Electronic supplementary information

A dual-responsive probe for the simultaneous monitoring viscosity and peroxynitrite with different fluorescence signals in living cells

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1. General Experimental Section

All chemical/biological reagents and solvents were obtained commercially and used as supplied. HepG2 cells (human hepatocellular carcinoma cell line) were grown in high glucose DMEM (Biological Industries) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 U mL$^{-1}$) and streptomycin (100 U mL$^{-1}$). Bruker 400NMR spectrometers were used for NMR. High-resolution mass spectrometry (HR-MS, ESI) spectra were obtained on a Bruker maxis UHRTOF instrument. A Shimadzu UV-1700 vis spectrophotometer and a HITACHI F-4600 fluorescence spectrophotometer were used to record absorption and fluorescence spectra. Cell imaging and in vivo imaging experiments were measured on a Leica SP8 confocal fluorescence spectrophotometer.

**Preparation of the solution**

Preparation of ONOO$^-$ solution: 2 mL of HCl (0.6 mM) was slowly added dropwise to a mixture of 2 mL of NaNO$_2$ (0.6 mM) and 2 mL of H$_2$O$_2$ (0.7 mM) under ice-bath conditions, and then pour 2 mL of cold NaOH solution (1.2 mM) into the mixed solution, calibrated to its final concentration by UV-1700 vis absorption spectroscopy, and stored at -20 °C.

The final concentration of the probe stock solution was 2×10$^{-3}$ M. metal ion solutions, reducing species solutions, and halide ion solutions were stock solutions at 20 mM. The metal ion and halide ion solutions were diluted to a concentration of 100 μM according to the experimental requirements. The reducing substance solution was diluted to a concentration of 1 mM. Oxidizing species solution and SO$_3^{2-}$ solution stock solution concentration was 10 mM. The oxidizing species and SO$_3^{2-}$ solution were diluted to a concentration of 100 μM according to the experimental requirements. The water used in the preparation process was ultrapure water, and the reagents used were analytical reagents.

**PH Effects**

MC-V-P solutions with different pH values were prepared by adding probe MC-V-P stock solution to 2 mL of mixed solution with different pH values, and the final concentration of probe was 10 μM.
Cytotoxicity assay

The in vitro cytotoxicity was measured using a standard methyl thiazolyl tetrazolium (MTT, MACKLIN) assay in HepG2 cell lines. Briefly, cells growing in log phase were seeded into 96-well cell-culture plate at $1 \times 10^6$ / well for 24h. Probe and compound 3 solutions of different concentrations (0, 5, 10, 15, 20 μM) prepared with cell culture medium, each concentration was three groups in parallel, after culturing in the incubator for 12 hours, the probe solution in the well plate was aspirated and discarded. Then add 200 μL of MTT solution to each well and continue to incubate for 4 hours. After 4 h, the remaining MTT solution was removed, and 100 μL of DMSO was added to each well to dissolve the formazan crystals, and the formazan crystals were dissolved by shaking gently for five minutes. The absorbance value at 490 nm wavelength was measured with a multifunctional microplate reader, and the cell viability was calculated.

2. Synthesis of probes

![Synthetic route of probe MC-V-P](image)

**Synthesis of Compound 2**

In an ice-water bath, 20 mL of dry chloroform and 5 mL of anhydrous N, N-dimethylformamide were added to a 100 mL round-bottomed flask, and phosphorus
tribromide (3.1 mL, 180 mmol) was slowly added under magnetic stirring. After stirring in an ice bath for 45 min, cyclohexanone (1.3 mL, 49 mmol) was slowly added dropwise, and then the mixture was transferred to room temperature and reacted for 16 hours. After the reaction, the reaction solution was slowly added dropwise to ice, the pH of the solution was adjusted to be neutral, and then extracted with ethyl acetate. The organic phase was dried with anhydrous NaSO\(_4\), filtered and concentrated in vacuo. Finally, the orange-red viscous compound 1 was obtained, which was directly used in the next reaction without purification.

Weigh the compound (755 mg, 4 mmol), 4-(diethylamino) salicylaldehyde (385 mg, 2 mmol) and cesium carbonate (2.0 g, 6 mmol) in a 50 mL round bottom flask and mix with 6 mL N, N-dimethylformamide was dissolved and reacted at room temperature for 48 hours. After the reaction, the reaction solution was filtered, the filtrate was washed and extracted with water and dichloromethane, the organic phase was dried with anhydrous NaSO\(_4\), filtered, and concentrated under reduced pressure. Purification by flash chromatography on silica gel (petroleum ether: ethyl acetate=5: 1) afforded 317 mg of compound 2 (56% yield). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 10.28 (s, 1H), 6.99 (d, \(J = 8.6\) Hz, 1H), 6.61 (s, 1H), 6.46-6.20 (m, 2H), 3.38 (q, \(J = 7.1\) Hz, 4H), 2.58-2.49 (m, 2H), 2.44 (t, \(J = 6.1\) Hz, 2H), 1.76-1.62 (m, 2H), 1.20 (t, \(J = 7.1\) Hz, 6H); \(^1^3\)C NMR (101 MHz, CDCl\(_3\)): \(\delta\) 187.16, 162.01, 154.19, 149.60, 128.10, 127.61, 123.14, 111.36, 110.28, 107.76, 97.18, 44.61, 29.86, 29.71, 21.64, 20.69, 12.61. HRMS:(ESI, m/z) Calculated for C\(_{18}\)H\(_{21}\)NO\(_2\) [M+H]\(^+\): 284.1645, found: 284.1664.

**Synthesis of Compound 3**

To a solution of compound 2 (283 mg, 1 mmol) and 4-pyridineacetonitrile (236 mg, 2 mmol) in anhydrous ethanol (5mL) was added a amount of piperidine, the mixture was stirred at 80 °C for 4 hours. After the reaction, the reaction solution was concentrated. Purification by flash chromatography on silica gel (petroleum ether: ethyl acetate = 2:1) afforded 125 mg of compound 3 (44% yield). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.59 (s, 2H), 8.17 (s, 1H), 7.51 (s, 2H), 6.99 (d, \(J = 8.6\) Hz, 1H), 6.58 (s, 1H), 6.42 (d, \(J = 8.6\) Hz, 1H), 6.35 (s, 1H), 3.41 (q, \(J = 7.0\) Hz, 4H), 3.02 (t, \(J = 5.6\) Hz, 2H), 2.44 (t, \(J = 5.6\) Hz, 2H), 1.76-1.62 (m, 2H), 1.20 (t, \(J = 7.1\) Hz, 6H).
Hz, 2H), 2.62-2.48 (m, 2H), 1.86-1.80 (m, 2H), 1.22 (t, \( J = 7.0 \) Hz, 6H); \(^{13}\)C NMR (101 MHz, CDCl\(_3\)): \( \delta \) 156.30, 154.83, 150.06, 149.54, 138.82, 127.52, 127.43, 123.84, 119.35, 110.66, 108.79, 108.02, 98.14, 96.94, 53.50, 44.60, 29.74, 29.50, 26.17, 21.25, 12.68. HRMS:(ESI, m/z) Calculated for C\(_{25}\)H\(_{25}\)N\(_3\)O \([\text{M+H}]^+\): 384.2070, found: 384.2056.

**Synthesis of probe MC-V-P**

To a solution of compound 3 (383 mg, 1 mmol) and 4-bromomethylphenylboronic acid pinacol (297 mg, 1 mmol) in toluene (6mL) was added a amount of piperidine. The reaction was carried out at 110 °C until a solid precipitated. The reaction solution was cooled to room temperature, filtered under reduced pressure and rinsed with toluene to obtain 204 mg of a dark green solid with a yield of 30%.

\(^{1}H \) NMR (400 MHz, CDCl\(_3\)): \( \delta \) 8.64 (d, \( J = 6.3 \) Hz, 2H), 8.40 (s, 1H), 7.93 (d, \( J = 6.9 \) Hz, 2H), 7.83 (d, \( J = 7.9 \) Hz, 2H), 7.43 (d, \( J = 7.9 \) Hz, 2H), 7.25-7.13 (m, 2H), 7.00 (d, \( J = 1.8 \) Hz, 1H), 6.66 (dd, \( J = 8.9, 2.1 \) Hz, 1H), 5.71 (s, 2H), 3.59 (q, \( J = 7.0 \) Hz, 4H), 3.07 (t, \( J = 5.9 \) Hz, 2H), 2.71-2.62 (m, 2H), 1.84 (s, 2H), 1.34 (s, 12H), 1.28 (t, \( J = 7.0 \) Hz, 6H);

\(^{13}\)C NMR (101 MHz, CDCl\(_3\)): \( \delta \) 163.40, 156.43, 153.28, 152.06, 141.74, 139.92, 136.86, 136.26, 135.90, 128.81, 128.03, 122.49, 119.61, 112.36, 112.26, 111.46, 97.32, 88.85, 84.11, 77.40, 62.15, 58.44, 45.31, 28.87, 25.87, 24.86, 21.06, 18.46, 12.80. HRMS:(ESI, m/z) Calculated for C\(_{38}\)H\(_{43}\)BN\(_3\)O\(_3\)^{3+} \([\text{M}]^+\): 600.3398, found: 600.3416.

**3. Reaction Mechanism Verification**

To understand the reaction mechanism, high-resolution mass spectrometry (HRMS) analysis of the probe MC-V-P and ONOO\(^-\) reaction mixture was performed. The reaction product was verified by high-resolution mass spectrometry. As shown in Figure S2, probe MC-V-P itself presents a positive ion peak with an obvious mass spectrum peak at m/z = 600.3416, and when the probe MC-V-P reacted with ONOO\(^-\), a clear product peak was detected at m/z = 384.2056. The result confirmed the sensing mechanism of the probe toward ONOO\(^-\), which resulted in the formation of compound 3.
4. Supplemental figures

Figure S2. (a) High-resolution mass spectrum of MC-V-P (b) High-resolution mass spectrum of the probe and ONOO⁻ response products.

Figure S3. (a) Fluorescence spectrum of the response product (10 μM) in PBS, λ<sub>ex</sub> / λ<sub>em</sub> = 485 /580 nm. (b) UV-visible absorption spectrum and fluorescence emission spectrum of MC-V-P (10 μM) in PBS. Abs / λ<sub>em</sub> = 640 /740 nm.

Figure S4. Stability of MC-V-P (10 μM) at different pH values
Figure S5. MTT assay of HepG2 cells in the presence of different concentrations of compound 3.

Figure S6. Test of photo-stability. Fluorescence images were achieved by means of time-sequential scanning of the MC-V-P-loaded (a) or compound 3-loaded (b) HepG2 cells for 10 min. Normalized fluorescence intensity from three regions from 0 to 600 s of time-sequential scanning.

Figure S7 (a) Fluorescence response spectra of MC-V-P (10 μM) to nystatin (10 μM), LPS (2 μg/mL), IFN-γ (50 ng/mL) and UA (100 μM) (b) Response fluorescence response spectra of the
product (10 μM) to Nystatin (10 μM), LPS (2 μg/mL), IFN-γ (50 ng/mL) and UA (100 μM)

5. $^1$H NMR, $^{13}$C NMR and HR MS spectra

Figure. S8 $^1$H-NMR spectrum of compound 2 in CDCl$_3$ (400 MHz).

Figure. S9 $^{13}$C-NMR spectrum of compound 2 in CDCl$_3$ (400 MHz).
Figure. S10 HR MS spectrum of compound 2

Figure. S11 $^1$H-NMR spectrum of compound 3 in CDCl$_3$ (400 MHz).
Figure S9 13C-NMR spectrum of compound 3 in CDCl$_3$ (400 MHz).

Figure S13 HR MS spectrum of compound 3
Figure. S14 $^1$H-NMR spectrum of MC-V-P in CDCl$_3$ (400 MHz).

Figure. S15 $^{13}$C-NMR spectrum of MC-V-P in CDCl$_3$ (400 MHz).
Figure S16 HR MS spectrum of MC-V-P