Supporting Information

A new long-wavelength fluorescent probe for imaging of peroxynitrite in live cells and inflammatory sites of zebrafish

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1. Synthetic experiment

Preparation and storage of the stock solution of ACDM-BE

**Synthesis of Compound M1**

3-Hydroxy-3-methyl-2-butanone (1.0 g, 9.8 mmol), malononitrile (1.3 g, 19.7 mmol) and sodium ethoxide (0.1 g, 1.47 mmol) were dissolved in ethanol (5 mL). Then the mixed solution was stirred for 1.5 h in ice bath under a N\textsubscript{2} atmosphere, followed by being heated up under the reflux of condensation for 1 h. After cooled down to the ambient temperature, the formed precipitate was separated by filtration and thereafter further purified by recrystallization using ethanol to generate a solid product M1 of pale green (1.23 g, 63%). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 2.37 (s, 3H), 1.63 (s, 6H). \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) \(\delta\) 182.86, 175.32, 111.10, 110.49, 109.03, 104.76, 99.90, 24.37, 14.28.

**Synthesis of Compound M2**

M1 (0.6 g, 3 mmol) and 4-aminobenzaldehyde (0.3 g, 2.5 mmol) were dissolved in ethanol (2 mL) and tetrahydrofuran (8 mL), and then ammonium acetate (77 mg, 1 mmol) was added into the preceding mixture. The mixture was reacted for 24 h under N\textsubscript{2} at room temperature and
dried by rotary evaporator after completion. The crude product was cleansed by silica gel column chromatography (PE/EA, 1/1, v/v) to gain purple solid (0.6897 g, 92%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.88 (d, $J = 15.8$ Hz, 1H), 7.67 (d, $J = 8.7$ Hz, 2H), 6.82 (d, $J = 15.8$ Hz, 1H), 6.66 (d, $J = 8.7$ Hz, 2H), 1.74 (s, 6H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 177.86, 176.14, 155.78, 150.36, 134.08, 122.51, 114.70, 113.98, 113.15, 112.54, 108.18, 98.56, 92.03, 51.23, 26.14. HRMS (ESI) calc. for C_{18}H_{14}N_4O 302.1168, found 325.1064 [M+Na]+.

Scheme S3. Synthesis of compound M2

Synthesis of ACDM-BE

Scheme S4. Synthesis of target probe ACDM-BE

2. Configuration of active oxygen

ROO•
APPH (1 M, 2,2’-azobis (2-amidinopropane) dihydrochloride) was dissolved in ultrapure water under stirring at the temperature of 37 °C for 0.5 h to produce ROO•.

•O$_2$–
KO$_2$ solution (0.25 M) and 18-crown-6 ether (2.5 equivalents) were dissolved in solution of DMSO to generate O$_2$–.

•HO
Hydroxyl radicals were generated by Fenton reaction. Hydrogen peroxide (H$_2$O$_2$, 10 equivalents) was supplemented to a solution of Fe (ClO$_4$)$_2$ to produce •HO.
\( ^1\text{O}_2 \)

NaMoO\(_4\) (20 mM) and H\(_2\)O\(_2\) (20 mM) were dissolved in ultrapure water, and then which were mixed equally to obtain \( ^1\text{O}_2 \) (10 mM).

**ONOO\(^-\)**

3 mL of sodium nitrite (0.6 M), 1.5 mL of hydrochloric acid (0.6 M), 1.5 mL of hydrogen peroxide (0.7 M) and 3 mL sodium hydroxide (3 M) were simultaneously mixed at 0 °C to obtain a resultant solution of pale yellow, and then the obtained solution was dispensed into 1 mL of centrifuge tubes and stored at -20 °C. The UV absorption value collected at 302 nm is measured by using Hitachi U-3900 UV/VIS spectrophotometer, and the corresponding concentration was obtained by Lambert Beer's law \( (\varepsilon = 1670 \text{ M}^{-1}\text{CM}^{-1}) \).

\( -\text{OCl} \)

The hypochlorite concentration was determined using Hitachi U-3900 UV/VIS spectrophotometer and the value of absorbance was collected at 292 nm. Then the corresponding concentration was obtained according to Lambert Beer's law \( (\varepsilon = 360 \text{ M}^{-1}\text{cm}^{-1}) \).

\( \text{H}_2\text{O}_2 \)

The hydrogen peroxide concentration was determined using Hitachi U-3900 UV/VIS spectrophotometer and the value of absorbance was measured at 240 nm. Then the corresponding concentration was obtained by Lambert Beer's law \( (\varepsilon = 43.6 \text{ M}^{-1}\text{cm}^{-1}) \).

3. **The detailed mechanism of the reaction between ACDM-BE and ONOO\(^-\)**
Figure S1. Proposed detailed principle of the reaction between ACDM-BE and ONOO⁻.

4. Absorbance spectra of probe ACDM-BE
Figure S2. Absorbance spectrum of ACDM-BE (5 μM) at different concentrations of ONOO⁻ (0-10 μM), 3 minutes wait in ACN/PBS buffer solution (0.1 M, pH 7.4, v/v, 3/20).

5. UV-Vis Analysis and fluorescence of ACDM-BE

Figure S3. Absorbance spectrum of probe ACDM-BE (5 μM) before (black curve) and after (red curve) addition of ONOO⁻ (10 μM) in ACN/PBS buffer solution (0.1 M, pH 7.4, v/v, 3/20). Inset: visual pictures of ACDM-BE (5 μM) in ACN/PBS buffer solution (0.1 M, pH 7.4, v/v, 3/20) with addition of ONOO⁻ (10 μM) under bright light.
6. Determination of the limit of detection (LOD) and comparisons of ACDM-BE with other fluorescent probes for detecting ONOO⁻

The limit of detection (LOD) was calculated by IUPAC and ACS method.

\[ \text{LOD} = \frac{3 \times \sigma}{s} \]

In this formula, 3 represents the confidence level, \( \sigma \) is the standard deviation (11 times) of blank samples, and \( s \) is defined as the sensitivity of analysis (the slope of the linear equation). \( \sigma \) was determined via the standard deviation of 11 times of independent fluorescence intensity of ACDM-BE (5 \( \mu \)M) without addition of ONOO⁻ at 604 nm, and the value was 1.084574. As shown in Figure 2, the linear relationship between fluorescence intensity of ACDM-BE at 604 nm and low range of concentration of ONOO⁻ (from 0 to 2.0 \( \mu \)M) meets the linear equation \( y = 152.532 x + 39.172 \), so \( s \) was the slope of this linear equation, and that is 152.532. Then we put the obtained values into above equation, and the LOD was calculated as about 21.47 nM.
<table>
<thead>
<tr>
<th>Probe</th>
<th>Fluorescence excitation wavelength</th>
<th>Fluorescence emission wavelength</th>
<th>Limit of detection (LOD)</th>
<th>Literature</th>
</tr>
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<tr>
<td><img src="image1.png" alt="Probe 1" /></td>
<td>430 nm</td>
<td>540 nm</td>
<td>2.5 μM</td>
<td>Chem. Commun., 2014, 50, 9353-9356.(^5)</td>
</tr>
<tr>
<td><img src="image2.png" alt="Probe 2" /></td>
<td>600 nm</td>
<td>638 nm</td>
<td>45 nM</td>
<td>Anal. Chem., 2017, 89, 10924-10931.(^6)</td>
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<tr>
<td><img src="image3.png" alt="Probe 3" /></td>
<td>425 nm</td>
<td>493 nm</td>
<td>150 nM</td>
<td>Chem. Commun., 2014, 50, 9947-9950.(^7)</td>
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<tr>
<td><img src="image4.png" alt="Probe 4" /></td>
<td>640 nm</td>
<td>700 nm</td>
<td>25 nM</td>
<td>Biosens. Bioelectro n., 2015, 64, 285-291.(^8)</td>
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<tr>
<td><img src="image5.png" alt="Probe 5" /></td>
<td>475 nm</td>
<td>515/635 nm</td>
<td>49.7 nM</td>
<td>Biosens. Bioelectro n., 2017, 91, 849-856.(^9)</td>
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<tr>
<td><img src="image6.png" alt="Probe 6" /></td>
<td>480 nm</td>
<td>510/606 nm</td>
<td>150.54 nM</td>
<td>Biosens. Bioelectro n., 2017, 90, 75-82.(^10)</td>
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<tr>
<td><img src="image7.png" alt="Probe 7" /></td>
<td>530 nm</td>
<td>578 nm</td>
<td>55 nM</td>
<td>Anal. Chem., 2017, 89, 5519-552511.(^11)</td>
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Table S1. Comparisons of the proposed fluorescent probe for detecting ONOO\(^{-}\) with a series of reported fluorescent probes.

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<thead>
<tr>
<th></th>
<th>540 nm</th>
<th>604 nm</th>
<th>21 nM</th>
<th>This work</th>
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7. pH titration of probe ACDM-BE

In this part, pH device PHB-4 (INESA Scientific Instrument Co., Ltd.) was used, and the calibration was carried out with a standard solution (pH= 6.86 and 4.00 or 9.18). The stock solution (1mM) of probe ACDM-BE for assay in vitro was diluted with ultrapure water to the final working concentration (5 \(\mu\)M), stored under argon or nitrogen at \(\leq -20^\circ\)C. The value of pH was adjusted with hydrochloric (1 M) acid and NaOH solution (1 M). After the pH value stable for 40 s, 3 mL of solution was taken in the cuvette, using Hitachi F-7000 fluorescence spectrometer and Hitachi U-3900 UV/VIS spectrophotometer. The pH titration curve was obtained by the maximum emission value acquired from the test and plotting the pH value. Thereby, the optimal biological test condition of the probe was obtained.

Figure S4. (a) Fluorescence intensities of only probe ACDM-BE (5 \(\mu\)M) and the probe ACDM-BE (5 \(\mu\)M) in the presence of ONOO\(^{-}\) (10 \(\mu\)M) at different value of pH from 3.19 to 10.99. (b) Scatter diagram of fluorescence intensities of only probe ACDM-BE (5 \(\mu\)M) and the probe ACDM-BE (5 \(\mu\)M) in the presence of ONOO\(^{-}\) (10 \(\mu\)M). \(\lambda_{\text{ex}} = 540\) nm. Slit widths: ex = 10 nm, em = 20nm.

8. Selectivity of probe ACDM-BE for various reactive oxygen species (ROS)
Figure S5. Absorbance spectrum of ACDM-BE (5 μM) after addition of ONOO⁻ (10 μM) and a series of interfering reagents (100 μM). From 1 to 28: blank, ClO⁻, Na₂SO₃, NaHSO₃, GSH, Hcy, Cys, H₂S, ROO•, GSH, •O₂⁻, •OH, TBHP, ¹O₂, H₂O₂, Ala, Arg, Asp, Gln, Glu, Gly, Lys, Met, Phe, Ser, Trp, Tyr, ONOO⁻.

Figure S6. Fluorescence spectrum of ACDM-BE (5 μM) after addition of ONOO⁻ (10 μM) and a series of amino acids (100 μM). From 1 to 14: blank, Ala, Arg, Asp, Gln, Glu, Gly, Lys, Met, Phe, Ser, Trp, Tyr, ONOO⁻.

9. Detailed protocols for MTT assay

The cytotoxicity of probe towards CHO-K1 cells applied to the subsequent imaging
experiments was determined by a MTT assay. CHO-K1 cells were planked in a 96-well plate at the density of 5000 cells in each well. After cells were cultured throughout the whole night, the complete medium in each well was removed and whereafter added with 100 µL fresh F-12 involving various concentrations of ACDM-BE (0 µM, 5µM, 10µM, 15µM, 20µM) for 12 h and 24 h, respectively. Cells in medium without ACDM-BE were used as the control group. After removing the medium, 100 µL of a solution of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 1 mg/mL) was added into per well of the 96-well microplate for another 3-4 h. After getting rid of the solution of MTT that no longer needed, 150 µL DMSO (Dimethyl sulfoxide) was added into each well of the plate and then the plates were oscillated in the shaker for 10 minutes. In addition, the final concentration of DMSO is 0.5% for the MTT assay. Finally, the survival rate of cells was obtained by calculating the absorbance at 490 nm through a microplate reader.

![Figure S7](image)

**Figure S7.** Cell viability of ACDM-BE (0, 5, 10, 15, 20 µM) in CHO-K1 cells at 12 h and 24 h. Error bars (SD) represent three independent experiments.

**10. NMR spectrum and HRMS**
Figure S8. $^1$H NMR spectrum of M1.

Figure S9. $^{13}$C NMR spectrum of M1.
Figure S10. $^1$H NMR spectrum of M2.

Figure S11. $^{13}$C NMR spectrum of M2.
Figure S12. HRMS spectrum of M2.
Figure S13. $^1$H NMR spectrum of probe ACDM-BE.

Figure S14. $^{13}$C NMR spectrum of probe ACDM-BE.
Figure S15. HRMS spectrum of probe ACDM-BE.
11. References


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