Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information

for

In vitro and in vivo imaging application of a 1,8-naphthalimide-derived Zn^{2+} fluorescent sensor with nuclear envelope penetrability

Changli Zhang,[a,b] Zhipeng Liu,[c] Yunling Li,[a] Weijiang He,*[,a] Xiang Gao,[d] 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] *School of Biochemical and Environmental Engineering, Nanjing Xiaozhuang College, Nanjing 211171, P. R. China;* [c] *College of Chemistry and Chemical Engineering, Liaocheng University, Liaocheng 252059, P. R. China;* [d] *Animal Model Research Center, Nanjing University, Nanjing 210093, PR China*

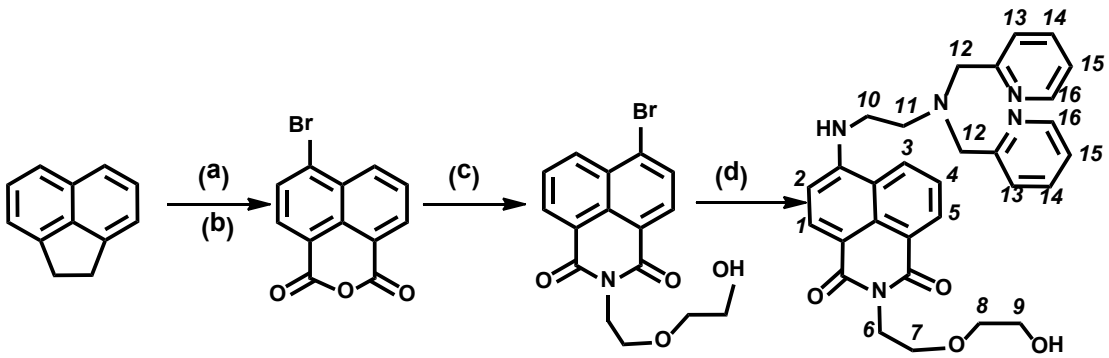
Email: heweij69@nju.edu.cn and zguo@nju.edu.cn.

1. Materials and Methods

Reagents and solvents for synthesis were of analytic grade. ^{1}H-NMR and ^{13}C-NMR were recorded on a Bruker DRX-500 spectrometer with TMS as standard. Mass spectra were measured on an LCQ electrospray mass spectrometer (ESMS, Finnigan). Melting points are uncorrected. All pH values were determined with a Sartorius pH-Meter PB-10. The solutions of different metal ions were prepared by dissolving NaCl, $CaCl_2$, KCl, $MgCl_2{\cdot}6H_2O$, $FeSO_4{\cdot}7H_2O$, $Zn(NO_3)_2{\cdot}6H_2O$, $NiCl_2{\cdot}6H_2O$, $CuCl_2$, $CdCl_2$, $MnCl_2{\cdot}4H_2O$, $CoCl_2{\cdot}6H_2O$, $AgNO_3$ and $Pb(NO_3)_2$ in double distilled water (all salts are of analytical grade). For the spectroscopic study, all solvents are of spectrum grade, and water is the double distilled water.

2. Sensor Synthesis

Scheme. S1. Synthesis of **Naph-BPEA**



Naph-BPEA

(**a**) NBS, DMF, rt, 2h; 77%. (**b**) $Na_2Cr_2O_7$, AcOH, reflux, 3h, NaOH, 50-55 °C, HCl; 36%. (**c**) $NH_2CH_2CH_2OCH_2CH_2OH$, EtOH, reflux, 2h; 84%. (**d**) methoxylethanol, BPEA, Et_3N, reflux, N_2 atmosphere, 5d; 37 %

Synthesis of 5-bromoacenaphthene[i]

A solution of N-bromosuccinimide (18.00 g, 0.1 mol) in DMF (50 mL) was added to a DMF suspension (50 mL) containing acenaphthene (15.40 g, 0.1 mol) with stirring at room temperature. After being stirred at room temperature for 2 h, then the solution was poured into cold water. The crude product (22.68 g) was obtained via filtration, and 17.78 g of pure 5-bromoacenaphthene were obtained via recrystallization from ethanol. Yield, 77 %. Mp: 51-52°C.

Synthesis of 4-bromo-1,8-naphthalic anhydride[ii]

Compound 5-bromoacenaphthene (17.78 g) was stirred with the mixture of glacial acetic acid (310 ml) and sodium dichromate (53.12 g) under reflux for 3 h. Then the dark green solution was diluted with cold water (65 mL). After cooled to the room temperature, yellow solid was obtained via filtration. Then the solid was stirred in 4% NaOH solution (300 mL) at 50 - 55 °C. After removing the solid via filtration, the filtrate was neutralised with 5 % hydrochloric acid and the white precipitate was formed. The solid obtained via filtration was then purified via recrystallization in conc. nitric acid, and 7.72 g white anhydride was obtained after desiccation. Yield, 36 %. Mp: 218 - 220 °C. ^{1}H-NMR (500 MHz, DMSO-d_6): δ (ppm) 8.66 (d, J = 8.65 Hz, 1 H), 8.63 (d, J = 7.35 Hz, 1 H), 8.39 (d, J = 7.8 Hz, 1 H), 8.29 (d, J = 7.85 Hz, 1 H), 8.06 (t, J = 7.85 Hz, 1 H).

Synthesis of 6-bromo-2-[2-(2-hydroxy-ethoxy)-ethyl]-benzo[de]isoquinoline-1,3-dione[iii]

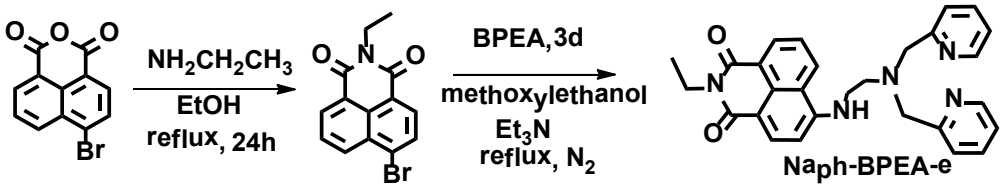
The condensation of 4-bromo-1,8-naphthalic anhydride (4.89 g, 17.6 mmol) and aminoethoxylethanol (1.86 g, 17.6 mmol) was carried out in the refluxing ethanol (98 ml) for 2 h with stirring. After being cooled to room temperature, the mixture was filtered to afford the yellow solid. The solid was purified by recrystallization in ethanol and 5.4 g pure product was obtained after filtration. Yield, 84 %. Mp: 133-134°C. ^{1}H-NMR (500 MHz, DMSO-d_6): δ (ppm) 8.57 (d, J = 7.5 Hz, 1 H), 8.54 (d, J = 8.5 Hz, 1 H), 8.33 (d, J = 8.0 Hz, 1 H), 8.22 (d, J = 7.5 Hz, 1 H), 8.00 (t, J = 8.0 Hz, 1 H), 4.23 (t, J = 6.5 Hz, 2 H), 3.66 (t, J = 6.5 Hz, 2 H), 3.47 (t, J = 4.5 Hz, 4 H), 2.0 (s, br, 1 H).

Synthesis of **Naph-BPEA**

BPEA (1.00 g, 4.16 mmol), 6-bromo-2-[2-(2-hydroxy-ethoxy)-ethyl]-benzo[*de*]iso quinoline-1,3-dione (1.86 g, 4.98 mmol), and triethylamine (0.42 mg, 4.16 mmol) were mixed and refluxed in methoxylethanol (30 ml) in nitrogen atmosphere for 5 days. Then the solvent was removed under reduced pressure. The crude product was purified by silica gel chromatography ($CHCl_3$/CH_3OH, 25:1, v/v) to afford 0.80 g **Naph-BPEA** as a yellow solid. Yield, 37 %. Mp: 157-159°C. ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 8.86 (d, J = 8.0 Hz, 1 H), 8.66 (d, J = 7.0 Hz, 1 H), 8.59 (d, J = 3.5 Hz, 2 H), 8.44 (d, J = 8.5 Hz, 1 H), 7.94 (s, 1 H), 7.71 (t, J = 8.0 Hz, 1 H), 7.58 (t, J = 7.5 Hz, 2 H), 7.42 (d, J = 7.5 Hz, 2 H), 7.17 (t, J = 6.0 Hz, 2 H), 6.56 (d, J = 8.5 Hz, 1 H), 4.46 (t, J = 6.0 Hz,

2 H), 4.06 (s, 4 H), 3.89 (t, J = 5.5 Hz, 2 H), 3.72 (t, J = 4.5 Hz, 4 H), 3.44 (t, J = 5.2 Hz, 2 H), 3.12 (t, J = 5.2 Hz, 2 H), 1.78 (s, br, 1 H). ^{13}C-NMR (500 MHz, $CDCl_3$): δ (ppm) 39.19, 40.94, 50.96, 54.03, 59.69, 61.88, 68.74, 72.25, 103.99, 108.69, 120.74, 122.42, 122.56, 123.35, 124.34, 127.93, 130.15, 131.32, 135.17, 136.74, 149.22, 149.35, 150.67, 158.71, 164.52, 165.30. ESI-MS (positive mode, m/z): 526.3 for $[M+H]^+$. Elemental analysis: Calcd. for $C_{30}H_{31}N_5O_4$: C, 68.55; H, 5.94; N, 13.32%. Found: C, 68.29; H, 6.25; N, 13.17%.

Scheme S2. Synthesis of **Naph-BPEA-e**.



Synthesis of 6-bromo-2-ethyl-1H-benzo[de]isoquinoline-1,3(2H)-dione[iii]

Compound 4-bromo-1,8-naphthalic anhydride (9.95 g, 35.0 mmol) was mixed with 3.0 mL ethylamine in 290 mL anhydrous ethanol with stirring. The mixture was refluxed with stirring for 1 day, and yellow precipitate was formed. After filtration and desiccation, the product (7.86 g) was obtained as yellow solids. Yield, 72 %. ^{1}H-NMR (500 MHz, DMSO-d_6): δ (ppm) 8.60(d, J = 7.5 Hz, 1H), 8.58(d, J = 8.6 Hz, 1H), 8.37(d, J = 7.85 Hz, 1H), 8.25(d, J = 7.7 Hz, 1H), 8.02(t, J = 7.75 Hz, 1H), 4.08(q, J = 7.1 Hz, 2H), 1.23(t, J = 7.0 Hz, 3H).

Synthesis of Naph-BPEA-e

A similar procedure for **Naph-BPEA** was adopted to synthesize **Naph-BPEA-e**. BPEA (1.00 g, 4.16 mmol), 6-bromo-2-ethyl-1H-benzo[de]isoquinoline-1,3(2H)-dione (1.52 g, 5.00 mmol), and triethylamine (0.42 mg, 4.16 mmol) were mixed and refluxed in methoxylethanol (30 mL) in N_2 atmosphere for 3 days. After removing the solvent in vacuo, the residue was then purified by silica gel chromatography ($CHCl_3$/CH_3OH, 90:1, v/v) to afford 0.72 g crude product. Then the product was purified further with thin layer chromatography ($CHCl_3$/CH_3OH, 50:1, v/v). Yield, 11 %. ^{1}H NMR(500 MHz, $CDCl_3$): δ (ppm) 8.82(d, J = 8.25 Hz, 1H), 8.64(d, J = 7.2 Hz, 1H), 8.59 (d, J = 4.15 Hz, 1H), 8.43(d, J = 8.35 Hz, 1H), 7.86(s, 1H), 7.72(d, J = 7.7 Hz,

1H), 7.57(t, J = 6.4 Hz, 2H), 7.39(d, J = 7.75 Hz, 2H), 7.16(t, J = 5.4 Hz, 2H), 6.56(d, J = 8.4 Hz, 1H), 4.24(q, J = 6.95 Hz, 2H), 4.01(s, 4H), 3.40(t, J = 4.25 Hz, 2H), 3.06(t, J = 5.4 Hz, 2H), 1.33(t, J = 7.0 Hz, 3H). ^{13}C-NMR (500 MHz, $CDCl_3$): δ 13.62, 35.26, 41.11, 51.19, 59.66, 104.09, 109.44, 120.96, 122.51, 123.13, 123.45, 124.43, 127.67, 130.19, 131.11, 134.90, 136.80, 149.40, 150.56, 158.96, 164.22, 164.88. ESI-MS (positive mode, m/z): 466.3 for $[M+H]^+$. Elemental analysis: Calcd. for $C_{28}H_{27}N_5O_2$: C, 72.24; H, 5.85; N, 15.04%. Found: C, 72.01; H, 6.12; N, 14.93%.

3. UV-vis and Fluorescence Titrations of Naph-BPEA with Zn^{2+} Solution

UV-vis and fluorescence titration of **Naph-BPEA** with Zn^{2+} solution were determined by a Lambda 35 UV-VIS spectrophotometer and a AMINCO Bowman series 2 luminescence spectrophotometer, respectively. The **Naph-BPEA** solutions for fluorescence (10 μM) and UV-vis titration (100 μM) were prepared with double distilled water in aqueous buffer (50 mM HEPES, 100 mM KNO_3, pH 7.2, 25 °C) containing 1 and 10 % DMSO, respectively. DMSO of spectrum grade was commercial available from TEDIA. The optical path length was 1 cm with a cell volume of 3.0 mL. The titration spectra were obtained by recording the spectrum after adding and mixing aliquots of Zn^{2+} solution (1.2 mM for fluorescence titration, 10 mM for UV titration) into the **Naph-BPEA** solution.

4. ^{1}H NMR Titration of Naph-BPEA with Zn^{2+} in CD_3OD

^{1}H NMR Zn^{2+} titration was carried out with Bruker DRX-500 (500 MHz) in CD_3OD at 25 ± 1 °C. Chemical shift was referenced to TMS (δ=0.00 ppm). Zn^{2+} solution (50 mM in CD_3OD) was added into **Nph-BPEA** solution (10 mM) step by step, and the final concentration of $Zn(NO_3)_2$ was varied from 0 to 30 mM.

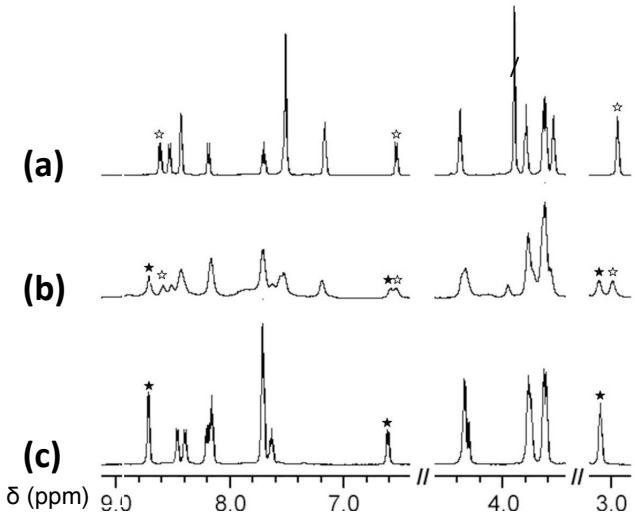


Fig. S1. ^{1}H NMR spectra of **Naph-BPEA** in CD_3OD upon Zn^{2+} titration. The spectra of apo-**Naph-BPEA** (**a**, $[Zn^{2+}]_{total}$/[**Naph-BPEA**]=0:1), of **Naph-BPEA** during Zn^{2+} titration (**b**, $[Zn^{2+}]_{total}$/[**Naph-BPEA**]=1:2), of Zn^{2+}/**Naph-BPEA** complex (**c**, $[Zn^{2+}]_{total}$/[**Naph-BPEA**]=1:1).

5. Fluorescent response of Naph-BPEA to different metal cations

The fluorescent response of **Naph-BPEA** to different metal cations was determined in 10 μM **Naph-BPEA** aqueous solution (1:99, DMSO/water, v/v; 50 mM HEPES, 100 mM KNO_3; pH = 7.40). Aliquots of metal cation solution (1.2 mM, 25 μl) were added to 3 ml of **Naph-BPEA** solution, and the fluorescence spectra were determined after each addition and complete mixing. The excitation wavelength was 450 nm. On the other hand, the fluorescent responses of **Naph-BPEA** to Zn^{2+} in the presence of 10 mM Ca^{2+}, Mg^{2+} or Na^+ were also determined.

6. Determination of Quantum Yields

Fluorescence quantum yields were determined with NBD-$NHCH_3$ (Φ = 0.38) in acetonitrile (λ_{ex} = 458 nm) as the reference. The quantum yields of **Naph-BPEA** and its zinc complex were calculated according to the following equation.

$$\Phi_x = \Phi_s(A_s S_x)/(A_x S_s)(n_x/n_s)^2$$

A_x and A_s are the absorbance of **Naph-BPEA** (or Zn^{2+}/**Naph-BPEA** complex) and NBD-$NHCH_3$. S_x and S_s are the integrated fluorescence emission corresponding to **Naph-BPEA** (or Zn^{2+}/**Nph-BPEA** complex) and the standard. n is the refractive index of solvent.

7. Binding Constant Determination

A series of buffered Zn^{2+} solutions were adopted for the determination of the dissociation constant of Zn^{2+}/**Naph-BPEA**. Therefore, HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) solutions (50 mM, pH 7.20, 0.1 M KNO_3) containing 10 mM of EGTA (ethylenebis(oxyethylenenitrilo)tetraacetic acid) were added with various amounts of $Zn(NO_3)_2$ (0 ~ 20 mM). The concentration of free Zn^{2+} was calculated with $[EGTA]_{total}$ and $[Zn^{2+}]_{total}$ using $K'_{Zn\text{-}EGTA}$ of 3.80×10^8 M^{-1}. The dissociation constant determination was carried out by recording the fluorescence intensity after adding 30 μL of **Naph-BPEA** solution (1 mM, DMSO as solvent) into the buffered Zn^{2+} solutions (3 mL). The final concentration of **Naph-BPEA** is around 10 μM. The dissociation constant was determined according to the varied emission intensity at 540 nm. The maximum emission intensity were obtained at $[Zn^{2+}]_{total}$ = 20 mM. All the fluorescence increment was normalized to 1 according the emission increment at $[Zn^{2+}]_{total}$ = 20 mM. The excitation wavelength is 450 nm.

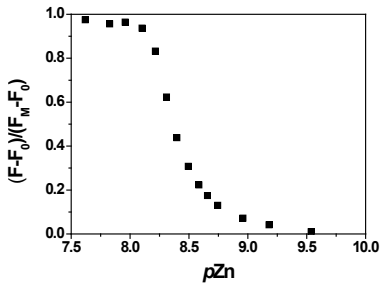


Fig. S2. Relative fluorescence intensity (at 540 nm) of **Naph-BPEA** as a function of $[Zn^{2+}]_{free}$ (pH 7.20, 50 mM HEPES, 100 mM KNO_3, 10 mM EGTA).

8. Fluorescent pH-dependence of Naph-BPEA

The fluorescent pH-dependence of **Naph-BPEA** was determined in aqueous solutions (1:99, DMSO/water, v/v; 100 mM KNO_3), and the fluorescence spectra were determined immediately after the solution pH values were adjusted to the desired pH by NaOH and HNO_3 solutions. The excitation wavelength was 450 nm. The experiments were carried out at 298 K.

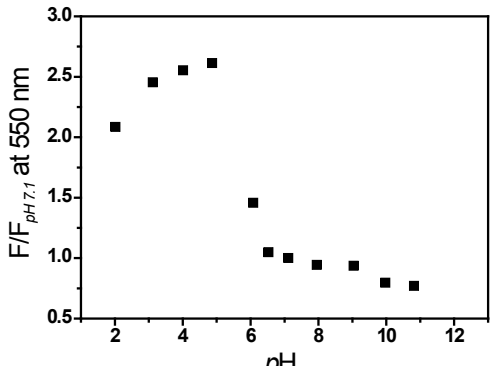


Fig. S3. pH-titration profile of **Naph-BPEA** according to the emission intensity at 540 nm. λ_{ex}, 450 nm.

9. Determination of detection limit.

The emission spectrum of free **Naph-BPEA** was collected for 20 times to determine the background noise σ. Then the solution was treated with various concentration of Zn^{2+} from 0 - 5 μM, and all fluorescence spectra were collected after thoroughly mixing. A linear regression curve was then fitted according to the emission intensity at 540 nm in the range of 0 – 5 μM, and the slope of the curve was obtained (Figure S4). The detection limit ($3\sigma slope^{-1}$) was then determined to be 57 nM.

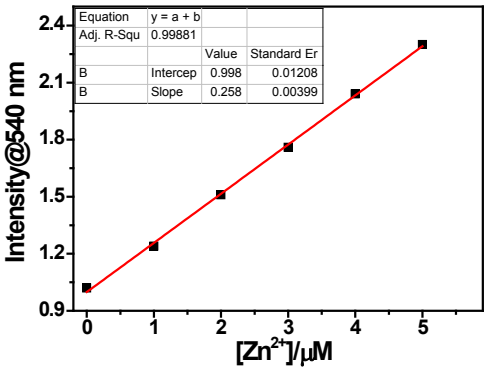


Fig. S4. Plot of fluorescence intensity of **Naph-BPEA** (10 μM) at 540 nm in HEPES buffer (50 mM, 100 mM KNO_3, pH 7.2) as a function of Zn^{2+} concentration in the range of 0-5 μM.

10. Cell Culture Procedure, Dyeing Method and Confocal Fluorescence Imaging

The **Naph-BPEA** dyeing solution for cell staining was prepared from a 5 mM **Naph-BPEA** aqueous stock solution by diluting with 1 × PBS to a final concentration

of 5 μM. HeLa and HepG2 cells were cultured in glass bottom dishes following the same procedure. The culture medium was Dulbucco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 3.7 mg/mL of $NaHCO_3$. For intracellular Zn^{2+} imaging of cells stained by **Naph-BPEA**, the cells were rinsed three times with 1 × PBS after removing the culture medium. Then the cells were incubated with **Naph-BPEA** solution (5 μM) for 20 min at room temperature. After removing the solution, the cells were washed three times with PBS and imaged. For the cells with exogenous Zn^{2+}, the exogenous Zn^{2+} was introduced by incubating the cells with 5 μM $ZnSO_4$/2-mercaptopyridine-*N*-oxide solution (1:1, 5 min), which was prepared by diluting 5 mM $ZnSO_4$ and 2-mercaptopyridine-*N*-oxide stock solution with 1 × PBS. Then the cells were dyed with **Naph-BPEA** in a procedure similar to that described above and imaged. Zn^{2+} scavenging in the **Naph-BPEA**-dyed HeLa cells of exogenous Zn^{2+} was carried out by TPEN incubation. Therefore, the cells incubated with Zn^{2+}/pyrithione were further treated with TPEN solution (25 μM in 1 × PBS) followed by washing with 1 × PBS and confocal imaging. For the co-staining experiments with **Naph-BPEA** and nucleus dye Hoechst 33342 (Invitrogen), the PBS-rinsed cells were dyed by Hoechst 33342 (5 μg/ml) via incubation at room temperature for 10 min. Then the rinsed cells (1 × PBS, two times) were dyed by **Naph-BPEA** in the procedure described above and imaged. After that, the cells were introduced with exogenous Zn^{2+} in the procedure described above and imaged. Finally, the cells with exogenous Zn^{2+} were deprived with TPEN in the procedure described above and imaged. A Leica TCS-SP5 microscope equipped with a 63 × oil-immersion objective was used for confocal imaging. The excitation wavelength for **Naph-BPEA** in co-staining experiments was 488 nm, and the band path was 500-600 nm. The excitation wavelength for Hoechst 33342 was 351 nm, and the band path was 420-470 nm.

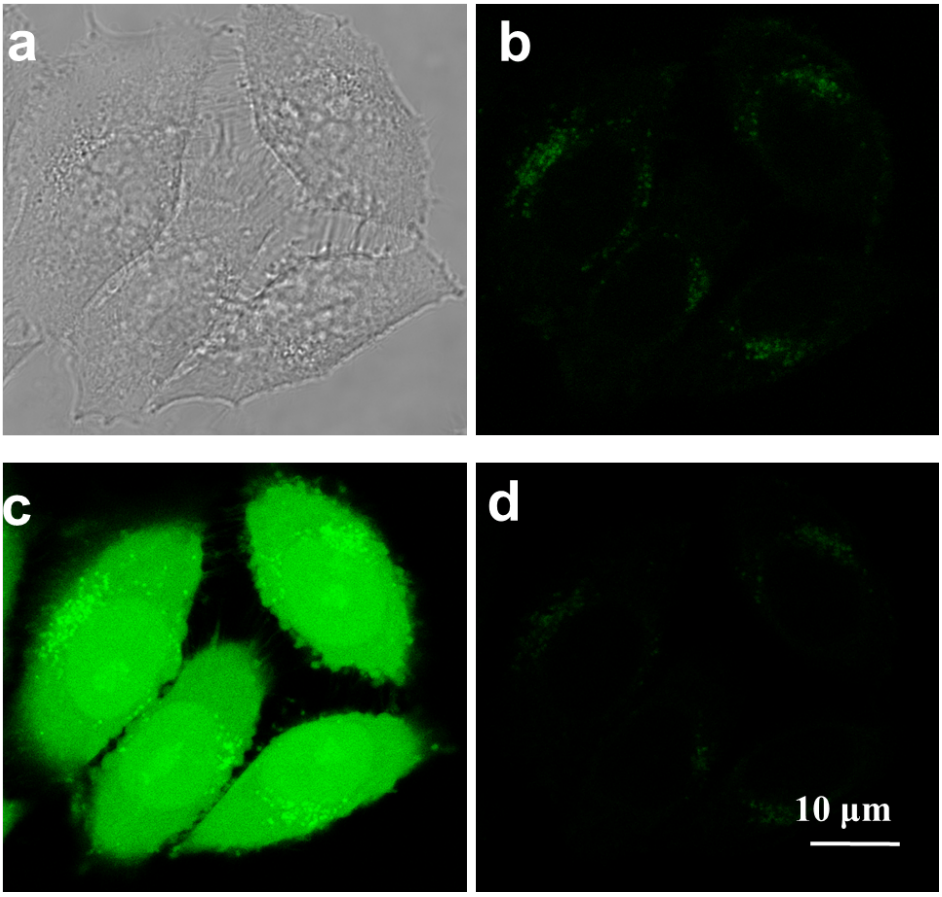


Fig. S5. Confocal fluorescence images of HepG2 cells stained by **Naph-BPEA** (5 μM, 20 min) at 25°C. (**a**) Bright field image of the stained cells; (**b**) fluorescence images of the stained cells; (**c**) fluorescence images of cells in (**b**) incubated with $ZnSO_4$/pyrithione (5 μM, 1:1); (**d**) fluorescence images of cells in (**c**) treated by 25 μM TPEN solution. λ_{ex}, 488 nm, band path 500 - 600 nm.

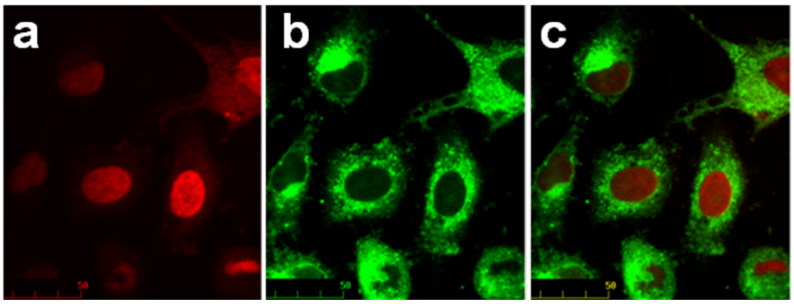


Fig. S6. Confocal fluorescence images of HeLa cells co-stained by Hoechst 33342 (5 μg/ml, 10 min at 25°C) and **NBD-BPEA** (5 μM, 20 min at 25°C). (**a**) Fluorescence image of fixed cells obtained from Hoechst channel (λ_{ex}, 351 nm, band path 420-470 nm); (**b**) fluorescence image of cells pre-incubated in 5 μM Zn^{2+}/pyrithione solution obtained from **NBD-TPEA** channel (λ_{ex}, 488 nm, band path 500-600 nm); (**c**) overlay of (**a**) and (**b**).

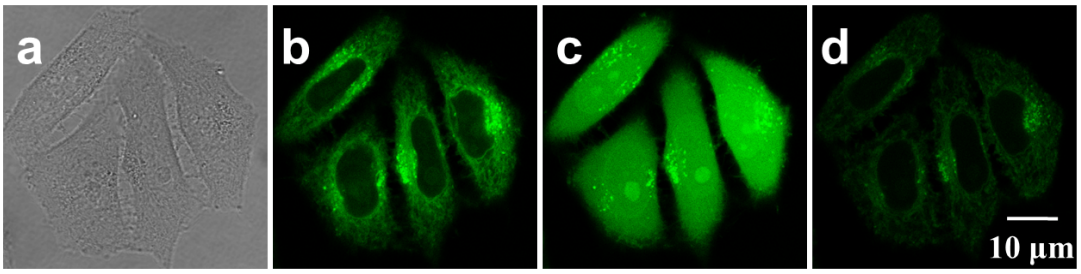


Fig. S7. Confocal fluorescence images of HepG2 cells stained by **Naph-BPEA-e** (5 μM, 20 min) at 25°C. (**a**) Bright field image of the stained cells; (**b**) fluorescence images of the stained cells; (**c**) fluorescence images of cells in (**b**) incubated with $ZnSO_4$/pyrithione (5 μM, 1:1); (**d**) fluorescence images of cells in (**c**) treated by 25 μM TPEN solution. λ_{ex}, 488 nm, band path 500 - 600 nm.

11. Zn^{2+} Imaging in Zebrafish Larva

Zebrafish embryos or larvae after fertilization were incubated at 28.5 °C in pure water from Milli-Q system. The 5-day-old zebrafish larvae were fed with 5 μM Zn^{2+} solution at 28.5 °C for 12 h, then the larvae were washed with 1 × PBS three times followed by incubation with 5 μM **Naph-BPEA** (or 5 μM **NBD-TPEA)** solution at 28.5 °C for 20 min. After rinse with 1 × PBS for three times, the larvae were then embedded in methyl cellulose for imaging. Non-Zn^{2+}-fed larvae were also imaged for comparison. A Leica MZ16F fluorescence stereomicroscope was utilized for imaging. Light from mercury lamp through GFP2 filter was used as the excitation light, and the exposure time was 1.0 s.

12. Cytotoxicity Assay

The cytotoxicity of **Naph-BPEA** was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the human cervical cancer cell line HeLa and the human hepatocarcinoma cell line HepG2 were seeded respectively in 96-well plates (5000 cells per well) with Dulbecco's modified Eagle's medium and incubated at 37 °C in the humidified atmosphere with 5% CO_2 for 24 h. The cells were then treated in triplicate with fresh medium containing 5 μM **Naph-BPEA** at 37 °C for 48 h. Aliquots of MTT (10 μL, 5 mg/mL) in PBS were added to each well. The supernatant was taken off after 4 h of

incubation and DMSO (150 µL) was added to each well, and the amount of the resultant MTT formazan in each well was determined at 570 nm using an ELISA plate reader. The cytotoxicity was calculated based on the data of three tests.

12. References

[i] S. D. Ross, M. Finkelstein, R. C. Petersen, *J. Am. Chem. Soc.,*1958, **80**, 4327.

[ii] A. T. Peters, Y. S. S. Behesti, *J. Soc. Dyers. Colourists*, 1989, **105**, 29.

[iii] Z.-F. Tao, X. Qian, J. Tang, *Dyes & Pigments.* 1996, **30**, 47.