Supporting information

pH/Glutathione-Responsive Release of SO_2 Induced Superoxide Radical Accumulation for Gas Therapy of Cancer

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EXPERIMENT

Reagents and Equipments

4-chlorocarbonylbenzoate, 2,4-Dimethylpyrrole, Triethylamine (Et_3N), Boron trifluoride etherate ($BF_3 \cdot OEt$), 4-Hydroxybenzaldehyde, Acetic Acid Glacial, Piperidine, N,N-Dimethylformamide (DMF), 2,4-Dinitrobenzenesulfonyl Chloride were purchased from Admas and used without further purification. $mPEG_{5K}$-PAE_{10K} was purchased from Xi'an ruixi Biological Technology Co., Ltd and used without further purification. ^1H NMR and ^{13}C NMR spectra were recorded on Bruker DRX NMR spectrometer with tetramethylsilane (TMS) as internal standard. The absorption spectra were measured on UV-3600 UV-vis spectrophotometer (Shimadzu, Japan). The cells fluorescence imaging was recorded by confocal laser scanning microscope (Olympus IX 70 inverted microscope) and inverted fluorescence microscope (Nikon ECLIPSE Ts2R).

Synthesis of BOD-Ester

4-chlorocarbonylbenzoate (3.96 g, 0.02 mol) was dissolved in 250 mL CH_2Cl_2 in nitrogen atmosphere at room temperature, then 2,4-Dimethylpyrrole (2.85 g, 0.03 mol) was added into the reaction mixture and stirred overnight. Next day, 10 mL Et_3N were added into the above at 0 °C and after 30 min, 10 mL $BF_3 \cdot OEt_2$ was injected. After 2 h, the mixture was washed with saturated saline and dried with Na_2SO_4. Then the solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography (PE:DCM=2:1) to afford an orange solid product. (1.31 g, yield: 23%) ^1H NMR (300 MHz, $CDCl_3$): δ 8.27-8.15 (d, J = 3.6 Hz, 2H), 7.50-7.40 (d, J = 3, 2H), 6.01 (s, 2H), 3.99 (s, 3H), 2.58 (s, 6H), 1.38 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.82, 164.81, 158.18, 154.77,

154.74, 137.14, 115.33, 112.35,101.16, 49.69, 45.35, 45.14, 44.93, 44.72, 44.51, 44.30, 44.09, 17.59.

Synthesis of BODEsOH

BOD-Ester (500 mg, 1.3 mmol) and 4-Hydroxybenzaldehyde (400 mg, 3.25 mmol) were dissolved in 20 mL DMF under N_2 atmosphere, then 0.5 mL acetic acid and 0.5 mL piperidine were added respectively, and the mixture was stirred for 6 h at 120 °C. The mixed solution above was extracted with ethyl acetate and water, then was washed with saturated saline and dried with Na_2SO_4. Afterward, the solvent was evaporated under reduced pressure and the crude product was purified by silica gel column chromatography (DCM:EA=3:2) to afford a dark golden yellow product (0.268 g, yield: 35%). (268 mg, yield: 35%) ^{1}H NMR (300 MHz, DMSO-D_6): δ 9.99 (s, 2H), 8.11-8.07 (d, *J* = 1.2, 2H), 7.59-7.55 (d, *J* = 1.2, 2H), 7.48-7.42 (d, *J* = 1.8, 6H), 7.35-7.28 (d, *J* = 2.1, 2H), 6.89 (s, 2H), 6.86-6.80 (d, *J* = 1.8, 4H), 3.96 (s, 3H), 1.33 (s, 6H). ^{13}C NMR (75 MHz, DMSO-D_6): δ 166.35, 159.65, 159.64, 153.09, 141.54, 137.92, 139.91, 136.59, 132.40, 130.43, 129.84, 129.68, 127.82, 118.70, 116.66, 52.95, 40.61, 40.40, 40.19, 39.98, 39.77, 39.56, 39.56, 39.35, 14.88.

Synthesis of BODS

BODEsOH (200 mg, 0.3 mmol) was dissolved in 30 mL CH_2Cl_2 and 100 μL Et_3N was added under N_2 atmosphere at 0 °C. Then 2,4-Dinitrobenzenesulfonyl Chloride (200 mg, 0.75 mmol) dissolved in CH_2Cl_2 was added dropwise into the above. After 5 h, the starting materials were found to be totally consumed by TLC monitoring. The H_2O was added to quench the reaction and CH_2Cl_2 was used to extracte mixed production, then the solution was washed with saturated saline and dried with Na_2SO_4. Subsequently, the solvent was

evaporated under reduced pressure and the crude product was purified by silica gel column chromatography (DCM) and recrystallzed by CH_2Cl_2/ CH_3OH to afford a purple solid product. (148 mg, yield: 47%) ^{1}H NMR (300 MHz, DMSO-D_6): δ 9.11-9.09 (d, *J* = 0.6, 2H), 8.60-8.55 (d, *J* = 1.5, 2H), 8.25-8.20 (d, *J* = 1.5, 2H), 8.13-8.08 (d, *J* = 1.5, 2H), 7.65-7.55 (d, *J* = 3.0, 8H), 7.50-7.40 (d, *J* = 3.0, 2H), 7.25 (d, 4H), 6.96 (s, 2H), 3.87(s, 3H), 1.36(s, 6H). ^{13}C NMR (75 MHz, DMSO-D_6): δ 166.29, 152.54, 152.13, 149.18, 148.66, 142.71, 139.27, 139.00, 136.58, 136.10, 134.25, 133.02, 131.06, 130.95, 130.55, 129.61, 129.56, 128.04, 123.38, 121.61, 119.85, 119.49, 52.99, 40.62, 40.41, 40.20, 39.99, 39.78, 39.57, 39.37, 14.94.

Preparation of BODS NPs

4 mg $mPEG_{5K}$-PAE_{10K} was dispersed in 10 mL DI water, then 2 mg BODS dissolved in 0.5 mL THF was added rapidly under ultrasound. After 20 min, the THF was removed to obtain BODS NPs.

After preliminary preparation of nanoparticles, uncoated and oversized particles were filtered and the unloaded compound was washed with dichloromethane (DCM). The UV absorption of the unloaded compound was measured and its encapsulation efficiency was calculated according to standard curve.

SO_2 Release Experiment

7-diethylaminocoumarin-3-aldehyde (DEACA) was used to measure SO_2 generation. In general, DEACA solution was added to BODS NPs PBS buffer solution, then added excess GSH. After that, fluorescence intensity of the mixture was measured by fluorescence spectrophotometer at intervals. (EX: 390 nm)

Cell Culture and Cytotoxicity Assay

HeLa and HepG2 cells were cultured in DMEM containing 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U/mL) at 37 °C in 5% CO_2.

HeLa and HepG2 cells were incubated with different concentration of BODS NPs for 24 h in 96-well plates. Then per well was added 20 μL MTT (5 mg/mL) and incubated for another 4 h. And the cells were tested by classical MTT assay to assess the cell viability.

Cellular uptake and Lysosomal escape

HeLa cell was incubated with 65 μg/mL BODS NPs for different time in 6-well plates, then washed with PBS for 2 or 3 times to remove excess material, the 500 μL lysosomal green fluorescent probe was added at 37 °C for 30min. The probe was removed and washed with PBS solution for 3 times again, and 2 mL PBS was used to keep the morphology of cell. The fluorescence imaging of the cells was observed by confocal laser scanning microscope. BODS NPs were excited at a wavelength of 570 nm and lysosomal green fluorescent probes were excited at 488 nm. (Cellular uptake experiment was conducted without probe)

ROS detection, Live-dead cell staining and JC-1 experiment

In general, the HeLa cells were pre-treated with BODS NPs, then different probes were added into plates at 37 °C for 30 min. The probe was removed and washed with PBS solution for 3 times again. The cells staining results were obtained through an inverted fluorescence microscope (Nikon ECLIPSE Ts2R).

SOD Activity, H_2O_2 detection and ATP assay

The HeLa cells were cultured in 6-well plates and treated with BODS NPs. After washing with PBS, the cells were tested according to the assay kit. The SOD activity and H_2O_2 detection were tested in 96-well plates with a Microplate reader. The ATP level tested in 96-

well plates with a luminometer.

Flow cytometer assay

The HeLa cells were incubated with 65 μg/mL BODS NPs for different incubation time (0 h, 8 h, 12 h, 24 h) in 6-well plates, collected with 1.5 mL centrifuge tubes and washed with PBS to reduce the influence of trypsin, then stained with Propidium Iodide (PI) and Annexin V-FITC. Finally, the signal of each sample was detected by a flow cytometer (PerkinElmer).

Electron spin-resonance

The ESR spectrum was divided into five groups. (1) supernatant collected from cell culture medium treated with BODS NPs after cell disruption with glass homogenizer (2) 300 μM BODS NPs, 50 μM Cu/Zn-SOD in pH 6.5 phosphate buffer (3) 300 μM BODS NPs, excessive GSH in pH 6.5 phosphate buffer (4) 50 μM Cu/Zn-SOD, 100 mM DMPO, excessive GSH and 300 μM BODS NPs in pH 7.4 phosphate buffer (5) 50 μM Cu/Zn-SOD, 100 mM DMPO, excessive GSH and 300 μM BODS NPs in pH 6.5 phosphate buffer and reacted for 5 hours respectively, then recorded at room temperature.

Hemolysis Assay

The erythrocyte was collected by washed blood with PBS, then treated with deionized, saline and different concentration of BODS NPs for 6 h at room temperature. After centrifugation (3000 rpm), the absorbance of the supernatants was recorded at 541 nm. Hemolysis equation: Hemolysis = $(A_{sample}-A_{negative})/(A_{positive}-A_{negative})$.

Fluorescence imaging

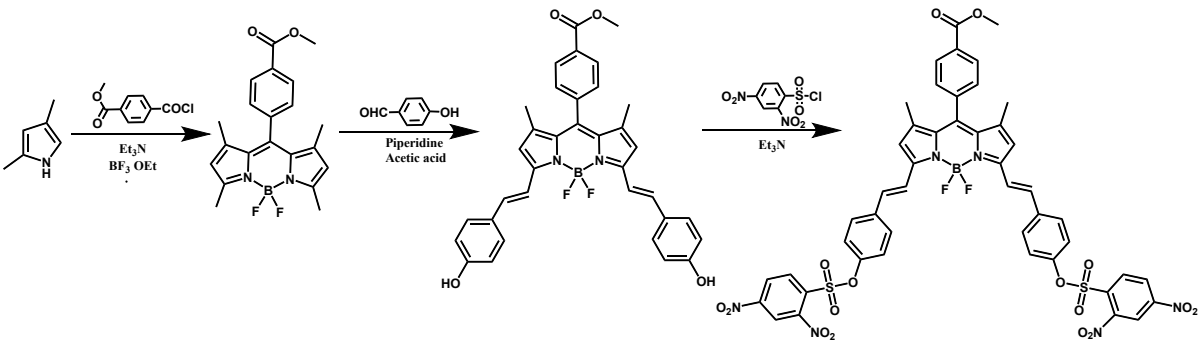
Nude mice are anesthetized with 10% chloral hydrate before injected intravenously with 170 μg/mL BODS NPs and the fluorescence distribution and intensity were recorded at different

time points (0, 2, 8, 12, 24, 36 h) by living fluorescence imager. After 36 h, the mice were sacrificed and the major organs were taken out to detect fluorescence intensity under 660 nm excitation wavelength.

In vivo gas therapy

All mouse studies were conducted in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals (Ministry of Health, China) and the protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Tech University. The tumor-bearing mice were subjected to indicated treatments and small-animal PET studies when the tumor volume reached 100 mm^3 and 100-300 mm^3, respectively. Ethical compliance with the IACUC protocol was maintained. None of the experiments did the size of tumor graft surpass 2 cm in any two dimensions (according to the limits defined by the IACUC protocol) and no mouse had severe abdominal distension (≥10% increase of original body weight).

The tumor-bearing model was constructed by subcutaneous injection with cell. When the tumor volumes reached about 100-110 mm^3, nude mice were divided into two groups, injected intravenously with 170 μg/mL BODS NPs and PBS every two days. The weight and tumour volume of mice were recorded. After treatment, the mice were sacrificed, and the major organs and tumor section were separated from bodies, then these tissue sections were collected for H&E staining.



Scheme S1. Synthetic routes of the BODS.

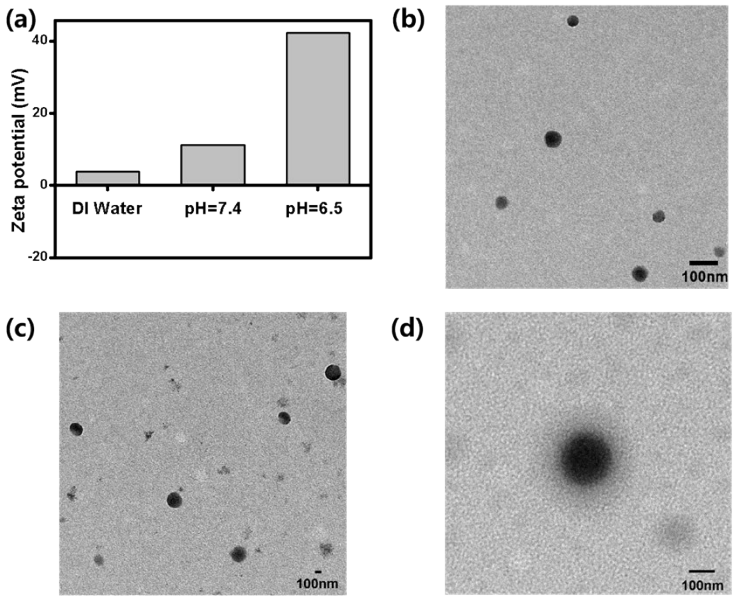


Figure S1. (a) Zeta potential of BODS NPs in DI water, pH=7.4 PBS and pH=6.5 PBS. (b) TEM image of the BODS NPs in pH=7.4 PBS. (c, d) TEM images of the BODS NPs in pH=6.5 PBS at different magnifications.

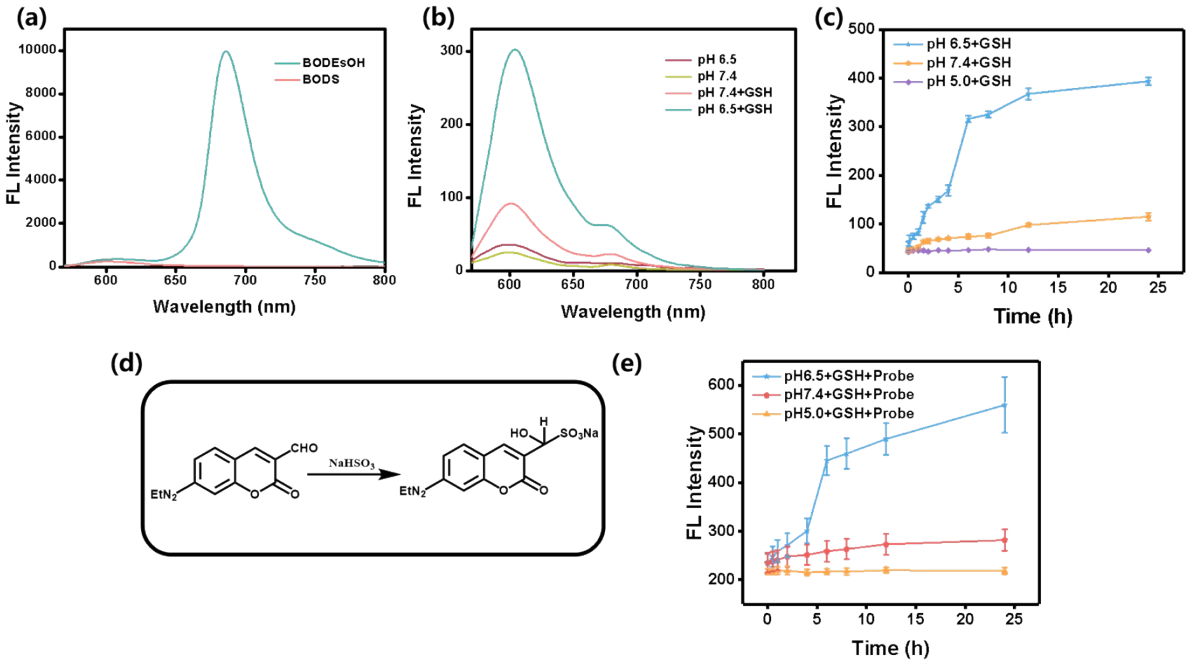


Figure S2 (a) Fluorescence spectrum of BODEsOH and BODS in DMSO. (b) Fluorescence spectrum of BODS NPs treated with or without GSH in pH=6.5 and 7.4 PBS. (c) Time-dependent fluorescence spectrum of BODS NPs treated with excess GSH in pH=7.4 6.5 and 5.0 PBS. (d) The mechanism of fluorescence probe for $HSO3^{-}$. (e) Time-dependent fluorescence spectrum of DEACA in the pH = 7.4, 6.5 and 5.0 PBS contained BODS NPs and excess GSH.

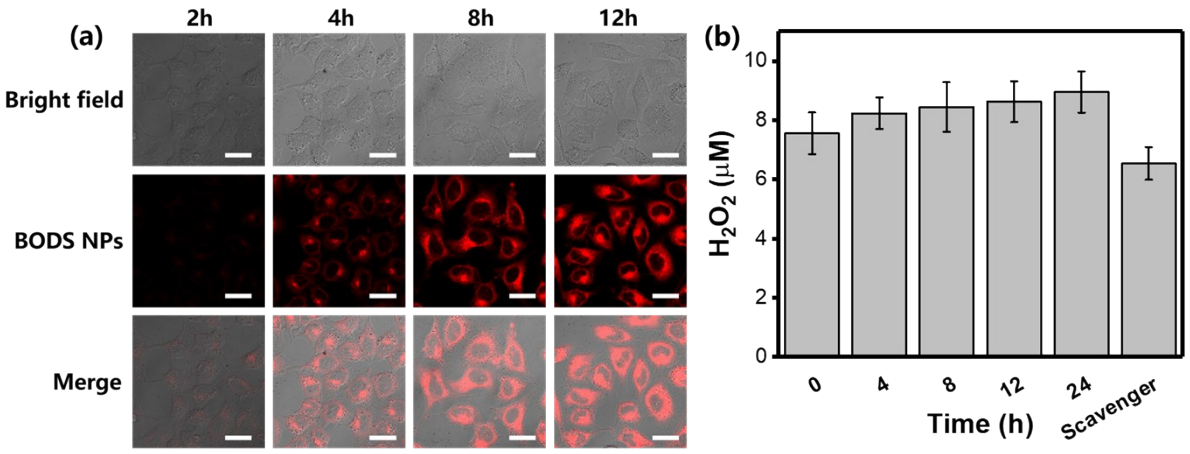


Figure S3. (a) Cell uptake experiment. (b) H_2O_2 concentration in HeLa cells pre-incubated with BODS NPs at different times.

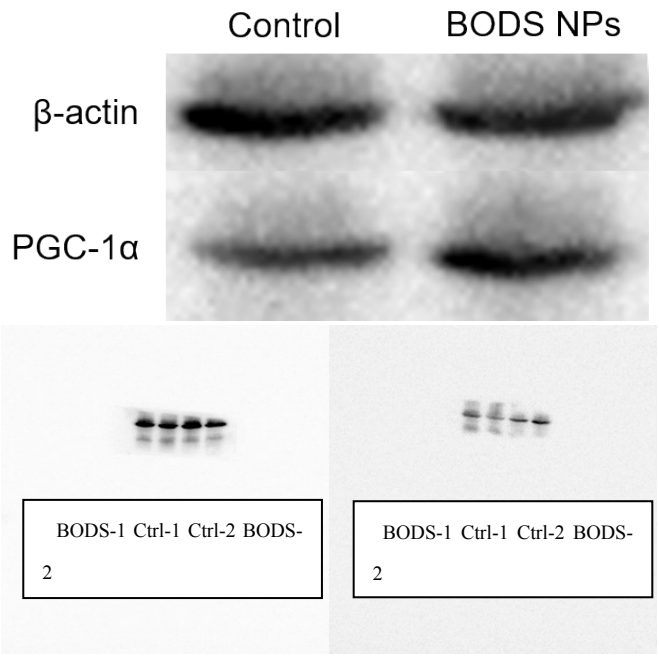


Figure S4. Effect of BODS NPs on protein expression of PGC-1.

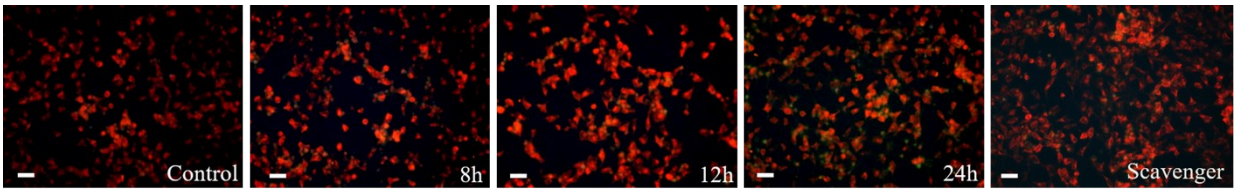


Figure S5. Fluorescence images of pre-incubated HeLa cells stained with JC-1 at different times.

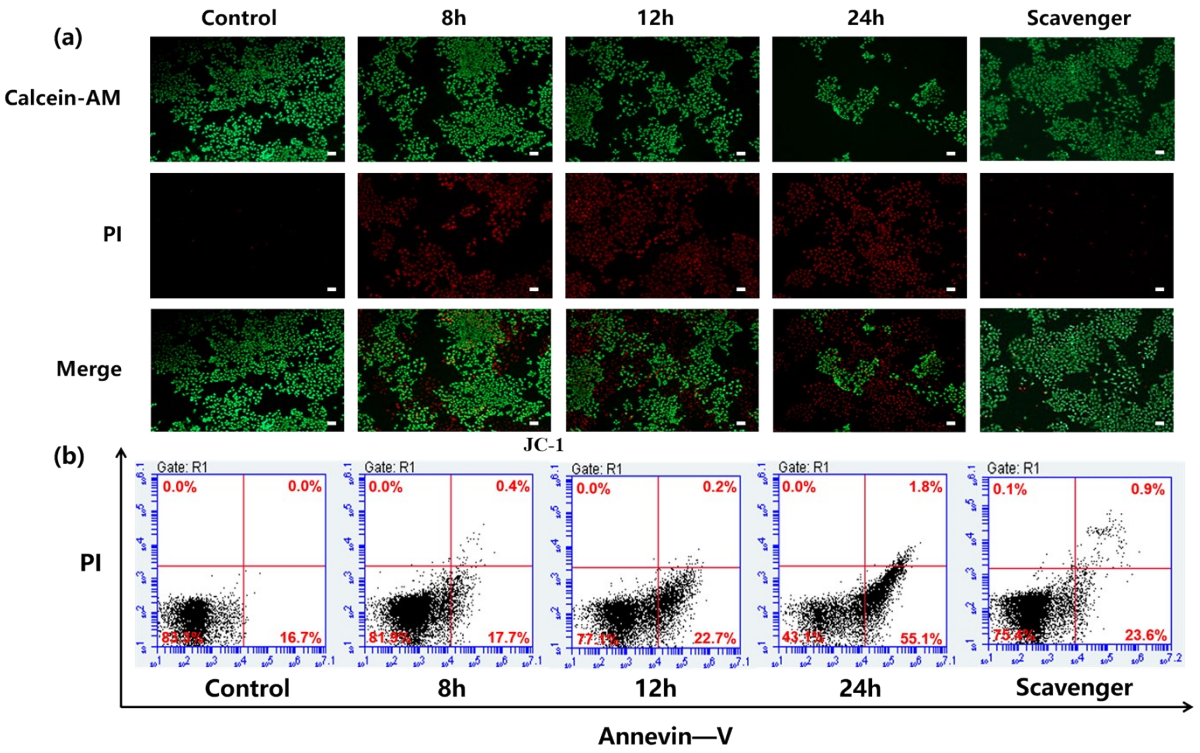


Figure S6. (a) Fluorescence images of pre-incubated HeLa cells co-stained with Calcein AM (green) and PI (red) at different times. (b) Flow cytometry assays of pre-incubated HeLa cells treated with Annexin V-FITC and PI dyes at different times.

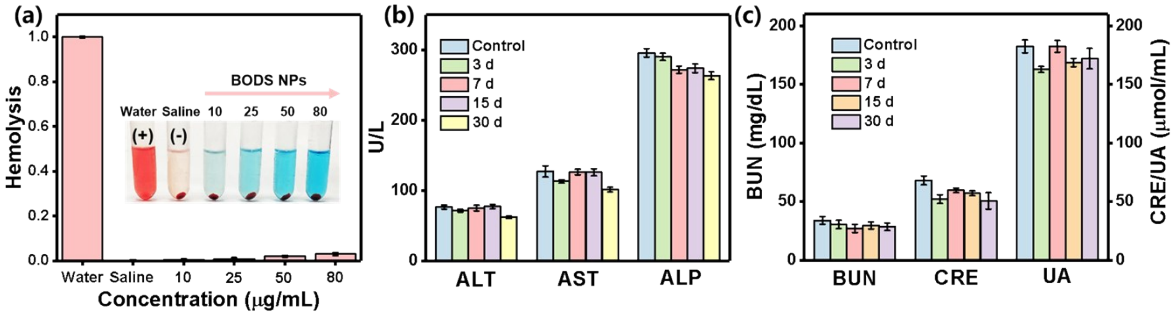


Figure S7. (a) Hemolysis assays of BODS NPs with different concentrations. (b) Blood biochemistry assays of liver function markers: ALT, AST, and ALP. (c) Blood biochemistry assays of kidney function markers: BUN, CRE and UA.

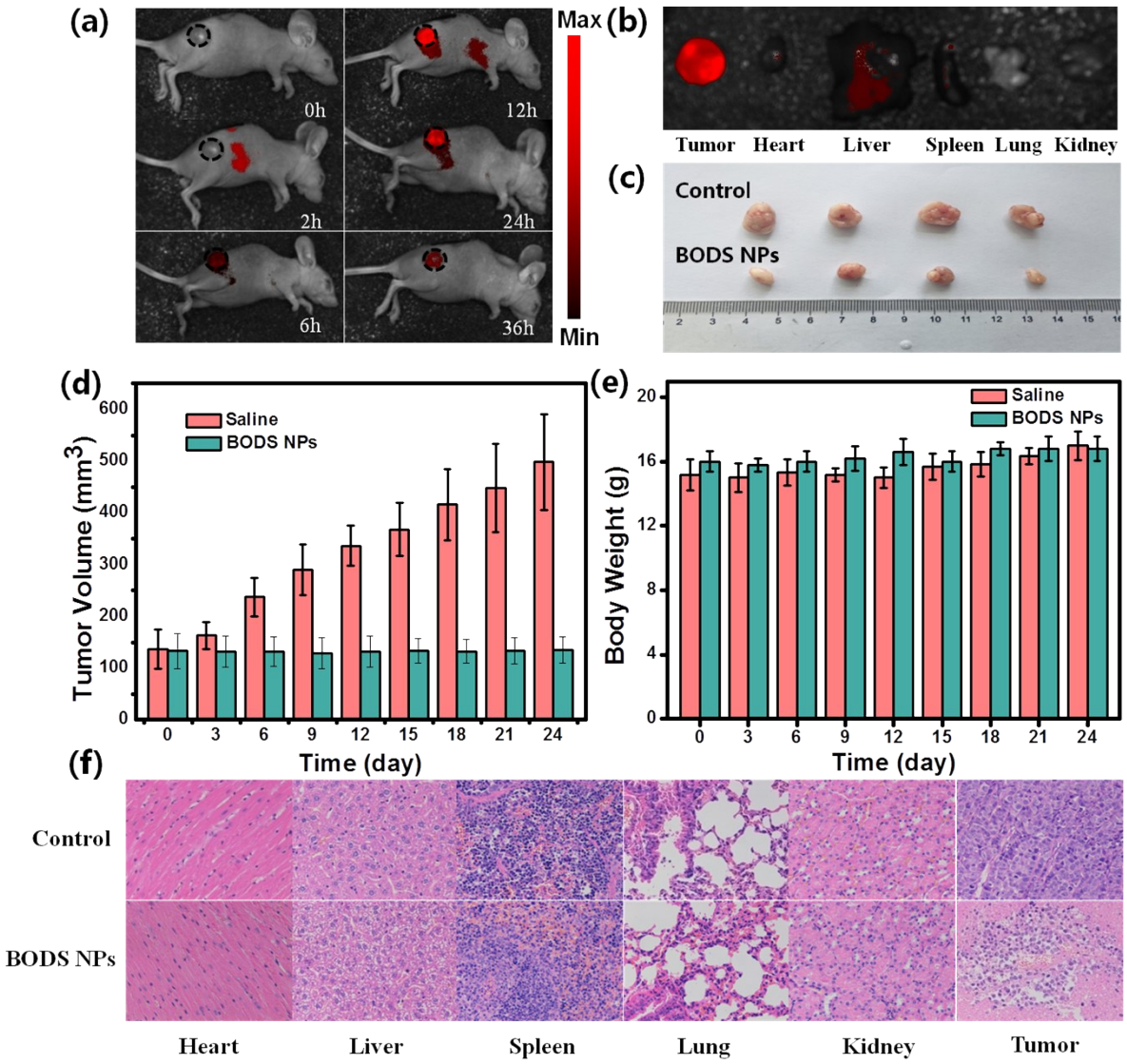


Figure S8. (a) Fluorescence images of mice at di□erent time after intravenous injection of BODS NPs. (b) Fluorescence images of organs taken from the sacrificed mice. (c) Photographs of tumors from sacrificed mice in different groups. (d) Body weight curves of mice in different groups during treatment. (e) Tumor volume curves of mice in different groups. (f) H&E staining of main organs and tumor tissues from sacrificed mice in different groups.

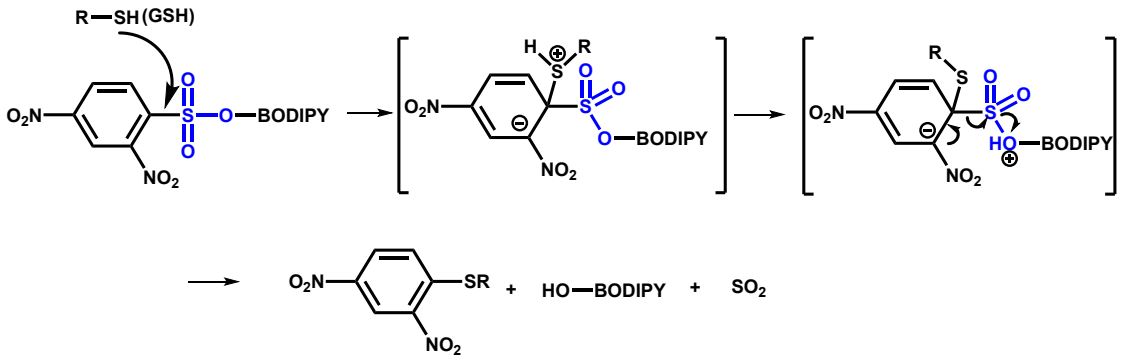


Figure S9. A possible mechanism of GSH mediated SO_2 release.

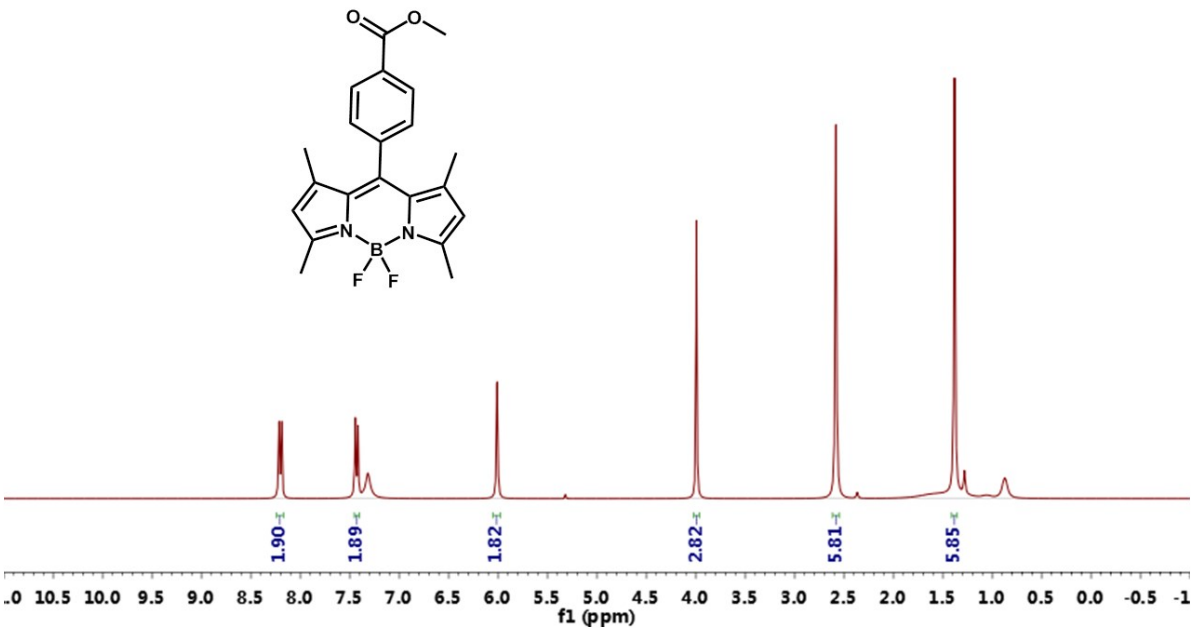


Figure S10. ^{1}H NMR of BOD-Ester.

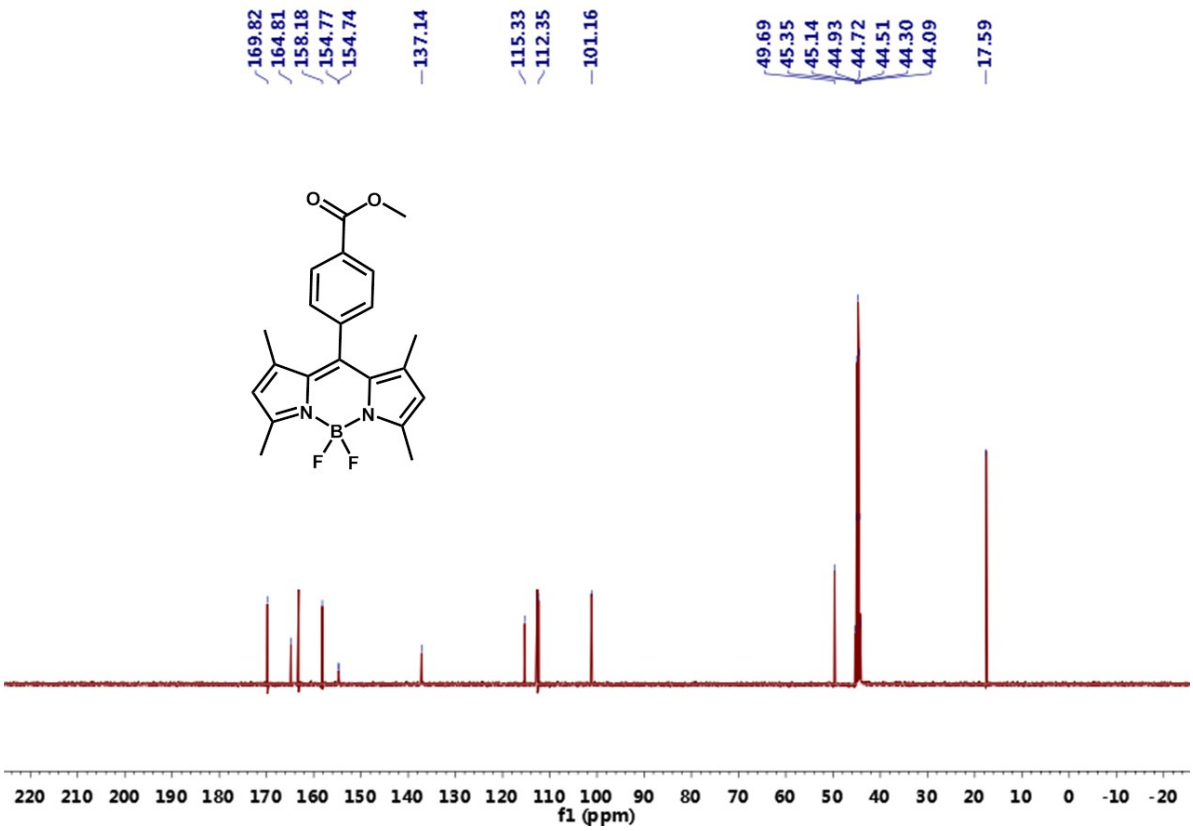


Figure S11. ^{13}C NMR of BOD-Ester.

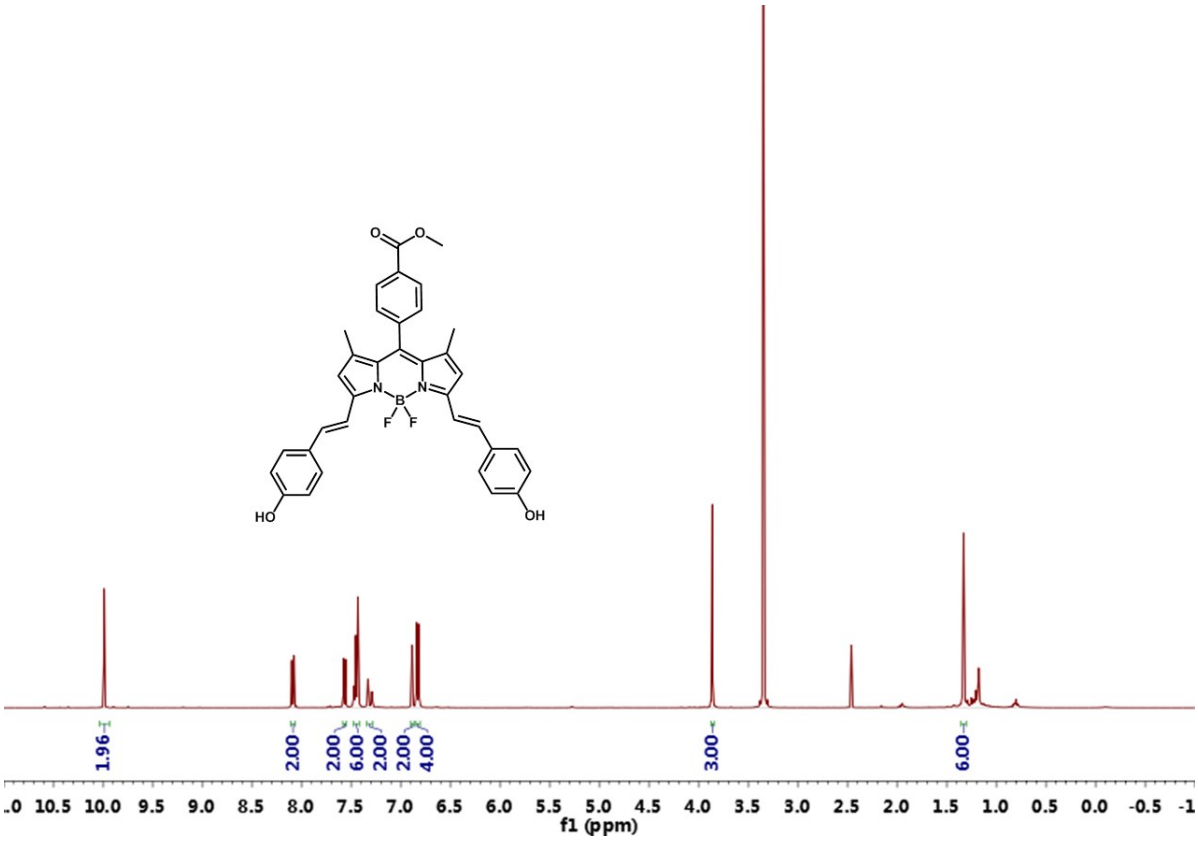


Figure S12. ^{1}H NMR of BODEsOH.

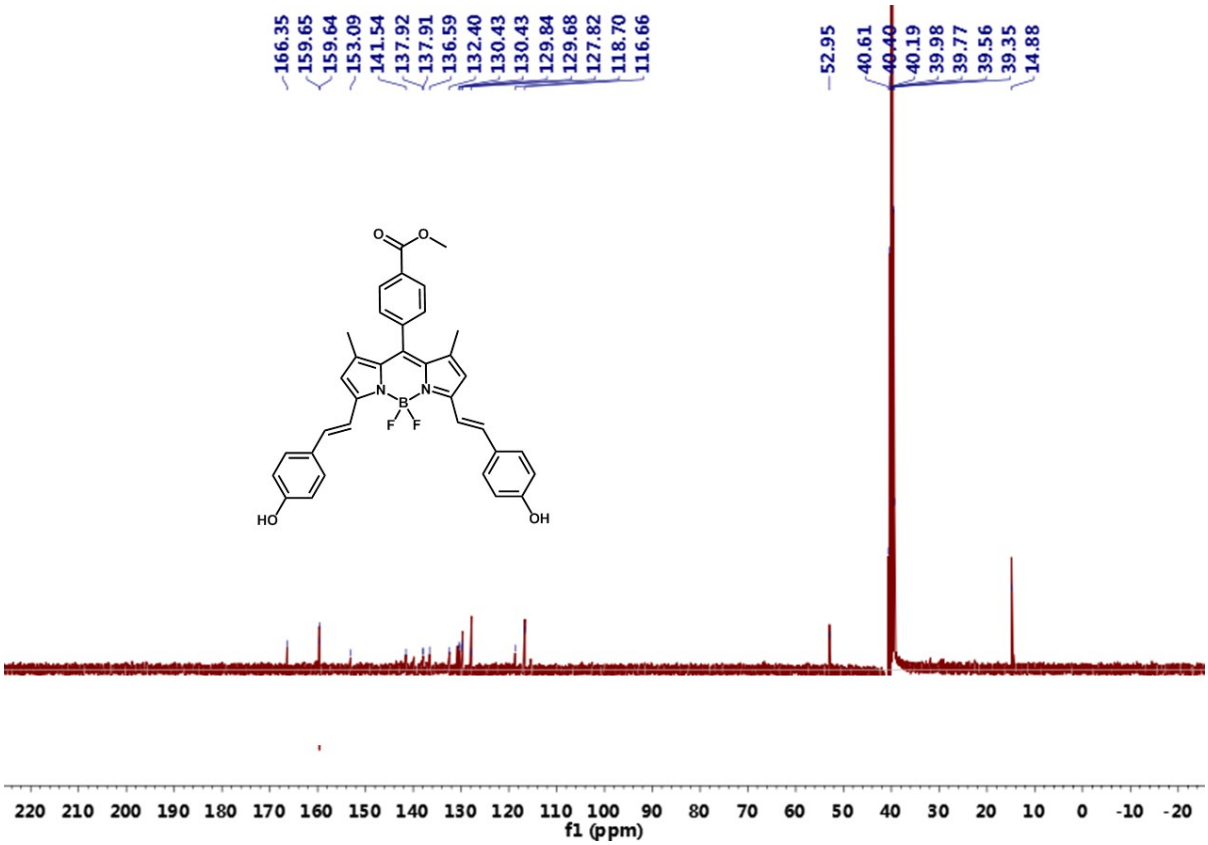


Figure S13. ^{13}C NMR of BODEsOH.

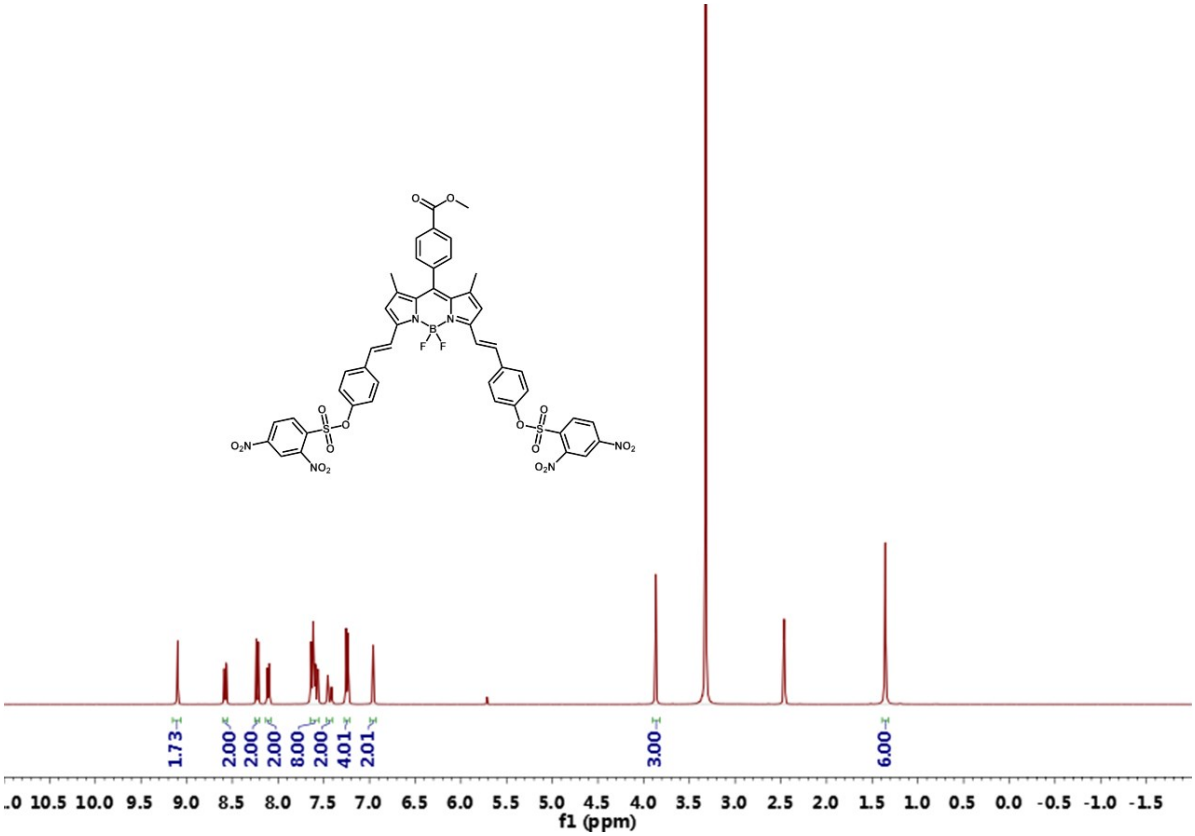


Figure S14. ^{1}H NMR of BODS.

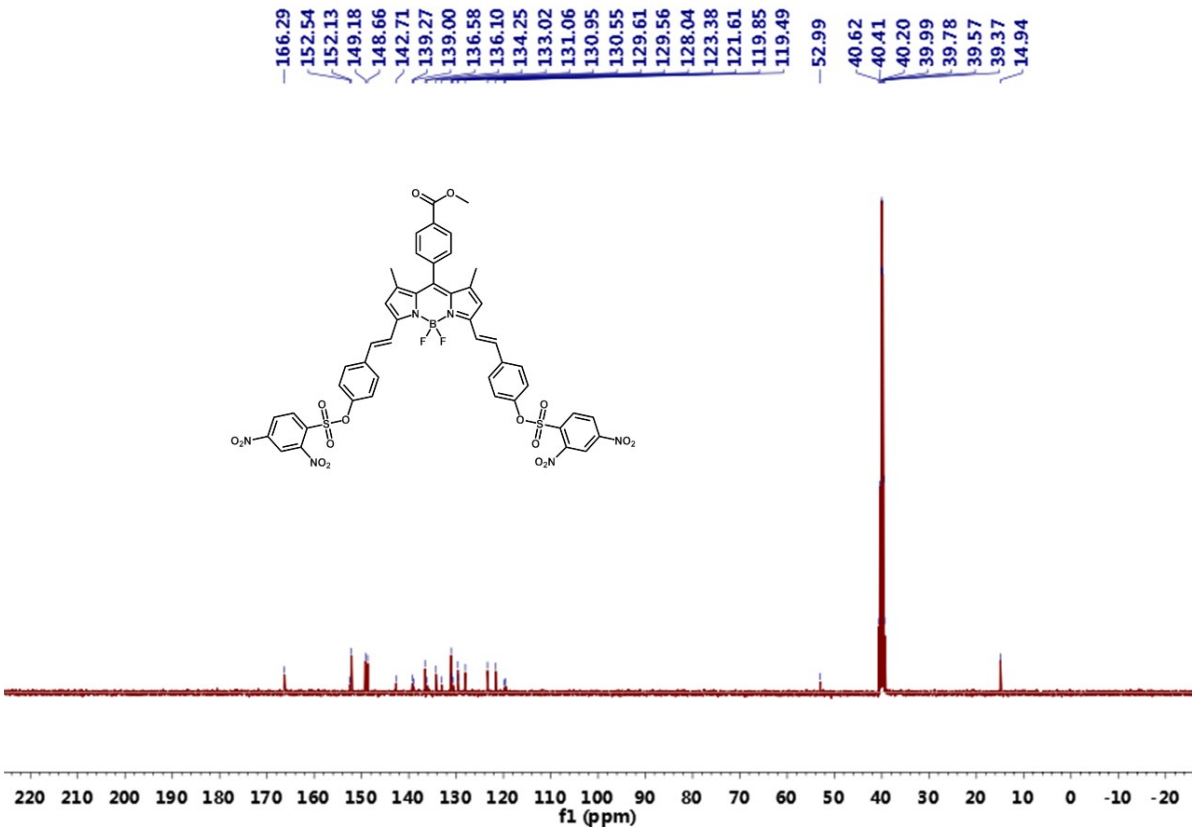


Figure S15. ^{13}C NMR of BODS.

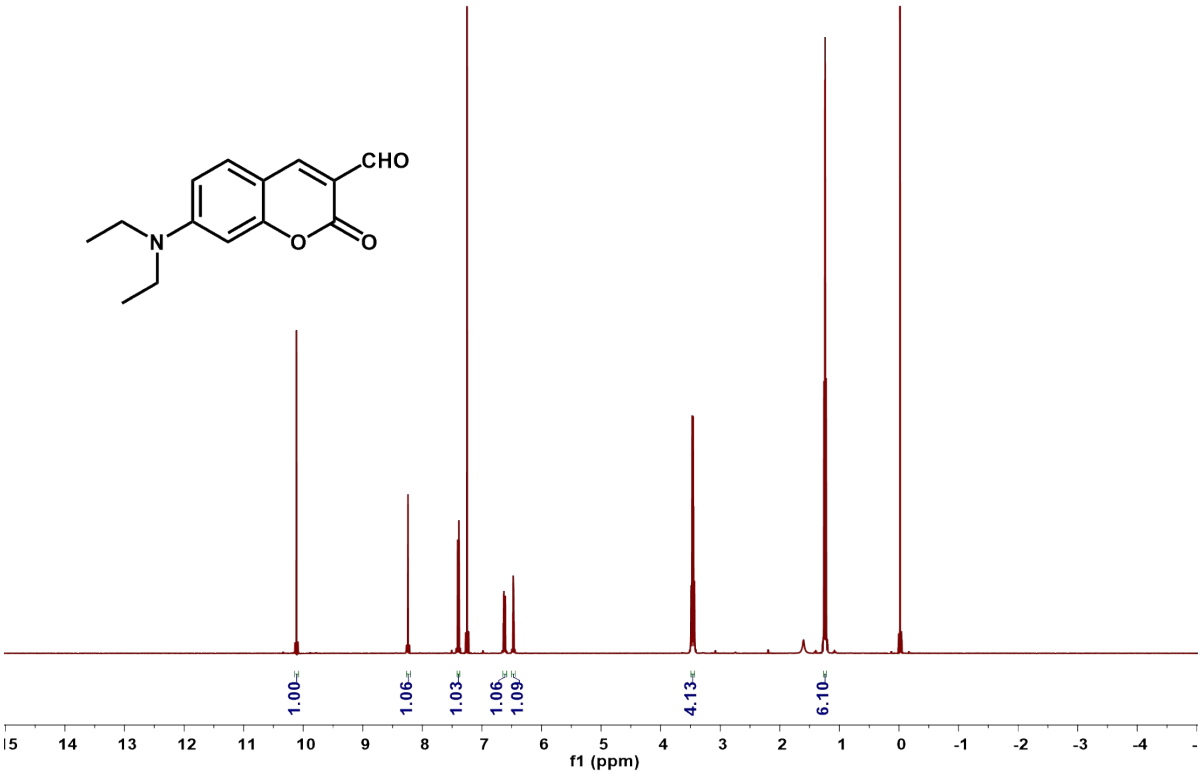


Figure S16. ^{13}H NMR of probe.

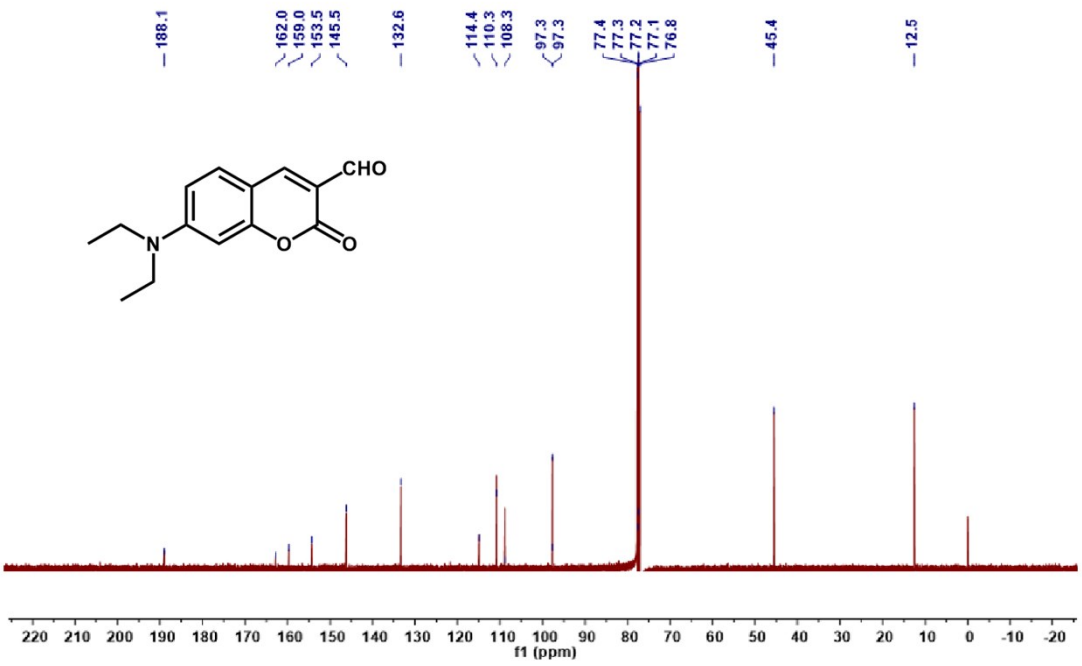


Figure S17. ^{13}C NMR of probe.

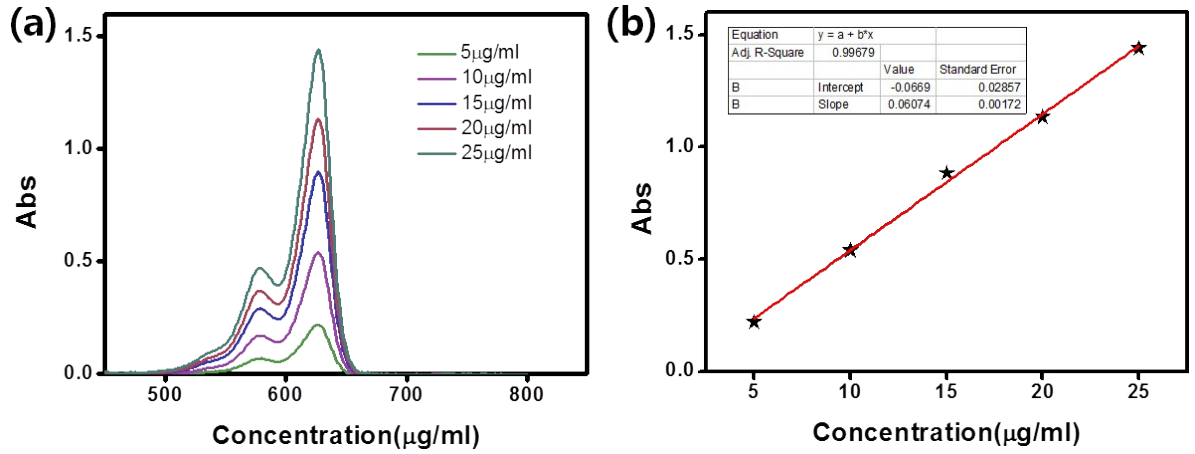


Figure S18. (a) Absorption spectra of BODS in CH_2Cl_2 with different concentration. (b) Linear fitting of BODS concentration and intensity.