Sub-20 nm Nontoxic Aggregation-Induced Emission Micellar Fluorescent Light-up Probe for Highly Specific and Sensitive Mitochondrial Imaging of Hydrogen Sulfide**

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Materials and methods

Poly (ethylene glycol) mono methyl ether (mPEG; Mn=2000 Da), 2,4-dihydroxybenzaldehyde were purchased from Sigma-Aldrich. 1-Bromohexadecane, hydrazine hydrate, acetonitrile, methanol, methylbenzene, Cs_2CO_3, $CuCl_2$, 4-toluene sulfonyl chloride (TsCl), dimethyl sulphoxide (DMSO), triethylamine (TEA) and tetrahydrofuran (THF) were obtained from Shanghai Chemical Co.

HeLa cells were purchased from the China Center for Type Culture Collection (Wuhan University) and cultured in DMEM medium supplemented with 4×10^{-3} M L-glutamine, 10% fetal bovine serum (FBS) and 1% antibiotics (100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) at 37℃ in a humidified atmosphere containing 5% CO_2.

Synthesis of compound 1.

1-Bromohexadecane (500 mg, 1.6 mmol) and 2,4-dihydroxybenzaldehyde (350 mg, 2.5 mmol) were dissolved in acetonitrile (20 mL), followed by addition of Cs_2CO_3 (815 mg, 2.5 mmol). After refluxed at 85℃ for 24 h, the mixture was cooled down and filtrated to obtain yellow solution, which was subsequently concentrated to remove organic phase and further separated by column chromatography (silica, petroleum ether : ethyl acetate = 10 : 1, v/v) to gain compound 1 as white solid (485 mg, 67%

yield). ^{1}H NMR ($CDCl_3$, 400 MHz):δ11.48 (s, 1H), 9.67 (s, 1H), 7.40 (d, 1H), 6.51 (d, 1H), 6.38 (s, 1H), 3.97 (t, 2H), 1.77 (d, 2H), 1.43 -1.24 (m, 26H), 0.86 (d, 3H);

Synthesis of compound 2.

The ethanol solution of compound 1 (217 mg, 0.6 mmol) and hydrazine hydrate (300 mg, 6 mmol) was prepared firstly, and then refluxed under N_2 atmosphere for 24 h. After cooled to 4℃ for 4 h, light-yellow precipitates were retained from the reaction mixture by filtrating, and washed with deionized water three times. Pure product of compound 2 as yellow powder was obtained for further reaction after drying under vacuum (237 mg, 96% yield). ^{1}H NMR ($CDCl_3$, 400 MHz):δ11.29 (s, 1H), 7.83 (s, 1H), 6.99 (d, 1H), 6.46 (m, 2H), 5.27 (s, 2H), 3.93 (t, 2H), 1.77 (d, 2H), 1.43 -1.24 (m, 26H), 0.88 (d, 3H);

Synthesis of compound 3.

mPEGOTs was synthesized as an intermediate product firstly. mPEG2000 (2×10^4 mg, 10 mmol) was dissolved in toluene (150 mL) and heated to 140℃for removing water in mPEG2000 using an oil-water separator. The solution was then cooled to room temperature after 4 h refluxing, and TsCl (2.5×10^3 mg, 13 mmol) and TEA (1.5×10^3 mg, 15 mmol) were added to the solution. After 24 h stirring at room temperature, the reaction mixture was filtered and concentrated to 50 mL. Then, ethyl ether (500 mL) was added into the solution to obtain mPEGOTs as white precipitate. mPEGOTs (5×10^3 mg, 2 mmol), 2,4-dihydroxybenzaldehyde (350 mg, 2.5 mmol) and Cs_2CO_3(815 mg, 2.5 mmol) were mixed in acetonitrile (50 mL) and stirred at 85℃for 24 h. The mixture was filtered and concentrated to 20 mL, and then was added to

ethyl ether (500 mL). The precipitates were collected and purified by column chromatography (silica, dichloromethane : methyl alcohol= 10 : 1, v/v), and finally dried under vacuum to afford compound 3 as white powder (3.64×10^3 mg, yield 69%). ^{1}H NMR ($CDCl_3$, 400 MHz):δ11.48 (s, 1H), 9.72 (s, 1H), 7.46 (d, 1H), 6.53 (m, 1H), 6.43 (s, 1H), 4.18 (t, 2H), 3.89-3.32 (m, 175H);

Synthesis of AIE-1.

Compound 2 (189 mg, 0.5 mmol) and compound 3 (1.18×10^3 mg, 0.55 mmol) were dissolved in ethanol (20 mL), and stirred at 80℃ for 24 h. The solution was cooled at 4℃ for 4 h and filtered. Ethanol was removed from the solution to obtain yellow precipitate. The solid was dissolved in water and dialysis against water (WMCO 3500 Da), and then lyophilized to afford AIE-1 as yellow powder (912 mg, yield 73%). ^{1}H NMR ($CDCl_3$, 400 MHz):δ11.75 (s, 1H), 8.59 (s, 1H), 7.20 (d, 1H), 6.51 (m, 2H), 4.16 (t, 2H), 3.93 (t, 2H), 3.89-3.32 (m, 175H), 1.77 (d, 2H), 1.43 -1.24 (m, 26H), 0.88 (d, 3H);

Preparation of solution containing micelles (AIE-M) or Cu^{2+}-adherent micelles (AIE-M-Cu).

To an aqueous solution (10 mL) was added AIE-1 (100 mg, 0.04 mmol), and then the solution containing micelles (AIE-M) was prepared by ultrasonic dispersion method for further use.

AIE-1 (100 mg, 0.04 mmol) and $CuCl_2 \cdot 2H_2O$ (17 mg, 0.1 mmol) were dissolved in THF (20 mL), and stirred at room temperature in dark for 2 days. Evaporation of the solvent under reduced pressure gave a black solid, which was dissolved in water and dialysis against water (WMCO 3500 Da) to afford aqueous solution containing Cu^{2+}-adherent micelles (AIE-M-Cu). At last, 5 mL AIE-M or AIE-M-Cu solution was

lyophilized to afford yellow or black powder for next utilizing.

Structural and fluorescent characterizations.

FT-IR spectra of the prepared compounds were collected on an FT-IR spectrometer (Perkin Elmer, USA). The samples were mixed with KBr, compressed to a plate, and evaluated over the spectral region of 400 cm^{-1} to 4000 cm^{-1}. ^1H nuclear magnetic resonance (^1H NMR) spectra were recorded on a Mercury VX-300 spectrometer at 300 MHz using $CDCl_3$ as the solvent and tetramethyl silane as an internal standard. Absorption spectral of AIE-M and AIE-M-Cu solution was measured by using a UV-Vis spectrometer (Lambda 35, PerkinElmer, USA). Quantum yield of AIE-M was measured according to an established procedure. The optical densities measured on the UV-vis spectrum were obtained on a UV-Vis spectrometer (Lambda 35, PerkinElmer, USA). Fluorescein disodium salt was chosen as a standard. Absolute value were calculated using the standard reference sample that has a fixed and known FL QY value. Fluorescence spectrum was measured by using a fluorescence spectrometer (RF-5301pc, SHIMADZU, JAPAN). HPLC analyses were performed with shimadzu HPLC system, equipped with a shimadzu LC-15C binary pump, a shimadzu SPD-15C detector (280 nm) and a waters C18 column.

Measurement of the critical micelle concentration (CMC).

A stock solution of pyrene (6.0×10^{-2} M) was prepared in acetone and stored at 5℃ until further use. For the measurement of CMC, the pyrene solution in acetone was added to deionized water to give a pyrene concentration of 12.0×10^{-7} M. The solution was then distilled under vacuum at 60℃ for 1 h to remove acetone from the solution. The acetone-free pyrene solution was mixed with the solution of polymeric micelles, the concentration of which ranged from 0.5 to 2×10^{-4}mg/mL. The final concentration

of pyrene in each sample solution was 6.0×10^{-7} M. The pyrene emission at 339 nm was recorded. The CMC was estimated by plotting I_{394}/I_{378} (I_{378} means intensity of the peak at 378 nm, I_{394} means intensity of the peak at 394) ratio from pyrene emission spectra against of the log of the micelle concentration.

Measurement of dynamic light scattering (DLS), transmission electron microscope (TEM) and polymer stability at different pH.

The nanoparticles hydrodynamic diameter were determined in PBS (0.01 M, pH 7.4) using Zetasizer nano ZS (Malvern Instruments Ltd., UK) with a He-Ne laser beam (633 nm). Briefly, the solution containing AIE-M (1 mg/mL) or AIE-M-Cu (1 mg/mL) was placed into a glass cuvette separately. The samples were measured at 25℃ and at a scattering angle of 173°. The average value was obtained from three replicate measurements for each sample. For stability test of two nanoparticles, there were two kind of PBS solution containing AIE-M (1 mg/mL) or AIE-M-Cu (1 mg/mL) which were incubated in shaking table at room temperature for 2 weeks, and the size of AIE-M or AIE-M-Cu was recorded by using DLS measurement every day during the test.

TEM was used to observe the nanoparticles morphology. Typically, a drop of AIE-M or AIE-M-Cu solution was placed on a copper grid with formvar film and stained with a 0.2% (w/v) solution of phosphotungstic acid. Measurement was performed using JEM-100CXII TEM at an acceleration voltage of 100kV.

AIE-1 aqueous solution was diluted to 1 mg/mL using acetate buffer (0.01 M, pH 5.0, 6.5 and 7.4) and shaken at 37 °C. After 24 h, each sample solution (1 mL) was lyophilized and then dissolved in acetonitrile–water solution (50:50, v/v, 10 mL). The stability of AIE-1 was determined by HPLC analyses using acetonitrile–water (50:50,

v/v) as the mobile phase eluted at 30 ℃ and 1.0 mL/min, and the chromatogram of compound 1 (D) and compound 3 (E) were also obtained for comparison under the same conditions.

Measurement of aggregation-induced-emission (AIE) characteristics.

Stock DMSO solution of AIE-1 with a concentration of 2 mM was prepared by putting AIE-1 (5 mg, 2 μmol) separately dissolved in DMSO (1 mL). An aliquot (0.2 mL) of the stock solution of DMSO was transferred to a 10 mL volumetric flask. After adding an appropriate amount of DMSO, water was added dropwise to furnish 40 μM DMSO/water mixtures with water fractions of 0-99 vol%. Each sample was determined by fluorescence measurement (excitation at 408 nm).

A range of AIE-M solution (40 μM) were prepared with additional $CuCl_2$ of different concentration (0-40 μM) together, and measured by fluorescence measurement (excitation at 408 nm) after stirring 30 min at room temperature.

For measurement of fluorescence spectrum with additional Na_2S of different concentration, a 100 μM stock solution of AIE-M-Cu and 1 mM stock solution of Na_2S was prepared in PBS firstly. Fluorescence measurement were performed by addition of 0.8 mL stock AIE-M-Cu solution and a proper amount of stock Na_2S solution to PBS (totally 2 mL) in a quartz cell.

AIE-M-Cu solution (40 μM) with addition of Na_2S (50 μM) was preparation in PBS, and incubated at 37℃ for 34 min. During incubation, 2 mL solution was transferred to a quartz cell for determining by fluorescence measurement at regular intervals (excitation at 408 nm).

Sensitive detection measurement of hydrogen sulfide.

A 400 μM stock solution of AIE-M-Cu was prepared in deionized water. Stock solution of anions (S^{2-}, Cl^-, CO_3^{2-}, NO_3^-, PO_4^{3-}, H_2PO^{4-}, $S_2O_3^{2-}$ and SO_3^{2-}) and amino

acid (DTT, GSH and Cys) in deionized water were also prepared. In a typical detection, test solutions were prepared by placing 0.2 mL AIE-M-Cu stock solution into a quartz cell, diluting the solution to 2 mL with an appropriate amount of anions or amino acid solution (final, 200 μM), and the fluorescence spectra were recorded after 1 h.

***In Vitro* Cytotoxicity.**

The cytotoxicity of AIE-M and AIE-M-Cu against HeLa cells were evaluated *in vitro* by the MTT assay. Briefly, HeLa cells were seeded into a 96-well plate at a density of 5.0×10^3 cells/well in 100 μL of DMEM containing 10% FBS. The cells were cultured for 1 day at 37℃ in 5% CO_2 atmosphere. Then, 100 μL solution of AIE-M or AIE-M-Cu in DMEM medium was added into the wells containing cells for further 24 h incubation. The final concentrations of nanoparticles ranged from 3.9 mg/L to 500 mg/L. After washing the cells with PBS, MTT stock solution (5 mg/mL in PBS, 20 μL) was added into each well and incubated for 4 h. The media were completely removed and 150 μL of DMSO was added into each well to dissolve the formazan blue crystal. The absorbance of the solution was measured using a microplate reader at 570 nm. Cell viability was expressed as follows: Cell viability (%) = $(A_{sample}-A_{0})/(A_{control}-A_{0}) \times 100\%$. Where A_{sample} and $A_{control}$ are the absorbance of the sample treated cells and the control cells treated with MTT, respectively. A_0 is the absorbance of the solution containing cells and complete DMEM without MTT and other sample. The A_{sample}, $A_{control}$, and A_0 were obtained after subtracting the absorbance of DMSO. Data are presented as average ± SD (n = 4).

Fluorescence imaging experiments of AIE-M.

HeLa cells were seeded in a glass bottom dish at a density of 1×10^5 cells per well and then incubated for 1 day prior to tests, solution of AIE-M was added at final

concentration of 40 μM or 80 μM and the HeLa cells were allowed to culture for further 4 h and 8 h, respectively. After washing with PBS for 3 times, the cells were observed under a laser scanning confocal microscopy (CLSM, Nikon C1-si TE2000, BD laser).

For subcellular localization of mitochondria/lysosomal with mitotracker/lysotracker and AIE-M, HeLa cells were seeded as previous method. Then AIE-M was added at final concentration of 40 μM and the cells were allowed to culture for further 8 h. After washing with PBS, the cells were treated with MitoTracker (200 nM) for 10 min or lysotracker (100 nM) for 1 h, and observed by using CLSM after washed again.

Fluorescence imaging experiments of AIE-M-Cu.

HeLa cells were seeded in a glass bottom dish at a density of 1×10^5 cells per well and then incubated for 1 day prior to tests. Intact HeLa cells were treated with a combination of AIE-M (40 μM) and $CuCl_2$ (40 μM) for 8 h and then washed quickly with PBS. The cells were then treated with Na_2S (0 μM, 5 μM, 10 μM, 20 μM, 40 μM and 80 μM) for 30 min, and washed with PBS before imaging.

HeLa cells were treated with a combination of AIE-M (40 μM) and $CuCl_2$ (40 μM) for 8 h and then washed quickly with PBS for imaging. After that Na_2S (final 50 μM) was added to incubate with cells, the cells were observed under a CLSM at 5 min, 10 min, 20 min and 30 min.

For subcellular localization of mitochondria/lysosomal with mitotracker/lysotracker and AIE-M-Cu, HeLa cells were seeded as previous method. Then AIE-M and $CuCl_2$ were added separately at final concentration of 40 μM and the cells were allowed to culture for further 8 h. After washing with PBS, the cells were treated with Na_2S (100 μM) for 20 min, followed by co-staining with mitotracker (200 nM) for 10 min or lysotracker (100 nM) for 1 h after washed again, and were observed under a CLSM at

last.

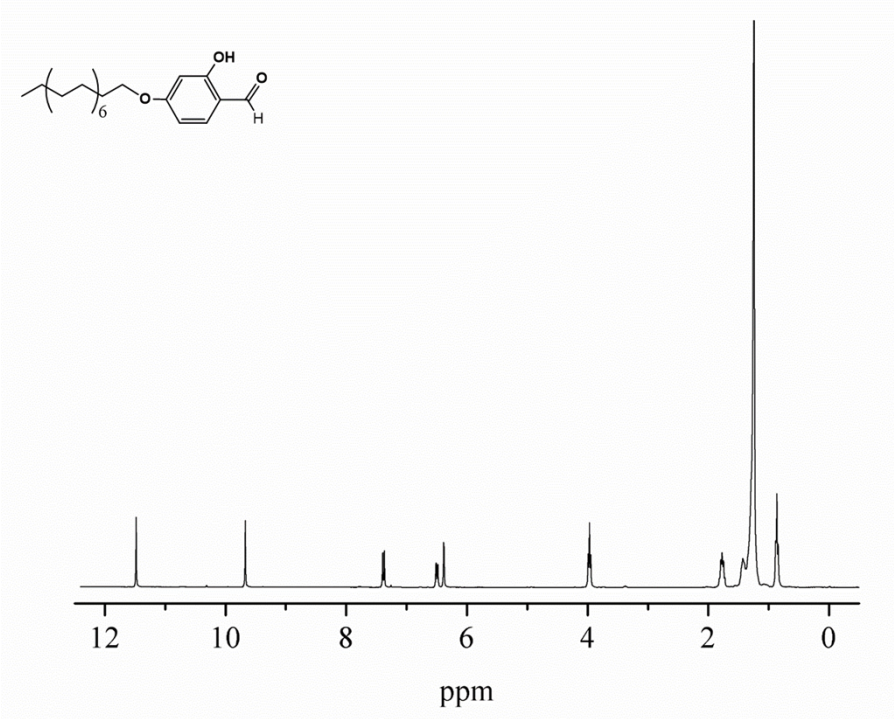

Figure S1. ^{1}H NMR spectrum of compound 1.

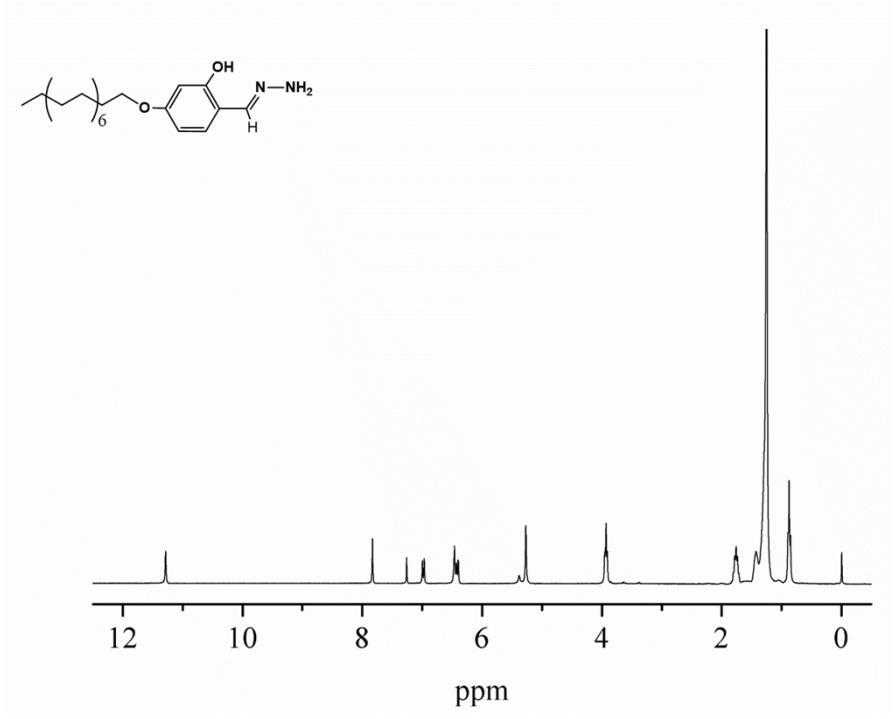

Figure S2. ^{1}H NMR spectrum of compound 2.

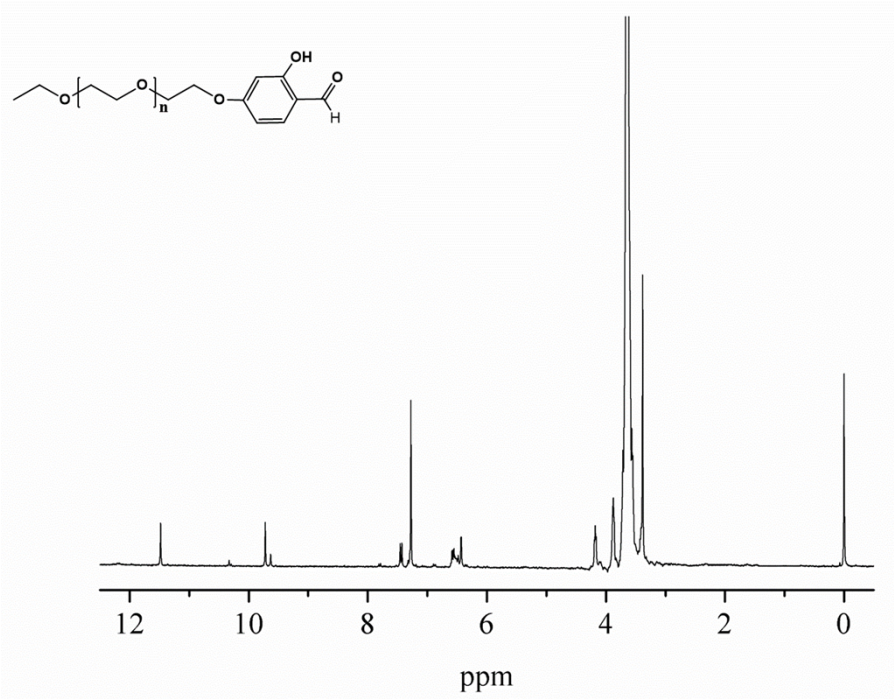

Figure S3. ^{1}H NMR spectrum of compound 3.

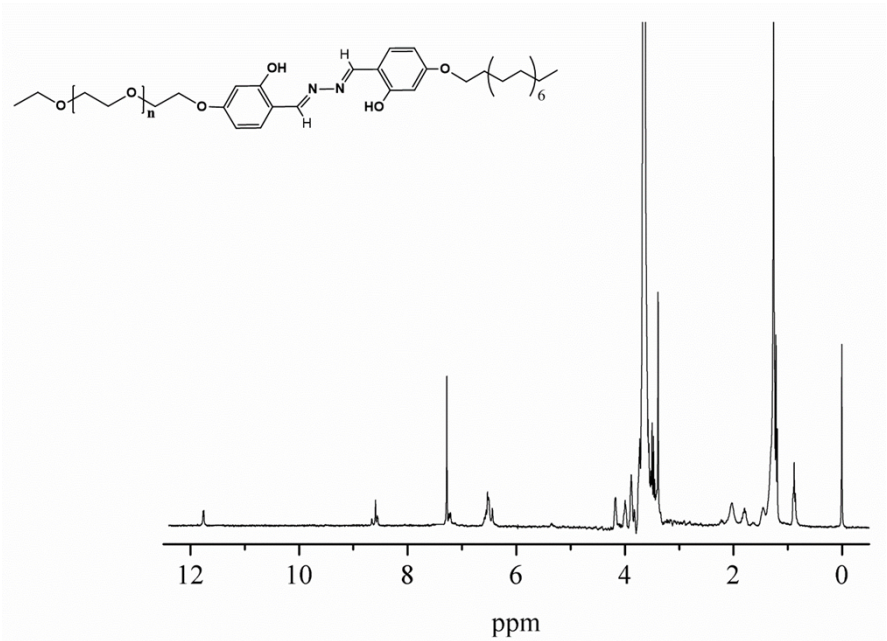

Figure S4. ^{1}H NMR spectrum of AIE-1.

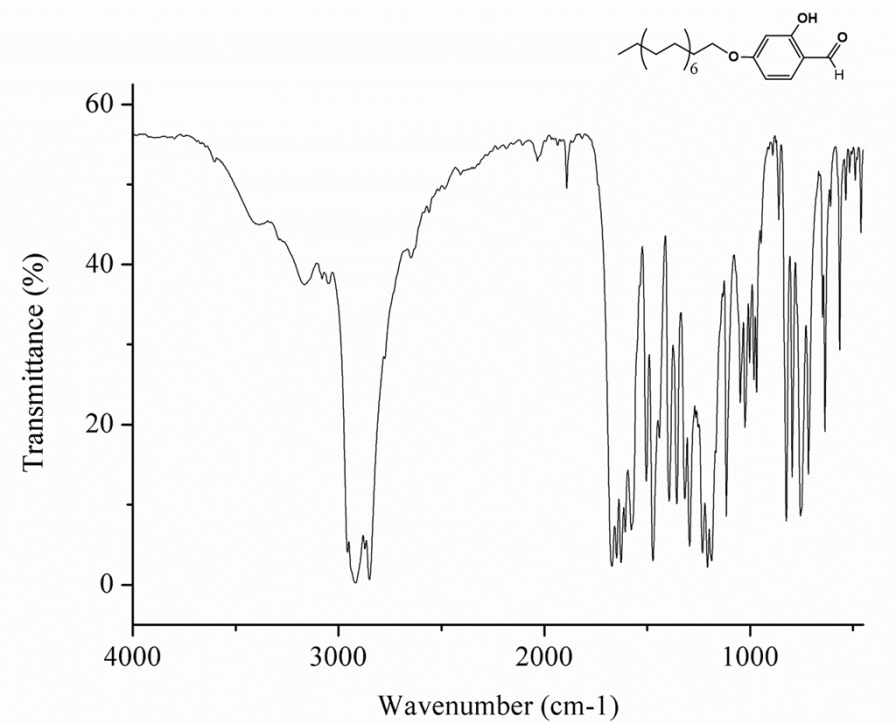

Figure S5. FT-IR spectrum of compound 1.

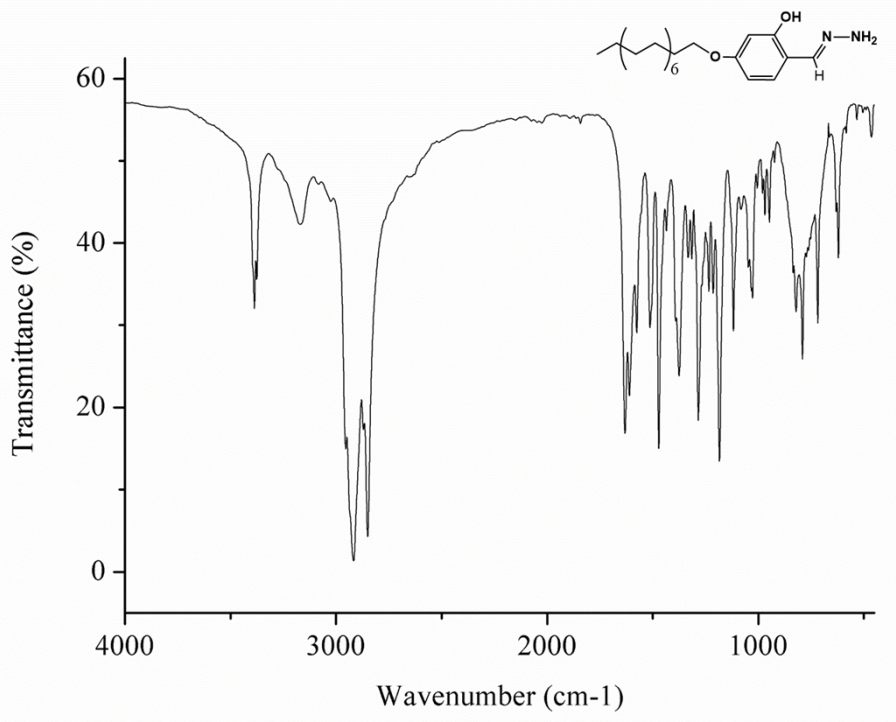

Figure S6. FT-IR spectrum of compound 2.

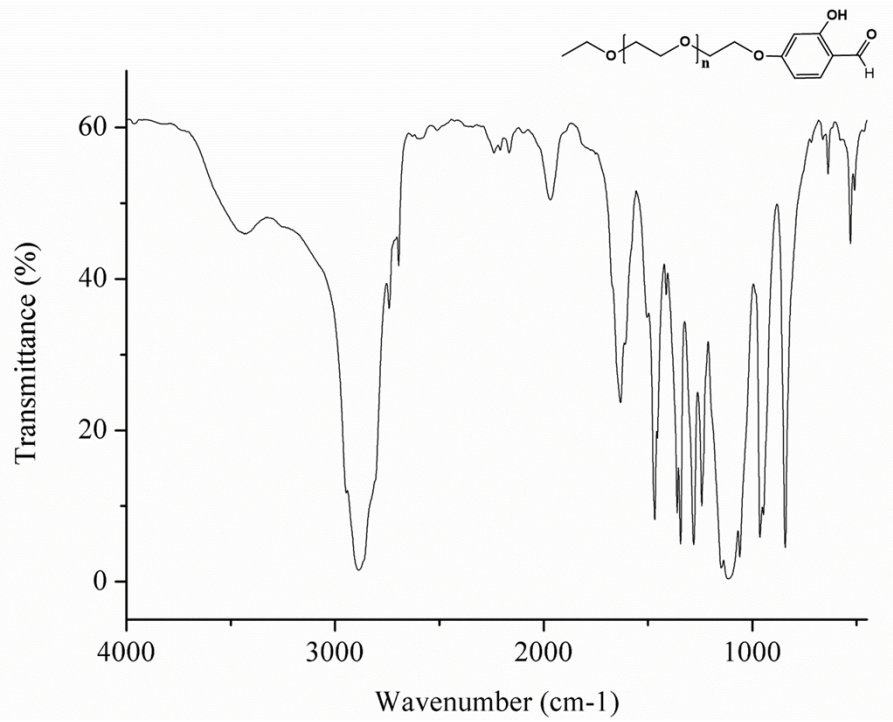

Figure S7. FT-IR spectrum of compound 3.

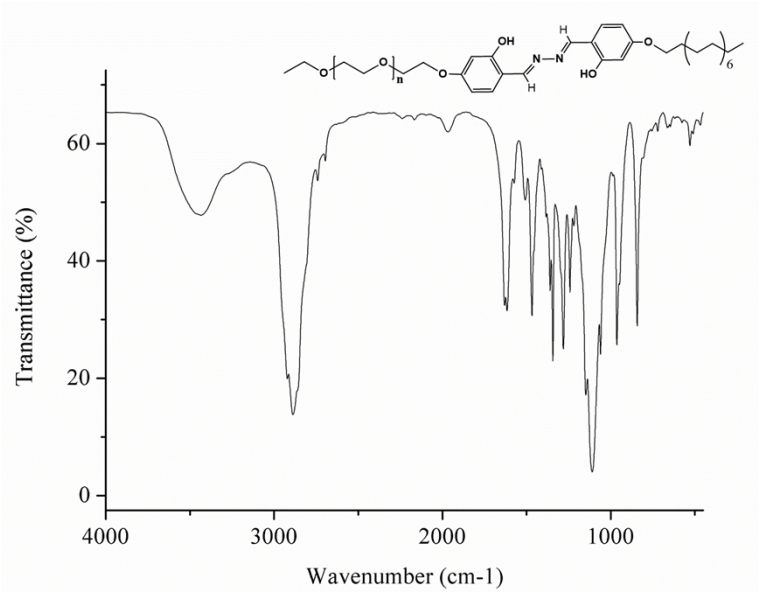

Figure S8. FT-IR spectrum of AIE-1.

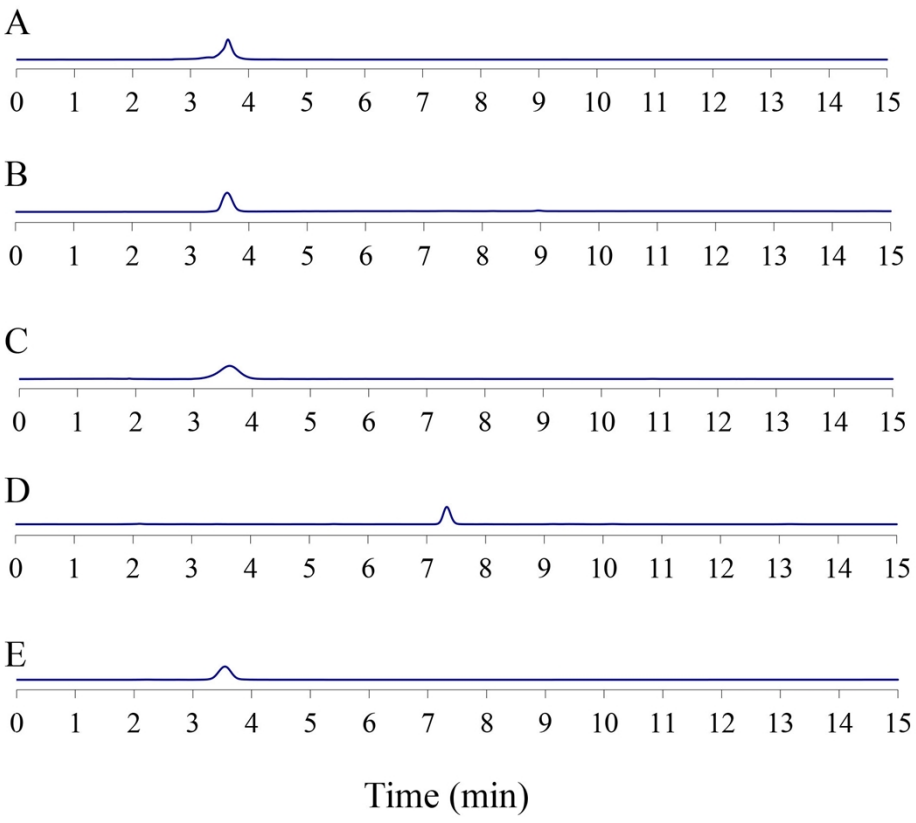

Figure S9. HPLC chromatogram of AIE-1 which was treated in sodium acetate buffer of pH 7.4 (A), 6.5 (B), 5.0 (C) for 24 h, and the chromatogram of compound 1 (D) and compound 3 (E) were also obtained for comparison.

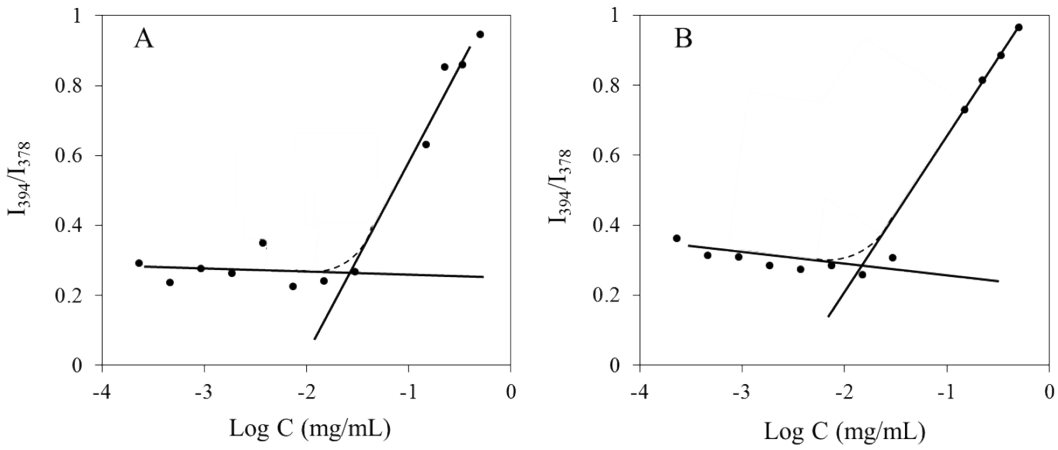

Figure S10. Plot of the I_{394}/I_{378} ratio from pyrene emission spectra versus log C of AIE-M (A) and AIE-M-Cu (B), and the CMC of AIE-M or AIE-M-Cu is 26 or 12

mg/L respectively.

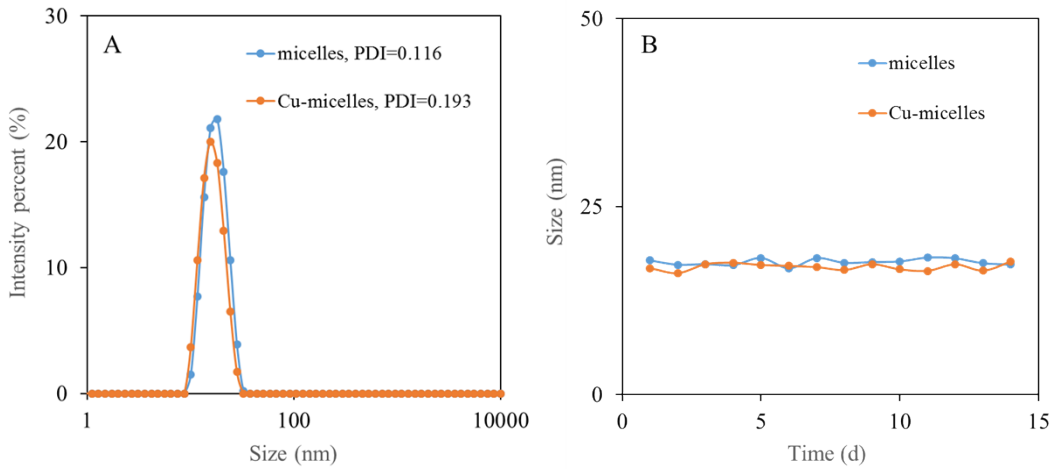

Figure S11. (A) The size distribution of AIE-M and AIE-M-Cu determined by DLS in PBS (pH=7.4, 0.01M). (B) Stability of two nanoparticles (size *vs* time) in PBS (pH=7.4, 0.01M) during 2 weeks.

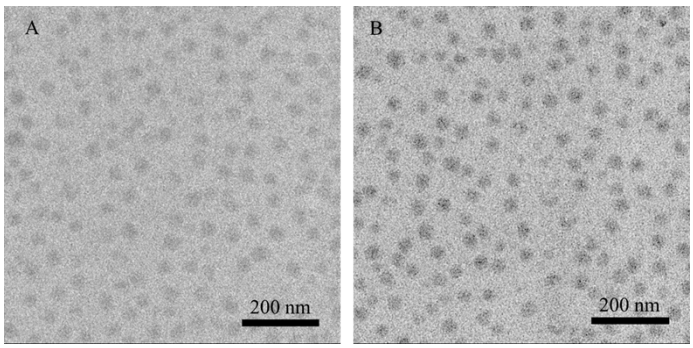

Figure S12. TEM images of AIE-M (A) and AIE-M-Cu (B).

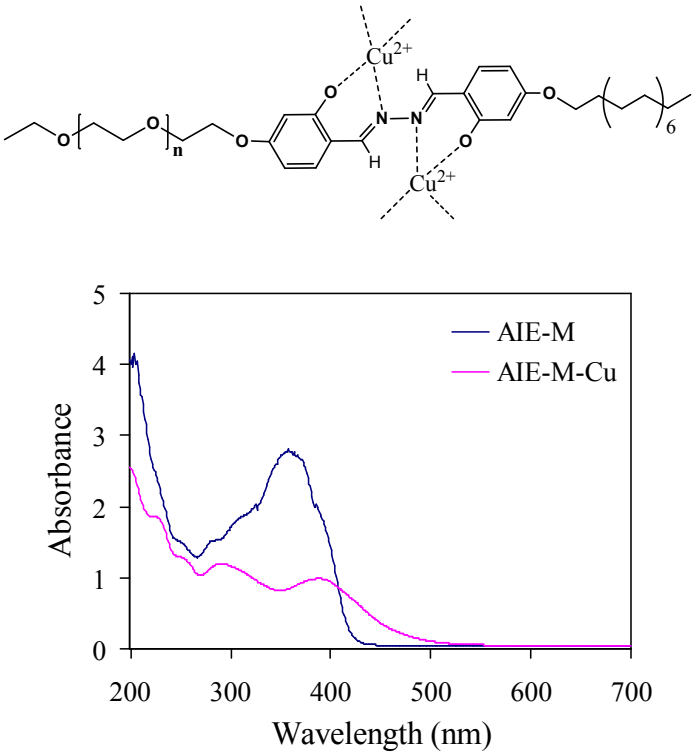

Figure S13. Structure of AIE-M-Cu and absorption spectral of AIE-M and AIE-M-Cu.

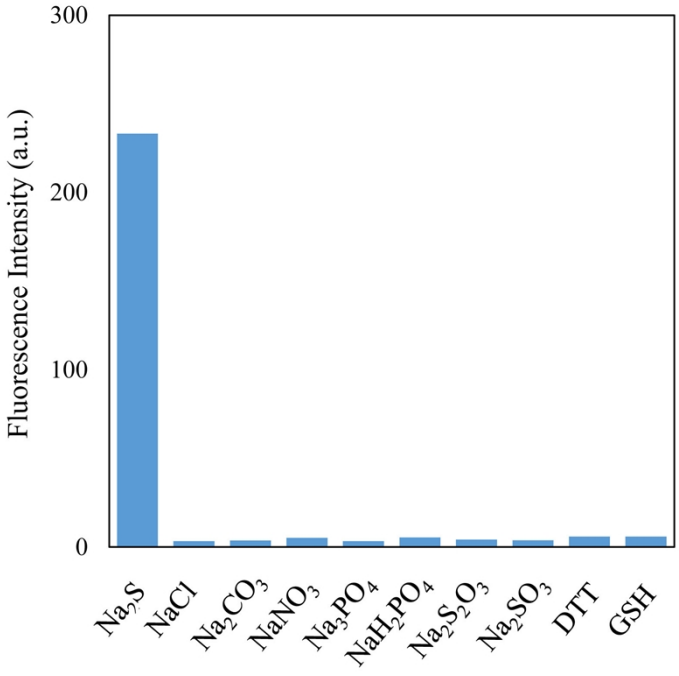

Figure S14. Fluorescence spectrum of 40 μM AIE-M-Cu in the presence of thiols, inorganic sulfur compounds, anions and amino acid. Bars represent fluorescence intensity 1 h after addition of Na_2S (200 μM), NaCl (200 μM), Na_2CO_3 (200 μM),

$NaNO_3$ (200 μM), Na_3PO_4 (200 μM), NaH_2PO_4 (200 μM), $Na_2S_2O_3$ (200 μM), Na_2SO_3 (200 μM), DTT (200 μM), GSH (200 μM) and Cys (200 μM).

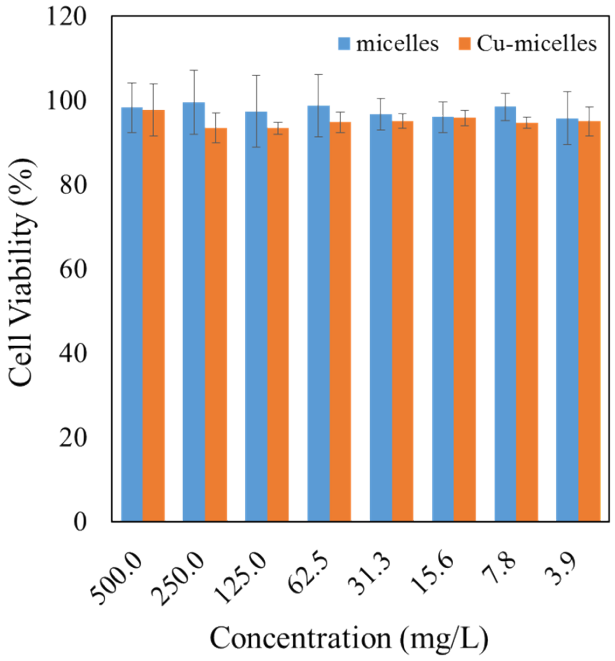

Figure S15. MTT assay of AIE-M and AIE-M-Cu in HeLa cells after incubation for 24 h.

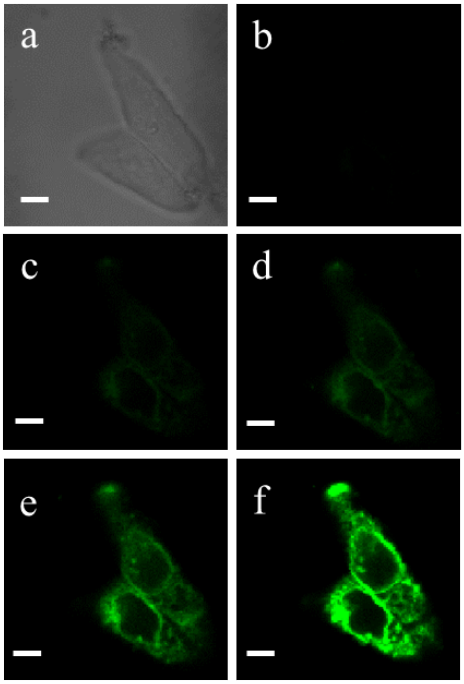

Figure S16. Time-dependent confocal microscopy images of HeLa cells after incubation with Na_2S (50 μM). Firstly, HeLa cells were incubated with AIE-M-Cu (40 μM) for 8 h, and after washed with PBS (pH 7.4, 0.01M), 50 μM of Na_2S was added to incubate with cells for 0 min (a, b), 5 min (c), 10 min (d), 20 min (e) and 30 min (f). λ_{ex} = 408 nm. Scale bar = 20 μm.

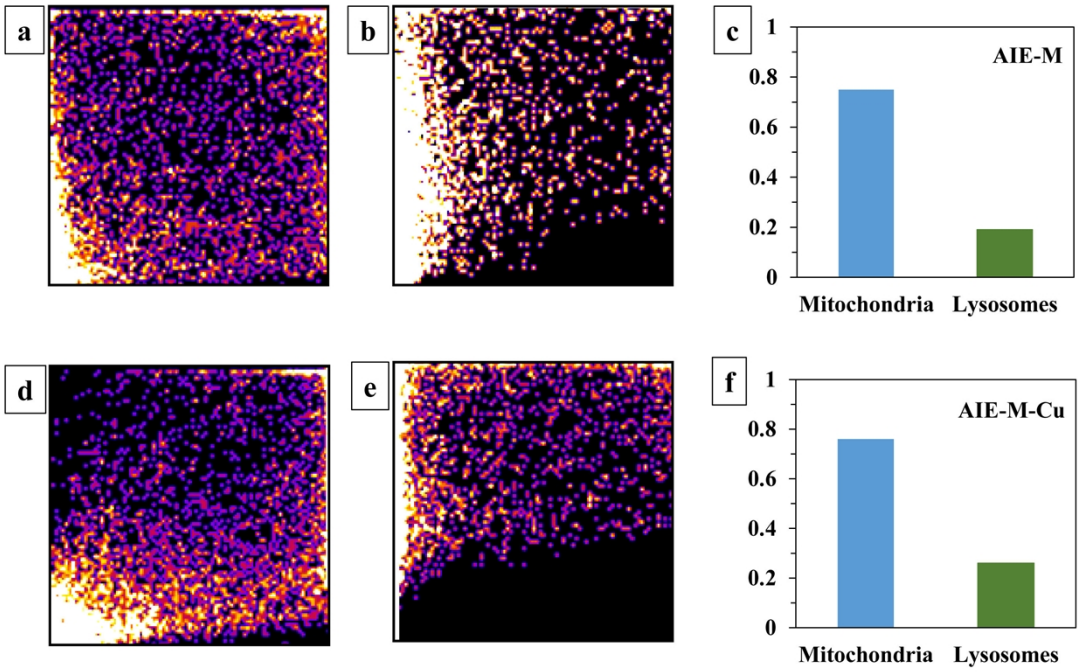

Figure S17. Colocalization scatter plot for AIE-M (a, b) or AIE-M-Cu (d, e) in mitochondria and lysosomes of HeLa cells; comparison of calculated Pearson's correlation coefficients for AIE-M (c) or AIE-M-Cu (f) in mitochondria and lysosomes of HeLa.