge-transfer (ICT). Moreover, the ratios of emission intensities at 550 and 435 nm displayed a large increase from 0 to 4.25 (Fig. 6a, inset). Furthermore, a new fluorescence emission peak was observed at 550 nm. This result suggests that a new compound was formed during the titration process. The reaction product TPE-TLE-O was confirmed using 1H NMR (Fig. S1). The AIE material TPE-TLE was capable of detecting H₂O₂ by an aggregation emission method.

Sensitivity was an important criterion for developing highly sensitive fluorescence imaging agents. Mainly reason was that the pathophysiologic ally relevant concentrations of H₂O₂ were in the low to medium micro molar concentrations. We further investigate sensitive of probe in order to prove whether TPE-TLE can detect H₂O₂ in intercellular environment. As shown in Fig. 6b, the detection limit of the probe was 6 ± 0.6 μM, indicating that the probe TPE-TLE exhibited highly sensitive to H₂O₂ by an aggregation emission method. We expect that the probe TPE-TLE was able to detect low micro molar concentrations of H₂O₂ and possessed potentially applicable in medical and biological.

To demonstrate the selectivity of TPE-TLE to H₂O₂, TPE-TLE was treated with a wide variety of cations, anions, and oxidants, and then fluorescence spectra were further measured. As shown in Fig. 6c and d, the probe...
TPE-TLE exhibited a significant red-shift of the fluorescence emission spectra in the presence of H$_2$O$_2$. Other representative species such as Ca$^{2+}$, Na$^+$, K$^+$, ClO$^-$, NO$^-$, NO$_2^-$, OH$^-$, O$_2$, H$_2$O$_2$, NO, TBHP, Cys, and GSH were added to TPE-TLE solutions. H$_2$O$_2$ elicited a large ratiometric signal with $I_{550}/I_{435}$. By contrast, other species induced a very low ratiometric response with $I_{550}/I_{435}$. This clearly indicated that the probe TPE-TLE had an excellent selectivity to H$_2$O$_2$ over the other analytes. The AIE material TPE-TLE was capable of highly selective detecting H$_2$O$_2$ by an aggregation emission method. In addition, the time-dependent fluorescence intensity changes from the probe TPE-TLE to H$_2$O$_2$ were further studied. As shown in Fig. S2, the reaction can be completed about 3 h.

We conducted another experiment condition to show that the probe can detect H$_2$O$_2$ in a ratiometric fashion in vitro. To examine the ratiometric fluorescence response of probe TPE-TLE to H$_2$O$_2$, the probe was titrated with different equiv. of H$_2$O$_2$ in 90% PBS buffer solution (containing 10% DMF). As shown in Fig. 4a and inset, the free probe displayed an emission peak with maximum at around 450 nm. By contrast, upon addition of H$_2$O$_2$, the intensity of the emission peak at 450 nm gradually decreased with the simultaneous appearance of a new blue-shifted emission band centered at 560 nm. This result was similar with response of probe TPE-TLE to H$_2$O$_2$ in 50% PBS buffer solution (containing 50% DMF). Moreover, as shown in Fig. S4b, the probe TPE-TLE exhibited higher sensitivity (Detection limit: 1 μM) in PBS buffer solution (containing 10% DMF) than 50% PBS buffer solution (containing 50% DMF). Furthermore, selectivity experiment indicated that the probe has high selectivity for H$_2$O$_2$ in 10% PBS buffer solution (containing 10% DMF) (Fig. S4c and d). The above results demonstrated that probe TPE-TLE could ratiometric detect H$_2$O$_2$ in 10% PBS buffer solution (containing 10% DMF).

We investigated influence of the probe TPE-TLE to H$_2$O$_2$ under different pH conditions. The results revealed that the pH value of solution had a notable influence on the probe response to H$_2$O$_2$. As shown in Fig. S4, in the presence of H$_2$O$_2$, the fluorescence intensity at 550 nm of probe response to H$_2$O$_2$ had no changes with the value increase of pH in the range of 5.0–8.0. In the absence of H$_2$O$_2$, the free probe TPE-TLE exhibited weak fluorescence intensity in the wide pH range. The above results demonstrated that the TPE-TLE can respond well to H$_2$O$_2$ without dramatic influenced by pH.
Living cell imaging studies in RAW264.7 macrophages. In order to be useful as ratiometric imaging agents, ratiometric fluorescent probe should have low cytotoxicity. Thus, we investigated the potential toxicities of TPE-TLE against a representative cell: RAW264.7 macrophages. The living cells were incubated with various concentrations of the new ratiometric probe for 24 h, and then the cell viability was determined by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays; The results indicated that the ratiometric probe do not exhibit marked cytotoxicity (Fig. S5).

As we know, H$_2$O$_2$ widely exists in RAW 264.7 macrophages and plays key role in apoptosis and oxidative stress. Thus, to prove the capability of the probe TPE-TLE for H$_2$O$_2$ ratiometric fluorescence imaging in living cells, TPE-TLE (8 µM) was incubated with living RAW 264.7 macrophages for 0.5 h at 37 °C, and then confocal imaging was carried out. As shown in Fig. 7, cells emitted a strong blue fluorescence signal and weak red fluorescence in the blue (Fig. 7b) and red channels (Fig. 7c), respectively. Because endogenous H$_2$O$_2$ will be produced by stimulating RAW264.7 macrophage cells using 12-myristate-13-acetate (PMA). Then TPE-TLE (8 µM) was incubated with living RAW 264.7 macrophages treated with PMA for 0.5 h at 37 °C, and further achieved cells imaging. Imaging results demonstrated that cells displayed a slight blue emission (Fig. 7e) and a strong red emission (Fig. 7f), consistent with the H$_2$O$_2$-induced ratiometric fluorescent response in vitro. Moreover, we further obtained ratiometric fluorescence imaging picture of both channels in living RAW 264.7 macrophages (Fig. 7g). The above results indicated that the probe TPE-TLE was capable of ratiometric imaging endogenous H$_2$O$_2$ in living RAW 264.7 macrophages by an aggregation emission method. The ratios of emission intensities profiles further prove that the TPE-TLE emitted stronger fluorescent intensity in living RAW 264.7 macrophages treated with PMA than RAW 264.7 macrophages untreated with PMA (Fig. 7h). This result was agreement with H$_2$O$_2$-induced ratiometric fluorescent response in vitro.

Living cell imaging studies in cancer cells HepG2. It has been known for many years that H$_2$O$_2$ plays a major role in the progression of human illnesses, perhaps most importantly, cancer. To demonstrate the probe TPE-TLE ratiometric imaging exogenous H$_2$O$_2$, the living cancer cells HepG2 and HepG2 treated with PMA were prepared. Imaging results indicated that cells untreated with PMA gave a strong blue fluorescence signal and a weak red signal in blue and red channels, respectively (Fig. 8a–c). However, images of cells treated with PMA results demonstrated that cells displayed a strong blue emission (Fig. 8e) and a strong red emission (Fig. 8f). Similarly with RAW 264.7 macrophages, ratiometric fluorescence imaging picture (Fig. 8g) and fluorescence emission profiles (Fig. 8h) were obtained. Thus, the above results establish that TPE-TLE is cell-membrane permeable ratiometric probe with AIE property, and suitable for the ratiometric imaging exogenous H$_2$O$_2$ in the living HepG2 cells by an aggregation emission method.
Compared to RAW 264.7 macrophages, both channels exhibited stronger fluorescence in living HepG2 cells than RAW 264.7 macrophages in the absence and presence of PMA. Moreover, the TPE-TLE probe emitted stronger red fluorescence in HepG2 cells treated with PMA than RAW 264.7 macrophages. Furthermore, the TPE-TLE exhibited higher ratio of emission intensities in HepG2 cells treated with PMA than RAW 264.7 macrophages. The above results demonstrated that cancer cells should contain more H₂O₂ than macrophages.

To the best of our knowledge, no reports to date have been published on the construction of ratiometric H₂O₂ fluorescent probes with AIE property for ratiometric fluorescent imaging endogenously H₂O₂ in RAW 264.7 macrophage and HepG2 cells.

**Conclusions**

In summary, we introduced a thiazole group and a borate moiety on the TPE-core to afford a novel ratiometric H₂O₂ probe TPE-TLE with AIE property for the first time. For sensing mechanism of TPE-TLE, in the presence of H₂O₂, the oxidation reaction of H₂O₂ to the borate moiety will provide TPE-TLE-O, which will decrease aggregation fluorescence signal of TPE-TLE to induce aggregation fluorescence signal of TPE-TLE-O. Furthermore, the TPE-TLE exhibits excellent properties including the well-resolved emission peaks, high sensitivity, high selectivity, low cytotoxicity, and good cell-membrane permeability. The above mechanism and attributes enable us to demonstrate, for the first time, the ratiometric imaging of endogenously produced H₂O₂ in macrophages and cancer cells. Moreover, by comparing to fluorescence and ratio of emission intensities of both cells, it is firstly found that cancer cells should contain much more endogenous H₂O₂ than macrophages. We expect that TPE-TLE will be useful fluorescent platform for the development of a variety of ratiometric fluorescent probes with AIE property, and further achieve unique biological applications.

**Methods**

**General procedure for the spectral measurement.** The stock solution of the probe TPE-TLE was prepared at 1 mM in DMSO. The PBS (pH = 7.4) solutions with 50% DMSO was prepared. The solutions of various testing species were prepared from NaCl, KCl, CaCl₂, glutathione (GSH), H₂O₂, homocysteine (Hcy), cysteine (Cys), NaNO₂, NaOH, t-butylhydroperoxide, NaClO, in the twice-distilled water. The test solution of the probe TPE-TLE (5 μM) were 50% PBS buffer solution (containing 50% DMF) and 90% PBS buffer solution (containing 10% DMF). The resulting solution was shaken before measuring the spectra. For titration and selectivity experiments of probe, both experiments were carried out using excitation wavelength of 405 nm, and the excitation and emission slit widths of the spectral measurement were 5 and 5 nm, respectively.
Quantum yields. The fluorescence quantum yields would be calculated by the following formula (1):

$$\Phi = \Phi_s \left( \frac{A_s}{A} \right) \left( \frac{\eta_s^2}{\eta^2} \right) \frac{I}{I_s}$$

(1)

In this formula, $s$ and $r$ stand for the sample and the reference, respectively. $\Phi$ stands for quantum yield; $F$ and $A$ stands for the integrated emission intensity and the absorbance, respectively; $n$ is refractive index.

Cytotoxicity assay. Living RAW264.7 cells were cultivated in per well of 96-well plate. After 24 h, the culture medium was changed using new culture medium containing different concentration (0 μM, 1 μM, 5 μM, 10 μM, 20 μM) TPE-TLE. After 8 h, the culture medium and the excess probes were removed, and then 10 μL MTT (5 mg/mL in PBS) was added to the above medium. Subsequently, the culture medium was removed, and 100 μL DMSO was added to the 96-well plate in order to dissolve the formazan crystal product. The plate containing Living RAW264.7 cells was shaken for 10 min, and then the plate containing RAW264.7 cells was measured at 490 nm using the microplate reader. OD_{490} sample denotes the RAW264.7 cells incubated with the probe TPE-TLE at different incubation times, OD_{490} control denotes the RAW264.7 cells without the probe TPE-TLE, OD_{490} blank denotes the wells containing only the culture medium. The cell viability would be calculated by the following formula: Cells viability (%) = (OD_{490} sample − OD_{490} blank)/(OD_{490} control − OD_{490} blank) × 100%.

Cells culture. Living RAW264.7 macrophages were prepared in H-DMEM (Dulbecco's Modified Eagle's Medium, High Glucose) supplemented with 10% fetal bovine serum in a 5% CO₂ incubator at 37 °C. Before the living cells imaging experiments, living RAW264.7 cells were seeded confocal dish (density of cells was $1 \times 10^5$).
mL). The cells were placed on glass coverslips and allowed to adhere for 48 h. Cells imaging experiments could be carried out as soon as the cells reached about 70% confluence.

Firstly, control experiment was further carried out. The culture medium of the cells was added to a fresh medium containing 50.0 μg/mL 12-myristate-13-acetate (PMA) and incubated for 3 h. Secondly, the medium was removed and washed three times with PBS in order to remove the excess PMA. After that, 1 mL of the medium containing 8 μM TPE-TLE−O was added and further incubated at 37 °C for 30 min. Finally, the confocal imaging was carried out in the blue (λex = 405 nm, λem = 425–475 nm) and green channels (λex = 405 nm, λem = 570–620 nm); (f) Images of cells treated with PMA in the red emission channel (λex = 405 nm, λem = 570–620 nm); (g) Ratiometric images of cells untreated with PMA. (h) The ratio of emission intensities profiles in HepG2 cells untreated and treated with PMA. Scale bar = 20 μm. Statistical analyses were performed with a Student’s t-test (n = 4). *P < 0.001 and the error bars represent standard deviation (±S.D.).

Figure 8. (a) Bright-field image of cells untreated with PMA. (b) Images of cells untreated with PMA in the blue emission channel (λex = 405 nm, λem = 425–475 nm); (c) Images of cells untreated with PMA in the red emission channel (λex = 405 nm, λem = 570–620 nm); (d) Bright-field images of cells treated with PMA; (e) Images of cells treated with PMA in the blue emission channel (λex = 405 nm, λem = 425–475 nm); (f) Images of cells treated with PMA in the red emission channel (λex = 405 nm, λem = 570–620 nm); (g) Ratiometric images of cells untreated with PMA. (h) The ratio of emission intensities profiles in HepG2 cells untreated and treated with PMA. Scale bar = 20 μm. Statistical analyses were performed with a Student’s t-test (n = 4). *P < 0.001 and the error bars represent standard deviation (±S.D.).

Synthesis of 1. The compound was synthesized by refer literature38.

Synthesis of TPE-TLE-O. In a round-bottomed flask (25 mL) equipped with a magnetic stirrer, a solution of the compound 1 (0.37, 1.0 mmol) and 2-aminothiophenol (0.15 g, 1.2 mmol) in methanol (10 mL) was prepared. Then, 30% H2O2 (6.0 mmol) and 37% HCl (3.0 mmol) were added and the mixture was stirred at room temperature for 2 h. The mixture was quenched by adding H2O (10 mL), extracted with EtOAc (3 5 mL), and the combined extracts were dried (Na2SO4). The corresponding benzothiazoles were obtained after removal of solvents and purified by silica gel chromatography (eluent: n-hexane/EtOAc = 4:1). The yield of TPE-TLE-O was 65%. 1H NMR (400 MHz, CDCl3) δ 12.47 (s, 1 H), 7.99–7.94 (m, 1 H), 7.85 (dd, J = 8.0, 1.1 Hz, 1 H), 7.50 (m, 1 H), 7.42–7.35 (m, 2 H), 7.20–7.02 (m, 16 H), 6.88 (d, J = 8.6 Hz, 1 H). 13C NMR (101 MHz, DMSO-d6) δ 155.39, 151.77, 143.78, 143.45, 140.99, 135.41, 135.07, 131.26, 131.12, 128.44, 128.38, 128.29, 127.16, 127.03, 126.94, 125.52, 122.54, 122.41, 116.95.
Synthesis of TPE-TLE. Compound 2 (0.24 g, 0.5 mmol) was added into a flask containing a mixture of 4-bromomethylphenyl boronic acid (0.15 g, 0.5 mmol), K2CO3 (0.07 g, 0.5 mmol), and 10 mL of DMF with nitrogen at room temperature for 6 h, then poured into H2O (500 mL) and extracted with EtOAc. The organic phase was separated, dried with MgSO4, and removed by vacuum distillation. The product was obtained as a yellow solid with a yield of 60% after purified by column chromatography with ethyl acetate/petroleum ether (2:1, v/v) as eluent. Melting point: 142–146 °C. 1H NMR (400 MHz, DMSO-d6) δ 8.10 (s, 1H), 8.05–8.07 (d, J = 8.0 Hz, 1H), 7.95–7.97 (d, J = 8.4 Hz, 1H), 7.71–7.73 (t, J = 7.6 Hz, 2H), 7.56–7.58 (d, J = 8.0 Hz, 2H), 7.48–7.57 (t, J = 7.8 Hz, 1H), 7.38–7.46 (t, J = 7.4 Hz, 1H), 6.99–7.16 (m, 17H), 5.40 (s, 2H), 1.30 (s, 12H). 13C NMR (101 MHz, DMSO-d6) δ 162.21, 154.88, 151.80, 143.63, 143.55, 143.37, 141.36, 139.89, 139.80, 138.30, 137.73, 135.77, 135.15, 135.08, 131.30, 131.21, 131.11, 128.42, 128.30, 127.96, 127.19, 125.73, 124.86, 122.25, 121.51, 113.71, 84.19, 70.82, 40.65, 40.44, 40.24, 40.03, 39.82, 39.61, 39.40, 25.16. HRMS (ESI) (m/z): [M+H]+ calcd for C46H40BNO3S: 698.3000, found, 698.3001.

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Acknowledgements
This work was financially supported by NSFC (51503077, 21472067, 21672083), Taishan Scholar Foundation (TS 201511041), and the startup fund of the University of Jinan (309-10004). Doctor start up fund of University of Jinan (160082102) and NSFSP (ZR2015PE001).

Author Contributions
W. Lin and Y. Liu conceived the idea and directed the work. Y. Liu and J. Nie designed the experiments and performed the organic synthesis and spectral measurements. Y. Liu, J. Niu and F. Meng performed the bioimaging. All authors contributed to data analysis, manuscript writing and participated in research discussions.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-07465-5

Competing Interests: The authors declare that they have no competing interests.

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