# Electronic Supplementary Information

# Trichromatic-emission and dual-ratio semiconducting polymer dots as fluorescent probes for simultaneous quantification of $Cu^{2+}$ and pH in vitro and in vivo

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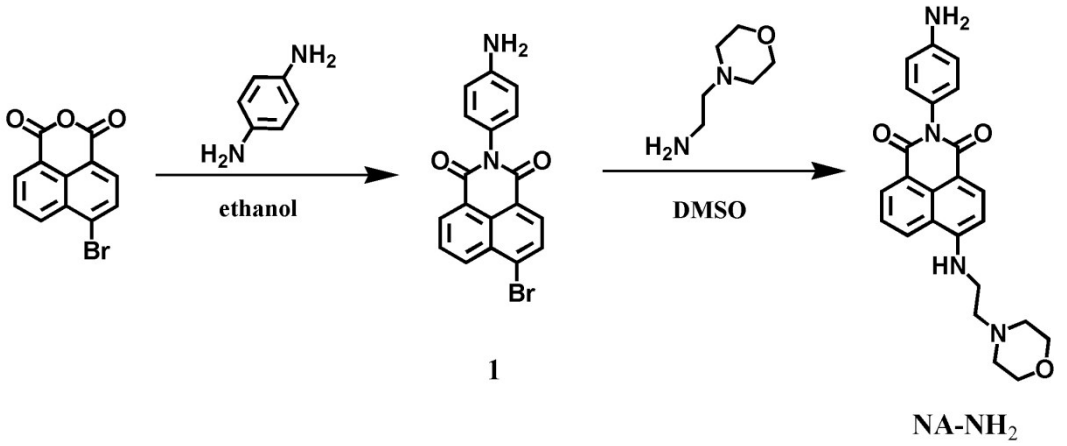
## Experimental Procedures

### 1. Instrumental and reagents.

Poly(9,9-diotylfluorene) (PFO, Mw=89000, polydispersity=2.3) was purchased from ADS Dyes, Inc. Tetraphenylporphyrin (TPP) and 4-(2-Aminoethyl) morpholine were purchased from TCI (Shanghai) Development Co., Ltd. Poly(styrene-co-maleic anhydride) (PSMA), dimethyl sulf-oxide (DMSO, anhydrous), tetrahydrofuran (THF, anhydrous) and 2-(4-(2-hydroxyethyl)-1-piperazinyl) ethanesulfonic acid buffer (HEPES) were purchased from Sigma-Aldrich. All other Reagents were purchased from commercial sources and used without further purification. The zebrafishes were acquired from Shanghai FishBio Co., Ltd.

$^{1}H$ NMR and $^{13}C$ NMR spectra were recorded on a BRUKER ASCEndtm400 MHz instrument. HRMS (high resolution mass spectrometry) spectra were obtained with an Agilent 6200 instrument. Transmission electron microscopic (TEM) images were obtained with a Hitachi HT 7700 TEM instrument. Fluorescence and UV absorption spectra were obtained with a PerkinElmer LS-55 fluorescence spectrophotometer and Hitachi U-3900 spectrophotometer, respectively. The confocal fluorescence images were obtained with a confocal laser scanning microscope (TCS SP8, Leica, Germany).

### 2. Preparation of TE probe.




**Scheme S1.** Synthesis of NA-$NH_2$

### 2.1 Synthesis of compound 1.

Compound 1 was synthesized according to the corresponding literature method.[1] Specifically, 4-bromo-1,8-naphthalic anhydride (1.1 g , 4 mmol) and 1,4-Diaminobenzene (0.648 g, 6 mmol) were added into 30 mL ethanol and then refluxed for 8 h. After cooling to room temperature, the precipitated solid was filtered and washed with ethanol and dried under a vacuum to afford an gray solid of compound 1 (1.24 g, 85%).

**2.2 Synthesis of NA-$NH_2$.**

Compound 1 (2- (4-Aminophenyl) -6-bromo-1H-benzo[df] isoquinoline-1, 3(2H) -dione) (1 mmol, 367 mg) and 4-(2-aminoethyl) morpholine (4 mmol, 0.54 mL) were dissolved in 10 mL of dry dimethyl sulfoxide. The mixture was stirred at 90 °C for 12 h. After completion of the reaction and cooling to room temperature, the resulting solution was quickly poured into 100 mL ice water, and a yellow precipitated solid was filtered to give as crude product. The crude product was further purified by silica gel column chromatography using eluent ($CH_2Cl_2$/ MeOH, v/ v = 100/ 8) to afford a yellow solid of NA-$NH_2$ (337 mg, 81%). $^1$H NMR (400 MHz, DMSO-d6): δ 8.68 (d, J = 8.8 Hz, 1H), 8.42 (d, J = 7.2 Hz, 1H), 8.25 (d, J = 8.4 Hz, 1H), 7.71 (t, J = 16.0 Hz, 1H), 7.65 (t, J = 11.6 Hz, 1H), 6.88 (d, J = 2.0 Hz, 1H), 6.86 (d, J = 2.0 Hz, 1H), 6.83 (d, J = 8.4 Hz, 1H), 6.64 (d, J = 2.0 Hz, 1H), 6.62 (d, J = 1.6 Hz, 1H), 5.21 (s, 2H), 3.61 (t, J = 8.8 Hz, 4H), 3.54 (m, 2H), 2.68 (t, J = 12.4 Hz, 2H). $^{13}$C NMR (100 MHz, DMSO): δ 164.76, 163.97, 150.88, 148.71, 134.65, 137.19, 130.22, 129.73, 128.87, 124.84, 123.02, 120.68, 114.14, 108.79, 104.37, 66.68, 56.65, 53.84. HRMS $[M+H]^+$ : m/z Calcd 417.1921, Found 417.1925.

**2.3 Synthesis of NA-PSMA.**

The NA-PSMA was synthesized from NA-$NH_2$ and PSMA polymer by carrying out an amidation reaction according to the previous reports.[2] In a detail procedure, PSMA polymer (9 mg, 0.0053 mmol) and NA-$NH_2$ (6 mg, 0.0144 mmol) were dissolved in 15.0 mL of anhydrous THF. The mixture was stirred at 65 °C for 48 h under nitrogen atmosphere. After completion of the reaction, the solvent was removed by rotary evaporation, and a yellow powders of NA-PSMA were afford, which was used for the next step without further purification.

**2.4 Preparation of Pdots.**

The trichromatic-emission Pdots were prepared using a modified nano-precipitation method. Typically, PFO, NA-PSMA polymer and TPP were dissolved into THF with a concentration of 1 mg $mL^{-1}$, respectively. Then 50 μL of PFO solution, 100 μL of NA-PSMA solution and 10 to 50 μL TPP solution were added into 2.0 mL THF, and the mixture was sonicated for 5 min to form a homogenous solution. The resulting solution was quickly injected into10 mL of distilled water in a bath sonicator and sonicated for 5 min. The THF was rotary evaporated under reduced pressure, and the resulting solution was filtered through a 0.22 μm membrane filter. The obtained trichromatic-emission Pdots colloidal solution was dispersed and fixed to 10 mL of deionized water for further use.

The NA@Pdots was prepared from PFO and NA-PSMA with a mass ratio of 1:2 through the same procedures for TE Pdots preparation. The prepared NA@Pdots was used only for co-localization experiments.

The experiments about the optimization of the TE Pdots composition was performed in pH 5.0 buffer solution since pH 5.0 is the physiological pH value of the lysosome. For optimizing the proportion of the three components, the amount of PFO and NA-PSMA are fixed, while the content of TPP is variable.

**3. Procedures for the simultaneous pH and $Cu^{2+}$ sensing.**

pH sensing: Typically, 160 μL of trichromatic-emission Pdots solution (20 μg $mL^{-1}$) was added into different pH of 10 mM HEPES in 2 mL Centrifuge tubes. The mixture of 800 μL was incubated for 5 minutes at 37 °C, after incubation, the fluorescence spectrum was recorded at an excitation wavelength of 365 nm.

$Cu^{2+}$ sensing: Typically, 800 μL of mixture of TE pdots (4 μg $mL^{-1}$) and $Cu^{2+}$ at different amounts was incubated at 37 °C for 5 min in 10 mM HEPES buffer (pH= 5.0). After incubation, the fluorescence spectrum was recorded at an excitation wavelength of 365 nm.

Simultaneous pH and $Cu^{2+}$ sensing: Typically, 800 μL of mixture of TE pdots (4 μg $mL^{-1}$) and $Cu^{2+}$ at different amounts (0-16 μM) was incubated at 37 °C for 5 min in various pH values HEPES buffer, respectively. After incubation, the fluorescence spectrum was recorded at an excitation wavelength of 365 nm.

**4. Cell imaging of pH and $Cu^{2+}$.**

Lysosomal pH imaging：HeLa cells were incubated with TE Pdots (2.0 μg $mL^{-1}$) for 1 h, and then chloroquine (100 μM) was added, after incubated for another 30 min.

For imaging various pH values in lysosomes，the cells were incubated with TE Pdots (2.0 μg $mL^{-1}$) for 1 h, then cells were rinsed with HEPES buffer (10 mM, pH= 7.2-7.4) three times. The treated cells were incubated another 30 min in different pH HEPES buffer containing 10 μM nigericin and 5.0 μM monensin.

Simultaneous imaging of pH and $Cu^{2+}$: For simultaneous fluorescence imaging experiment of pH and $Cu^{2+}$ in lysosomes, we conducted four sets of experiments: For the control experiments, HeLa cells were pre-treated with 50 μM $Cu^{2+}$ for 30 min, and then 100 μM chloroquine was added for changing lysosomal pH, respectively. The fluorescence images of TE Pdots were collected 410-460 nm (blue channel), 500-550 nm (green channel) and 630-680 nm (red channel) upon excitation with 405nm.

**5. Zebrafish culture and imaging of pH and $Cu^{2+}$.**

Zebrafish Culture: 4-day-old zebrafishes were used for bio-imaging in vivo. The zebrafish embryos were incubated in E3 embryo media (15 mM NaCl, 0.5 mM KCl, 1 mM $MgSO_4$ , 1 mM $CaCl_2$ , 0.15 mM $KH_2PO_4$ , 0.05 mM $Na_2HPO_4$, 0.7 mM $NaHCO_3$, $10^{-5}$% methylene blue, pH 7.5) at 28 °C for four days. Then the 4-day-old zebrafishes were used for subsequent imaging experiments.

Simultaneous Zebrafish imaging of pH and $Cu^{2+}$: Typically, 4-day-old zebrafishes were pre-treated with 50 μM $Cu^{2+}$ for 30 min in E3 embryo media, then the zebrafishes were washed three time to remove $Cu^{2+}$ of vitro. The pre-treated zebrafishes and control were incubated with TE Pdots (2.0 μg $mL^{-1}$) in E3 embryo media at 28 °C for 1 h. Finally, the washed zebrafishes were transferred into 10 mM pH 4.0, 5.0 and 6.0 HEPES buffer solution for fluorescence imaging, respectively. The fluorescence images of TE Pdots were collected 410-460 nm (blue channel), 500-550 nm (green channel) and 630-680 nm (red channel) upon excitation with 405 nm.

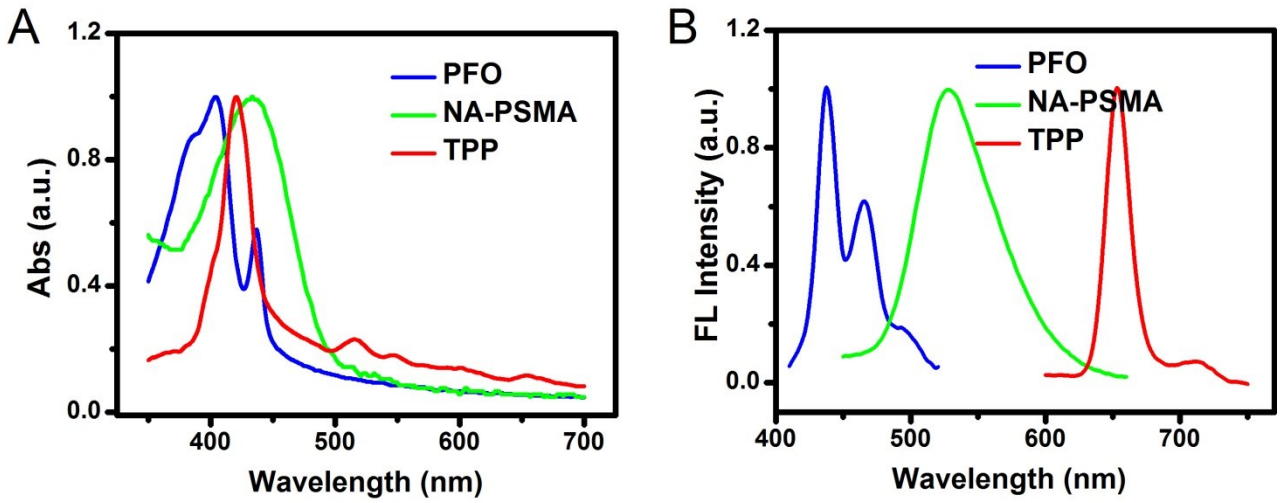



**Fig. S1** UV-vis absorption spectra (A) and fluorescence spectra (B) of PFO (blue line), NA-PSMA (green line) and TPP (red line).

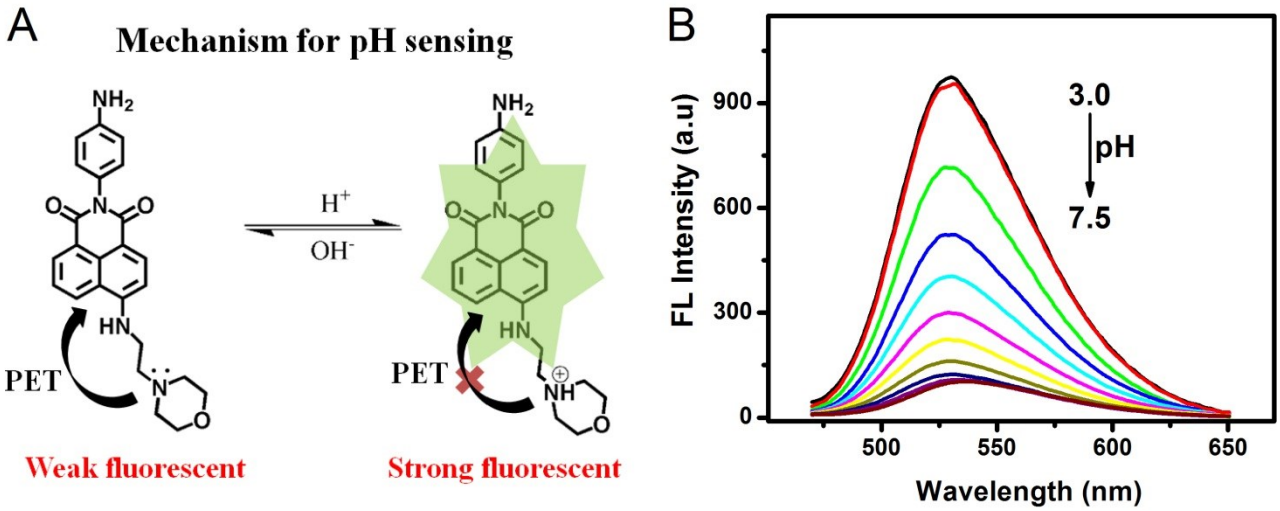



**Fig. S2** (A) Schematic mechanism of NA-$NH_2$ for pH sensing. (B) Fluorescence responses of NA-$NH_2$ to various pH values.

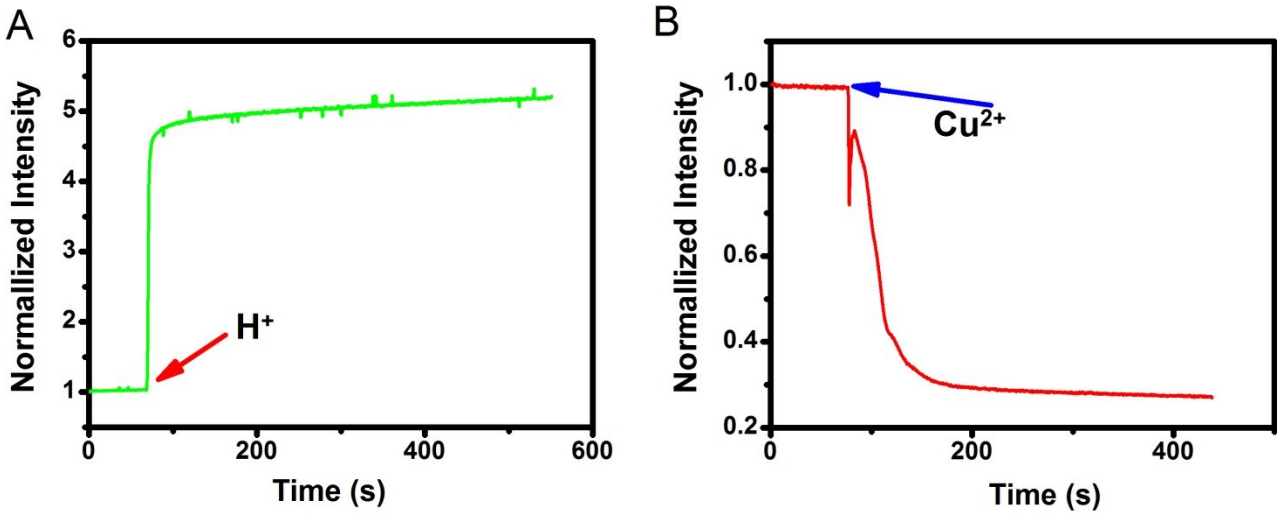



**Fig. S3** Time-dependent fluorescence of TE Pdots (4 μg mL$^{-1}$) recorded at 530 nm for pH (A) and 655 nm $Cu^{2+}$ (B) sensing, respectively.

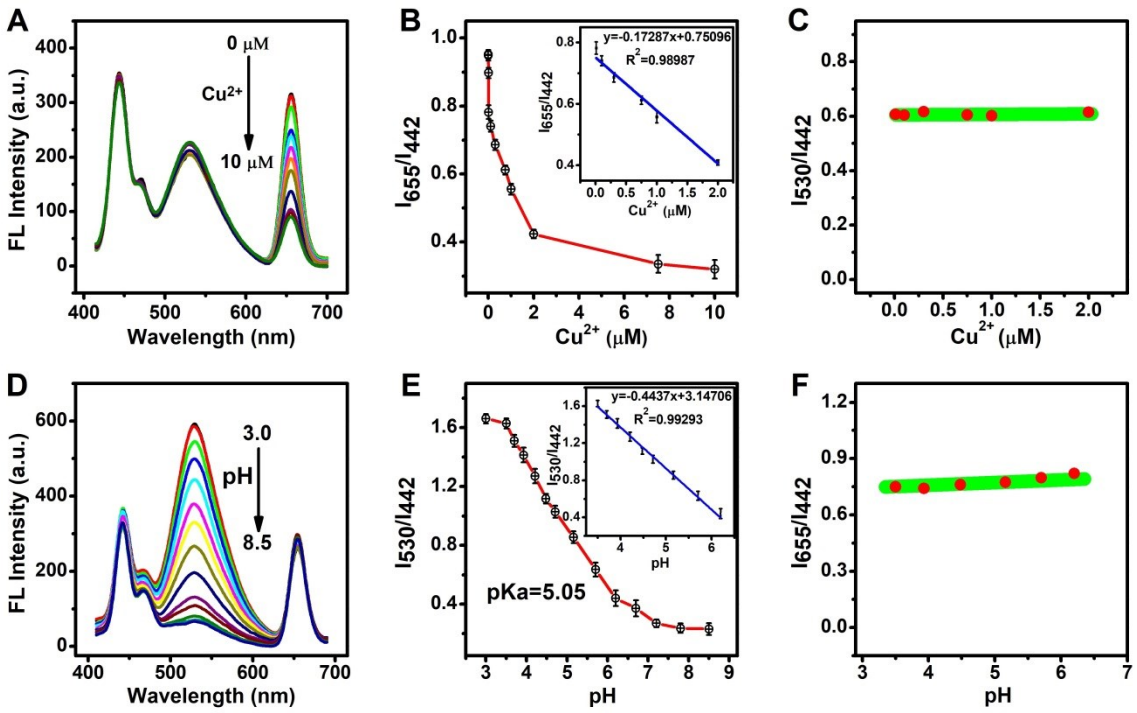



**Fig. S4** The sensing systems using the TE Pdots for $Cu^{2+}$ and pH detection in vitro. (A) Fluorescence spectra of Pdots in pH 5.0, 10 mM HEPES buffer with various $Cu^{2+}$ concentrations. (B) Calibration curve of $I_{655}/I_{442}$ against various $Cu^{2+}$ concentrations. (C) The ratiometric signals of $I_{530}/I_{442}$ against different $Cu^{2+}$ concentrations in pH 5.0, 10 mM HEPES buffer. (D) Fluorescence spectra of TE Pdots in 10 mM HEPES buffer with various pH values. (E) Calibration curve of $I_{530}/I_{442}$ ratios against various pH values. (F) The ratiometric signals of $I_{655}/I_{442}$ against different pH values. The used concentration of TE Pdots is 4 μg mL$^{-1}$.

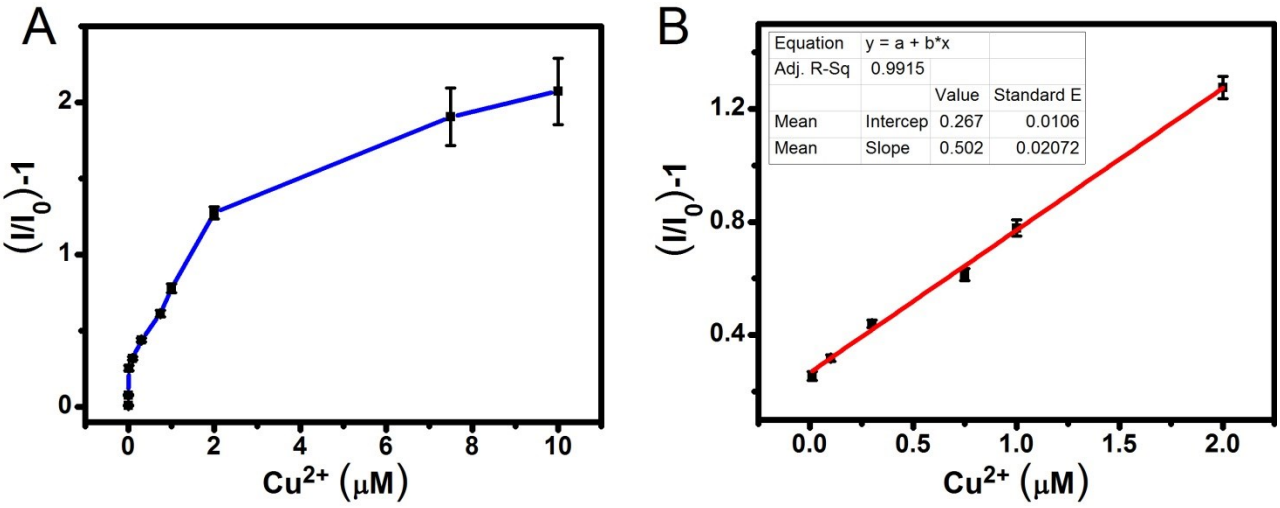



**Fig. S5** The plots (A) and linear responses (B) of $I_0/I$ ratios against $Cu^{2+}$ concentrations. Herein $I_0$ and $I$ are the fluorescence intensity at 655 nm in the absence and presence of $Cu^{2+}$, respectively.

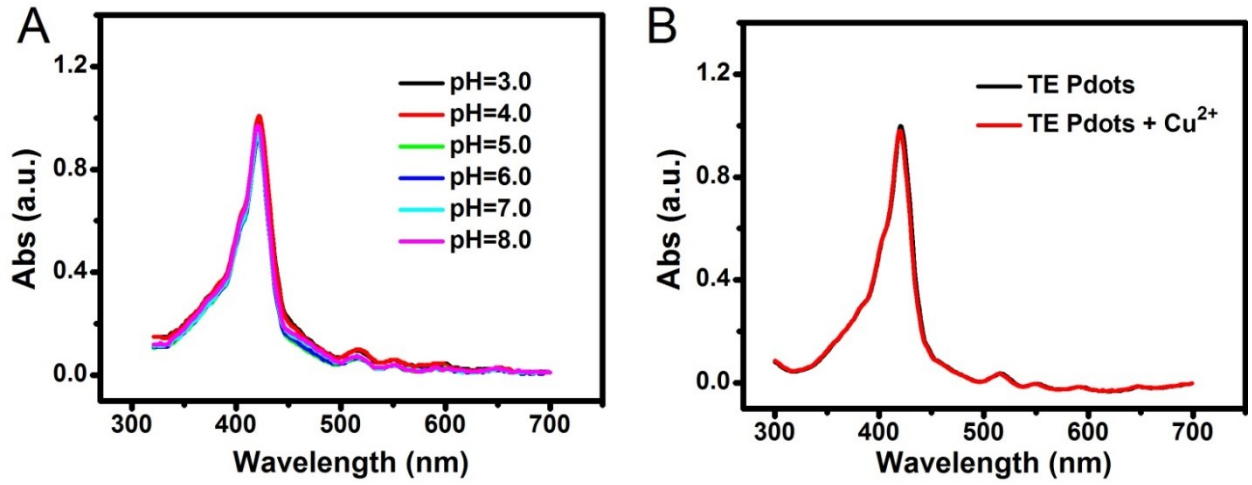



**Fig. S6** UV-vis absorption spectrum of 4 μg $mL^{-1}$ TE Pdots with various pH values (A) and 20 μM $Cu^{2+}$ (B).

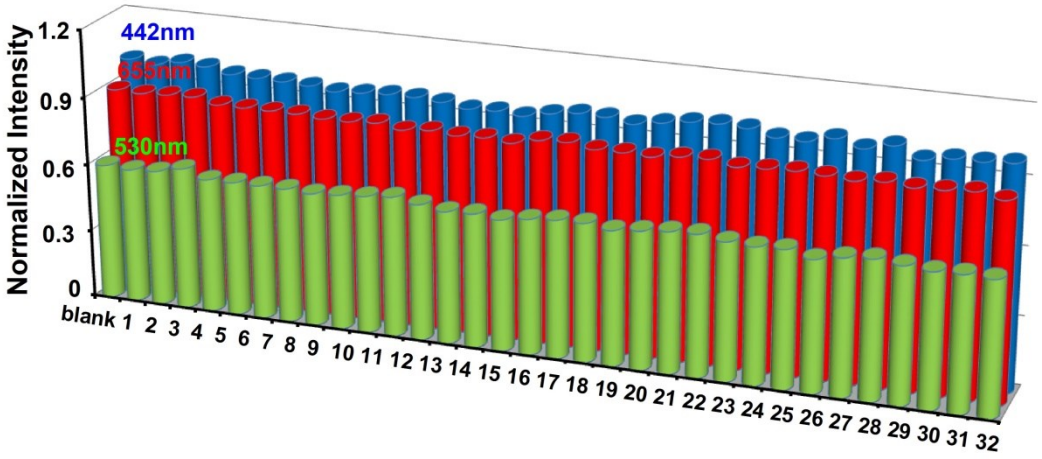



**Fig. S7** Fluorescence emission of 4 μg $mL^{-1}$ TE Pdots at 442nm (blue column), 530nm (green column) and 655nm (red column) in the presence of various potential interfering species, respectively. 1. $Na^+$ (1mM); 2. $K^+$ (1mM); 3. $Mg^{2+}$ (100μM); 4. $Zn^{2+}$ (100μM); 5 $Ca^{2+}$ (100μM); 6. $Mn^{2+}$ (100μM); 7. $Co^{2+}$ (100μM); 8. $Ba^{2+}$ (100μM); 9. $Fe^{3+}$ (50μM); 10. $Fe^{2+}$ (50μM); 11. Glucose (1mM); 12. Sucrose (1mM); 13. AA (1mM); 14. GSH (1mM); 15. Cys (1mM); 16. Hcy (1mM); 17. L-Arginine (100μM); 18. L-Tryptophan (100μM); 19. L-Aspartic acid (100μM); 20. L-Proline (100μM); 21. L-Asparagine (100μM); 22. L-Hydroxyproline (100μM); 23. DL-Threonine (100μM); 24. L-Aspartic acid (100μM); 25. DL-Phenylalanine (100μM); 26. $Na_2S$ (100μM); 27. $NaHSO_3$ (100μM); 28. $H_2O_2$ (100μM); 29. NaClO (100μM); 30. $NaNO_2$ (100μM); 31. $ONOO^-$ (100μM); 32. $Cu^+$ (50μM).

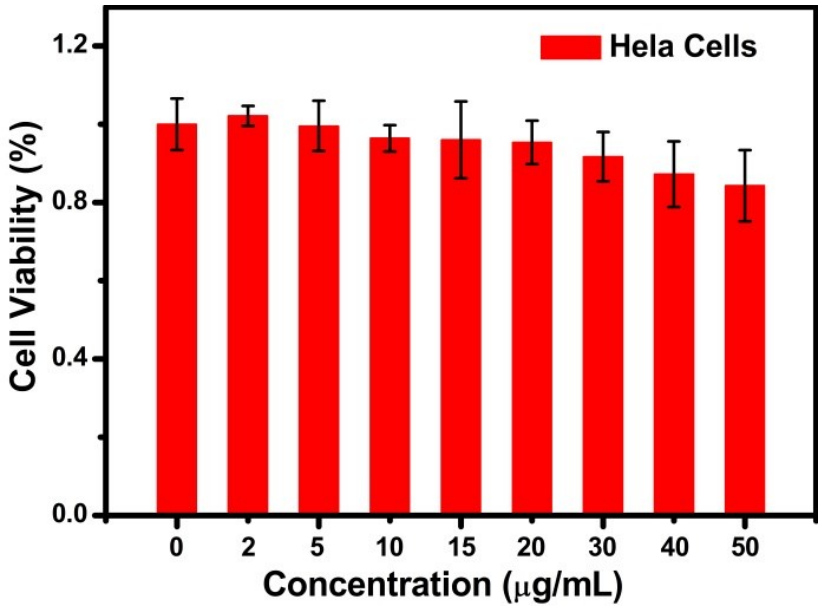



**Fig. S8** The viability of HeLa cells treated by TE Pdots with various concentrations for 24 h.

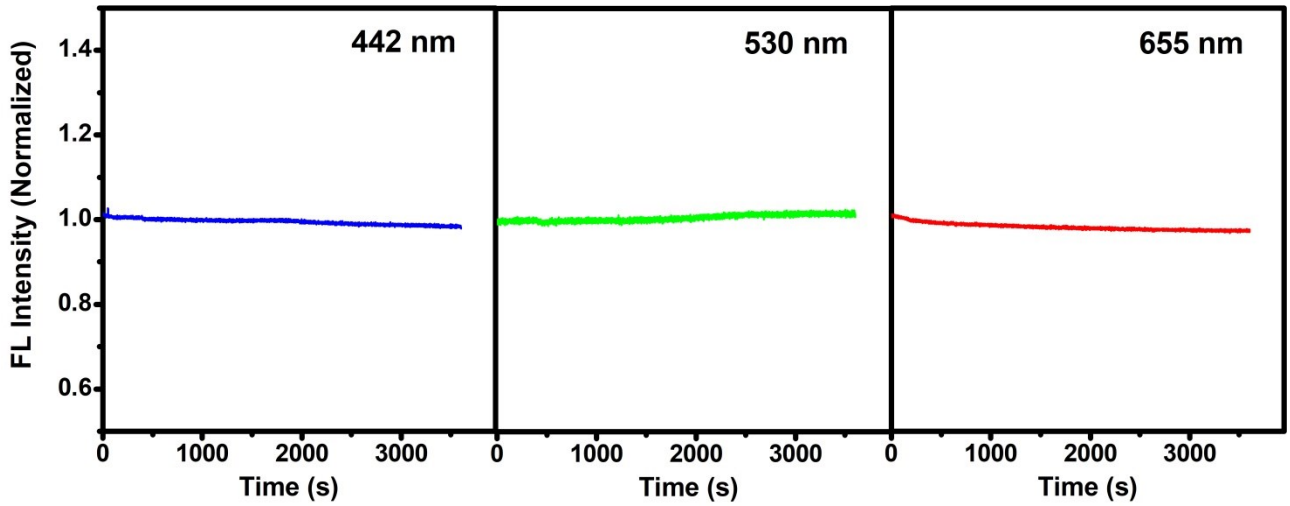



**Fig. S9** The fluorescence intensity of TE Pdots at 442 (left side, blue line), 530 (middle side, green line) and 655 (right side, red line) nm with the excitation wavelength of 405 nm for continuous excitation 1 h. voltage of xenon lamp: 700V, slit: 15 × 15, TE Pdots: 4 μg $mL^{-1}$.

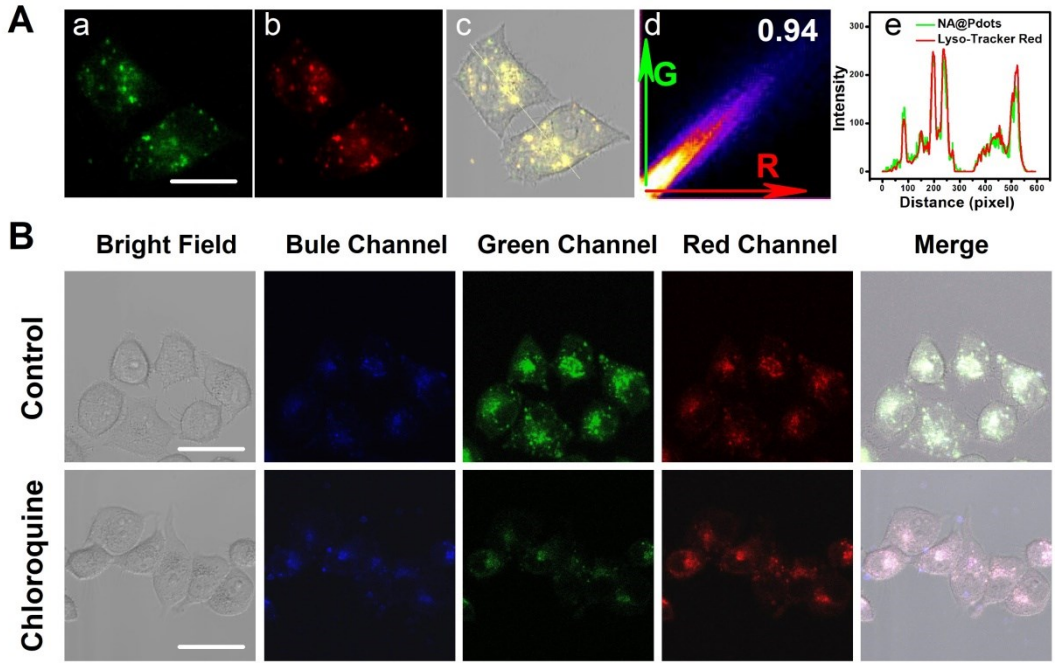



**Fig. S10** (A) Colocalization images of NA@Pdots in green channel (a), Lyso-Tracker Red in red channel (b), merging a, b and bright field (c) in Hela cells, colocalization scatter plot of NA@Pdots and Lyso-Tracker Red (d), and intensity profile of the yellow line in the green and red channel (e). Ex = 488 nm, scale bar =20 μm. (B) Fluorescence images of HeLa cells incubated with TE Pdots and then treated with (below) or without (upper) 100 μM chloroquine. Ex = 405 nm; scale bar = 50 μm. The concentration of Pdots used in imaging experiments is 2.0 μg $mL^{-1}$.

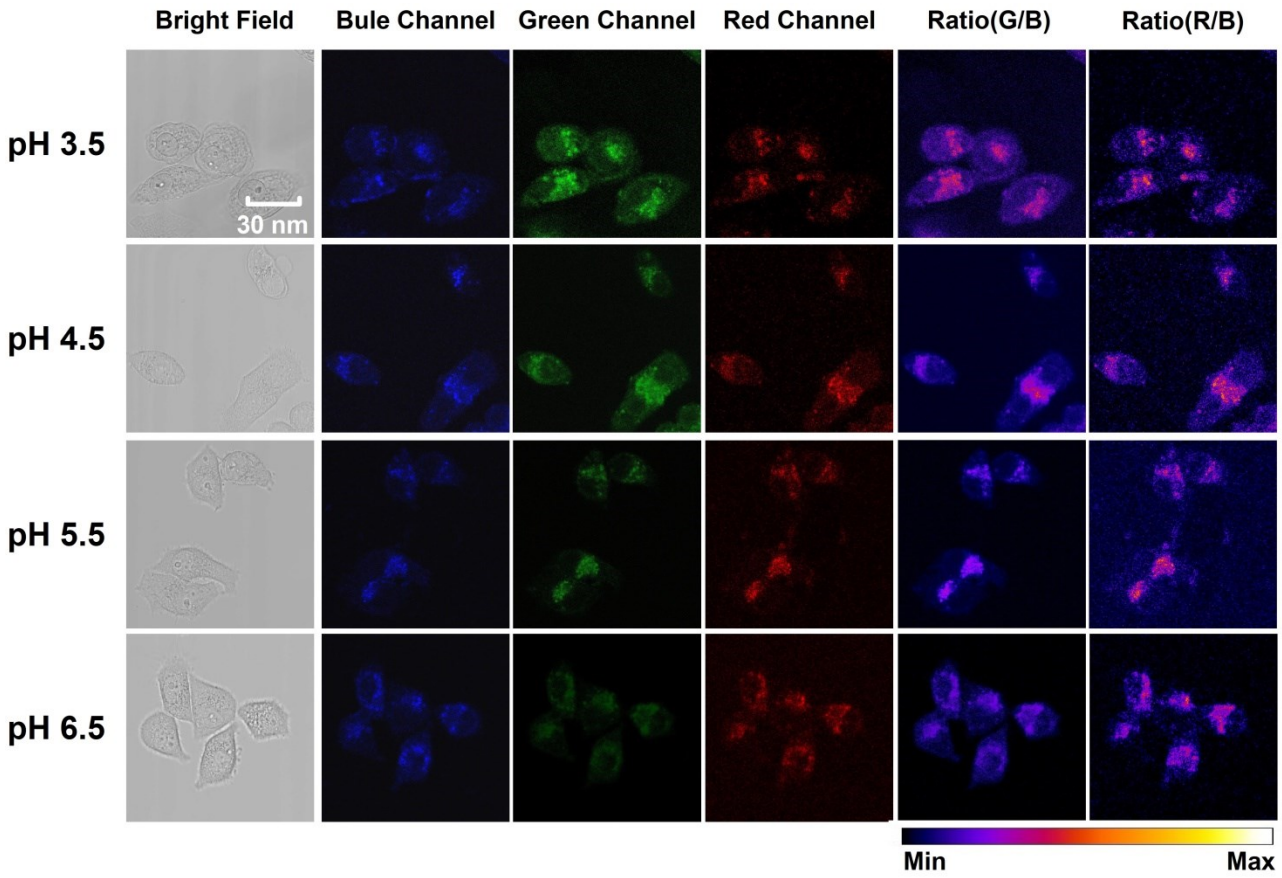



**Fig. S11** Fluorescence images for TE Pdots (2 μg $mL^{-1}$) in Hela cells at different pH (pH=3.5, 4.5, 5.5, 6.5. 10 mM HEPES buffers): blue channel (PFO) = 410-460 nm, green channel (NA) =500-550 nm, red channel (TPP) = 630-680 nm, Ex=405nm. Ratio (G/B) images and Ratio (R/B) images represent the ratios of green channel and blue channel, red channel and blue channel, respectively. Scale bar =30 μm.

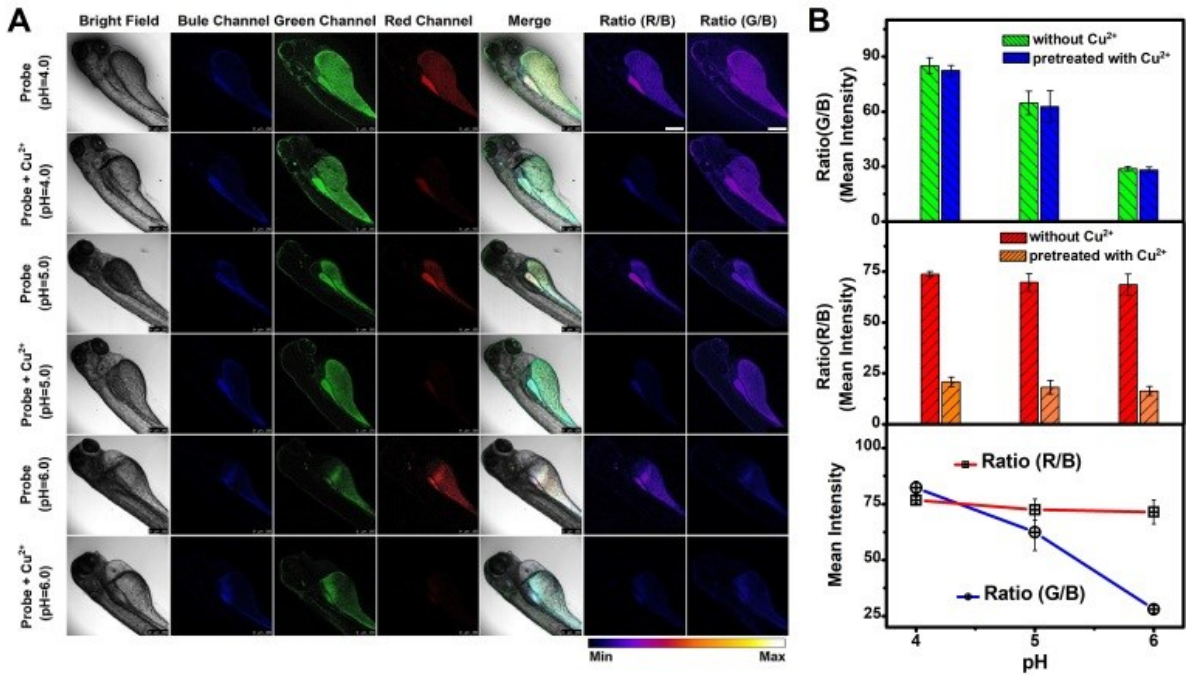



**Fig. S12** (A) Simultaneous visual monitoring the variations of $Cu^{2+}$ and pH (pH = 4.0, 5.0, 6.0) in 4-day-old zebrafishes. (B) Quantification of the relative mean fluorescence levels of zebrafishes from the images of R/B, G/B ratios. The concentration of TE Pdots and $Cu^{2+}$ used in vivo experiments is 2.0 μg $mL^{-1}$ and 50 μM, respectively. Ex = 405 nm. Scale bar = 250 μm

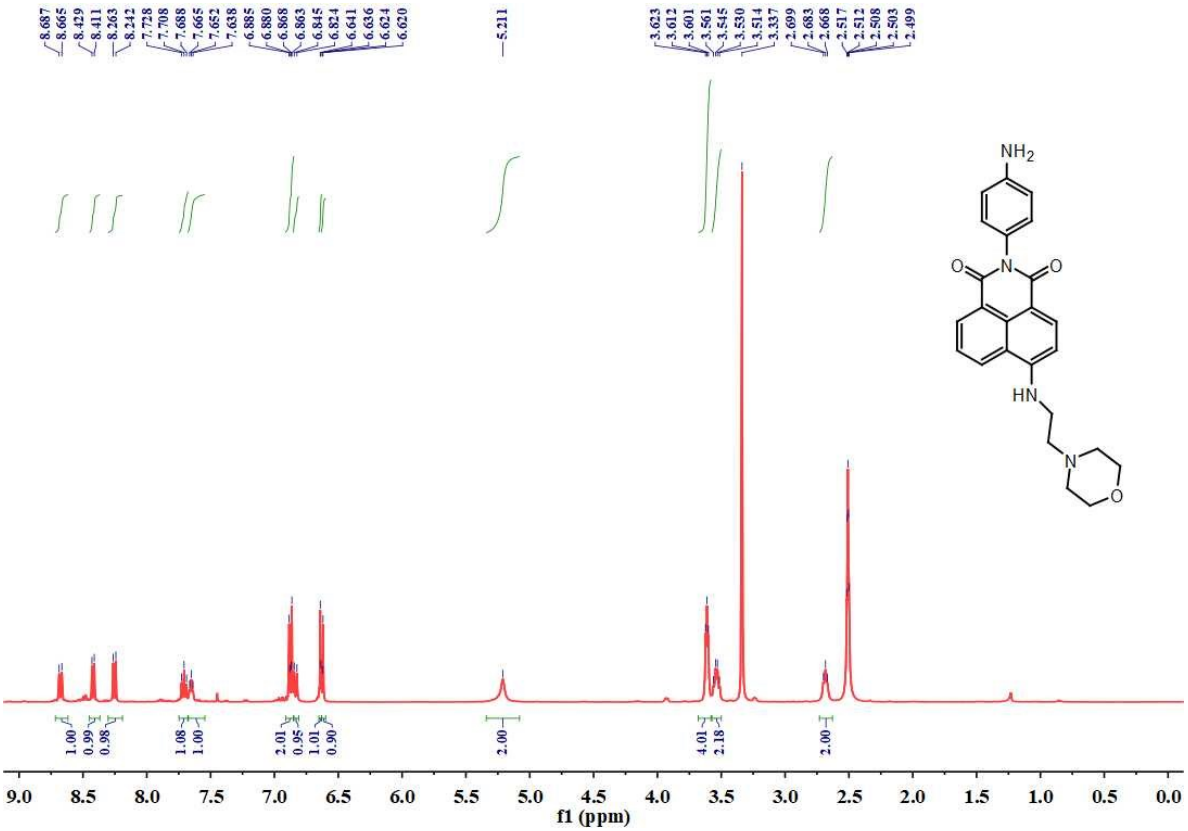



**Fig. S13.** $^{1}$H NMR of NA-$NH_2$

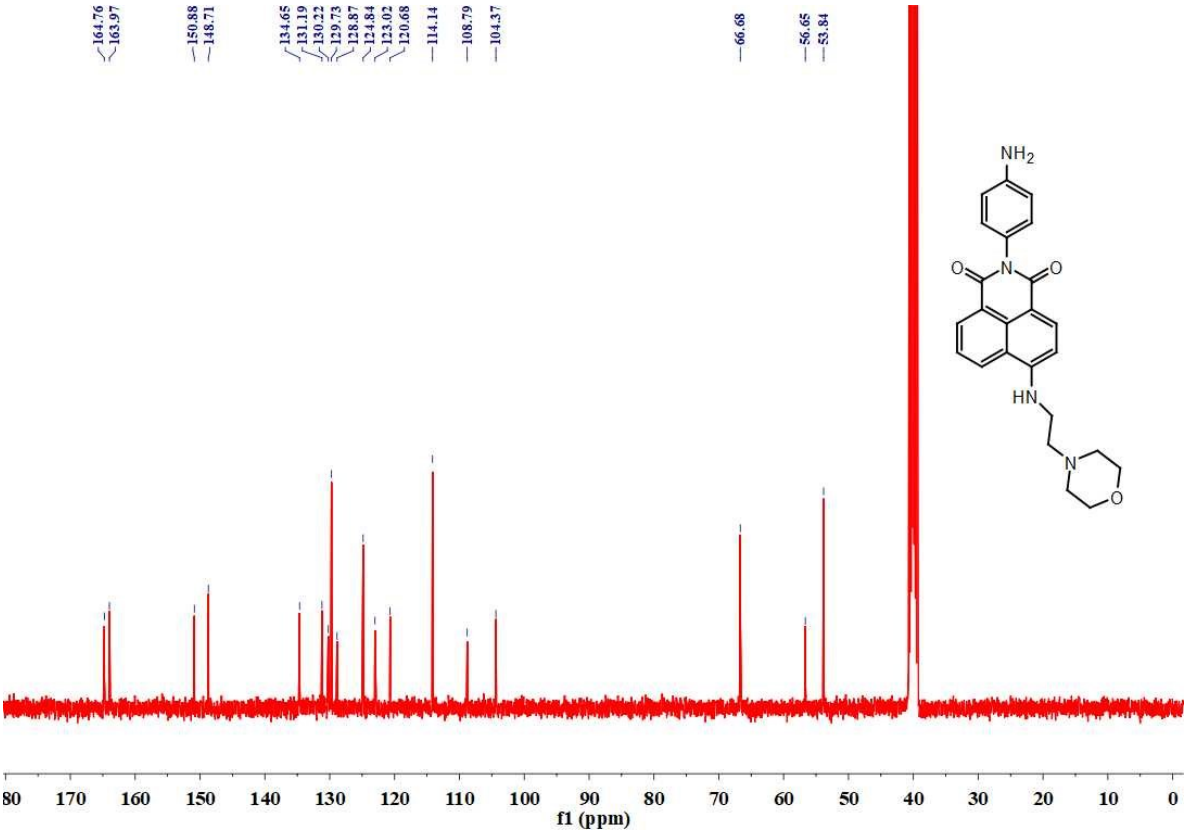



**Fig. S14.** $^{1}$C NMR of NA-$NH_2$

**Supplementary References**

(1) P. Zhang, J. Li, B. Li, J. Xu, F. Zeng, J. Lv and S. Wu, *Chem. Commun.* 2015, **51**, 4414–4416.

(2) Q. Li, J. Zhang, W. Sun, J. Yu, C. Wu, W. Qin and D. T. Chiu, *Langmuir* 2014, **30**, 8607−8614.