Electronic Supplementary Information

Two-Channel Responsive Fluorescent Probe with AIE Characteristics for Selective Imaging Superoxide Anion in Living Cells

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1. Materials and Instrumentation and cell culture.

Materials. All the chemicals and reagents are commercially available and used without further purification. DCM, THF and toluene are distilled under nitrogen before use. 4,4’-dimethoxybenzophenone, 4-bromobenzophenone, 4-hydroxybenzyl alcohol, phosphinic chloride, carbon tetrabromide, triphenylphosphine, zinc powder, titanium tetrachloride and tetrakis(triphenylphosphine) palladium are purchased from TCI. Potassium carbonate, triethylamine, L-buthionine sulfoximine (BSO), phorbol-12-myristate-13-acetate (PMA), lipopolysaccharide (LPS) are purchased from Sigma Aldrich. Fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) are purchased from Invitrogen. PBS buffer (pH = 7.4, 10mM) is prepared with pure water from a Millipore filtration system.

Instrumentation. $^1$H and $^{13}$C NMR spectra is measured on Bruker ARX 400 NMR spectrometer using DMSO as solvent, and tetramethylsilane (TMS; $\delta = 0$ ppm) was chosen as internal reference. Photoluminescence spectra are measured on PerkinElmer LS 55 spectrofluorometer and UV spectra are measured on Biochrom Libra S80PC double beam spectrometer. High-resolution mass spectra (HRMS) are measured on a GCT Premier CAB 048 mass spectrometer operating in MALDI-TOF mode. Particle sizes are measured using a Brookhaven ZetaPlus potential analyzer (Brookhaven instruments corporation, USA). Confocal lasing scanning microscopic (CLSM) images and fluorescence spectra are obtained on confocal microscope (Zeiss Laser Scanning Confocal Microscope; LSM7 DUO) using ZEN 2009 software (Carl Zeiss).
**Sample preparation.** The stock solution of probe was prepared at 1.0 mM in dimethyl sulfoxide (DMSO). Solution of GSH (1.0 mM), NaClO (1.0 mM), H$_2$O$_2$ (1.0 mM), SIN-1 (1.0 mM) and FeSO$_4$ (1.0 mM) were prepared with H$_2$O. NOC-5 was prepared as a solution (1.0 mM) in aqueous NaOH (10.0 mM). KO$_2$ was used as a solution (1.0 mM) in DMSO. The hydroxyl radical was produced by mixing H$_2$O$_2$ (20 μM) with Fe$^{2+}$ (200 μM). Single oxygen (1$^1$O$_2$) was obtained by addition H$_2$O$_2$ (50 μM) to NaOCl (50 μM). Nitric oxide and Peroxynitrite were generated by using 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5) and 3-morpholinosydnonimine hydrochloride (SIN-1), respectively.

**Titration.** Different volume of KO$_2$ were added to PBS buffer (0.9 mL) and probe (10.0 μL, 10.0 μM) in a 1.5 mL centrifugal tube. Then corresponding volume of DMSO was added to the mixture until 1 mL, which contains 10% DMSO. The mixture was equilibrated and placed at 25 °C for 5 min before fluorescence (FL) spectra were measured. The excitation wavelength was 390 nm.

**Cell Culture and Fluorescence Imaging.** HepG2 cells were provided by American Type Culture Collection (ATCC). They were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing penicillin (100U/mL), 10% heat-inactivated fetal bovine serum (FBS), and streptomycin (100 μg/mL) and were maintained in humidified incubator at 37 °C under 5% CO$_2$ environment. The cells were incubated with 10 μM probe at 37°C for 2 h. After that, the medium was removed and the cells were rinsed with PBS. The probe-loaded cells were treated with PMA for 0.5 h, LPS for 3 h and L-BSO for 0.5 h, respectively. After that, the media was removed and washed with PBS buffer. The
cells were imagined by confocal laser scanning microscope. The excitation wavelength was 405 nm, and the emission filter was 510-560 nm and 575-625 nm.

**Cytotoxicity assay.** HepG2 cells were provided by American Type Culture Collection (ATCC). They were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing penicillin (100U/mL), 10% heat-inactivated fetal bovine serum (FBS), and streptomycin (100 μg/mL) and were maintained in humidified incubator at 37 °C under 5% CO2 environment. After removal of the medium, cells were incubated with various concentrations of probe for 12 h and 24 h respectively. The cytotoxicity of the probe was assessed by MTT assay according to ISO 10993-5. For each independent experiment, the assays were performed in eight replicates. And the statistic mean and standard derivation were utilized to estimate the cell viability.

2. **Synthetic procedures**

Compound 1 was synthesized according to the procedures reported in literature.1

**Synthesis of compound 2.** To a solution of 4-hydroxybenzyl alcohol (1 mmol, 124.14 mg) and triethylamine (0.5 mL) in distilled THF (20 mL), phosphinic chloride (1 mmol, 236.63 mg) was added dropwise under nitrogen at 0 °C. The reaction was stirred at room temperature overnight. The resulting solution was quenched with 5mL water. The solvent was evaporated under reduced pressure. The crude product was extracted with DCM for 3 times and purified by silica gel chromatography with hexane/ethyl acetate (2:1) as eluent to afford the desired product (230mg) as white solid. Yield = 70%. 1H NMR (400 MHz, DMSO):δ = 7.939-7.885 (m, 4H), 7.622-7.600 (m, 2H), 7.573-7.524 (m, 4H), 7.241 (s, 4H), 5.143 (t, J = 5.6 Hz, 1H), 4.411 (d, J = 5.6
Hz, 2H) ppm; $^{13}$C NMR (100 MHz, DMSO): $\delta = 149.867, 149.785, 139.402$ (4C), 133.178, 133.150, 132.049 (2C), 131.946 (2C), 130.648, 129.427, 129.298, 128.304, 120.665, 120.616, 62.702 ppm; HRMS (ESI, m/z) Calcd. For [C$_{19}$H$_{17}$O$_3$P]: 324.0915, found: 325.0992.

**Synthesis of compound 3.** To a solution of compound 2 (1 mmol, 324.32 mg) and carbon tetrabromide (1 mmol, 331.63 mg) in distilled DCM (20 mL), triphenyl phosphine (1 mmol, 262.3 mg) was added under nitrogen at 0 °C. The reaction was stirred at room temperature overnight. The reaction was quenched with 5 mL saturated sodium bicarbonate solution. The crude product was extracted with DCM for 3 times and purified by silica gel chromatography with hexane/ethyl acetate (2:1) as eluent to afford the desired product (150mg) as white solid. Yield = 40%. $^1$H NMR (400 MHz, DMSO): $\delta = 7.942$-$7.887$ (m, 4H), $7.632$-$7.607$ (m, 2H), $7.580$-$7.531$ (m, 4H), $7.412$-$7.391$ (d, $J = 8.4$ Hz, 2H), $7.276$-$7.252$ (m, 2H), 4.641 (s, 2H) ppm; $^{13}$C NMR (100 MHz, DMSO): $\delta = 150.993, 150.913, 134.933$ (4C), 133.296, 133.269, 132.020 (2C), 131.917 (2C), 131.369, 130.458, 129.487, 129.356, 121.252, 121.205, 34.195 ppm; HRMS (ESI, m/z) Calcd. For [C$_{19}$H$_{16}$BrO$_2$P]: 386.0071, found: 387.0138.

**Synthesis of probe.** Compound 1 (0.1 mmol, 47 mg) and compound 3 (0.1 mmol, 39 mg) was heated to reflux at 80 °C in acetonitrile (15 mL). After removing the solvent, the crude product was purified by silica gel chromatography with methanol/DCM (1/10, by volume) as eluent to afford the desired product (77 mg) as red solid. Yield = 90%. $^1$H NMR (400 MHz, DMSO): $\delta = 9.0885$ (d, $J = 6.8$ Hz, 2H), 8.4385 (d, $J = 6.8$ Hz, 2H), 7.9195 (d, $J = 6.8$ Hz, 2H), 7.907-7.876 (m, 4H), 7.655-7.611 (m, 2H), 7.579-7.533
(m, 4H), 7.485 (d, J = 8.8 Hz, 2H), 7.360-7.338 (m, 2H), 7.208-7.142 (m, 5H), 7.012-6.992 (m, 2H), 6.937 (d, J = 8.8 Hz, 2H), 6.889 (d, J = 8.8 Hz, 2H), 6.723 (t, J = 8.8 Hz, 4H), 5.717 (s, 2H), 3.689 (s, 6H) ppm; $^{13}$C NMR (100 MHz, DMSO): $\delta$ = 158.119, 157.958, 154.154, 151.261, 151.182, 148.283, 144.554, 143.232, 141.688, 137.502, 135.183, 135.161, 132.861, 132.828, 132.142, 132.054, 131.956, 131.501, 131.409, 131.197, 130.929, 130.886, 130.806, 130.639, 130.438, 129.848, 129.030, 128.898, 128.065, 127.713, 126.573, 124.289, 121.2, 121.151, 113.426, 113.187, 61.308, 54.970, 54.943 ppm; HRMS (ESI, m/z) Calcd. For $[C_{52}H_{43}NO_4P-Br]$: 776.2924, found: 776.2929.

References

Scheme S1. Synthetic route to the target fluorescent probe I.
Figure S1. $^1$H NMR spectrum of compound 2 in DMSO. The solvent peaks are marked with asterisks.
Figure S2. $^{13}$C NMR spectrum of compound 2 in DMSO. The solvent peaks are marked with asterisks.
Figure S3. $^1$H NMR spectrum of compound 3 in DMSO. The solvent peaks are marked with asterisks.
Figure S4. $^{13}$C NMR spectrum of compound 3 in DMSO. The solvent peaks are marked with asterisks.
Figure S5. $^1$H NMR spectrum of probe in DMSO. The solvent peaks are marked with asterisks.
Figure S6. $^{13}$C NMR spectrum of probe in DMSO. The solvent peaks are marked with asterisks.
Figure S7. High resolution mass spectrum (HRMS) of compound 2.

Figure S8. HRMS of compound 3.
Figure S9. High resolution mass spectrum (HRMS) of Probe I.
Figure S10. (A) Fluorescence (FL) spectra of probe in DMSO/water mixtures with different water fractions ($f_w$). (B) Plot of relative peak intensity ($I/I_0$) vs $f_w$. Concentration of the probe: 10 μM; Excitation wavelength: 390 nm.
Figure S11. Plot of fluorescence (FL) peak intensity (I/I₀) versus pH value. The data are extracted from Figure 1A. Excitation wavelength: 390 nm. Probe I concentration: 10 μM in PBS buffer solution (10 mM, containing 1% DMSO.)
**Figure S12.** Size distribution of probe I in PBS buffer containing 1% DMSO at 25 °C.
Concentration: 10 µM
Figure S13. UV-visible spectra of probe I (10 μM) in PBS buffer containing 1% DMSO, treated with (black curve) and without (red curve) superoxide anion at 25°C.
Figure 14. Relative fluorescence intensity response ($I_{525}/I_{615}$) of probe I (10 μM) to KO$_2$ (100 μM) and 500 μM of various metal ions. Excitation wavelength: 390 nm; incubation time: 10 min.
Figure S15. Real time fluorescence spectra of the probe in HepG2 cells. (A) Cells loaded probe (10 μM) without further treatment; (B) cells loaded probe (10 μM) treated with PMA (5 μg/mL) for 30 min.
Figure S16. MTT assay of probe I on HepG2 cells.
Figure S17. Fluorescence microscopy images of probe-loading HepG2 cells taken 3h after washing.