Supplementary Information

for

A Zn^{2+} fluorescent sensor derived from 2-(pyridin-2-yl)benzoimidazole with ratiometric sensing potential

Zhipeng Liu[a], Changli Zhang[a], Yunling Li[a], Fang Qian[a], Zhengyi Wu[a], Xiaoliang Yang[a], Weijiang He*,[a] Xiang Gao[b] and Zijian Guo*,[a]

[a] *State Key Laboratory of Coordination Chemistry, Coordination Chemistry Institute, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, P. R. China*

[b] *Animal Model Research Center, Nanjing University, Nanjing 210093, P. R. China*

Email: zguo@nju.edu.cn, heweij69@nju.edu.cn

***Contents*:**

1. Materials and general methods

2. Synthesis of PBITA and its zinc complex Zn^{2+}/PBITA

3. Absorption and emission spectra

4. Determination of the dissociation constant of Zn^{2+}/PBITA complex

5. Zinc titration of PBITA by ^1H NMR spectroscopy

6. Determination of quantum yields

7. Fluorescence of PBITA at different pH in DMSO–H_2O

8. Theoretical modeling

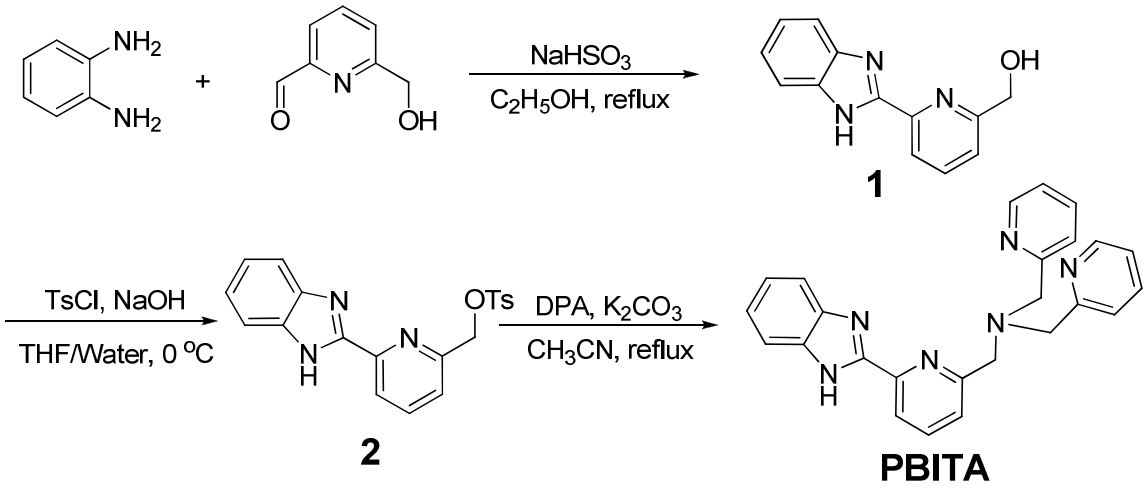
9. Cell culture methods and confocal imaging

10. References

1. Materials and general methods

All the solvents were of analytic grade. The stock solutions of metal ions for fluorescence discrimination were prepared from $MnCl_2$, $PbCl_2$, $CoCl_2 \cdot 6H_2O$, Zn $(NO_3)_2 \cdot 7H_2O$, $CaCl_2$, NaCl, $CuSO_4$, $NiCl_2 \cdot 6H_2O$, KCl, $CdCl_2 \cdot 2.5H_2O$, $HgCl_2$, $MgCl_2 \cdot 6H_2O$ using doubly distilled water. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DRX-300 spectrometer with TMS as internal standard in $CDCl_3$. Mass spectrometric data were determined with a LCQ□ESI-MS□Therso Finnigan□ mass spectrometer. Fluorescence measurements were performed on an AMINCO Bowman series 2 with 3 nm slit for both excitation and emission. Absorption spectra were measured on a Shimadzu UV-3100 or an UV-VIS-NIR spectrophotometer. All pH measurements were determined by a Model PHS-3C meter.

2. Synthesis of PBITA and its zinc complex Zn^{2+}/PBITA



Scheme 1

Synthesis of compound 1

The ethanol solution (50 mL) containing 6-(hydroxymethyl) pyridine-2-carbaldehyde[1] (1.0 M) was added slowly at room temperature to the mixture of *o*-phenylenediamine (3.92 g, 20 mmol) and $NaHSO_3$ in ethanol (200 mL). The resulting mixture was refluxed with stirring for 6 h. Then, the mixture was cooled to room temperature. After the removal of solvent *in vacuo*, the residue was dissolved in CH_2Cl_2 and washed with brine. The organic layer was dried over anhydrous $MgSO_4$ and evaporated under reduced pressure after filtering off $MgSO_4$. The crude product was purified by column chromatography (silica gel, EtOAc: hexane = 5: 2, R_f □ 0.4), and pure compound **1** was

obtained as a white solid in 64% yield. M.p. 253-254 ^{o}C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.88 (s, 2H, -CH_2), 7.30 (m, 4H, Ar-H and Py-H), 7.67 (m, 2H, Py-H), 7.81(t, 1H, *J* = 7.8, Py-H), 8.34 (d, 1H, *J* = 7.8, Py-H). ESI-MS (*m/z*): Calcd. 248.24, found: 248.03 for $[M+ Na]^+$.

Synthesis of PBITA

Compound **1** (0.190 g, 0.137 mmol) and 0.5 mL aqueous solution containing NaOH (0.6 g, 0.15 mmol) were mixed in THF (10 mL) when cooled with an ice-water bath. Then, 5 mL THF solution containing 0.153 g TsCl (0.617 mmol) was added to the mixture by a syringe at 0 ^{o}C. After stirring for 1 h at this temperature, the mixture was washed with brine and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure and the residue as compound **2** was used for the preparation of PBITA without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.31 (s, 3H, -CH_3), 5.16 (s, 2H, -CH_2), 7.27-7.35 (m, 5H, Ar-H and Py-H), 7.65-7.80 (m, 5H, Ar-H and Py-H), 8.34 (d, 1H, *J* = 7.8, Py-H).

Compound **2** (103 mg, 0.27 mmol), 48.9 mg BPA (0.25 mmol) and 40 mg K_2CO_3 (0.29 mmol) were mixed in 10 ml anhydrous CH_3CN and refluxed for 5 h under a flow of dry N_2 gas.[2] After the reaction mixture was cooled to room temperature, the solvent was removed under reduced pressure. Then, the residue was dissolved in CH_2Cl_2 and washed with brine. The organic layer was dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, EtOAc: MeOH = 50: 1, R_f □ 0.35) to give pure PBITA as a white solid in 46 % yield. M.p. 73-74 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.90 (s, 2H, -CH_2), 3.99 (s, 4H, -CH_2), 7.20 (t, 2H, *J* = 6.9, Py-H), 7.29-7.31 (m, 4H, Ar-H and Py-H), 7.59-7.78 (m, 8H, Ar-H and Py-H), 8.27 (d, 1H, *J* = 8.1, PBI-H), 8.63 (d, 1H, *J* = 5.7, Py-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 57.32, 59.88, 119.55, 122.25, 122.48, 122.95, 123.11, 123.68, 123.96, 136.61, 136.77, 137.58, 147.90, 149.02, 151.92, 156.79, 159.35. ESI-MS (*m/z*): Calcd. 407.20, found: 407.25 for $[M+H]^+$. Element analysis (%) Calcd. for $C_{25}H_{22}N_6$: C, 73.87; H, 5.56; N, 20.67. Found: C, 73.79; H, 5.45; N, 20.72.

Synthesis of Zn^{2+}/PBITA complex

$ZnCl_2 \cdot H_2O$ (7.3 mg, 0.048 mmol) dissolved in MeOH (1 mL) was added to 2 mL methanol dissolved with 20 mg PBITA (0.048 mmol) and stirred at room temperature for 2 h. Then, ether

was added to the mixture. The resulting precipitate was filtered and washed with a small volume of Et_2O, and 19 mg dry solid was obtained as the Zn^{2+}/PBITA complex. Yield, 85%. ESI-MS (*m/z*): Calcd. 469.11, found: 469.25 for $[M+Zn-H]^+$; Calcd. 235.05, found: 235.17 for $[M+Zn]^{2+}$.

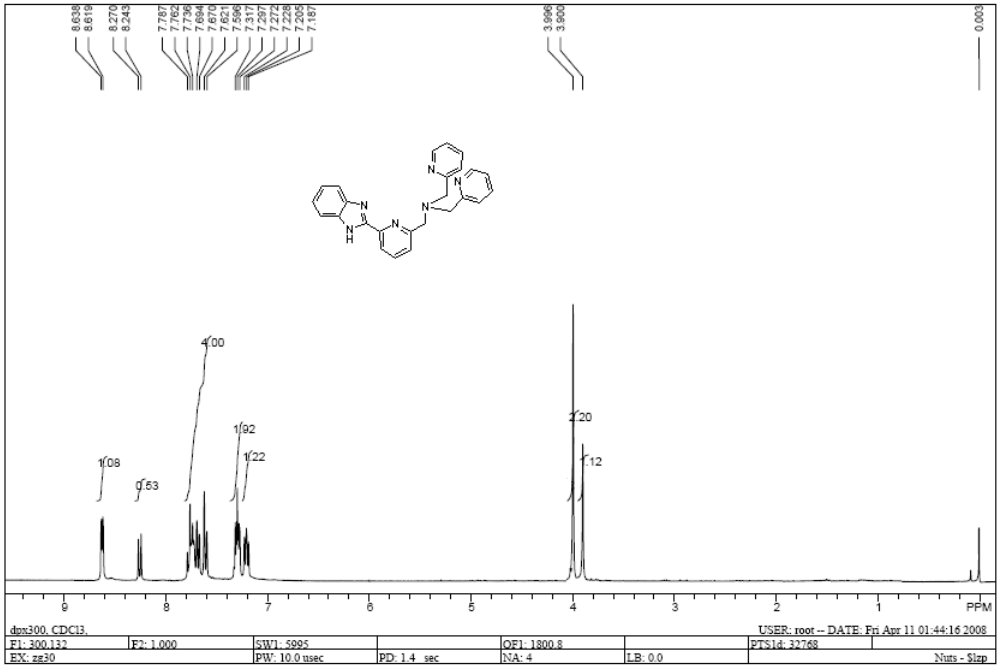


Figure S1 The ^1H NMR spectrum of PBITA in $CDCl_3$.

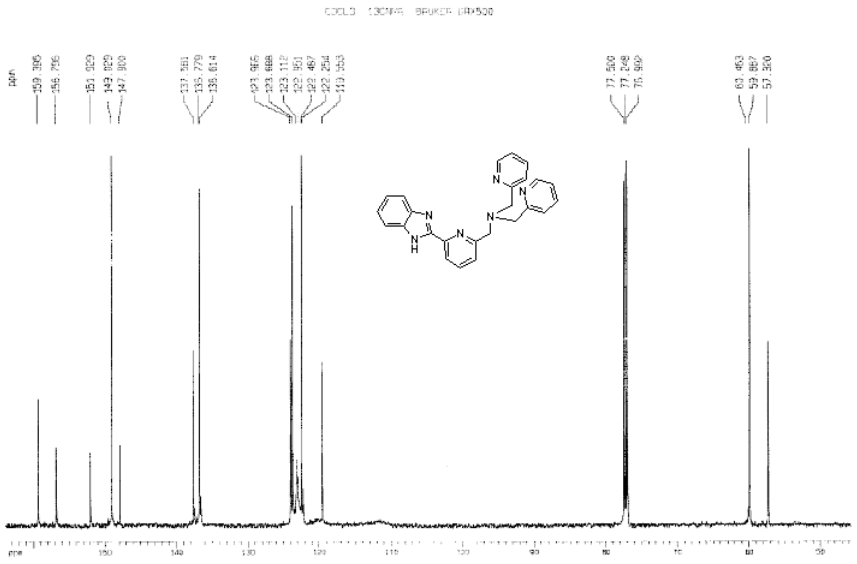


Figure S2 The ^{13}C NMR spectrum of PBITA in $CDCl_3$.

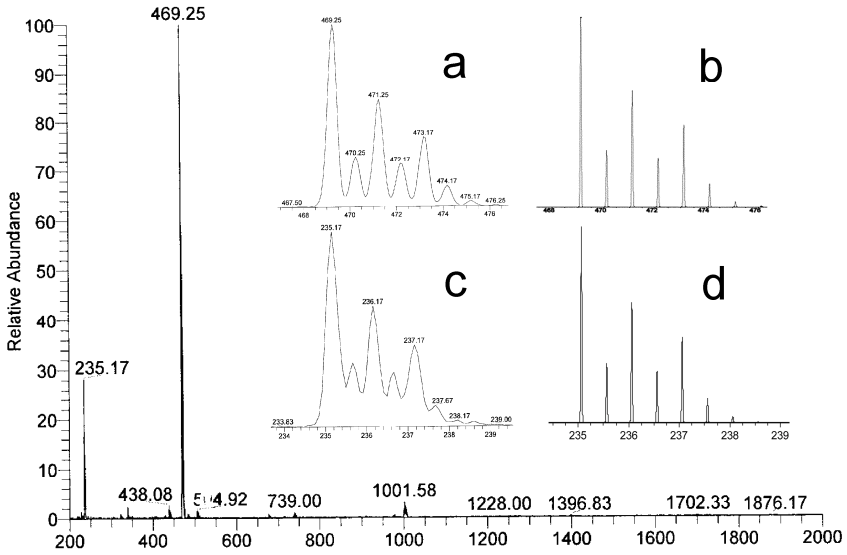


Figure S3 The MS spectrum of Zn^{2+}/PBITA complex. The determined isotopic distribution patterns of the peaks with *m*/*z* of 469.25 and 235.17 are shown as insets a and c. The corresponding simulated ones for $[M+Zn-H]^+$ and $[M+Zn]^{2+}$ are shown as insets b and d.

3. Absorption and emission spectra of PBITA

The PBITA stock solution (1×10^{-4} M) was prepared by directly dissolving the sensor in DMSO. For the spectroscopic determination, the stock solution was diluted with HEPES buffer to the desired concentration (1×10^{-5} M). For Zn^{2+} titration, aliquots of 1 μl aqueous Zn^{2+} solution Zn $(NO_3)_2$ **(**1×10^{-3} M) were added to 3 mL diluted PBITA solution. The measurements were carried out in 1 min. after the addition. All experiments were carried out at 298 K.

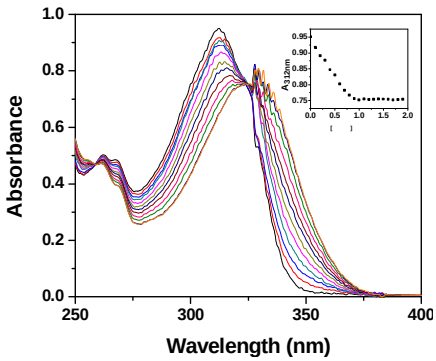


Figure S4. Absorption spectra of PBITA (1×10^{-5} M) in HEPES solution (DMSO/water = 1 : 9, v/v, pH = 7.2) when titrated with Zn^{2+}.

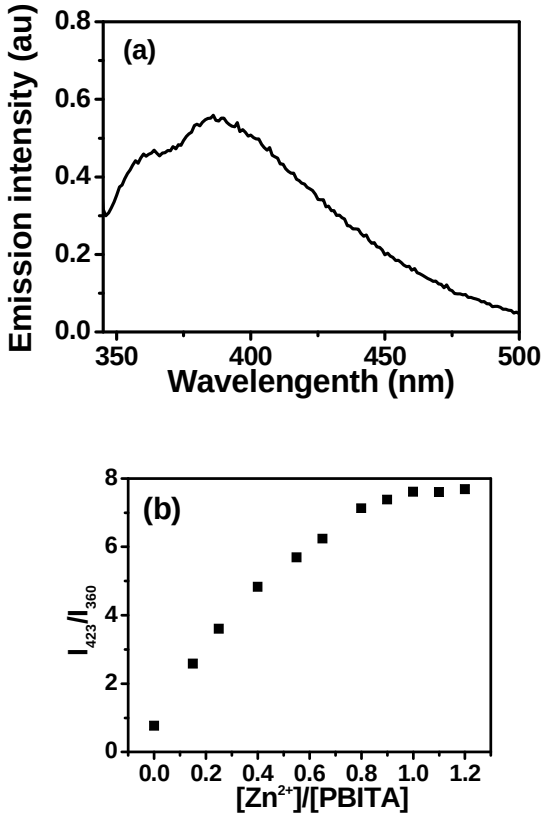


Figure S5. (a) Emission spectrum of PBITA (1×10^{-5} M) in HEPES solution (DMSO/water = 1 : 9, v/v, pH = 7.2) When excited at 336 nm. (b) The Zn^{2+} titration profile of PBITA (1×10^{-5} M) in HEPES solution (DMSO/water = 1 : 9, v/v, pH = 7.2) according the I_{423}/I_{360}, when excited at 336 nm.

4. Determination of the dissociation constant of Zn^{2+}/PBITA complex.

The HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) buffer (50 mM, pH 7.20, 0.1 M KNO_3) containing $Zn(NO_3)_2$ (0 ~ 9 mM) and 10 mM of EGTA (ethylenebis(oxyethylenenitrilo)tetraacetic acid) were prepared, and the concentration of free Zn^{2+} was calculated using reported method (Eq. 1).[3]

$$K'_{\text{Zn-EGTA}} = K_{(\text{ZnL})}(1+10^{(\text{pKLMH-pH})}/((1+10^{(\text{pH-pKZn})})(1+10^{(\text{p}K_1\text{-pH})}+10^{(\text{p}K_1+\text{p}K_2\text{-2pH})})) \quad \text{(Eq. 1)}$$

at pH = 7.20. Thus for EGTA, pK_1 = 9.40, pK_2 = 8.79, pK_3 =2.70, pK_3 =2.70, pK_{LMH} = 9.40, log $K_{(\text{ZnL})}$ = 12.6 (25 °C, μ = 0.1 M).[4] The protonation constants were corrected upward by 0.11 when working in 0.1 M ionic strength. Thus, $K'_{\text{Zn-EGTA}}$ value at pH 7.20, 0.1 M ionic strength is $3.80 \times 10^8\ M^{-1}$. The calculated $[Zn^{2+}]_{\text{free}}$ concentration of each solution is:

$[Zn^{2+}]_{\text{total}}$	(mM)	0.03	0.05	0.1	0.5	0.8	1	2	3	5	6	7	8	9
$[Zn^{2+}]_{\text{free}}$	(nM)	0.008	0.013	0.026	0.14	0.23	0.29	0.66	1.1	2.6	4	6.1	11	24

Stock solutions of PBITA in DMSO (1×10^{-4} M) were diluted to the same final concentration of 1

$\times 10^{-5}$ M with 50 mM HEPES buffer (pH 7.2, 0.1M KNO_3) containing 10 mM EGTA and 0 - 9 mM $Zn(NO_3)_2$. The fluorescence ratio F_{423}/F_{360} of each solution was measured, and fitted to the following equation (eq 2[5]) which gave a K_d of 7.9×10^{-12} M.

$$F = (F_{min} K_d + F_{max} [Zn^{2+}])/(K_d + [Zn^{2+}]) \text{ (Eq. 2)}$$

In the above equation, F is the fluorescence intensity, F_{max} is the maximum fluorescence intensity, F_{min} is the fluorescence intensity with no addition of Zn^{2+}, and $[Zn^{2+}]_{free}$ is the free Zn^{2+} concentration.

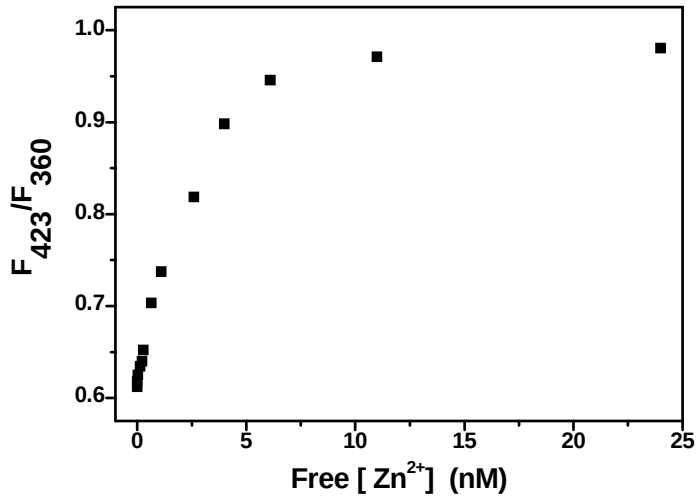


Figure S6. Fluorescence ratio of PBITA (1×10^{-5} M) as a function of the concentration of free Zn^{2+} in 50 mM HEPES buffer (pH 7.2, 0.1 M KNO_3) with 10 mM EGTA and 0 - 9 mM Zn^{2+}. All data were expressed as the fluorescence ratio (423 nm/360 nm). These data were fitted according to ref. 9a to give the K_d constant.

5. Zinc titration of PBITA by ^{1}H NMR spectroscopy

The ^{1}H NMR titrations were carried out by adding aliquots of 1 μl solutions of $Zn(NO_3)_2\cdot7H_2O$ in CD_3OD (9.0×10^{-2} M) to the solutions of PBITA in CD_3OD (2.7×10^{-2} M). The spectra were determined after 1 min. of shaking upon each addition. The experiments were carried out at 298 K.

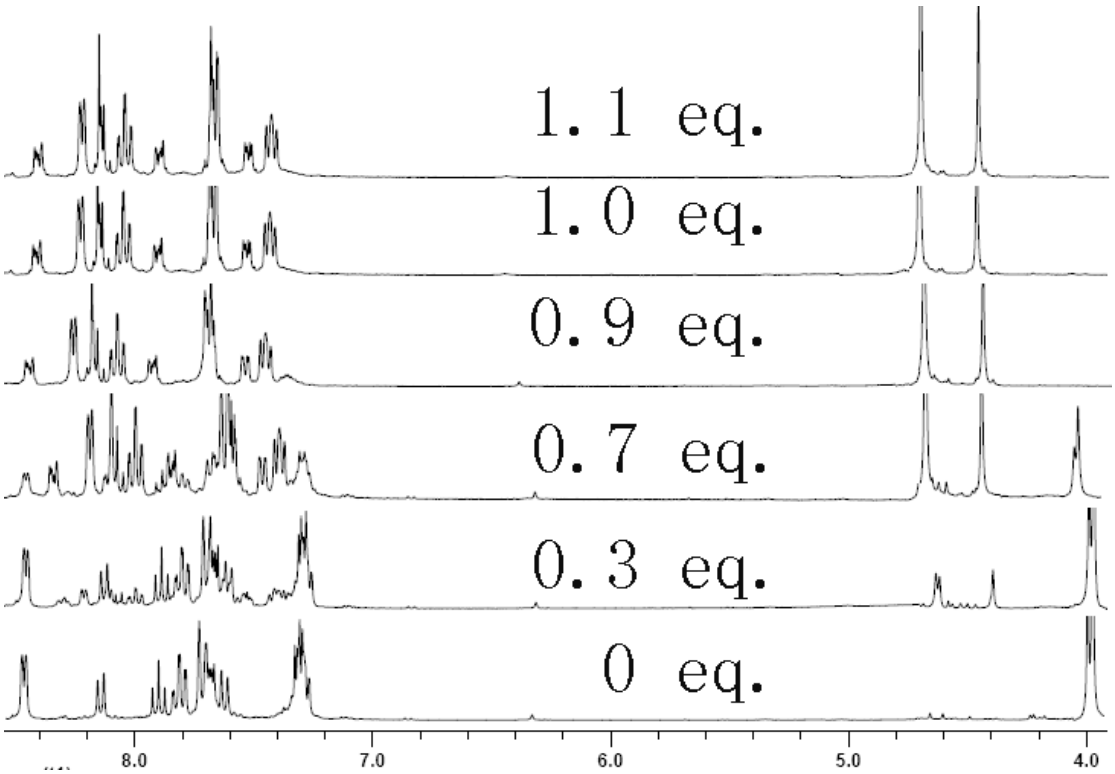


Figure S7. ^{1}H NMR spectra of PBITA (initial c = 2.7 × 10^{-2} M) in CD_3OD upon Zn^{2+} titration (c = 9.0 × 10^{-2} M in CD_3OD). (**a**) PBITA; (**b**) PBITA + 0.3 eq. Zn^{2+}; (**c**) PBITA + 0.7 eq. Zn^{2+}; (**d**) PBITA + 0.9 eq. Zn^{2+}; (**e**) PBITA + 1.0 eq. Zn^{2+}; **(f)** PBITA + 1.1 eq. Zn^{2+}.

6. Determination of quantum yields

Fluorescence quantum yield of PBITA and Zn^{2+}/PBITA complex were determined in aqueous solutions (100 mM KNO_3, 50 mM HEPES, pH = 7.2, DMSO/water = 1:9, v/v) by using quinine sulfate solution (Φ_f = 0.546, 0.5 M H_2SO_4) as references. The quantum yields were calculated using Eq.3:

$$\Phi_u = [(A_s F_u n^2)/ (A_u F_s n_0^2)]\Phi_s. \quad \text{(Eq.3)}$$

Where A_s and A_u are the absorbance of the reference and sample solution at the reference excitation wavelength, F_s and F_u are the corresponding integrated fluorescence intensity, and n and n_0 are the refractive indexes of the solvents of the sample and the reference, respectively. Absorbance of samples and references at their respective excitation wavelengths was controlled to be lower than 0.05.

7. Fluorescence of PBITA at different pH in DMSO–H_2O

Stock solutions of PBITA in DMSO (1×10^{-4} M) were diluted to a final concentration of 1×10^{-5} M with water. These solutions were adjusted to the suitable pH by KOH and HNO_3 solutions. The

experiments were carried out at 298 K.

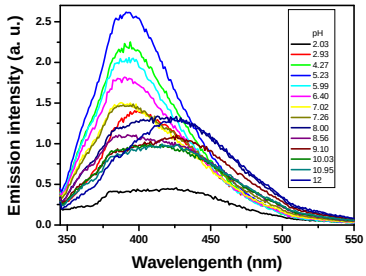


Figure S8. Fluorescence emission spectra of PBITA (1×10^{-5} M) in aqueous (9 : 1, water/DMSO, v/v) solutions of different pH. λ_{ex}, 336 nm.

8. Theoretical modeling

The geometry optimizations of PBITA and its zinc complex were performed in vacuum using the hybrid density functional Becke-3-Lee-Yang-Parr (B3LYP) potential in conjuration with a 6-31G(d. p) basis set for H, C, N, O and Zn atoms, as implemented in GAUSSIAN 03 software package.[6] This level is often estimated to be adequate for the geometry optimization of aromatic compounds with metal interactions.

9. Cell culture methods and confocal imaging

HeLa cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10 % fetal bovine serum, penicillin (100 units/ml), streptomycin (100 mg/ml) and 5% CO_2 at 37 °C. After removing the incubation media and rinse with 1× PBS for three times, the cells were stained by PBITA by incubating the cells in 10 μM PBITA solution for 20 min at room temperature. Then the cells was washed three times with PBS and imaged with a Leica TCS S2 microscope equipped with a 63 × oil-immersion objective. For the imaging of HeLa cells with exogenous Zn^{2+}, the exogenous Zn^{2+} was introduced by incubating the cells with 5μM $ZnSO_4$/2-mercaptopyridine-*N*-oxide solution (Prepared by diluting 5 mM $ZnSO_4$/2-mercaptopyridine-*N*-oxide stock solution with 1× PBS). Then the cells were dyed with PBITA solution in a similar procedure described above and imaged. After the imaging, the cells of exogenous Zn^{2+} were further treated with 50 μM TPEN solution (prepared by diluting the TPEN stock solution with 1× PBS) to scavenge the intracellular Zn^{2+}. Then the cells were rinsed with 1× PBS and imaged. For all imaging, the samples were excited at 356 nm, and the band pass is 410 – 450 nm.

10. References

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