

# **Supporting Information**

## **BODIPY-Based Ratiometric Fluorescent Probe for Sensing Peroxynitrite in Inflammatory Cells and Tissues**

**Qian Wu, Ziwei Hu, Guoyang Zhang, Yulong Jin \* and Zhuo Wang \***

State Key Laboratory of Chemical Resource Engineering, College of Chemistry,  
Beijing University of Chemical Technology, Beijing 100029, China

\* Correspondence: jinyulong@buct.edu.cn (Y.J.);  
wangzhuo77@mail.buct.edu.cn (Z.W.)

## Reagent and Materials

All reagents were commercially obtained and used without further purification unless otherwise specified. Deionized water was used for all experiments. 4-hydroxybenzaldehyde, 2,4-dimethylpyrrole, trifluoroacetic acid (TFA), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), triethylamine (Et<sub>3</sub>N), boron trifluoride diethyl etherate (BF<sub>3</sub>·OEt<sub>2</sub>), N,N-dimethylformamide (DMF), phosphorus oxychloride (POCl<sub>3</sub>), 1,2,3,3-tetramethyl-3H-indolium iodide (Indo), Rhodamine 123 (Rh 123), lipopolysaccharide (LPS), carbonyl cyanide-3-chlorophenylhydrazone (CCCP),  $\lambda$ -carrageenan ( $\lambda$ -carr), dimethyl sulfoxide (DMSO), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydrochloric acid (HCl), sodium nitrite (NaNO<sub>2</sub>), and sodium hydroxide (NaOH) were purchased from Beijing Innochem Technology Co., Ltd. Thin-layer chromatography (TLC) plates and silica gel were supplied by Qingdao Ocean Chemical.

All cell culture reagents were sterile and handled in a biosafety cabinet to ensure a contamination-free environment. High-glucose Dulbecco's Modified Eagle's Medium (DMEM), heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin, and phosphate-buffered saline (PBS) were purchased from Hyclone. The human blood sample was provided by Beijing China-Japan Friendship Hospital. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit was obtained from KeyGEN BioTECH (Jiangsu, China).

## Instruments

<sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded on a Bruker Avance III spectrometer (400 MHz, Germany). UV-vis spectra were recorded using a UV-vis spectrophotometer (HITACHI, U3900H, Japan), and fluorescence spectra were obtained on a fluorescence spectrophotometer (HITACHI, F-4600, Japan). A laser scanning confocal microscope (Leica SP8, Germany) was used for cell imaging. Cell viability measurements were recorded using a microplate reader (PerkinElmer Enspire, USA). Fluorescence signals from the mice were captured using an IVIS Lumina IV system (PerkinElmer Enspire, USA).

## Sample Preparation and Testing.

The probe MOBDP-I was dissolved in DMSO to prepare a stock solution (5 mM). The stock solution of ONOO<sup>-</sup> was prepared by simultaneously adding 0.6 M NaNO<sub>2</sub>, 0.6 M HCl, and 0.7 M H<sub>2</sub>O<sub>2</sub> to a 1.5 M NaOH solution at 0 °C. The concentration of ONOO<sup>-</sup> was determined by measuring its absorbance at 302 nm, using an extinction coefficient of 1670 M<sup>-1</sup>·cm<sup>-1</sup>. Fluorescence and absorption measurements were conducted in PBS (10 mM, pH 7.4) with a final concentration of 10  $\mu$ M for MOBDP-I.

## Cell Culture

HeLa cells and RAW264.7 cells were purchased from the Chinese National Infrastructure of Cell Line Resource. HeLa cells and RAW264.7 cells were cultured in high-glucose DMEM supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). The cells were incubated at 37 °C in a 5% CO<sub>2</sub> environment. Before cell staining, all cells were plated in a confocal petri dish and cultured for 24 h.

## Cytotoxicity Assays

The effects of MOBDP-I on cell viability were assessed using the standard MTT assay. HeLa cells and RAW264.7 cells in the logarithmic growth phase were seeded into 96-well plates at a density of approximately 1  $\times$  10<sup>4</sup> cells per well. After 24 h of cell attachment, the original medium was removed, and the cells were washed with PBS buffer (pH 7.4). 100  $\mu$ L of MOBDP-I (0 - 40  $\mu$ M) was added to each well and incubated for 24 h. Subsequently, 50  $\mu$ L of MTT solution was

added to each well. After 4 h of incubation, the culture medium was removed, and 150  $\mu$ L of DMSO was added to dissolve the resulting formazan crystals. After 10 min, absorbance was measured at 490 nm using a microplate reader. Cell viability (%) was calculated using the formula:

$$\text{Cell viability (\%)} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}} \times 100 \quad (\text{S1})$$

OD<sub>sample</sub>, OD<sub>control</sub>, and OD<sub>blank</sub> denote the cells incubated with various concentrations of the MOBDP-I, the cells without the MOBDP-I, and the wells containing only the culture media, respectively.

### Hemolysis Experiment

Blood samples from mice were collected using vacuum blood collection tubes. 2 mL PBS (pH = 7.4) was added to 1 mL blood sample, centrifuged at 8000 rpm for 10 min, and red blood cells in serum were separated. Then the red blood cells were washed with PBS for 5 times and resuspended in 10 mL PBS. 0.2 mL of the hemoglobin dispersion was mixed with (a) 0.8 mL of PBS as a negative control, (b) 0.8 mL of deionized water as a positive control, and (c) 0.8 mL of different concentrations of probe MOBDP-I. After thorough mixing, the samples were allowed to stand at room temperature for 4 h. The samples were then centrifuged at 12000 rpm for 5 min, and hemolysis was assessed by measuring the UV absorbance of the supernatant at 540 nm using a microplate reader. The hemolysis rate was calculated using the following formula:

$$\text{HR} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{PBS}}}{\text{Abs}_{\text{H}_2\text{O}} - \text{Abs}_{\text{PBS}}} \times 100\% \quad (\text{S2})$$

### Cell Imaging Experiment

In cell imaging experiments of exogenous ONOO<sup>-</sup>, HeLa cells ( $3 \times 10^5$ ) were inoculated into confocal culture dishes and incubated for 24 h. The experiment was divided into six groups. The first group of HeLa cells was incubated with MOBDP-I (10  $\mu$ M) for 30 min. The remaining five groups of cells were first incubated with MOBDP-I (10  $\mu$ M) for 30 min, and then co-incubated with different concentrations of ONOO<sup>-</sup> (2, 4, 6, 8 and 10  $\mu$ M) for 30 min. Confocal fluorescence imaging of HeLa cells was measured green and red fluorescence channel.

In the cell imaging experiments of endogenous ONOO<sup>-</sup>, HeLa cells or RAW264.7 cells ( $3 \times 10^5$ ) were inoculated into confocal culture dishes and incubated for 24 h. The experiment was divided into four groups. In the first group, cells were incubated with MOBDP-I (10  $\mu$ M) for 30 min. In the second group, cells were pretreated with LPS (3  $\mu$ g/mL) for 6 h, followed by incubation with MOBDP-I (10  $\mu$ M) for 30 min. The third group was pretreated with LPS (3  $\mu$ g/mL) for 12 h, then incubated with MOBDP-I (10  $\mu$ M) for 30 min. In the fourth group, cells were pretreated with LPS (3  $\mu$ g/mL) for 6 h, treated with NAC (200  $\mu$ M) for 2 h, and finally incubated with MOBDP-I (10  $\mu$ M) for 30 min. Subsequently, the effects of different concentrations of LPS on cells were explored. HeLa cells were incubated with different concentrations of LPS (0, 1, 3 and 5  $\mu$ g/mL) for 3 h, and then co-incubated with MOBDP-I for 30 min. HeLa cells without LPS were used as the control group, and HeLa cells with different concentrations of LPS were used as the experimental group.

### Fluorescence Imaging *in vivo*

C57BL/6 mice (6 - 7 weeks, black, female) were used for fluorescence imaging *in vivo*. The mice were purchased from Beijing Huaifukang Biotechnology Co., Ltd. All animal protocols were approved by the Animal Care Committee of China-Japan Friendship Hospital, with approval number zryhy12-20-10-2. According to the relevant literature, a certain concentration of  $\lambda$ -

Carrageenan ( $\lambda$ -carr) can produce  $\text{ONOO}^-$  at the joints of mice and induce rheumatoid arthritis[1], while a certain concentration of LPS can generate  $\text{ONOO}^-$  in the abdominal area, leading to peritonitis[2]. Additionally, LPS at specific concentrations can cause  $\text{ONOO}^-$  production in the brain of mice, inducing an inflammatory response.[3] Before performing *in vivo* fluorescence imaging, the hair at the imaging site was removed to minimize interference with the imaging results. For the mouse rheumatoid arthritis and peritonitis models, MOBDP-I was injected directly at the site of inflammation, while for the brain inflammation model, MOBDP-I was administered intravenously. The imaging process utilized 5% isoflurane gas with an oxygen flow rate of 1.0 L/min. Fluorescence intensity from the imaging area was analyzed using Living Image software (Life Software 4.5.5).

**Imaging of Arthritis:** the mice were divided into two groups. The left limb of each mouse was used as a control, with an in-situ injection of PBS (50  $\mu\text{L}$ , pH = 7). The right limb was treated with an in-situ injection of  $\lambda$ -carr (50  $\mu\text{L}$ , 5 mg/mL) in the right hind limb joint to induce arthritis. Eight hours later, one mouse received an in-situ injection of NAC (50  $\mu\text{L}$ , 30 mg/kg) into the right hind limb joint. One hour after that, all mice received an in-situ injection of MOBDP-I (20  $\mu\text{M}$ , 50  $\mu\text{L}$  in 1:9 DMSO/PBS) into the hind limb joints, followed by anesthesia for *in vivo* fluorescence imaging.

**Imaging of Peritonitis:** The mice were divided into three groups. The control group received an intraperitoneal injection of PBS (50  $\mu\text{L}$ , pH 7.4). Experimental groups A and B were injected intraperitoneally with LPS (50  $\mu\text{L}$ , 2 mg/mL) to induce peritonitis. Eight hours later, mice in experimental group B were administered an intraperitoneal injection of NAC (50  $\mu\text{L}$ , 30 mg/mL). One hour after NAC administration, all mice received an intraperitoneal injection of MOBDP-I (20  $\mu\text{M}$ , 50  $\mu\text{L}$  in 1:9 DMSO/PBS). The abdominal region of each mouse was then imaged using a 3D quantitative imaging system.

**Imaging of Brain Inflammation:** The mice were randomly assigned to two groups. The control group received an intraperitoneal injection of PBS (100  $\mu\text{L}$ , pH 7), while the experimental group was injected intraperitoneally with LPS (100  $\mu\text{L}$ , 2 mg/mL) to induce brain inflammation. After 8 hours, all mice were injected with MOBDP-I (20  $\mu\text{M}$ , 100  $\mu\text{L}$  of 1:9 DMSO/PBS v/v) via the tail vein. Subsequently, the mice were euthanized, and their brains were harvested for *in vitro* fluorescence imaging.

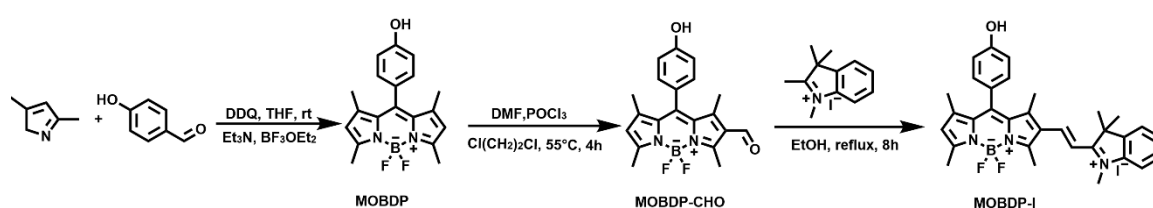


Figure S1. The synthetic route of MOBDP-I.

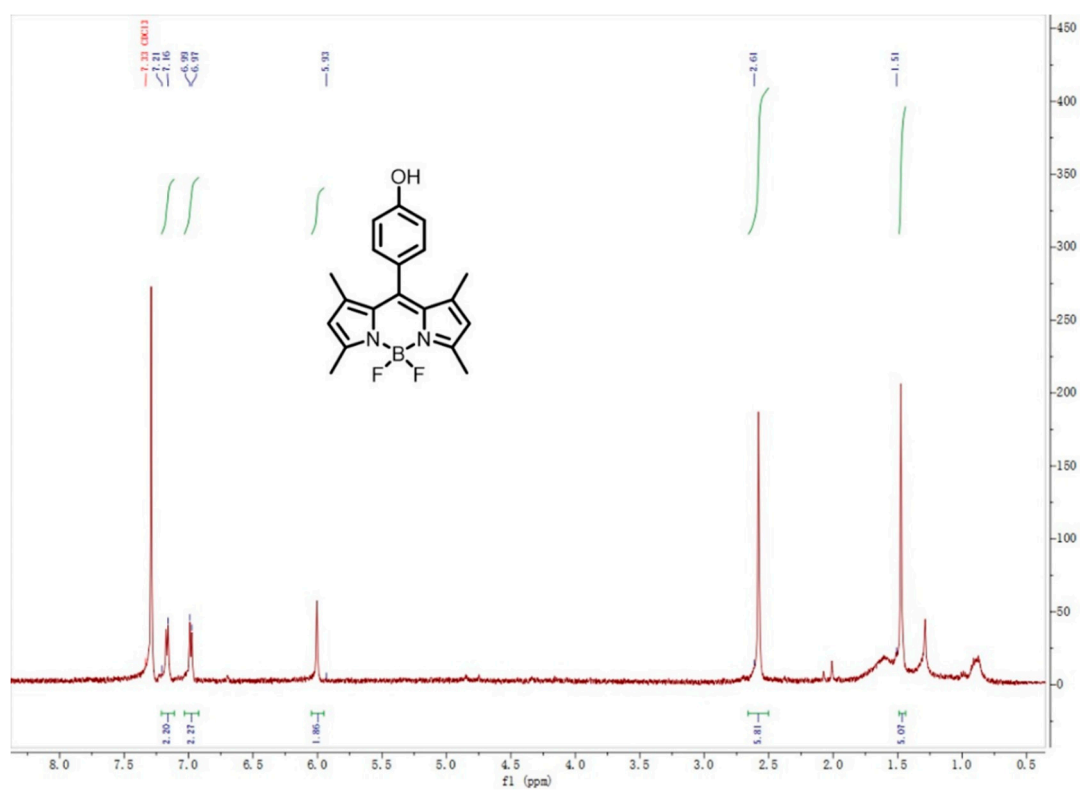


Figure S2. <sup>1</sup>H NMR spectrum of MOBDP in CDCl<sub>3</sub>-d<sub>4</sub>.

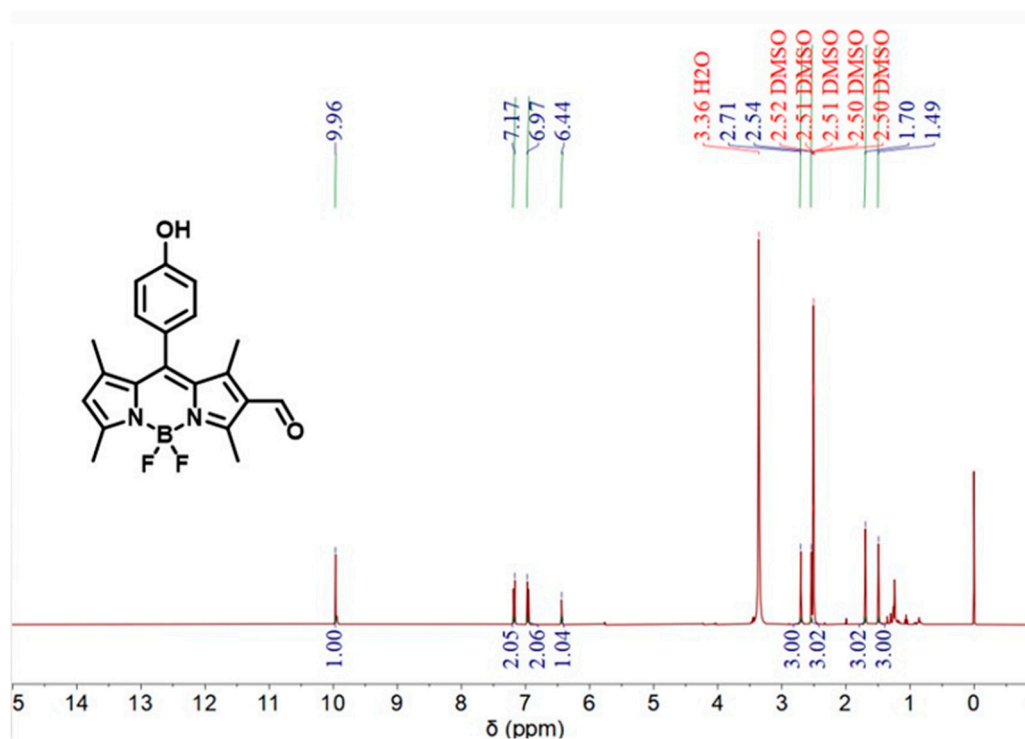


Figure S3. <sup>1</sup>H NMR spectrum of MOBDP-CHO in CDCl<sub>3</sub>-d<sub>4</sub>.

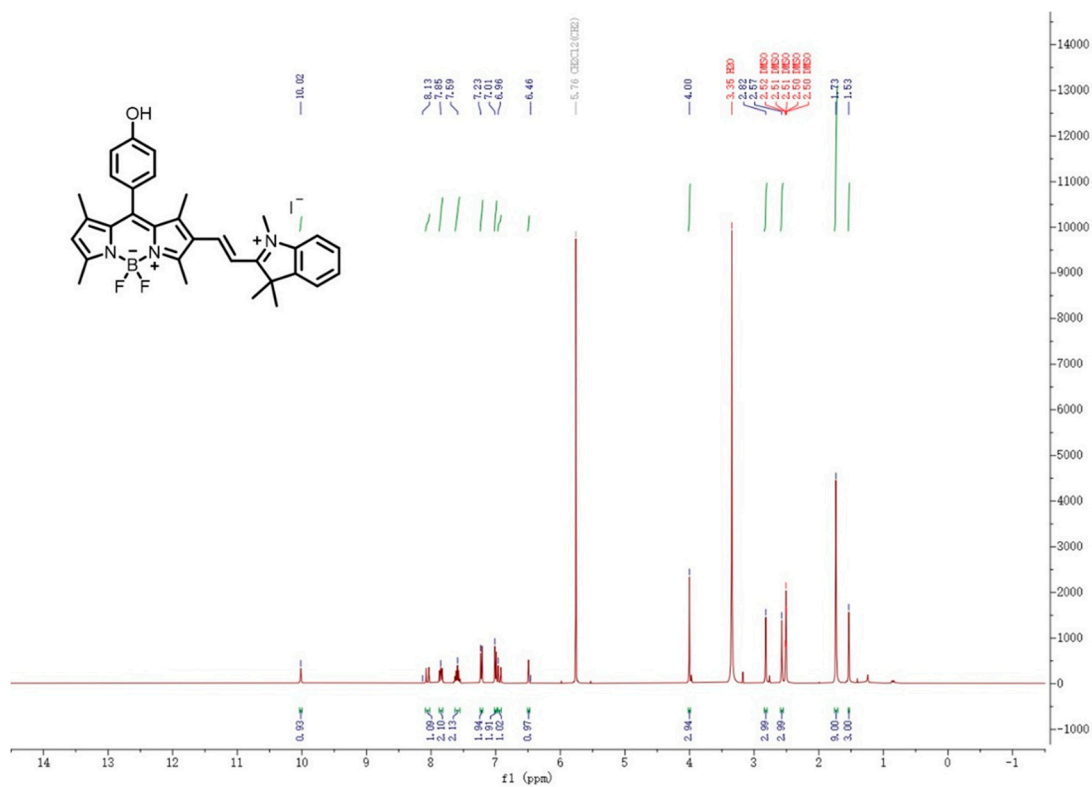


Figure S4. <sup>1</sup>H NMR spectrum of MOBDP-I in DMSO-*d*<sub>6</sub>.

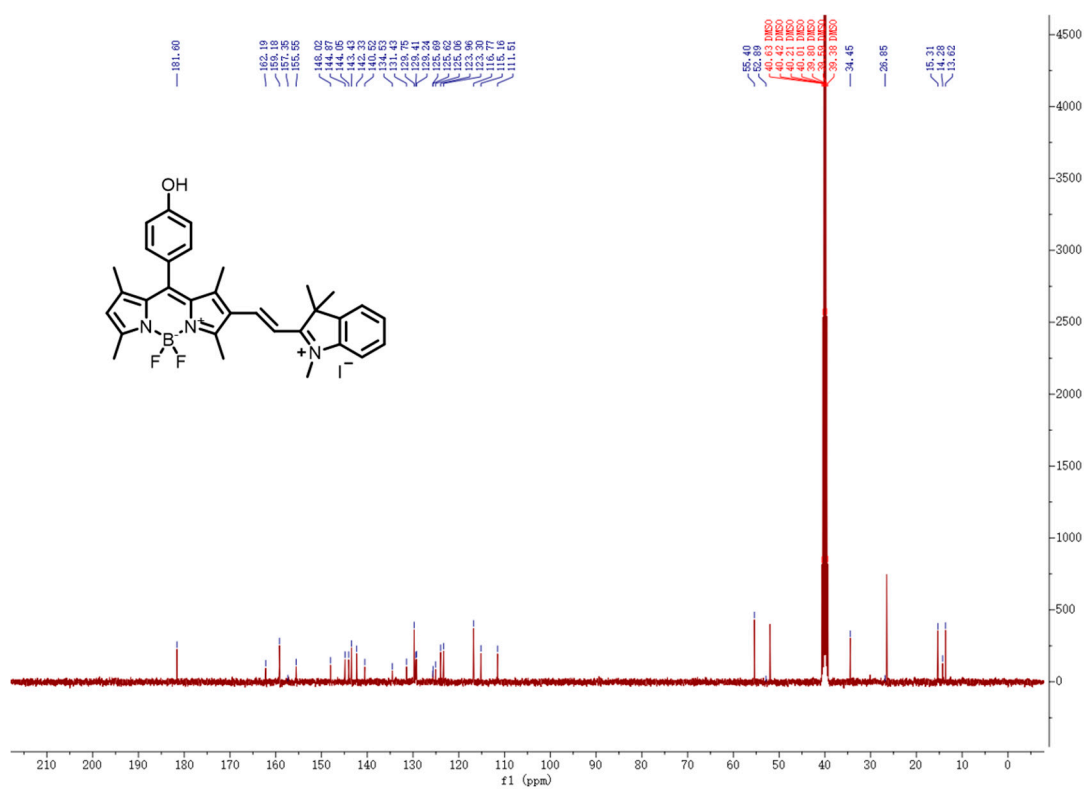


Figure S5. <sup>13</sup>C NMR spectrum of MOBDP-I in DMSO-*d*<sub>6</sub>.

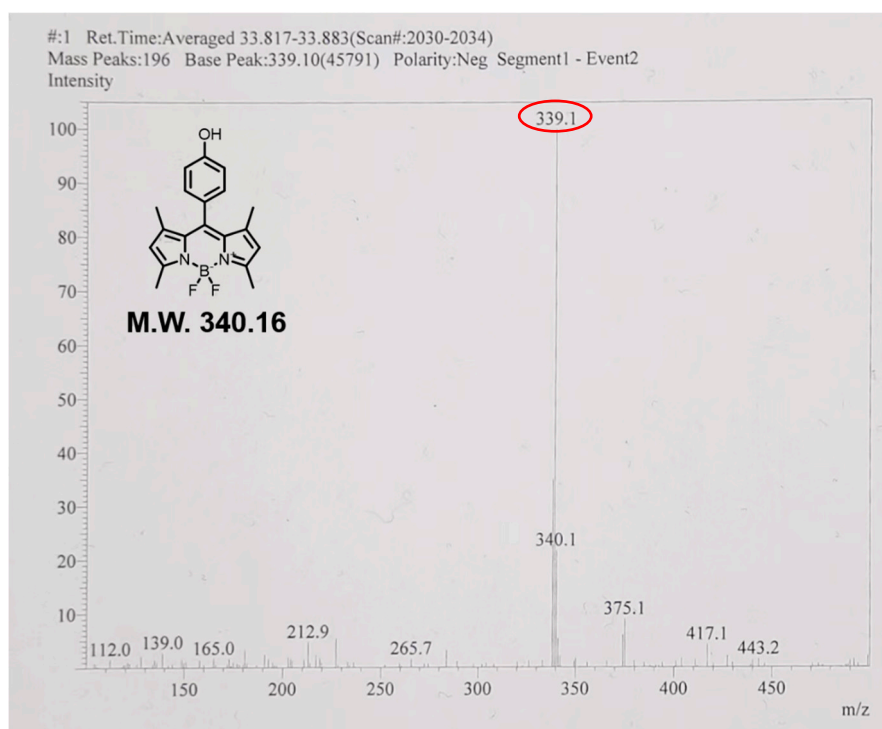


Figure S6. The mass spectrum (ESI) of MOBDP.

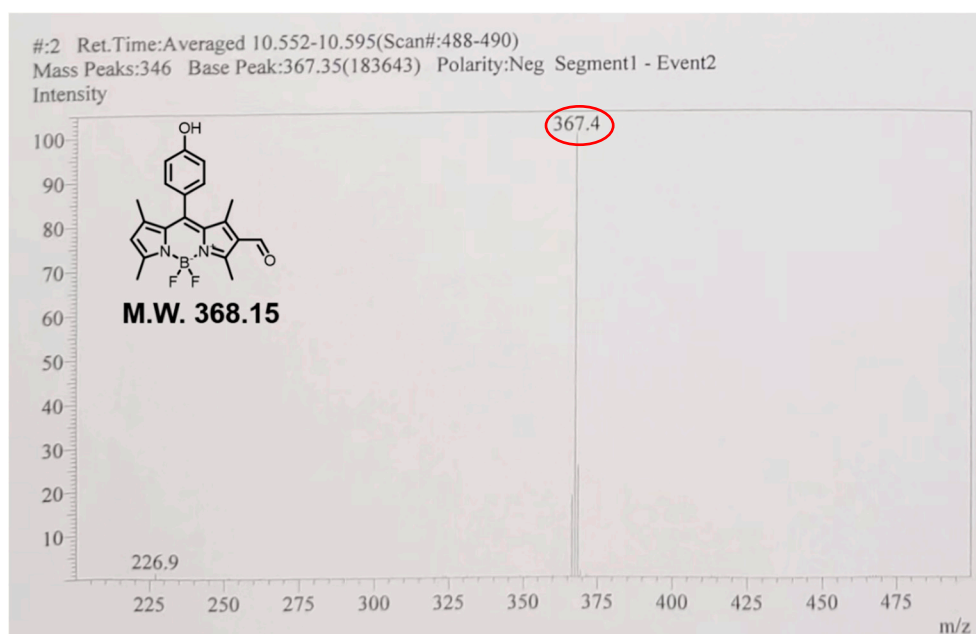


Figure S7. The mass spectrum (ESI) of MOBDP-CHO.

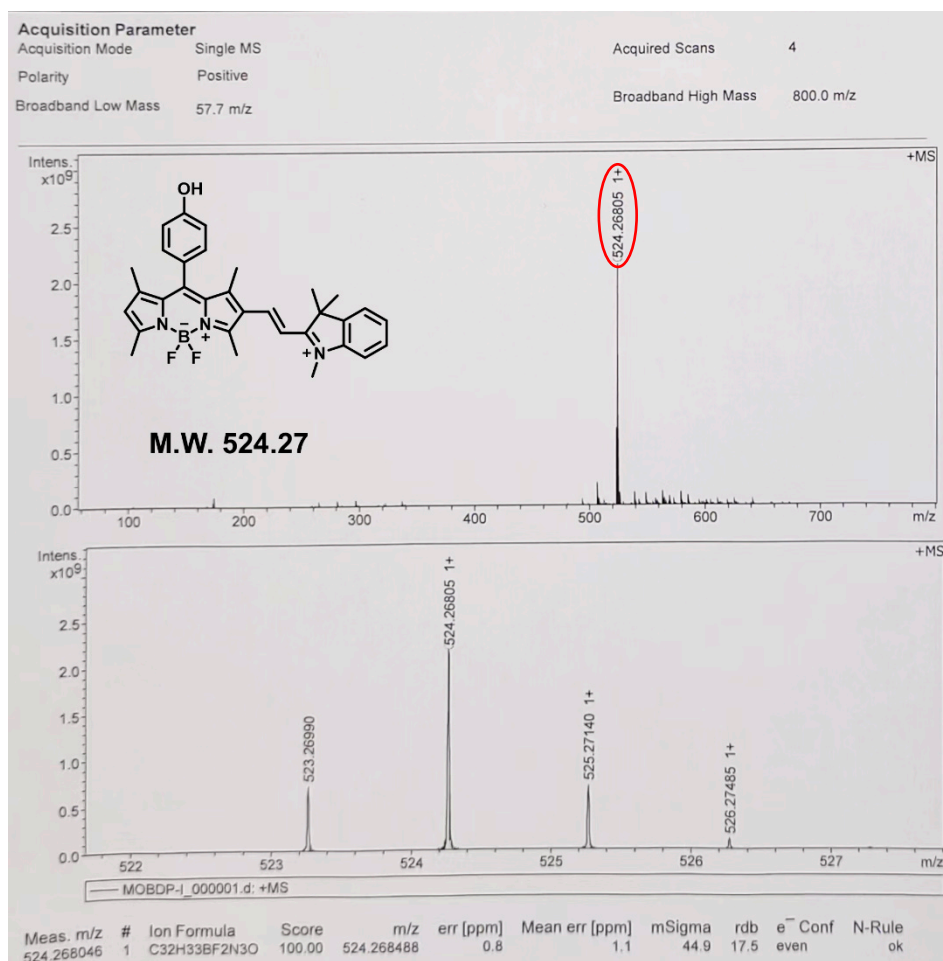


Figure S8. The mass spectrum (HRMS) of MOBDP-I. HRMS (ESI, positive) m/z. calcd for C<sub>32</sub>H<sub>33</sub>BF<sub>2</sub>N<sub>3</sub>O<sup>+</sup> ([M]<sup>+</sup>): 524.2679; found: 524.2680.

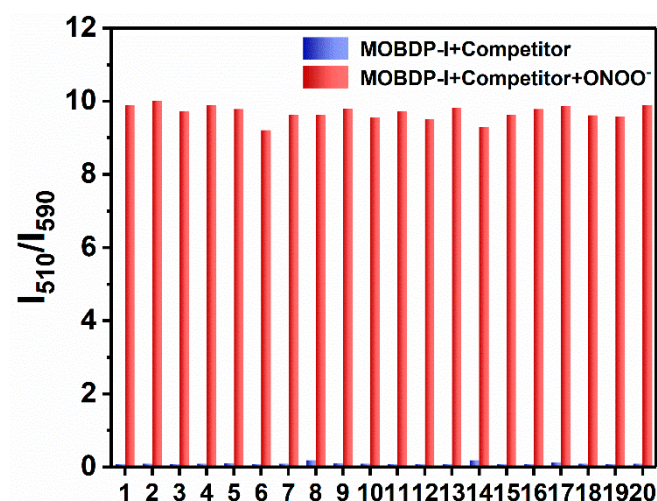


Figure S9. The fluorescence response intensity ( $I_{510}/I_{596}$ ) of MOBDP-I to various analytes (50  $\mu$ M) and ONOO<sup>-</sup> (10  $\mu$ M) + analytes (50  $\mu$ M). Analytes (1-20): <sup>1</sup>O<sub>2</sub>, ClO<sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, ROO<sup>-</sup>,  $\cdot$ OH, NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, H<sub>2</sub>S, SO<sub>3</sub><sup>2-</sup>, HSO<sub>3</sub><sup>-</sup>, GSH, Cys, K<sup>+</sup>, Na<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup>, Ni<sup>2+</sup>, Co<sup>2+</sup>.



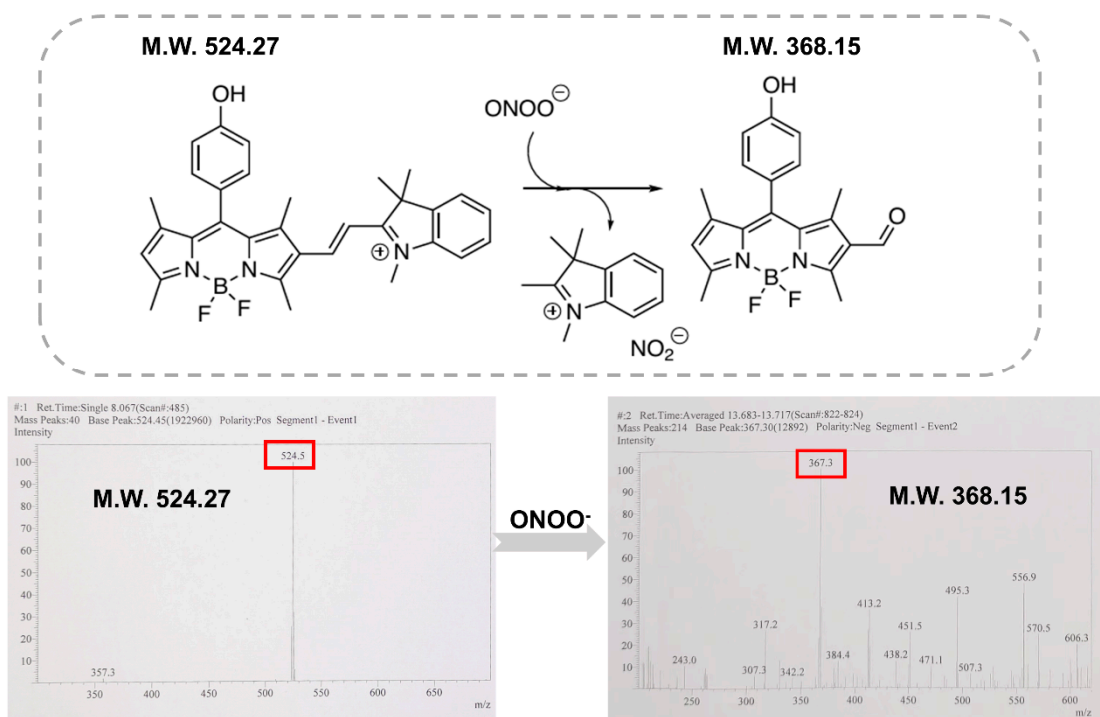


Figure S10. The reaction mechanism of MOBDP-I with  $\text{ONOO}^-$  and mass spectra (ESI) of MOBDP-I and MOBDP-CHO. Note that MOBDP-I was analyzed in positive ion mode. The detected charged species was  $[\text{M}]^+$ , as itself is a cation. The product MOBDP-CHO was analyzed in negative ion mode. The detected charged species should be  $[\text{M}-\text{H}]^-$ .

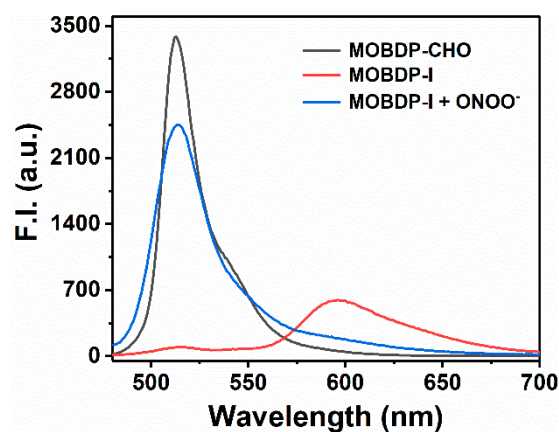


Figure S11. Fluorescence spectra of MOBDP-CHO (10  $\mu\text{M}$ ), MOBDP-I (10  $\mu\text{M}$ ) and MOBDP-I (10  $\mu\text{M}$ ) in response to  $\text{ONOO}^-$  (10  $\mu\text{M}$ ) in PBS (pH = 7.4)

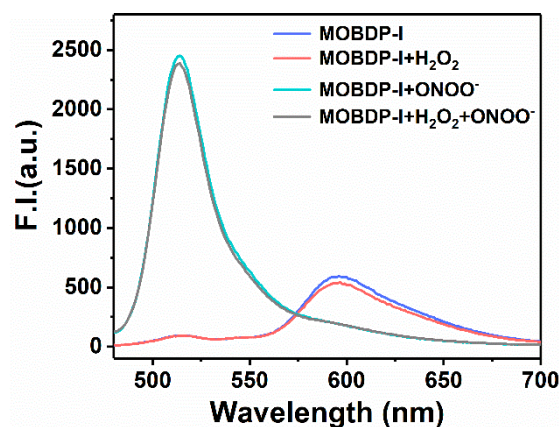


Figure S12. Fluorescence spectra of MOBDP-I (10 μM), MOBDP-I (10 μM) + H<sub>2</sub>O<sub>2</sub> (50 μM), MOBDP-I (10 μM) + ONOO<sup>-</sup> (10 μM) and MOBDP-I (10 μM) + H<sub>2</sub>O<sub>2</sub> (50 μM) + ONOO<sup>-</sup> (10 μM).

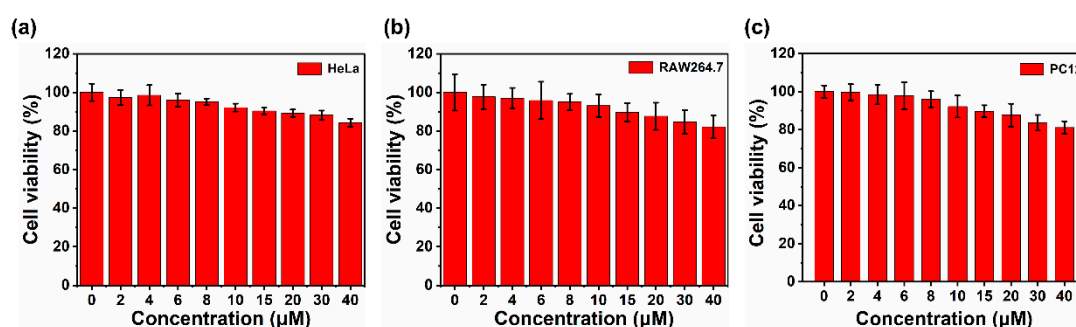


Figure S13. MTT assay of (a) HeLa cells, (b) RAW264.7 cells and (c) PC12 cells in the presence of different concentrations of MOBDP-I (0 ~ 40 μM). The error bars represent ± standard deviation (SD) (n = 3).

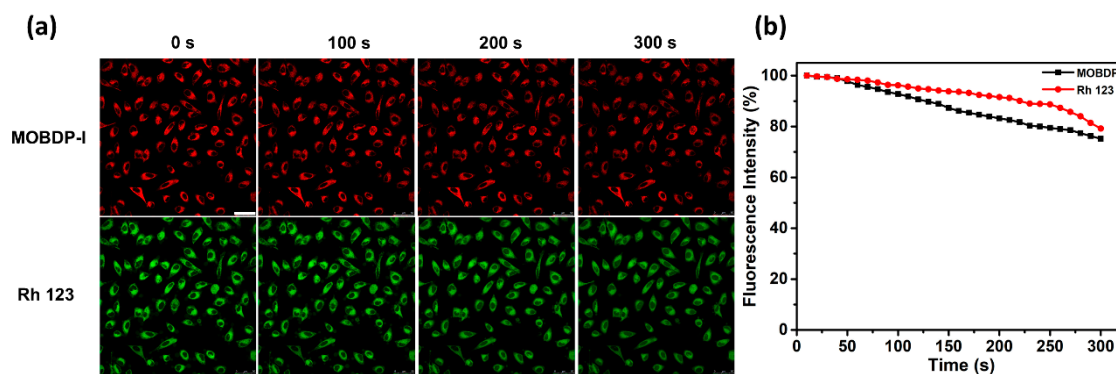


Figure S14. (a) Photostability of MOBDP-I (10 μM) and Rhodamine 123 (5 μM) incubated with HeLa cells, the signal intensity varies with the laser bleaching time. (Red channel:  $\lambda_{\text{ex}}=543$  nm,  $\lambda_{\text{em}}=580-650$  nm. Green channel:  $\lambda_{\text{ex}}=488$  nm,  $\lambda_{\text{em}}=510-540$  nm, scale bar = 50 μm). (b) Normalized intensity profiles of average intracellular signals calculated from the confocal fluorescence images in (a) by applying ImageJ.

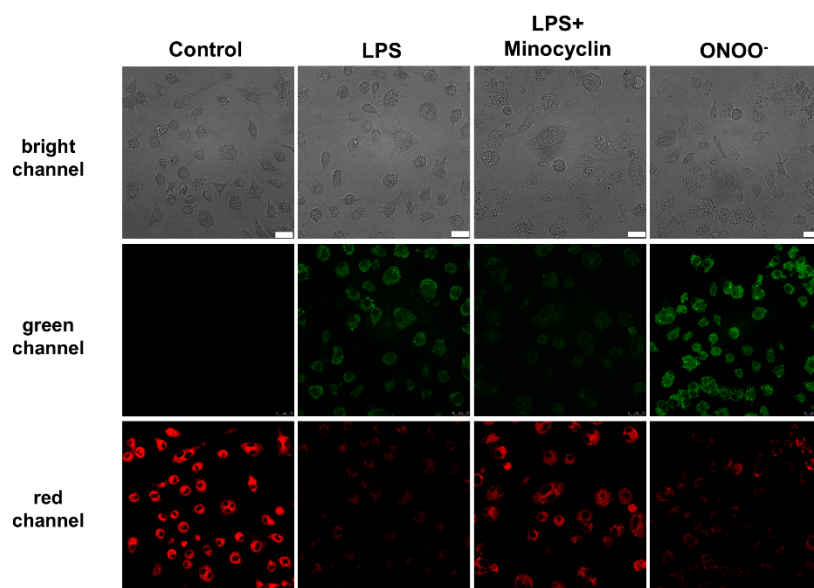


Figure S15. Confocal fluorescence imaging of ONOO<sup>-</sup> in RAW264.7 cells using the probe MOBDP-I (10  $\mu$ M, 30 min). Control group: MOBDP-I only; LPS group: LPS (1  $\mu$ g/mL, 6 h) then MOBDP-I; LPS + Minocycline group: LPS (1  $\mu$ g/mL, 6 h), minocycline (300  $\mu$ M, 2 h), then MOBDP-I; ONOO<sup>-</sup> group: exogenous ONOO<sup>-</sup> (10  $\mu$ M, 30 min), then MOBDP-I. Green channel:  $\lambda_{\text{ex}}$  = 488 nm,  $\lambda_{\text{em}}$  = 510 - 540 nm. Red channel:  $\lambda_{\text{ex}}$  = 543 nm,  $\lambda_{\text{em}}$  = 600 - 650 nm, scale bar: 75  $\mu$ m.

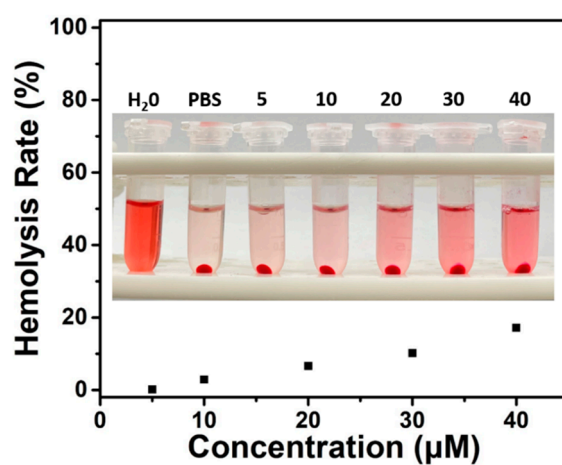


Figure S16. Hemolysis rate at different concentrations of MOBDP-I (0 - 40  $\mu$ M).

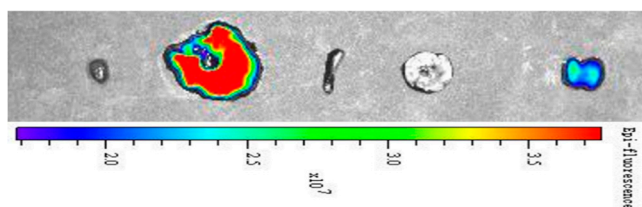


Figure S17. Fluorescence imaging in the major organs (heart, liver, spleen, lung, and kidney) isolated from mice.

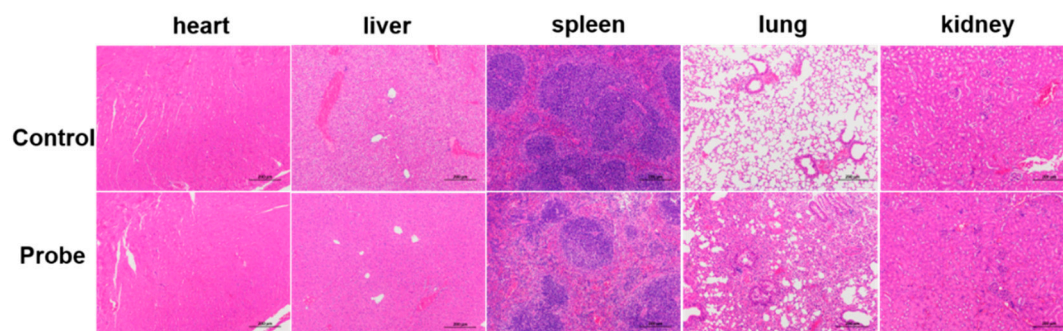
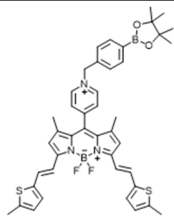
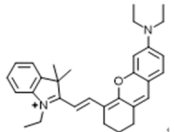
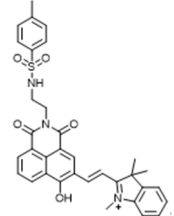
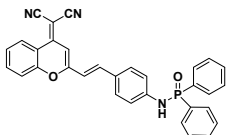
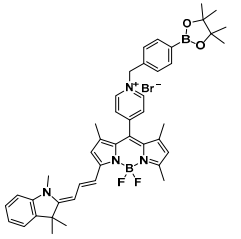
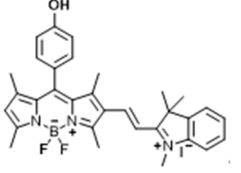


Figure S18. Hematoxylin and eosin (H&E) staining of major organs (heart, liver, spleen, lung, and kidney) collected from mice.

**Table S1.** Comparison of the proposed probe with other reported fluorescence probes for detecting peroxynitrite

No.	Probe	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	SNR (Fold)	LOD	Response time	Biological applications	Ref
1		620/694 nm	66	120 nM	2 min	Imaging in cells and mouse peritonitis	[4]
2		360/487 nm 710/742 nm	436	170 nM	150 s	Imaging in cells and mouse peritonitis	[5]
3		420/527 and 634 nm	22	930 nM	3 min	Imaging in cells and zebrafish	[6]
4		512/685 nm	4.5	96 nM	10 min	Imaging in cell and mice epilepsy model	[7]

5		660/740 nm	247	50 nM	15 s	Imaging in cell and mice epilepsy model	[8]
6		460/510 and 590 nm	44	9.6 nM	1 s	Imaging in cell and mouse models of rheumatoid arthritis, peritonitis and brain inflammation	<b>This work</b>

## References

1. Xu, W.; Yang, Q.; Zeng, J.; Tan, L.; Zhou, L.; Peng, L.; Zhou, Y.; Xie, C.; Luo, K.; Zhang, Z. A biomarker (ONOO<sup>-</sup>)-activated multicolor fluorescent probe for early detection and assessment of arthritis. *Sens. Actuators B Chem.* **2022**, *359*, doi:10.1016/j.snb.2022.131565.
2. Wang, Z.; Wang, W.; Wang, P.; Song, X.; Mao, Z.; Liu, Z. Highly Sensitive Near-Infrared Imaging of Peroxynitrite Fluxes in Inflammation Progress. *Anal. Chem.* **2021**, *93*, 3035-3041, doi:10.1021/acs.analchem.0c05118.
3. Sun, Y.; Tang, X.; Li, X.; Kong, X.; Tian, M.; Wang, Y.; Dong, B. PET-ESIPT-based fluorescent probes for revealing the fluctuation of peroxynitrite (ONOO<sup>-</sup>) in living cells, zebrafishes and brain tissues. *Sens. Actuators B Chem.* **2022**, *353*, doi:10.1016/j.snb.2021.131121.
4. Qin, S.; Ran, Y.; He, Y.; Lu, X.; Wang, J.; Zhao, W.; Zhang, J. Near-Infrared Fluorescence Probe for Visualizing Fluctuations of Peroxynitrite in Living Cells and Inflammatory Mouse Models. *Chemosensors* **2023**, *11*, doi:10.3390/chemosensors11060316.
5. Zhou, D.Y.; Li, Y.; Jiang, W.L.; Tian, Y.; Fei, J.; Li, C.Y. A ratiometric fluorescent probe for peroxynitrite prepared by de novo synthesis and its application in assessing the mitochondrial oxidative stress status in cells and in vivo. *Chem Commun (Camb)* **2018**, *54*, 11590-11593, doi:10.1039/c8cc07389a.
6. Liu, Y.; Ma, Y.; Lin, W. Construction of a bi-functional ratiometric fluorescent probe for detection of endoplasmic reticulum viscosity and ONOO<sup>-</sup> in cells and zebrafish. *Sens. Actuators B Chem.* **2022**, *373*, doi:10.1016/j.snb.2022.132742.
7. Luo, X.; Cheng, Z.; Wang, R.; Yu, F. Indication of Dynamic Peroxynitrite Fluctuations in the Rat Epilepsy Model with a Near-Infrared Two-Photon Fluorescent Probe. *Anal. Chem.* **2021**, *93*, 2490-2499, doi:10.1021/acs.analchem.0c04529.
8. Han, R.; Ma, X.; Wang, J.; Zhang, B.; Ruan, M.; Jiao, J.; Zhao, W.; Zhang, J. Novel BODIPY-based NIR fluorescent probe with appropriate Stokes shift for diagnosis and treatment evaluation of epilepsy via imaging ONOO<sup>-</sup> fluctuation. *Sens. Actuators B Chem.* **2025**, *423*, doi:10.1016/j.snb.2024.136766.