Supporting Information

A two-photon fluorescence probe for colorimetric and ratiometric monitoring of mercury in live cells and tissues

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1. General information

Unless otherwise noted, materials were purchased from commercial suppliers and used without further purification. All the solvents were treated according to general methods. Flash column chromatography was performed using 200-300 mesh silica gel. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet), coupling constants (Hz) and integration. $^{13}$C NMR spectra were recorded at 75 MHz with complete proton decoupling. Fluorescence emission spectra were obtained using a RF-5301/PC spectrofluorophotometer (Shimadzu). UV absorption spectroscopy measurements were carried out on Scinco S-3100 using a 1 cm optical path length cell at room temperature. The high resolution mass spectra (HRMS) were measured on a Bruker Ultraflex Xtreme MALDI-TOF/TOF mass spectrometer by ESI.
2. Synthetic pathway of probe NAP-PS.

Scheme S1. Synthetic pathway of probe NAP-PS.

Synthesis of compound 1: \(^1\)

Compound 1 was first synthesized in 69% yield: 4-bromo-1,8-naphthalic anhydride (1.1 g, 4.0 mmol, 1.0 eq) and \(n\)-butylamine (0.5 mL, 4.8 mmol, 1.2 eq) were dissolved in 30 mL anhydrous EtOH and heated to reflux. After the total completion of the reaction monitored by TLC plate, the mixture was cooled down and the solid was slowly precipitated down from the viscous solution. The product was filtered off and washed with cooled EtOH.

Compound 1: \(\delta\) \(^1\)H NMR (300 MHz, CDCl\(_3\)): 8.73-8.63 (1 H, m), 8.63-8.55 (1 H, m), 8.43 (1 H, d, \(J = 7.9\)), 8.06 (1 H, d, \(J = 7.9\)), 7.90=7.80 (1 H, m), 4.19 (2H, dd, \(J = 8.5, 6.7\)), 1.83 – 1.64 (2 H, m), 1.47 (2 H, dq, \(J = 14.7, 7.3\)), 1.00 (3 H, t, \(J = 7.3\)).

Synthesis of compound 2: \(^1\)

Compound 2 was synthesized in 67% yield: Compound 1 (665 mg, 2.0 mmol, 1.0 eq) and K\(_2\)CO\(_3\) (1.40 g, 10.0 mmol, 5.0 eq) were dissolved in 30 mL anhydrous MeOH. The mixture was heated to reflux and stirred overnight. After the total consumption of compound 1 (monitored by TLC), the reaction was cooled down. The precipitated solid was filtered and washed with distilled water for several time.

Compound 2: \(\delta\) \(^1\)H NMR (300 MHz, DMSO): 8.57 – 8.54 (1 H, m), 8.53-8.50 (1 H, m), 8.49-8.43 (1 H, m), 7.85-7.75 (1 H, m), 7.33 (1 H, d, \(J = 8.4\)), 4.13 (3 H, s), 4.09 – 3.86 (2 H, m), 1.67 – 1.52 (2 H, m), 1.35 (2 H, dq, \(J = 14.4, 7.3\)), 0.90 (3 H, dt, \(J = 18.4, 7.2\)); \(\delta\) \(^{13}\)C NMR (125 MHz, DMSO): 165.94, 164.29, 163.66, 161.05, 134.02, 131.77, 128.98, 127.14, 123.50, 122.65, 114.99, 107.03, 79.88, 57.35, 30.43, 20.51, 14.42.

Synthesis of compound NAP-OH: \(^1\)

Compound NAP-OH was then synthesized in 63% yield: Compound 2 (283 mg, 1.0 mmol) was dissolved in 20 mL concentrated HI aqueous solution (57%). The reaction was heated to reflux and stirred overnight. After cooling down, NaOH aqueous solution (10%) was added to the mixture until the pH near
to neutral, the solid was then slowly precipitated. The precipitate was filtered and washed by water to give compound NAP-OH.

Compound NAP-OH: δ ¹H NMR (300 MHz, DMSO): 11.87 (1 H, s), 8.58-8.51 (1 H, m), 8.50-8.44 (1 H, m), 8.3 (1 H, d, J = 8.2), 7.83-7.70 (1 H, m), 7.25-7.10 (1 H, m), 4.26 – 3.73 (2 H, m), 1.58 (2 H, dd, J = 14.9, 7.6), 1.34 (2 H, dd, J = 14.9, 7.4), 0.92 (3 H, t, J = 7.3); δ ¹³C NMR (125 MHz, DMSO): 164.38, 163.71, 160.97, 134.26, 131.83, 129.90, 129.59, 126.32, 123.11, 122.54, 113.32, 110.69, 30.46, 20.52, 14.43.

Synthesis of compounds probe NAP-PS:

Probe NAP-PS was synthesized in 71 % yield according to a modified procedure of the reported literature: Compound NAP-OH (135 mg, 0.5 mmol, 1.0 eq) was dissolved in 20 mL anhydrous THF (20 mL) under argon. PhPCl₂ (0.36 mL, 2.0 mmol, 4.0 eq) and triethylamine (0.5 mL) in 5 mL THF was added dropwise to the reaction over a period of 5 min. The mixture was stirred at room temperature for 3 hours. Elemental sulfur (65 mg, 2.0 mmol, 4.0 eq) in 5 mL THF was then added dropwise to the mixture at room temperature under argon. The mixture was heated to reflux and monitored by TLC plate. After the total consumption of compound NAP-OH, the solvent was removed and purified by aluminum chloride neutral column chromatography.

Probe NAP-PS: δ ¹H NMR (300 MHz, CDCl₃): 8.65-8.55 (m, 1H), 8.45 – 8.33 (m, 2H), 8.14 – 7.99 (m, 4H), 7.75-765 (m, 1H), 7.65 – 7.49 (m, 6H), 7.4-7.3 (m, 1H), 4.21 (t, J = 7.5 Hz, 2H), 1.69 (dq, J = 15.0, 8.5, 6.4 Hz, 2H), 1.43 (dq, J = 14.7, 7.3 Hz, 2H), 0.96 (t, J = 7.3 Hz, 3H); δ ¹³C NMR (125 MHz, CDCl₃): 164.35, 163.74, 134.31, 133.43, 132.93, 132.01, 131.92, 131.61, 131.52, 129.76, 129.21, 129.10, 128.90, 127.10, 126.21, 126.17, 123.05, 119.25, 117.53, 117.48, 40.46, 30.44, 20.61, 14.07; HRMS (ESI) m/z = 486.1302, calcd for [M+H]⁺ C₂₈H₂₄NÖ₃PS = 486.1293.
3. Linearship of concentration and fluorescence ratios.

Figure S1. Fluorescence intensity ratios of probe NAP-PS at F_{550}/F_{450} to mercury concentration (0–90 μM). Spectra were recorded after incubation with different concentrations of mercury ion for 30 min; λ_{ex} = 380 nm; slits: 3/5 nm.

4. Detection limit of probe NAP-PS.

Figure S2. Detection limit of probe NAP-PS (10 μM) towards mercury; λ_{ex} = 380 nm; Spectra were recorded after incubation with different concentrations of mercury for 30 min.
5. Pseudo-first-order rate constant study.

Figure S3. Pseudo first-order kinetic plot of probe NAP-PS (10 μM) with the addition of mercury in HEPES buffer (1.0 mM, pH = 7.4);

6. Reaction mechanism of probe NAP-PS reaction with mercury.

6.1 HRMS study

Figure S4. Reaction mechanism of probe NAP-PS and Hg$^{2+}$. 
6.2 Fluorescence comparision

Figure S5. Fluorescence spectra of the mercury induced reaction mixture, probe NAP-PS and product NAP-OH in HEPES buffer (pH = 7.4, 1.0 mM); λ<sub>ex</sub> = 380 nm, Slits: 3/5 nm.
6.3 Absorption comparison

Figure S6. UV-Vis spectra of the mercury induced reaction mixture, probe NAP-PS and product NAP-OH in HEPES buffer (pH = 7.4, 1.0 mM).
7. Solid state study

Figure S7. Photographs of solid probe NAP-PS before A) and after B) ground with mercury(II) perchlorate.

Figure S8. Photographs of solid probe NAP-PS before A) and after B) ground with mercury(II) perchlorate under 365 nm UV hand-held lamp.
8. Two-photon cross section

The two-photon cross section ($\delta$) was determined by using femto second (fs) fluorescence measurement technique as described. Probes ($1.0 \times 10^{-5}$ M) was dissolved in HEPES buffer (1.0 mM, pH = 7.4) and the two-photon induced fluorescence intensity was measured at 720–1000 nm by using rhodamine 6G as the reference. The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using $\delta = \delta_r(S_r\Phi_r\phi_r c_r)/(S_s\Phi_s\phi_s c_s)$: where the subscripts s and r stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as S. $\Phi$ is the fluorescence quantum yield. $\phi$ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as c. $\delta_r$ is the TPA cross section of the reference molecule.

Figure S9. Two-photon spectra of NAP-PS and NAP-OH in HEPES buffer (1.0 mM, pH 7.4).
9. Cell viability study

MTT kit (AbCareBio CL) assay was performed to assess the cytotoxicity. HeLa cells were cultured in 96-well plate for 24 h, and then each different concentration of probes were added. After incubation for 2 h, the cultured medium was replaced with serum free medium containing 10 % MTT, and further incubated for 2 h. MTT containing medium was removed and DMSO was added to dissolve the formed precipitate. Absorbance was measured at 600 nm.

![Viability of HeLa cells in the presence of NAP-PS and NAP-OH as measured by using MTT assays. The cells were incubated with 0–100 μM of probes for 2 h.](image)

Figure S10. Viability of HeLa cells in the presence of **NAP-PS** and **NAP-OH** as measured by using MTT assays. The cells were incubated with 0–100 μM of probes for 2 h.
10. Staining experiment

HeLa cells were cultured on 20 mm glass bottomed dishes (NEST) using MEM (WelGene) containing 10% FBS, streptomycin (100 μg mL\(^{-1}\)) and penicillin (100 units mL\(^{-1}\)) under 5% CO\(_2\), 37 °C and humidified atmosphere for 2 days. Before TPM imaging, the culture medium was changed with serum free MEM, then treated NAP-PS (5 μM) and incubated for 30 min. Hg(ClO\(_4\))\(_2\) and TPEN were pretreated for 30 min before staining with NAP-PS and then washed.

Rat liver slices were prepared from the liver of 14 days old male SD rat and cut into 800 μm thickness using a vibrating-blade microtome in DPBS (Gibco). Slices were incubated with NAP-PS (50 μM) for 1 h under 5% CO\(_2\), 37 °C and humidified atmosphere. Then slices were washed two times with DPBS and transferred to glass bottomed dishes. Hg(ClO\(_4\))\(_2\) (250 μM) was pretreated for 30 min before staining NAP-PS, and then washed two times. The TPM images were acquired at about 50–220 μm depth.

10.1 Real-time staining monitoring

![Image](Figure S11. (a) Pseudo colored ratiometric TPM images and bright field images of HeLa cells incubated with NAP-PS (5 μM). Cells were pretreated with Hg(ClO\(_4\))\(_2\) (0, 5, 12.5, 25, 37.5, and 50 μM) for 30 min before labeling with NAP-PS; (b) Plot of average \(F_{\text{yellow}}/F_{\text{blue}}\) ratios in the TPM images. Images were acquired using 740 nm excitation and emission windows of 400–450 nm (blue) and 500–600 nm (yellow); Scale bars = 20 μm.)
10.2 Suitable two-photon excitation source and cell spectrum

Figure S12. TPM images of HeLa cells incubated with (a) probe NAP-PS (5 μM) and (b) Dye (NAP-OH) (5 μM) for 30 min at different excitation wavelength from 720 nm to 920 nm; (c) TPEF intensity corresponding to upper TPM images; (d) TPEF spectrum of probe NAP-PS and Dye (NAP-OH) in HeLa cells. Images were acquired at 380–680 nm emission windows; Scale bars = 50 μm.
11. Photostability study

Figure S13. TPM image of HeLa cells labeled with probe NAP-PS (5 μM) for 30 min; (b) TPEF intensity from A–C in Figure (a) as a function of time. The digitized intensity was recorded with 2.00 sec intervals for the duration of 1 h; Image and digitized intensity were acquired using 740 nm excitation and emission windows of 380–680 nm; Scale bars = 50 μm.
12. Cell calibration study

Figure S14. Pseudo colored ratiometric TPM images and bright field images of HeLa cells incubated with NAP-PS (5 μM). Cells were pretreated with Hg(ClO$_4$)$_2$ (0, 5, 12.5, 25, 37.5, and 50 μM) for 30 min before labeling with NAP-PS; (b) Plot of average $F_{\text{yellow}}/F_{\text{blue}}$ ratios in the TPM images. Images were acquired using 740 nm excitation and emission windows of 400–450 nm (blue) and 500–600 nm (yellow); Scale bars = 20 μm.
13. Copies of $^1\text{H}$ NMR, $^{13}\text{C}$ NMR and Mass Spectra

Figure S15. $^1\text{H}$ NMR spectrum (CDCl$_3$) of compound 1.

Figure S16. $^1\text{H}$ NMR spectrum (DMSO-$d_6$) of compound 2.
Figure S17. $^{13}$C NMR spectrum (DMSO-$d_6$) of compound 2.

Figure S18. $^1$H NMR spectrum (DMSO-$d_6$) of compound NAP-OH.
Figure S19. $^{13}$C NMR spectrum (DMSO-d$_6$) of compound NAP-OH.

Figure S20. $^1$H NMR spectrum (CDCl$_3$) of compound NAP-PS.
Figure S21. $^{13}$C NMR spectrum (DMSO-$d_6$) of compound NAP-PS.

Figure S22. HRMS spectrum of compound NAP-PS.
14. References

