Supplementary Information

Nitric Oxide-Activatable NIR-II Organic Small Molecule for Fluorescence Imaging-Guided Synergistic Photodynamic and Photothermal Therapy

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Content

1. Experimental Section

Materials and measurements.

All reagents used in experiments were purchased from Derthon Optoelectronics Materials Science Technology Co. Ltd. (Shenzhen, China), Bide Pharmatech Co. Ltd. (Shanghai, China) or J&K Chemical Ltd. (Beijing, China) and directly used without further purification. PS_{1000}-PEG_{2000} was purchased from Ruixi Biological Technology Co. Ltd. (Xi'an, China). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI-1640), Penicillin-Streptomycin (P/S) and 0.25% (1 ×) Trypsin were obtained from Gibco. 10 × phosphate buffer solution (PBS) was purchased from Sangon Biotech Co. Ltd. (Shanghai, China). Ultrapure water was collected from the Milli-Q reference system.

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance 400 MHz spectrometers. MALDI-TOF-MS was measured on a Bruker Maxis II mass spectrometer or Bruker Microflex LT /LRF MALDI-TOF mass spectrometer. The UV-vis absorption spectra were taken on a Shimadzu UV-2600 spectrophotometer. The fluorescence emission spectra were recorded on a HORIBA Fluorolog-QM spectrophotometer system. All NIR-II fluorescent images were performed by the NIROPTICS Infrared Region II in vivo imaging system. Visible fluorescent images were taken with the Bruker In-Vivo Xtreme II imaging system or Xenogen IVIS 200 imaging system. The size distribution was measured on the Malvern Zetasizer 3000HS dynamic light scattering (DLS) system. TEM images were taken with Thermo Fisher FEI Tecnai G2 F20 Field emission transmission electron microscope. Cell numbers were quantitated using an Accuri C6 flow cytometer (Becton 110 Dickinson, Franklin Lakes, NJ). The heating processed were measured by using a thermal imager (Fluke 480 pro). The absorbance for MTT analysis was performed on a SpectraMax M5 microplate reader.

Synthesis of CTBA

Synthesis of Compound 1

5 mL of concentrated sulfuric acid was added dropwise to 5 mL of concentrated hydrochloric acid under an ice bath. Then, benzothiadiazole (294 mg, 1 mmol) was

added slowly and in batches. After stirring at room temperature for about 24 h, the mixture was poured into ice water. Then the precipitated solid was collected and washed with water to obtain compound 1 (370 mg, 95%, yellow powder). MALDI-TOF-MS m/z: calcd for $C_6Br_2N_4O_4S^+$ 378.8 $[M + H]^+$; found 378.4 $[M + H]^+$ **(Figure S1)**.

Synthesis of Compound 2

Compound 1 (192 mg, 0.5 mmol) and tributyl(thiophen-2-yl)stannane (560 mg, 1.5 mmol) were dissolved in ultra-dry toluene, then the solution was degassed with N_2 for 30 min. Subsequently, $Pd(PPh_3)_4$ (57 mg, 0.05 mmol) was added to the mixture and stirred overnight at 110 ℃. After the reaction solution was cooled to room temperature, the residual toluene in the reaction solution was removed under reduced pressure. Then the mixed solution was extracted with dichloromethane and washed three times with saturated NaCl solution. Then dry the organic phase with anhydrous $MgSO_4$ and concentrated under vacuum. The crude product was separated and purified by silica gel column chromatography (eluent: petroleum ether/dichloromethane (V/V = 5/1)), and then dried in vacuo to obtain compound 2 (165 mg, 85%, orange solid). The ^1H NMR spectrum is shown in **Figure S2**. ^1H NMR (400 MHz, $CDCl_3$): δ 7.74 (d, J = 4 Hz, 2H), 7.52 (d, J = 4 Hz, 2H), 7.24 (m, 2H). MALDI-TOF-MS m/z: calcd for $C_{14}H_6N_4O_4S_3^+$ 390.96 $[M + H]^+$; found 390.96 $[M + H]^+$ **(Figure S3)**.

Synthesis of Compound 3

Compound 2 (117 mg, 0.3 mmol) was dissolved in 10 mL $CHCl_3$ and N-bromosuccinimide (270 mg, 1.5 mmol) solution in CH_3CN was added. After stirring at room temperature for about 24 h. The mixed solution was extracted with dichloromethane and washed three times with saturated NaCl solution. Then dry the organic phase with anhydrous $MgSO_4$ and concentrated under vacuum. The crude product was separated and purified by silica gel column chromatography (eluent: petroleum ether/dichloromethane (V/V = 5/1)), and then dried in vacuo to obtain compound 3 (136 mg, 83%, orange-red solid). The ^1H NMR spectrum is shown in **Figure S4**. ^1H NMR (400 MHz, $CDCl_3$): δ 7.45 (d, J = 4 Hz, 2H), 7.32 (d, J = 4 Hz, 2H). MALDI-TOF-MS m/z: calcd for $C_{14}H_4Br_2N_4O_4S_3^+$ 548.77 $[M + H]^+$; found

548.77 $[M + H]^+$ **(Figure S5)**.

Synthesis of Compound 4

Compound 3 (110 mg, 0.2 mmol) and 2-trimethyltin-4,4-bis(2-ethylhexyl)-4H-cyclopenta[2,1-b;3,4-b']dithiophene (282 mg, 0.5 mmol) were dissolved in ultra-dry toluene, then the solution was degassed with N_2 for 30 min. Subsequently, $Pd(PPh_3)_4$ (24 mg, 0.02 mmol) was added to the mixture and stirred overnight at 110 ℃. After the reaction solution was cooled to room temperature, the residual toluene in the reaction solution was removed under reduced pressure. Then the mixed solution was extracted with dichloromethane and washed three times with saturated NaCl solution. Then dry the organic phase with anhydrous $MgSO_4$ and concentrated under vacuum. The crude product was separated and purified by silica gel column chromatography (eluent: petroleum ether/dichloromethane (V/V = 10/1)), and then dried in vacuo to obtain compound 4 (155 mg, 65%, navy-blue solid). The ^1H NMR spectrum is shown in **Figure S6**. ^1H NMR (400 MHz, $CDCl_3$): δ 7.47 (m, 2H), 7.23- 7.20 (d, J = 4 Hz, 2H), 7.21, (m, 4H), 6.96 - 6.95 (m, 2H), 1.18 - 1.94 (m, 8H), 1.03 - 0.99 (m, 20H), 0.94 - 0.92 (m, 12H), 0.87 - 0.89 (m, 6H), 0.77 - 0.74 (m, 12H), 0.64 (t, J = 4 Hz, 6H), 0.61 (t, J = 4 Hz, 6H). MALDI-TOF-MS m/z: calcd for $C_{64}H_{78}N_4O_4S_7^+$ 1091.41 $[M + H]^+$; found 1091.41 $[M + H]^+$ **(Figure S7)**.

Synthesis of CTBA

Compound 4 (120 mg, 0.1 mmol) was dissolved in 5 mL CH_2Cl_2 and 5 mL 90% CH_3OH. Then zinc (390 mg, 6 mmol) and ammonium chloride (97 mg, 1.8 mmol) were added. After stirring at 50℃ overnight, the mixed solution was extracted with dichloromethane and washed three times with saturated NaCl solution. The organic phase was dried with anhydrous $MgSO_4$ and concentrated under vacuum. The crude product was separated and purified by silica gel column chromatography (eluent: petroleum ether/dichloromethane (V/V = 8/1)), and then dried in vacuo to obtain CTBA (80 mg, 70%, orange solid). The ^1H NMR spectrum is shown in Figure S8. ^1H NMR (600 MHz, $CDCl_3$): δ 7.31-7.29 (t, J = 6 Hz, 2H), 7.28-7.26 (t, J = 6 Hz, 2H), 7.16-7.15 (d, J = 6 Hz, 2H), 7.12 (s, 2H), 6.95-6.94 (m, 2H), 4.49 (s, 4H), 1.93 - 1.86 (m, 8H), 1.04-0.98 (m, 20H), 0.96-0.94 (m, 8H), 0.90-0.88 (m, 6H), 0.78-0.76 (t, J = 12 Hz, 6H),

0.71-0.68 (m, 4H), 0.65-0.60 (m, 12H). The ^{13}C NMR spectrum is shown in Figure S9. ^{13}C NMR (151 MHz, CDCl3) δ 158.31, 157.73, 150.65, 140.37, 139.28, 136.63, 136.51, 136.07, 133.28, 129.48, 124.81, 122.76, 122.36, 119.40, 119.26, 107.06, 77.25, 77.04, 76.83, 53.72, 43.30, 43.24, 35.15, 35.10, 34.20, 34.06, 28.63, 28.59, 27.36, 27.26, 22.86, 22.79, 14.18, 14.11, 10.68. MALDI-TOF-MS m/z: calcd for $C_{64}H_{82}N_4S_7^+$ 1136.46 $[M + H]^+$; found 1136.46 $[M + H]^+$ (Figure S10).

DFT calculations

The geometry of all molecules was optimized by density functional theory (DFT). All the DFT computations were performed by the B3LYP/6-31G levels. All structures were generated using CYL view. The SMD solvation model was used to account for the effects of THF solution. All these calculations were performed with Gaussian 16 software package.

Preparation and characterizations of CTBA-NPs[1]

According to the related literatures, the nanoparticles were prepared by self-assembly. Briefly, CTBA (1 mg) and PS_{1000}-PEG_{2000} (20 mg) were dissolved in 1 mL THF and ultrasonicated for 5 min. Under the ultrasonic ice bath condition, the above solution was quickly added into 10 mL water, and continued with the ultrasound for 30 min. Next, THF was removed by injecting nitrogen into the mixture to obtain nanoparticles aqueous. Then, the solution was filtered through a membrane filter (diameter =0.22 μm) for subsequent experiments.

In vitro NO response effect test of CTBA-NPs

DEA NONOate as NO donor was dissolved in water. Then, different concentrations of NO were added into CTBA-NPs solution. The mixed solution was incubated at room temperature for 1-2 h. The UV absorption spectra and fluorescence spectra of the solution were measured and NIR-II fluorescence imaging were performed. In all cases, the fluorescent intensity was quantified by ImageJ.

Calculation of the photothermal conversion efficiencies

The photothermal conversion efficiencies (η) were measured according to the reported method:

$$\eta = \frac{hS(T_{Max} - T_{surr}) - Q_{Dis}}{I(1 - 10^{-A_{808}})}$$

$$hS = \frac{mC}{\tau}$$

$$t = -\tau \ln(\theta)$$

$$\theta = \frac{T - T_{surr}}{T_{Max} - T_{surr}}$$

η represents the photothermal conversion efficiency, h denotes the heat transfer coefficient, S is the surface area of the container, T_{max} and T_{Surr} are the maximum steady state temperature and the environmental temperature, respectively. Q_{Dis} represents heat dissipated from the laser mediated by the solvent and container, I means the laser power. A_{808} is the absorbance of CTBA-NPs solution at 808 nm. τ represents the system time constant of samples, m and C are the mass of the irradiated solution and the heat capacity of the solvent, respectively. t is the time of the cooling period and θ is the ratio of the real-time temperature change in the cooling period to the maximum temperature change. T_{Max} was 39℃. T_{Surr} was 17.8℃. hS was calculated as 0.0223 J/℃. Q_{Dis} was calculated as 0.0714 J.

Cell experiments

HUVEC (Human umbilical vein endothelial cells) and HepG2 cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin at 37 ℃ with 5% CO_2 in an incubator. H22 (Hepatoma-22) cells were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin at 37 ℃ with 5% CO_2 in an incubator.

Cytotoxicity by MTT assay[2]

The cytotoxicity of CTBA-NPs against cells was evaluated by MTT assay. Briefly, cells were seeded with a density of 7000-8000 cells per well in 96-well plates. After incubating 24 h, the incubation medium was removed and the added 100 μL medium containing different concentrations of CTBA-NPs and NO. After incubating for about 8 h, 808 nm laser was applied to the corresponding group. After incubating for another 12 h, MTT (5 mg/mL, 10 μL) was added into each well and then the cells were

incubated for 4 h, then the medium containing MTT was removed. After adding DMSO (100 μL) into each well to dissolve the produced formazan, shaking the plate for 10 min, the absorbance at 490 nm was measured via a microplate reader.

Live-dead cell staining experiments

HUVEC/ HepG2/ H22 cells were seeded in 24-well plates with a density of about 10000 cells per well. After incubating for 12 h, the incubate was replaced with 300 μL medium containing CTBA-NPs and NO. After incubating for about 8 h, 808 nm laser was applied to the corresponding group. After the cells were incubated for another 4 h, the old culture medium was discarded and washed with PBS for 3 times. Then, the cells were incubated with medium containing Calcein AM (4 μM) and PI (9 μM) for 20 min. Finally, the fluorescence images of cells were obtained by an inverted fluorescence microscope.

Animal management

All animal experiments were conformed in accordance with the protocols approved by the university's institutional animal care and use committee (Shaanxi Normal University, CE-2024-0012-BG6). BALB/c mice (female, 20±5 g), were purchased from Shaanxi Normal University Laboratory Animal Center. They were feed with standard diets and free water drinking, under about 25 ℃, 40-60% humidity and 12 h dark/illumination alternation. At the end of experiments, the animals were executed euthanasia by carbon dioxide.

Biodistribution of CTBA-NPs in vivo[3]

The 5-week-old BALB/c mice were injected with CTBA-NPs solution (50 μM, 200 μL) through the tail vein. After 1, 4,12, 24 and 48 h, the mice were dissected and the heart, liver, spleen, lung, kidney, stomach, intestine and colon of each mouse were obtained. Then the fluorescent imaging was carried out by the Bruker In-Vivo Xtreme II imaging system (exposure time = 5 s). The corresponding quantitative data were recorded with the software equipped with the Bruker Xtreme II system.

In vivo safety studies in mice

The preliminary in vivo safety experiments were conducted in BALA/c mice. Healthy BALB/c mice were randomly divided into three groups (n = 3). The control

group was given intravenous injection of PBS every 2 days, while the other groups were given intravenous injection of CTBA-NPs at different concentrations (25, 50 or 100 μM) at the same time. After each injection, the mice were weighed and observed for signs of illness. After 14 days, the blood was obtained by blood collection through the retro-orbital venous plexus, partly by adding anticoagulant EDTA-K2 and partly by centrifugation to obtain serum for complete blood-cell counting and biochemical indicator test. Then the mice were euthanized and the major organs were isolated. The collected organs were fixed with 4% formaldehyde for H&E staining.

In vivo NIR-II fluorescent imaging and therapy of mice

All animal experiments were conformed in accordance with the protocols approved by institutional animal care and use committee of Shaanxi Normal University (CE-2024-0012-BG6). The mouse tumor model was established by subcutaneous injection of 1×10^7 H22 cells into the hind legs of mice. In vivo NIR-II fluorescent imaging experiments were performed when the tumor volume was about 70-100 mm^3. The therapy experiments were performed when the tumor volume was about 200-400 mm^3. Tumor size was measured with a caliper and the tumor volume was calculated with the equation: $V = ab^2/2$, where V is the tumor volume, and a and b are the length and width of the tumor, respectively.

Statistical analysis

Experimental conditions were performed at least in three sets of independent experiments and performed in duplicates, unless stated otherwise. Data was expressed as the means ± SD. For the comparison of two samples, the Student's t-test was used. A value of $P<0.05$ was considered to be statistically significant. (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$. IBM SPSS Statistics software was used for two-sided Student's t-testing (P value).

2. Supporting Figures

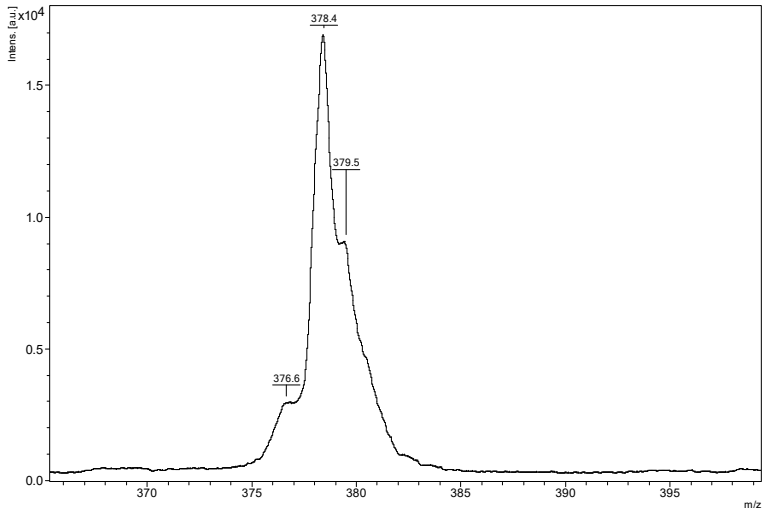

Fig. S1 MALDI-TOF MS spectrum of compound 1.

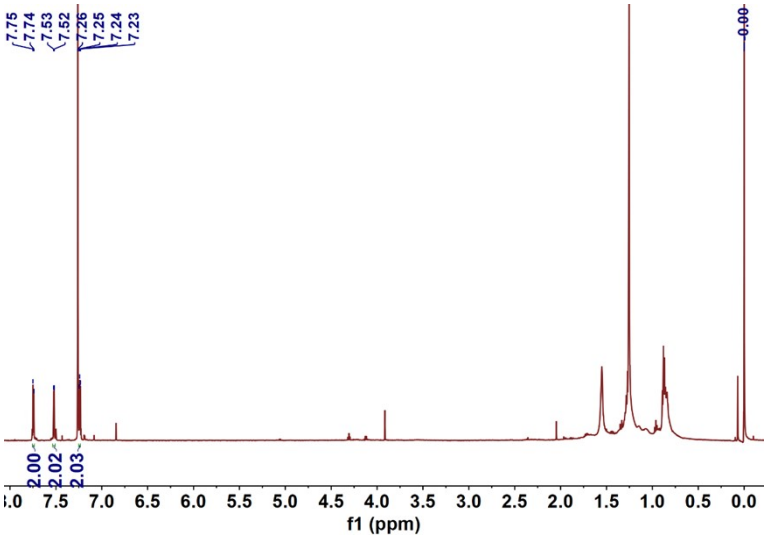

Fig. S2 ^{1}H-NMR spectrum of compound 2 in $CDCl_3$.

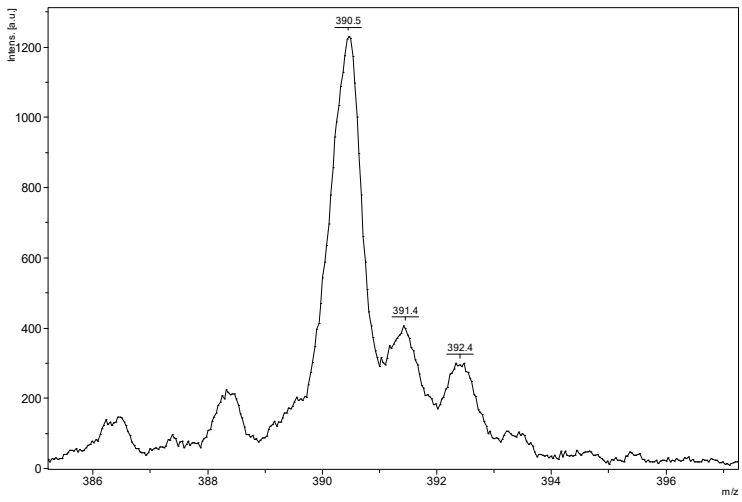

Fig. S3 MALDI-TOF MS spectrum of compound 2.

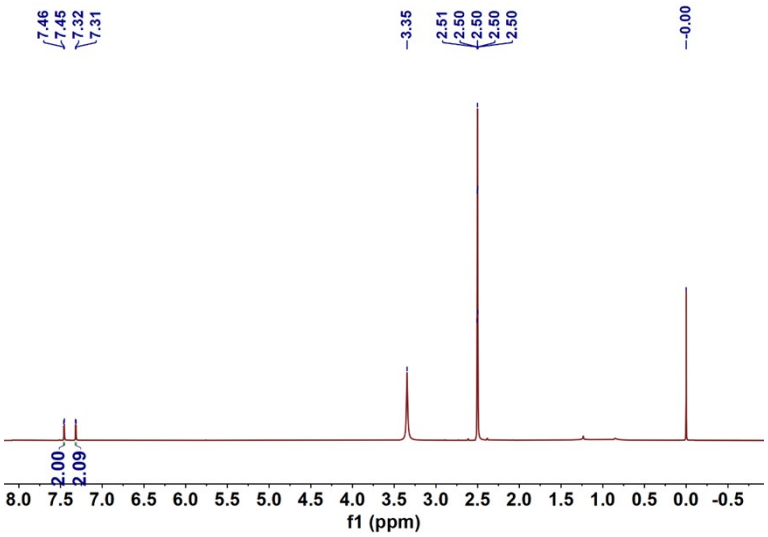

Fig. S4 ^{1}H-NMR spectrum of compound 3 in DMSO-d_6.

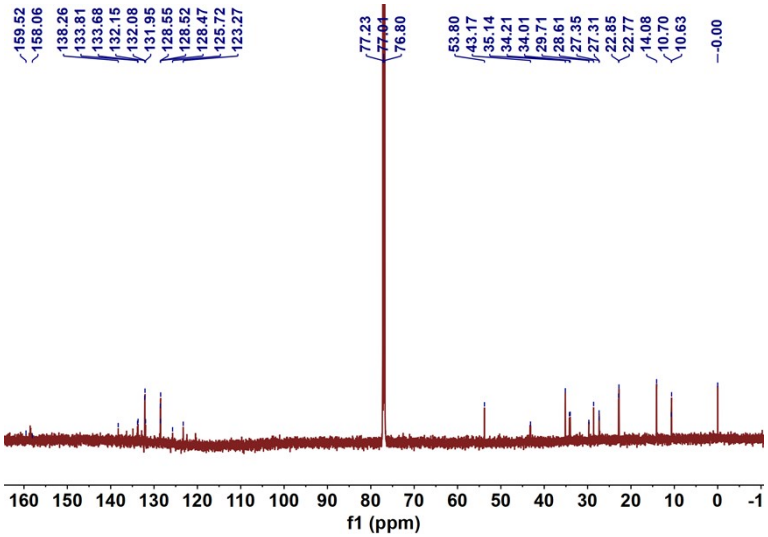

Fig. S5 ^{13}C-NMR spectrum of compound 3 in DMSO-d_6.

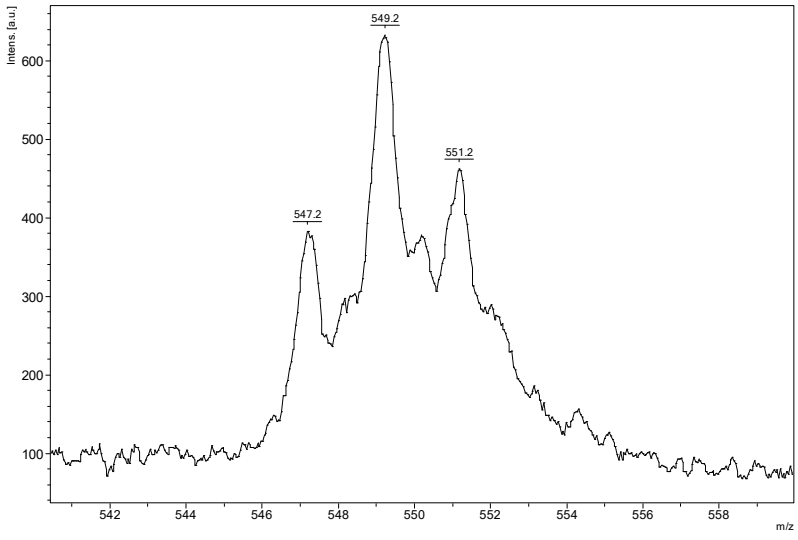

Fig. S6 MALDI-TOF MS spectrum of compound 3.

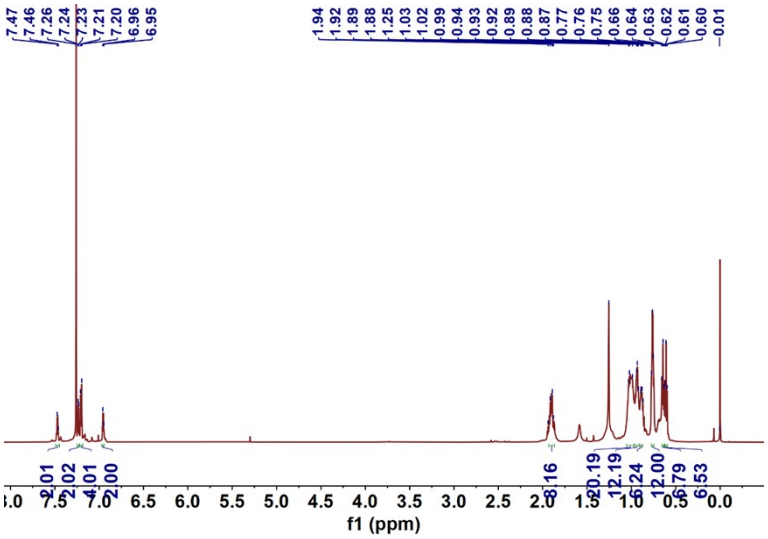

Fig. S7 ^{1}H-NMR spectrum of compound 4 in $CDCl_3$.

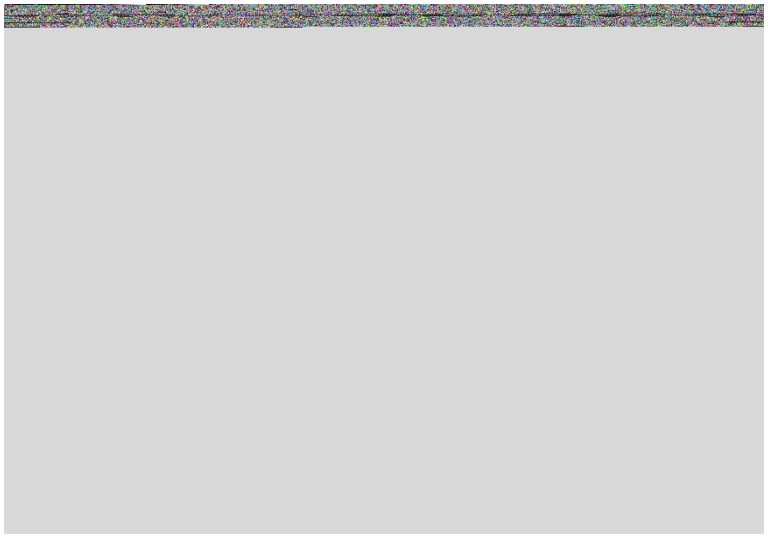

Fig. S8 ^{13}C-NMR spectrum of compound 4 in $CDCl_3$.

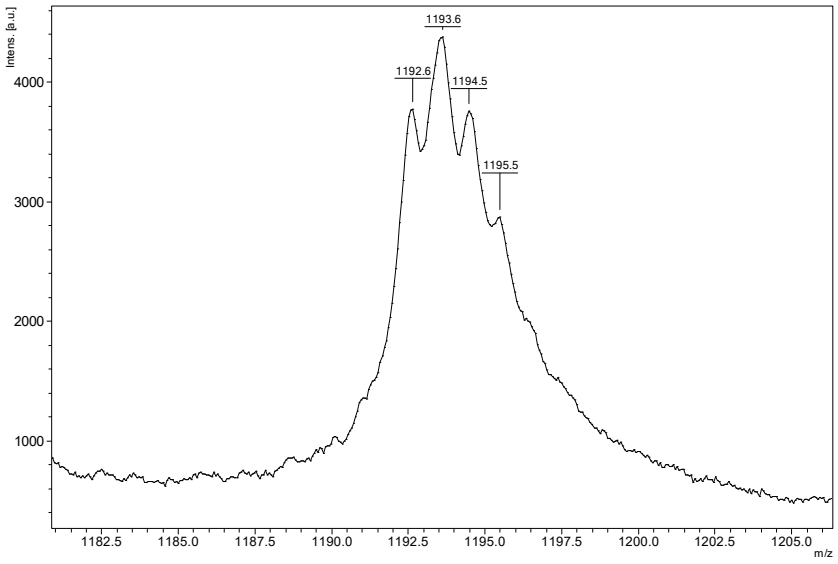

Fig. S9 MALDI-TOF MS spectrum of compound 4.

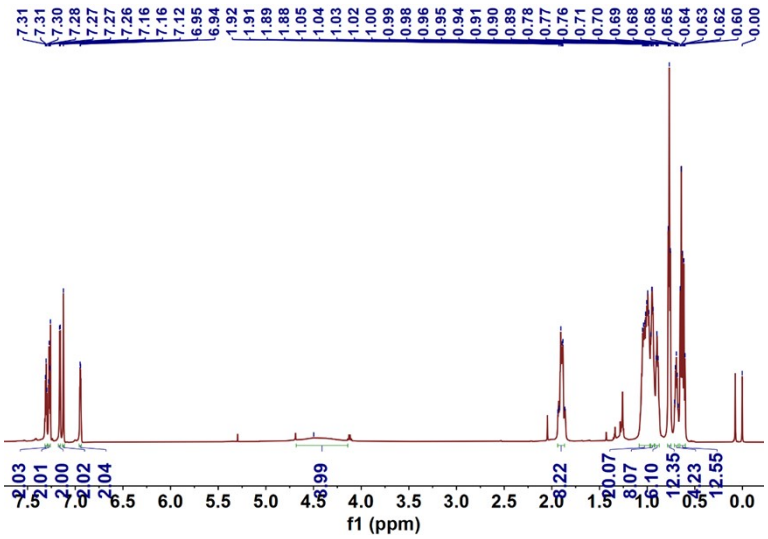

Fig. S10 ^{1}H-NMR spectrum of CTBA in $CDCl_3$.

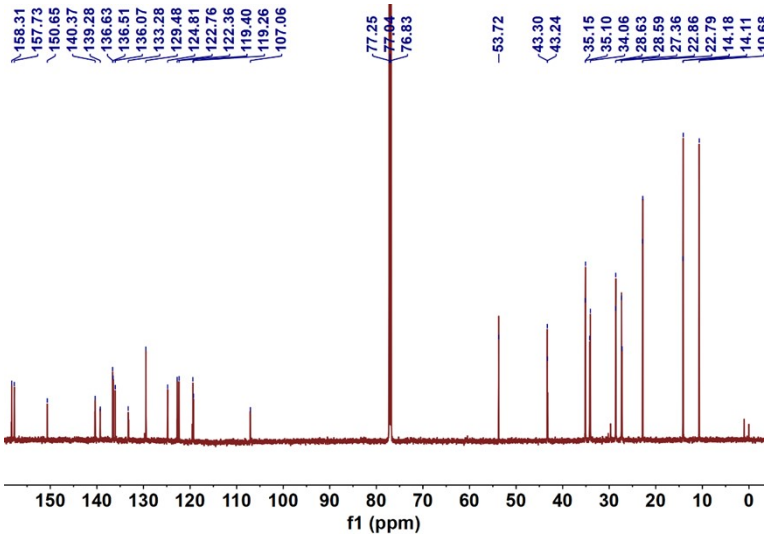

Fig. S11 ^{13}C-NMR spectrum of CTBA in $CDCl_3$.

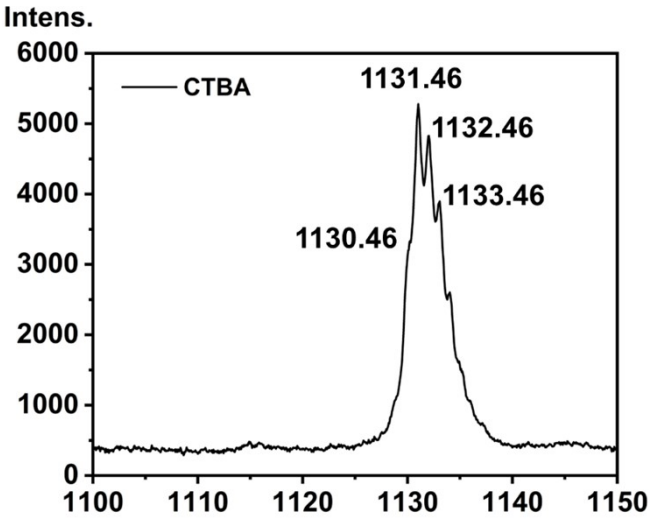

Fig. S12 MALDI-TOF-MS spectrum of CTBA

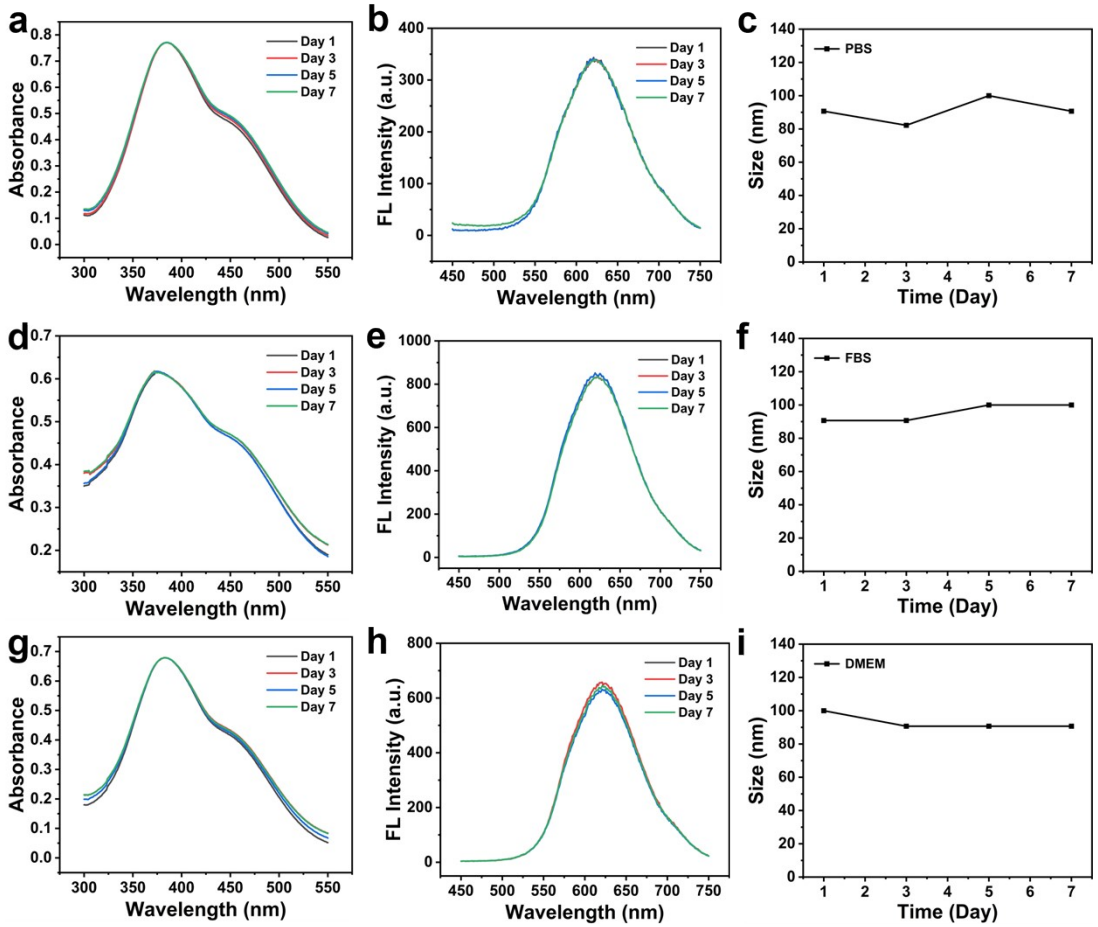

Fig. S13 Absorption spectra (a), fluorescence spectra (b) and hydrated diameter (c) of CTBA-NPs stored in PBS for 7 days. Absorption spectra (d), fluorescence spectra (e) and hydrated diameter (f) of CTBA-NPs stored in FBS for 7 days. Absorption spectra (g), fluorescence spectra (h) and hydrated diameter (i) of CTBA-NPs stored in DMEM for 7 days.

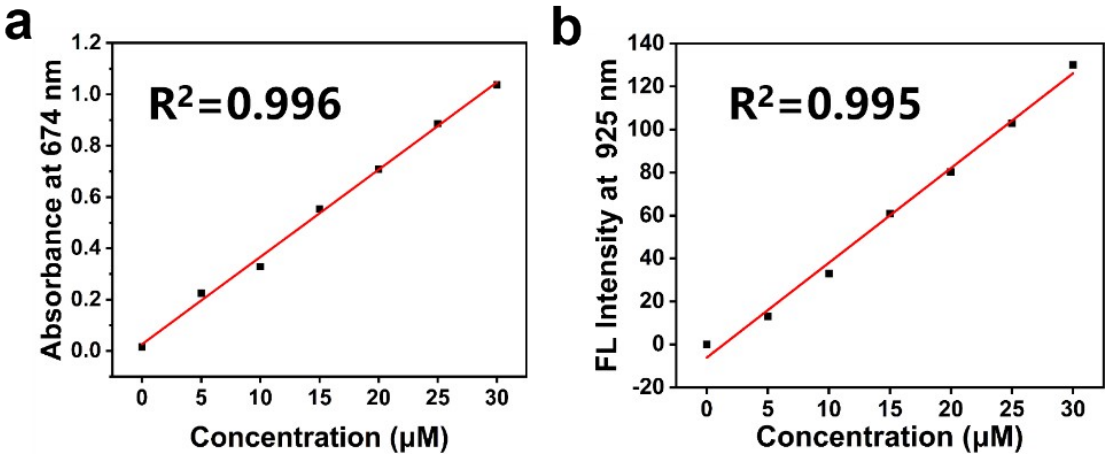

Fig. S14 (a) Plot of the absorbance at 695 nm ratio of CTBA-NPs to NO concentration. (b) Plot of the fluorescence intensity at 925 nm ratio of CTBA-NPs to NO concentration.

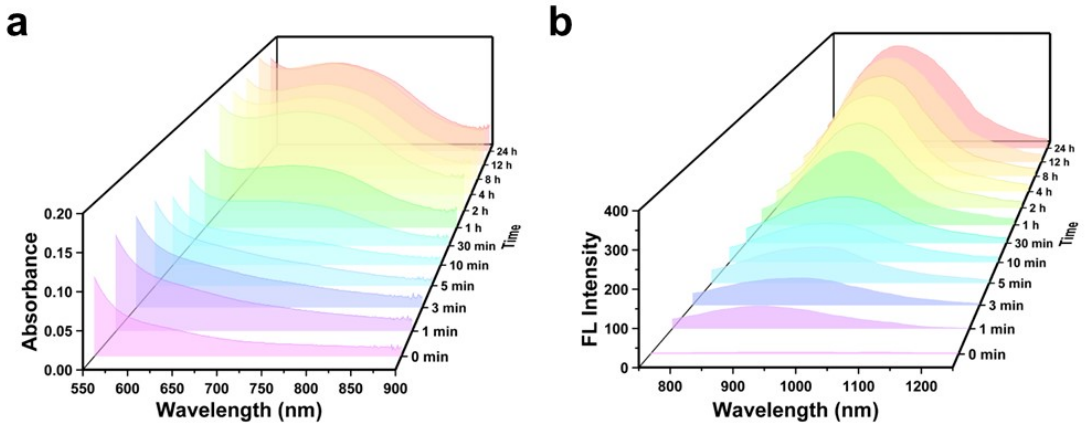

Fig. S15 (a) Absorbance spectra and (b) fluorescence spectra of CTBA-NPs (25 μM) responsive to NO of different time.

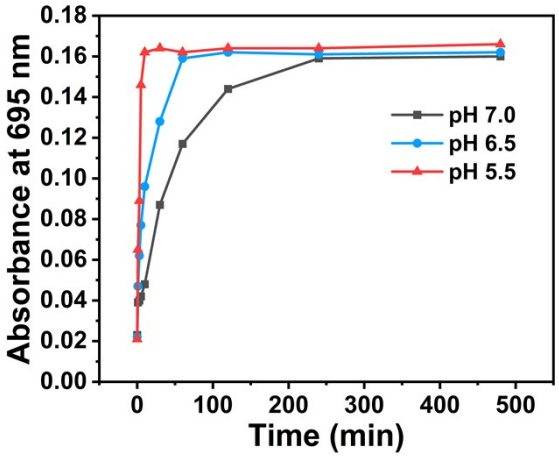

Fig. S16 The absorbance at 695 nm of CTBA-NPs at different time under different pH conditions.

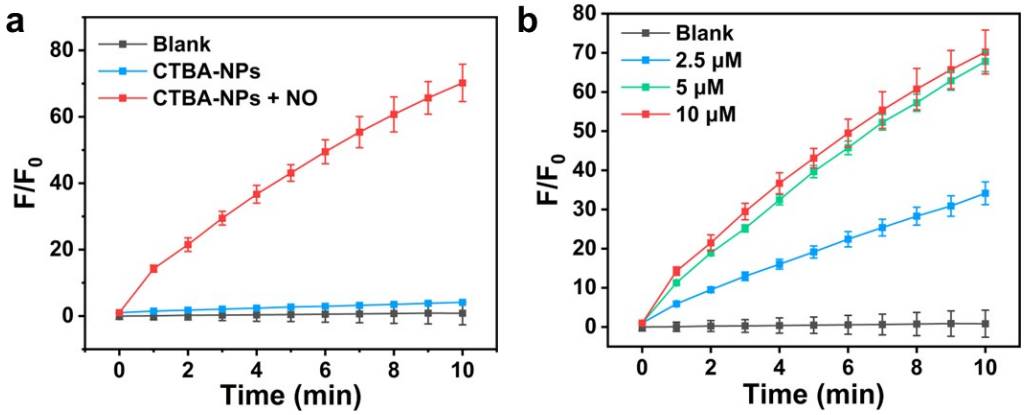

Fig. S17 (a) ROS generation of CTBA-NPs with and without NO under 808 nm laser (0.8 W/cm^2) for 10 min. (b) ROS generation of CTBA-NPs of different concentrations with NO under 808 nm laser (0.8 W/cm^2) for 10 min.

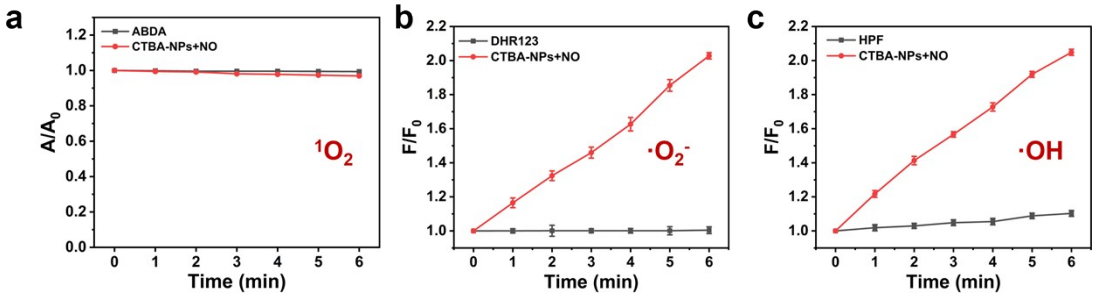

Fig. S18 (a) Absorbance changes of the ABDA probe (40 μM) to detect the generation of $^1O_2$. (b) FL intensity changes of the DHR123 probe (5 μM, λ_{ex} = 495 nm, λ_{em} = 526 nm) to detect the generation of $\cdot O_2^-$. (c) FL intensity changes of the HPF probe (5 μM, λ_{ex} = 490 nm, λ_{em} = 521 nm) to detect the generation of ·OH.

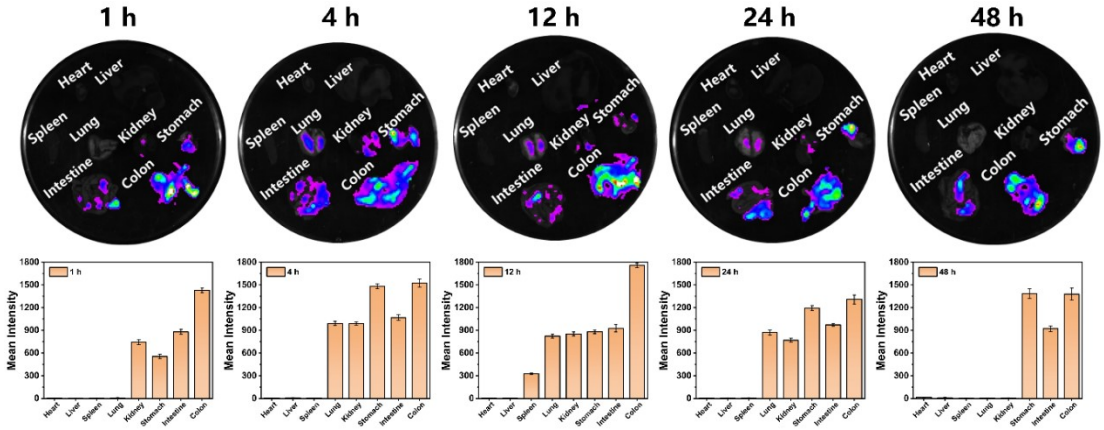

Fig. S19 After injection of CTBA-NPs (50 μM, 200 μL) into the mice via tail vein for 1 h, 4h, 12 h, 24 h and 48 h, the IVIS spectrum imaging system is conducted to detect the fluorescence signals in the liver, heart, spleen, lung, kidney, stomach, intestine and colon. Corresponding quantitative data of different organs. The fluorescence images of CTBA-NPs were collected at 535 nm (λ_{ex}: 410 nm).

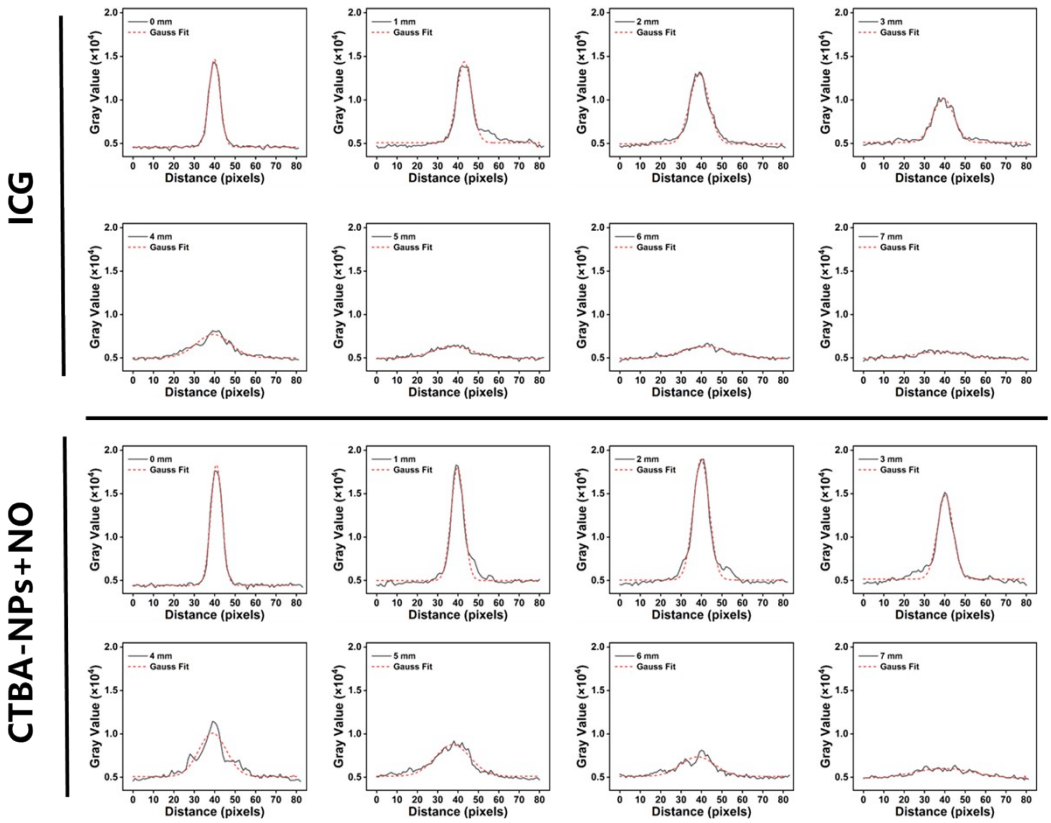

Fig. S20 The cross-section fluorescence intensity and its Guass fit curve of CTBA-NPs (a) and ICG (b) covered with different thicknesses (0-7 mm) of chicken breast tissue.

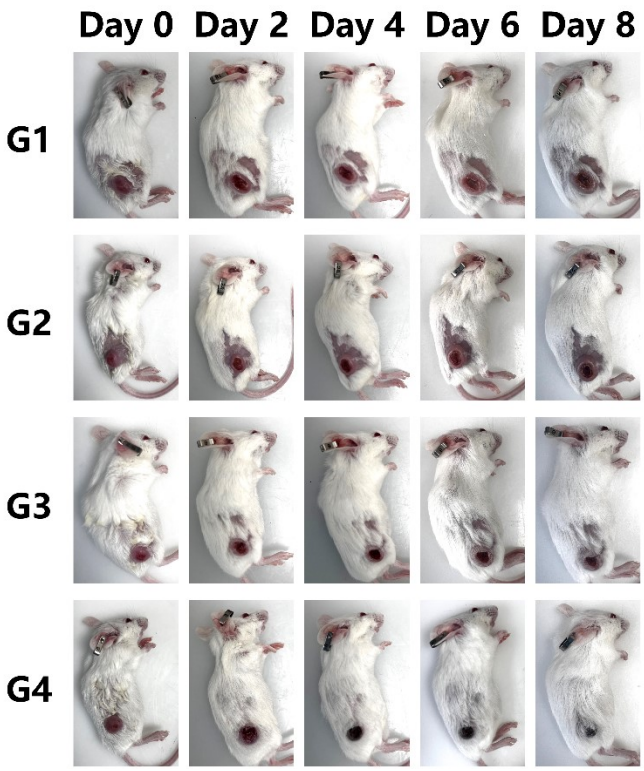

Fig. S21 Representative photos of mice in each group during treatment.

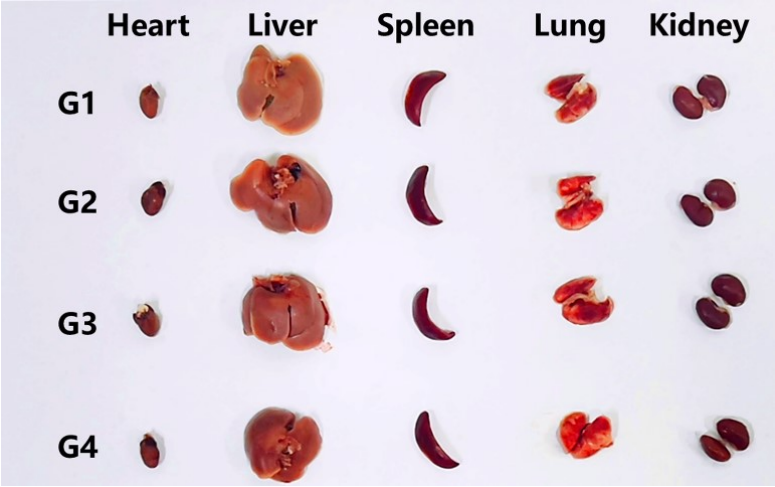

Fig. S22 The organs of each group of mice photographed after treatment.

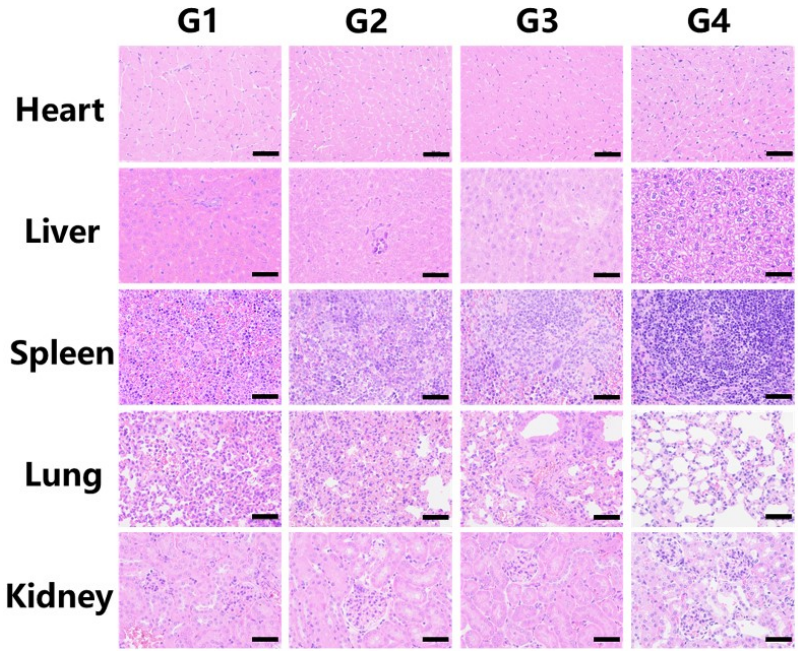

Fig. S23 Hematoxylin and eosin (H&E) staining for the main organs extracted from the mice in each group after treatment. Scale bars: 100 μm.

3. References

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