Supporting Information

Visualization of peroxynitrite/GSH cross-talk in the oxidant-antioxidant balance by a dual-fluorophore and dual-site based mito-specific fluorescent probe

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**Materials and methods.** All the reagents used in this work were got from suppliers without additional purification. $^{13}$C NMR and $^1$H NMR spectra were all recorded on the JNM-ECS-400 MHz. Mass spectrums (ESI) were performed on Bruker Micro TOF ESI-TOF Mass Spectrometer. The high-resolution mass spectrometry (HRMS) was carried out by a Bruker microTOF-Q II mass spectrometer. Fluorescence spectrums were measured by a Hitachi F-7000 spectrofluorophotometer with both excitation and emission slit widths set at 5.0 nm. The UV-vis spectrums were performed on the Agilent Technologies Cary 5000 Series UV-vis-NIR spectrophotometer. The two-photon absorption was measured using the integrating sphere on FLS920 of Edinburgh Instruments. Cell imaging was got from the Olympus confocal fluorescence microscopy (FV1000 MPE).

**UV-vis and fluorescence spectral measurements.** The stock solution of **CD-NA** ($10^{-3}$ mol/l) was prepared in DMSO. ROS and RSS were all prepared by the reported papers in water. In the process of testing, the solution of **CD-NA** (10 µM, 2 mL) diluted in PBS (0.01 M, 20% EtOH, pH 7.4) was filled into a quartz optical cell. The other specimens to be tested were added respectively by the micropipettes. The excitation of measurements was provided at 420 nm or 600 nm, and the excitation/emission slit widths were all set as 5.0 nm.

The procedures for the preparing ROS and RSS are listed below[1].

**Peroxynitrite (ONOO⁻):** To a mixture of H$_2$O$_2$ (30 %, 0.3 ml) and concentrated H$_2$SO$_4$ (98 %, 0.08 ml) in redistilled H$_2$O (5 ml), the solution of NaNO$_2$ (0.2 g) in ultrapure water (5 ml) was added rapidly. Then the obtained mixture was poured into NaOH aqueous solution (0.5 g, 10 mL) immediately and MnO$_2$ (0.08 g) was introduced to remove excess H$_2$O$_2$. The resulting solution was filtered to afford ONOO⁻.

**Hydrogen peroxide (H$_2$O$_2$):** The H$_2$O$_2$ was purchased from commercial suppliers and diluted by water.

**Hypochlorite (ClO⁻):** The ClO⁻ was obtained from sodium hypochlorite which was purchased from commercial suppliers and diluted by water.

**Superoxide (O$_2$⁻):** O$_2$⁻ was generated from KO$_2$ which was dissolved in DMSO.
Hydroxyl radical (•OH): •OH was prepared by the mixture of H₂O₂ and 10 eq. (NH₄)₂Fe(SO₄)₂.

Nitric Oxide (NO): NO was generated from the aqueous solution of Na₂ [Fe (CN)₅ NO]•2H₂O.

Nitrite (NO₂⁻): NO₂⁻ was prepared from the aqueous solution of sodium nitrite (NaNO₂).

Sulfuretted Hydrogen (H₂S): The H₂S was prepared by sodium sulphide (Na₂S) in deionized water.

Bisulfite ion (HSO₃⁻): The HSO₃⁻ was prepared by sodium hydrogen sulfite (NaHSO₃) in deionized water.

Thiosulfate (S₂O₃²⁻): The S₂O₃²⁻ prepared by sodium thiosulfate (Na₂S₂O₃) in deionized water.

Sulfate Radical (SO₄²⁻): The SO₄²⁻ was prepared by sodium sulfate (Na₂SO₄) in deionized water.

Glutathione (GSH), Cysteine (Cys), Homocysteine (Hcy), Histidine (His), Isoleucine (Iso), Leucine (Leu), Lysine (Lys), Methionine (Met), and Serine (Ser): These amino acid were all prepared in deionized water.

The spectrophotometric method for determining the precise concentration of ONOO⁻ has also been listed[2].

Peroxynitrite (ONOO⁻): The absorbance of the solution was measured at 302 nm, and the concentration of ONOO⁻ was further obtained by the formula [ONOO⁻] = Abs₃₀₂ nm / 1.67 (mM).

Cytotoxicity study. The MTT assay was conducted to evaluate the cytotoxicity. The solutions of CD-NA at different concentrations (0, 2, 5, 10, 20 and 50 µM) were all evaluated in HepG2 cells for 24 h according to the published literature.

Cell fluorescence imaging. HepG2 cells were selected to assess the applicability of CD-NA. The cells were incubated in the atmosphere of 5% CO₂ for 8 h and then treated with CD-NA (10 µM) for 30 min. For the investigation of CD-NA for GSH, the cells were incubated with CD-NA (10 µM) for 30 min and then treated with GSH (3 mM) for 10 min. To investigate the generating of endogenous ONOO⁻, the cells were firstly incubated with bacterial endotoxin lipopolysaccharide (LPS) (1 µg/mL) for 14 h, followed by the addition of the CD-NA (10 µM) for another 30 min. As
the control experiment, the HepG2 cells were also pretreated with 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) (300 µM) for 30 min and then incubated with LPS (1 µg/mL) for 14 h and CD-NA (10 µM) for 30 min. To explore the imaging performance of CD-NA at the organelle level, the cells were incubated with CD-NA (10 µM) for 30 min and treated with Mito-tracker Green or Lyso-tracker Green (2 µM) for another 10 min. All the cells were washed with PBS buffer (pH 7.4, 0.1 M) for three times to remove the free dye before imaging. The HepG2 cells loaded with CD-NA were excited at 850, 559 or 405 nm. The cells stained by Mito-tracker Green and Lyso-tracker Green were excited at 488 nm. The emission was collected in the red channel (620-670 nm) and green channel (445-495 nm) using the Olympus FV1000 laser confocal scanning microscope.

In the end, we examined the ability of CD-NA to visualize the production of ONOO$^-$ in a drug-induced hepatotoxicity model in HepG2 cells. The cells were incubated with CD-NA (10 µM) for 30 min and then incubated with antipyretic acetaminophen (APAP) (100 µM) for 30 min. Furthermore, as the control experiment, the HepG2 cells were pretreated with N-acetyl cysteine (NAC) (100 µM) for 30 min before incubated with APAP (500 µM).

**Response mechanism.** Based on the reported literature, the speculative response mechanism of CD-NA to ONOO$^-$ and GSH was proposed and displayed (Fig. S1, S4). When adding ONOO$^-$, the oxonium group of CD-NA suffered from nucleophilic reaction, denitration and oxidative cleavage, and finally got a carboxylic acid product. The mass spectrum of CD-NA in the presence of ONOO$^-$ was shown in figure S2. The mass spectrum of the carboxylic acid product (MS: 600.16) and the simulated data (MS: 600.16) were shown in figure S3. When adding GSH, the sulfoxide group of CD-NA suffered from nucleophilic got a reduce product. The mass spectrum of CD-NA in the presence of GSH was shown in figure S5. The mass spectrum of the reduce product (MS: 873.31) and the simulated data (MS: 873.31) were shown in figure S6.
Synthetic procedures of CD-NA.

Figure S1. The synthetic procedures of CD-NA.

**Synthesis of CD-NA.** These compounds a, b, c and e have been provided in the literatures [3-6]. The compound e was synthesised by the published literatures. e (1 mmol) was added into the CH$_2$Cl$_2$ solution (20 ml) of e (2 mmol), HATU (2 mmol) and trimethylamine (40 μl), and then stirred for 4 h under argon protection. Then, the reaction mixture was evaporated under reduced pressure and purified by silica gel to give the pure product CD-NA as a mulberry solid (0.29 g, 33 %). $^1$H NMR (400 MHz, ) δ 8.70 (d, J = 7.6 Hz, 1H), 8.58 (d, J = 7.2 Hz, 1H), 8.45 (t, J = 8.2 Hz, 1H), 8.18 (d, J = 7.8 Hz, 1H), 8.09 (d, J = 9.1 Hz, 1H), 7.83 – 7.74 (m, 1H), 7.72 – 7.63 (m, 1H), 7.40 (d, J = 7.5 Hz, 1H), 7.30 (s, 1H), 7.16 (d, J = 9.4 Hz, 1H), 7.13 – 7.06 (m, 1H), 6.99 (d, J = 9.1 Hz, 1H), 6.94 (dd, J = 9.5, 2.1 Hz, 1H), 4.51 – 4.44 (m, 1H), 3.74 (d, J = 5.5 Hz, 1H), 3.62 (dd, J = 14.0, 6.9 Hz, 1H), 3.55 (d, J = 4.0 Hz, 1H), 2.85 – 2.78 (m, 1H), 1.32 (t, J = 7.0 Hz, 1H). $^{13}$C NMR (101 MHz, CDCl3) δ 169.47, 166.67, 166.00, 165.88, 163.48, 163.35, 163.28, 161.58, 158.29, 154.94, 154.78, 147.79, 139.66, 136.14, 133.25, 131.95, 131.28, 131.21, 130.81, 130.54, 129.85, 129.40, 129.19, 128.55, 128.47, 128.43, 128.22, 128.13, 127.50, 125.08, 123.69, 123.36, 117.35, 117.15, 117.13, 116.02, 115.19, 114.08, 108.71, 96.87, 77.16, 52.78, 46.34, 46.19, 46.05, 44.80, 40.40, 39.80, 36.91, 31.60, 12.67. HRMS (M$^+$) found, 889.3043; calculated for C$_{52}$H$_{46}$FN$_4$O$_7$S$^+$, 889.3066.
**Figure S2.** The proposed reaction mechanism of CD-NA and ONOO$^-$. 

**Figure S3.** The mass spectrum of CD-NA in the presence of ONOO$^-$. 
Figure S4. The mass spectrum of CD-NA in the presence of ONOO⁻ and its simulated data.

Figure S5. The proposed reaction mechanism of CD-NA with GSH.
Figure S6. The mass spectrum of CD-NA in the presence of GSH.

Figure S7. The absorption spectrum of CD (red) and the emission spectrum of NA (black).
Figure S8. Fluorescence spectra of CD-NA (10 µM) with NO (0-200 µM) and GSH (0-5 mM) in PBS (0.01 M, 20% EtOH, pH 7.4)

Figure S9. The cytotoxicity test of CD-NA.
**Figure S10.** Two-photon excitation action cross-section of CD-NA.

**Figure S11.** Images of HepG2 cells only incubated with CD-NA (5 µM) for 10 min by the one-photon and two-photon confocal fluorescence microscopy. Scale bar: 10 µm.
Figure S12. $^1$H NMR spectrum of CD-NA (chloroform-d).

Figure S13. $^{13}$C NMR spectrum of CD-NA (chloroform-d).
**Figure S14.** The HRMS spectrum of CD-NA.

**References**


