Electronic Supplementary Information

A Turn-on Probe for Detecting Antituberculotic Drug-induced Liver Injury in Mice via NIR-II Fluorescence/Optoacoustic Imaging

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General experimental procedures

1. Reagents and Materials

Piperazine, 4-nitrophenylglyoxylic acid, oxalyl chloride and triethylamine were purchased from Aladdin Reagents and used without further purification. N, N-dimethyl-formamide (DMF), dichloromethane (DCM) and methanol were analytical grade reagents. RAW264.7 cells were purchased from KeyGen Biology Co. Ltd. H_2O_2 and isoniazid (INH) were purchased from Sigma Aldrich. The water used throughout the experiments was the triple-distilled water.

2. Apparatus

^{1}H NMR spectra were obtained with a Bruker Avance 600 MHz NMR Spectrometer. High resolution mass spectrometer (MS) was measured on a Bruker MAXIS IMPACT mass spectrometer. Absorption spectra were recorded on a Hitachi U-3010 spectrophotometer. The NIR-II Fluorescence spectra were recorded on a NIRQUEST512 spectrometer (excitation: 808 nm laser, emission range: 900-1700 nm). The NIR-II Fluorescence imaging in vivo and ex vivo was performed on NIR-II in Vivo Imaging System (Series II 808/900-1700, Suzhou NIR-Optics Technologies Co., Ltd.). Optoacoustic imaging was conducted with inVision128 multispectral optoacoustic tomographic (MSOT) imaging system (iThera Medical GmbH).

3. Synthetic Procedures and computational method

Synthesis of compound 1:

Compound 1 was synthesized according to the previous report [1].

Synthesis of TC-NN:

Compound 1 (223 mg, 0.40 mmol) and piperazine (138 mg, 1.60 mmol) were dissolved in 10 mL DMF under nitrogen atmosphere. The mixture was stirred at room temperature for 12 h. After that, the crude product was extracted with ethyl acetate and water for three times. Furthermore, the combine organic was purified by silica gel chromatography with CH_2Cl_2 / MeOH (25:1, v/v) as eluent to obtain the golden-brown solid. Yield: 160 mg (68%). ^{1}H NMR (600 MHz, DMSO-d6) δ 7.88-7.86 (d, J = 13.3 Hz, 2H), 5.90-5.88 (d, J = 13.5 Hz, 2H), 3.63-3.61 (t, J = 5.0 Hz, 4H), 3.35-3.34 (t, J = 5.2 Hz, 4H), 2.41-2.39 (t, J = 6.5 Hz, 4H), 1.68 – 1.66 (m, 2H), 1.54 (s, 12H), 1.23 (s, 1H). HR-MS (ESI, m/z) calcd for $C_{34}H_{31}N_8O_2$ $[M]^-$

583.2570, found 583.2584.

Synthesis of TC-H_2O_2:

Under a nitrogen atmosphere, 4-nitrophenylglyoxylic acid (39 mg, 0.20 mmol) and oxalyl chloride (53 μL, 0.60 mmol) were dissolved in 3 mL dichloromethane and stirred at 0°C for 3 min, then two drops of DMF was added and refluxed at 45 °C. After 1 h, the solvent and excess oxalyl chloride were removed via rotary evaporation. The residue was added with TC-NN (60 mg, 0.1 mmol), triethylamine (56 μL, 0.4 mmol) and 4 mL of dichloromethane. The mixture was stirred at room temperature for 3 h, which was then evaporated under reduced pressure to remove solvent. The crude product was purified by silica gel column chromatography with CH_2Cl_2 / MeOH (20:1, v/v) as eluent and the blackish green solid was obtained (35 mg, yield: 44%). ^{1}H NMR (600 MHz, DMSO-d6): δ 8.47 - 8.46 (d, *J* = 8.8 Hz, 2H), 8.22 - 8.20 (d, *J* = 8.8 Hz, 2H), 8.06 - 8.04 (d, *J* = 13.4 Hz, 2H), 5.88 - 5.86 (d, *J* = 13.6 Hz, 2H), 3.93-3.91 (m, 2H), 3.64-3.62 (m, 2H), 3.58-3.56 (m, 2H), 3.40-3.39 (m, 2H), 2.42-2.40 (t, *J* = 6.4 Hz, 4H), 1.68 – 1.66 (m, 2H), 1.53 (s, 12H). HR-MS (ESI, m/z) calcd for $C_{42}H_{34}N_9O_6$ $[M]^-$ 760.2632, found 760.2638.

4. Measurement of absorption and fluorescence spectra

The probe TC-H_2O_2 was prepared in a stock solution (1 mM) in DMSO. For dose-dependent experiments, H_2O_2 with varied doses from 0 – 50 μM was added into the probe TC-H_2O_2 solution (final TC-H_2O_2 concentration: 10 μM) in PBS solution (pH=7.4, 10% DMSO). The solutions were kept at 37 ℃ for 50 min and the absorption and fluorescence spectra of each of the solutions were measured. For time-dependent experiments, the solutions were kept at 37 ℃ for different time periods before spectral measurements. For selectivity and anti-interference experiments, H_2O_2 and /or other substances were added into the probe TC-H_2O_2 (10 μM) in PBS solution (pH=7.4, 10% DMSO). Afterwards the NIR-II fluorescence spectra and optoacoustic intensity of each solution were measured. The NIR-II fluorescence emission spectra were subjected to 808 nm laser irradiation at 70 $mW \cdot cm^{-2}$.

5. Cell culture

RAW264.7 cells were purchased from KeyGen Biology Co. Ltd (Nanjing, China). RAW264.7 cells were incubated in Dulbecco's modified eagle medium (DMEM) supplemented with 10%

FBS, 1% penicillin and streptomycin at 37 ℃ in an incubator (containing 5% CO_2 gas).

6. Cell viability assays

The cytotoxicity of the probe TC-H_2O_2 was evaluated by standard methyl thiazolyl tetrazolium (MTT) assay in RAW264.7 cells. Briefly, the cells were seeded in 96-well plates at 5000 cells per well and cultured for 24 h. The culture medium was washed three times with PBS and incubated with various concentrations (0, 5, 10, 20, 30, 50 μM) of the probe for an additional 24 h. After that the medium was discarded, and the cells were washed with PBS twice. Then the new medium containing 0.5 mg/mL MTT solution were added and incubated for another additional 4 h. Finally, the medium was washed with PBS solution three times and replaced with 150 μL DMSO to dissolve the precipitates. A Thermo MK3 ELISA reader was used to measure the absorbance at 570 nm and evaluate the viability of cells.

7. Cell imaging

The RAW264.7 cells were seeded in the 6-well plates and incubated for 24 h. After cells adhered to plates, the cells were washed with PBS and then incubated in medium supplemented with 10% FBS containing with the probe TC-H_2O_2 (30 μM) at 37 ℃ under 5% CO_2 for 1 h. For imaging exogenous H_2O_2 in RAW264.7 cells, the cells were pretreated with H_2O_2 (100 μM) for 1 h follow by washing with sterile PBS buffer for three times and then incubated with TC-H_2O_2 (30 μM) for 1 h. For imaging endogenously generated H_2O_2 in RAW264.7 cells, in the first group, the cells were pretreated with phorbol-12-mysirate-13-acetate (PMA) (1.0 μg/mL) for 30 min and then treated with TC-H_2O_2 (30 μM) for 1 h; in the second group, the cells were pretreated with phorbol-12-mysirate-13-acetate (PMA) (1.0 μg/mL) for 30 min and then incubated with NAC (a H_2O_2 scavenger, 500 μM) for 1 h, and further treated with TC-H_2O_2 (30 μM) for 1 h. After that, the treated cells were washed, trypsinized, centrifuged, suspended in PBS and then subjected to NIR-II fluorescence imaging using NIR-II in Vivo Imaging System (Series II 808/900-1700, Suzhou NIR-Optics Technologies Co., Ltd.) (excitation: 808 nm laser with 20 $mW \cdot cm^{-2}$, emission filter: 900-1700 nm) and MSOT imaging (excitation: 800 nm). For cell experiments in MSOT imaging, the control or the treated cells suspended in PBS was fully filled in modified commercial Wilmad NMR tubes respectively and fixed on the holder of the imaging instrument.

8. Phantom optoacoustic imaging

The test solutions containing 10 μM TC-H_2O_2 in PBS (pH=7.4, 10% DMSO) and varied concentrations of H_2O_2 (0, 5, 10, 20, 30, 40, 50 μM) were stirred for 50 min at 37 ℃. Then the solutions were added into commercial Wilmad NMR tubes for phantom optoacoustic imaging which was conducted with MSOT system (in Vision 128, iThera Medical GmbH).

The OA images were reconstructed at 800 nm with the back-projection reconstruction method.

9. Animal experiments

The BALB/c male mice (male, 6-7 weeks old) were purchased from Guangdong Medical Laboratory Animal Center (GDMLAC) and kept in the Laboratory Animal Center of South China Agricultural University. The animal experiments were approved by and conducted in compliance with the regulations of Ethics Committee of Laboratory Animal Center of South China Agricultural University. All animal operation was carried out conforming to the regulations on the management of laboratory animals of China and the Regulations on the Administration of Laboratory Animals of Guangdong Province. Mice were divided randomly to establish animal models and support subsequent experimental investigations. In the case of lethal experiment procedures, mice were euthanized by exposure to carbon dioxide gas. Before the imaging experiments, the hair of the mice was removed using the depilatory cream.

10. Mouse model of INH (isoniazid)-induced liver injury

The BALB/c male mice (male, 6-7 weeks old) were randomly divided into four groups (n=5 per group). For INH (isoniazid)-induced liver injury model groups, the mice were treated with varied doses of isoniazid (100 $mg \cdot kg^{-1}$ or 200 $mg \cdot kg^{-1}$) via intraperitoneal injection. For liver rehabilitation group, the mice were intraperitoneally injected with 200 $mg \cdot kg^{-1}$ isoniazid followed by oral gavage with N-acetylcysteine (NAC) (100 $mg \cdot kg^{-1}$). For the control group, the mice were treated with isovolumic saline via intraperitoneal injection.

11. NIR-II Fluorescence imaging

In the experiments, four hours after different groups of mice undergoing different treatments (the INH induced liver injury model was successfully established), the mice were anesthetized with 2% isoflurane in oxygen and were given an intravenous injection of 3.45 $mg \cdot kg^{-1}$ TC-H_2O_2 for vivo NIR-II fluorescence imaging (The following time points were selected for

imaging: 0 min, 5 min, 15 min, 30 min, 45 min, 60 min, 4 h, 24 h).

For ex-vivo (organ) NIR-II fluorescence imaging, four hours after different groups of mice undergoing different treatments, at 60 min after the probe injection, the mice were sacrificed by CO_2 asphyxiation and then the major organs such as heart, liver, spleen, lung and kidney were harvested for NIR-II fluorescence imaging. The NIR-II fluorescence images were taken with NIR-II in Vivo Imaging System (Series II 808/900-1700, Suzhou NIR-Optics Technologies Co., Ltd.) (excitation:808 nm laser with 30 $mW \cdot cm^2$, emission filter: 900-1700 nm)

12. Optoacoustic imaging

For in vivo optoacoustic experiment, mice were given an injection of probe TC-H_2O_2 (3.45 $mg \cdot kg^{-1}$) via tail vein. Then the mice were anesthetized with continuous isoflurane with oxygen flow and placed in prone position in animal holder for imaging at several time periods post i.v. injection of probe. The following wavelengths were selected for correspondence with the major turning points in the absorption spectra of TC-NN and hemoglobin: 700 nm, 730 nm, 750 nm, 765 nm, 780 nm, 800 nm, 850 nm and 900 nm (background). 10 individual frames at each wavelength were recorded. Cross-sectional images were acquired with a step size of 0.3 mm spanning through the whole liver region.

For ex vivo optoacoustic experiment, at 60 min post the probe injection, mice were sacrificed by CO_2 asphyxiation and the major organs were harvested for MSOT imaging. Guided ICA spectral unmixing was utilized to separate signal from the activated probe and those from the photo-absorbing elements in tissues in the body (e.g. hemoglobin).

13. Tissue histological evaluation

For the study of biosafety, mice were i.v. injected with the probe TC-H_2O_2 (3.45 $mg \cdot kg^{-1}$) or isovolumic saline daily for 7d. Then the major organs such as heart, liver, spleen, lung and kidney were excised for histological analyses via hematoxylin and eosin (H&E) staining.

For the evaluation of INH-induced liver injury, 4 h after the treatment of isoniazid via i.p. injection, the mice in four different groups were euthanized and the liver was excised and subjected for histological analyses via H&E staining.

14. Mice weight assessment

Two groups of BALB/c mice were injected with either normal saline (control group) or probe TC-H_2O_2 (3.45mg/kg^{-1}) via tail vein daily for 7 days. Then, the body weight of the mice was measured daily for 7 days.

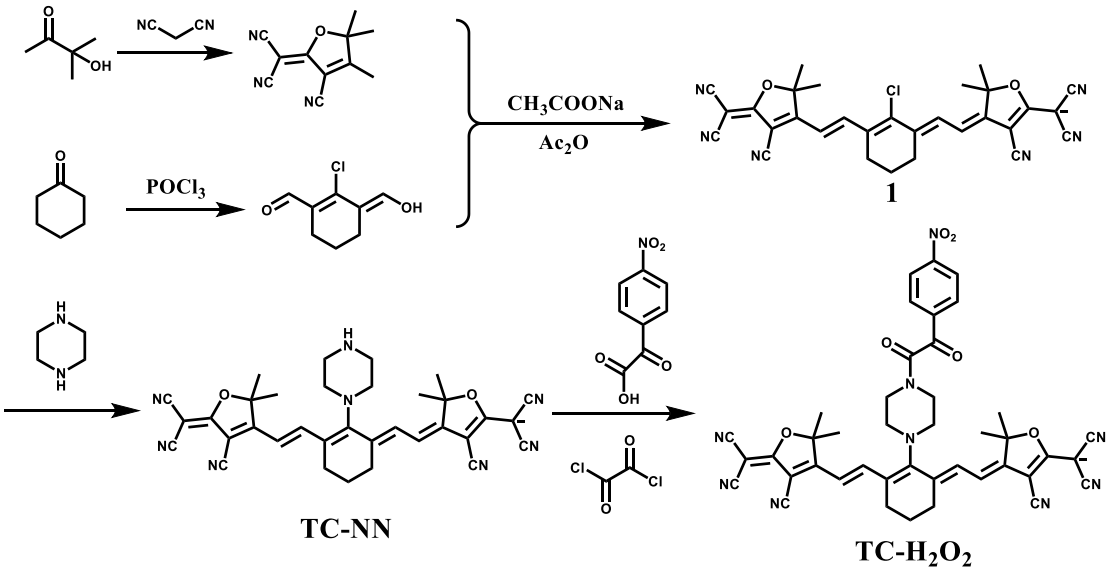
15. Serum biochemistry assessment

Serum biochemistry index alanine aminotransferase (ALT) was measured by Elisa Kits.

16. Relative OA intensity

Relative OA intensity was calculated with the equation:

Relative OA intensity=[$(OA_{800})_{H_2O_2}$-$(OA_{800})_{control}$]/ $(OA_{800})_{control}$



Scheme S1. Synthetic route of TC-H_2O_2.
(The counter cation of compound 1, TC-NN and TC-H_2O_2 is sodium ion)

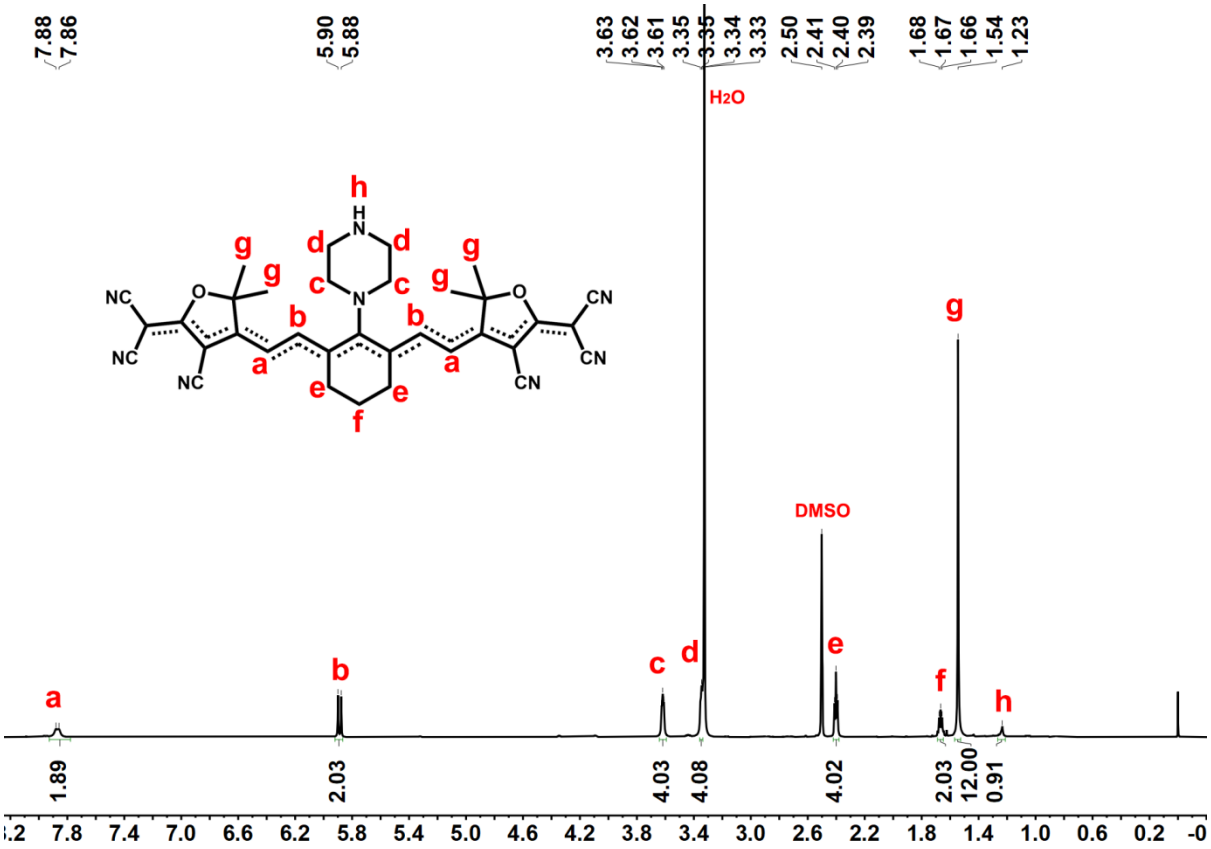


Fig. S1 ^{1}H NMR spectrum of TC-NN (600 Hz, DMSO-d_6).

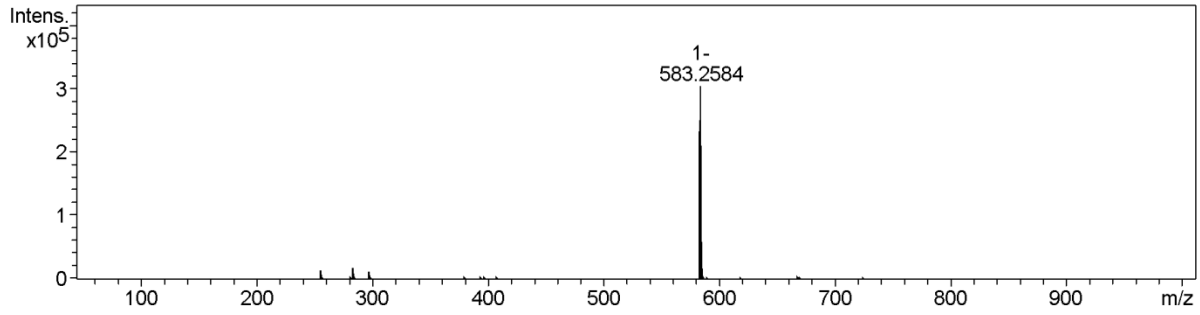


Fig. S2 HR Mass spectrum of TC-NN. m/z $[M]^-$ 583.2584

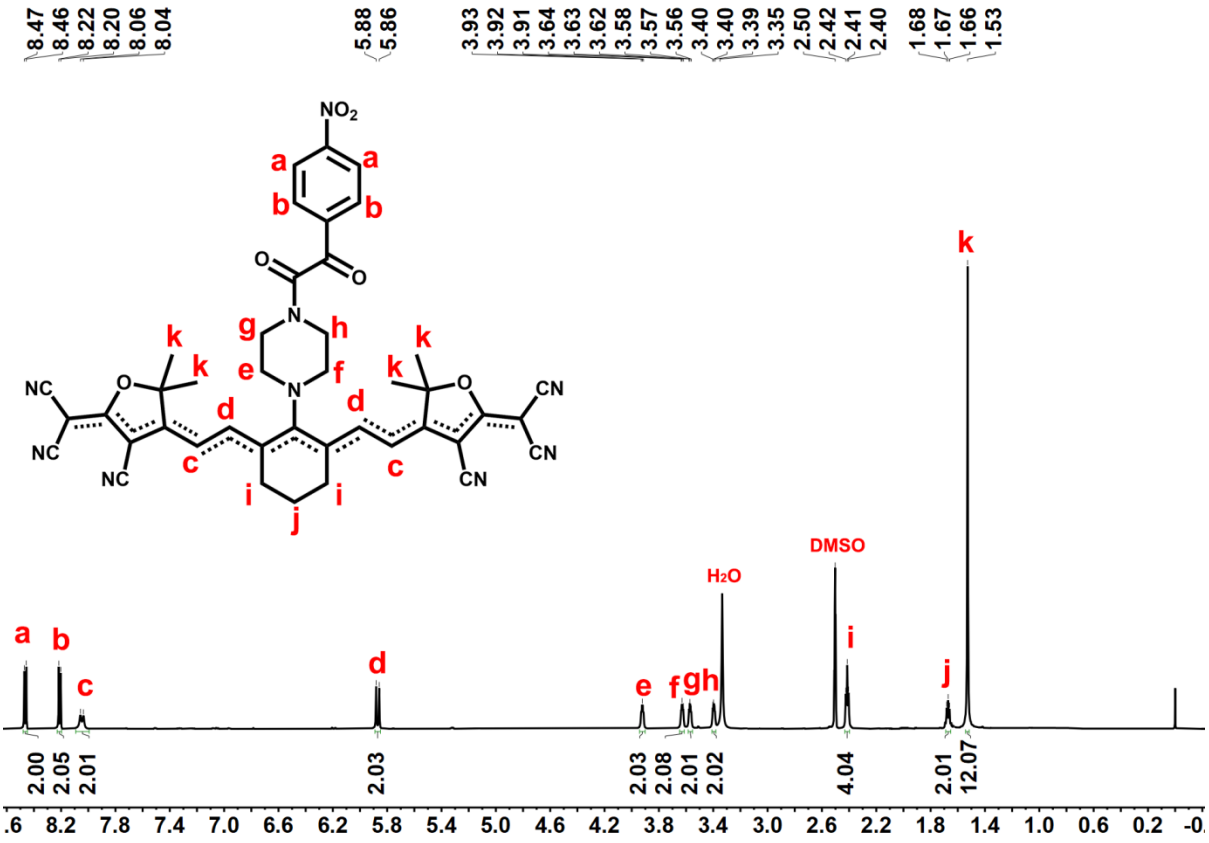


Fig. S3 ^{1}H NMR Spectrum of TC-H_2O_2 (600 Hz, DMSO-d_6).

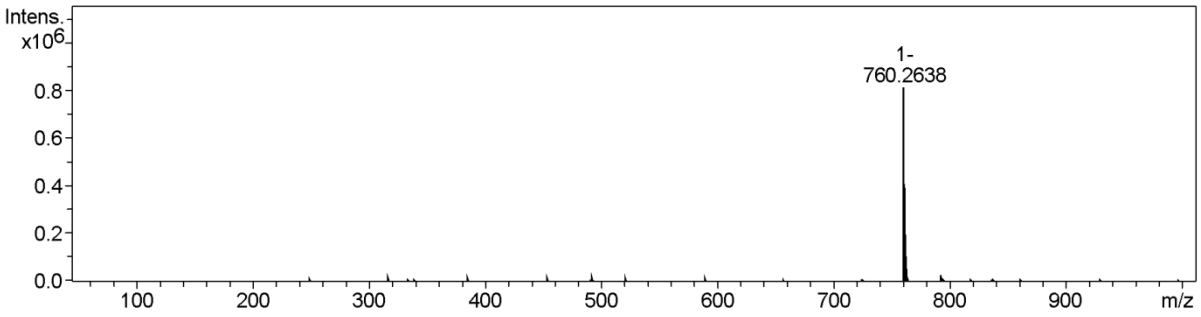


Fig. S4 HR Mass spectrum of TC-H_2O_2. m/z $[M]^-$ 760.2638

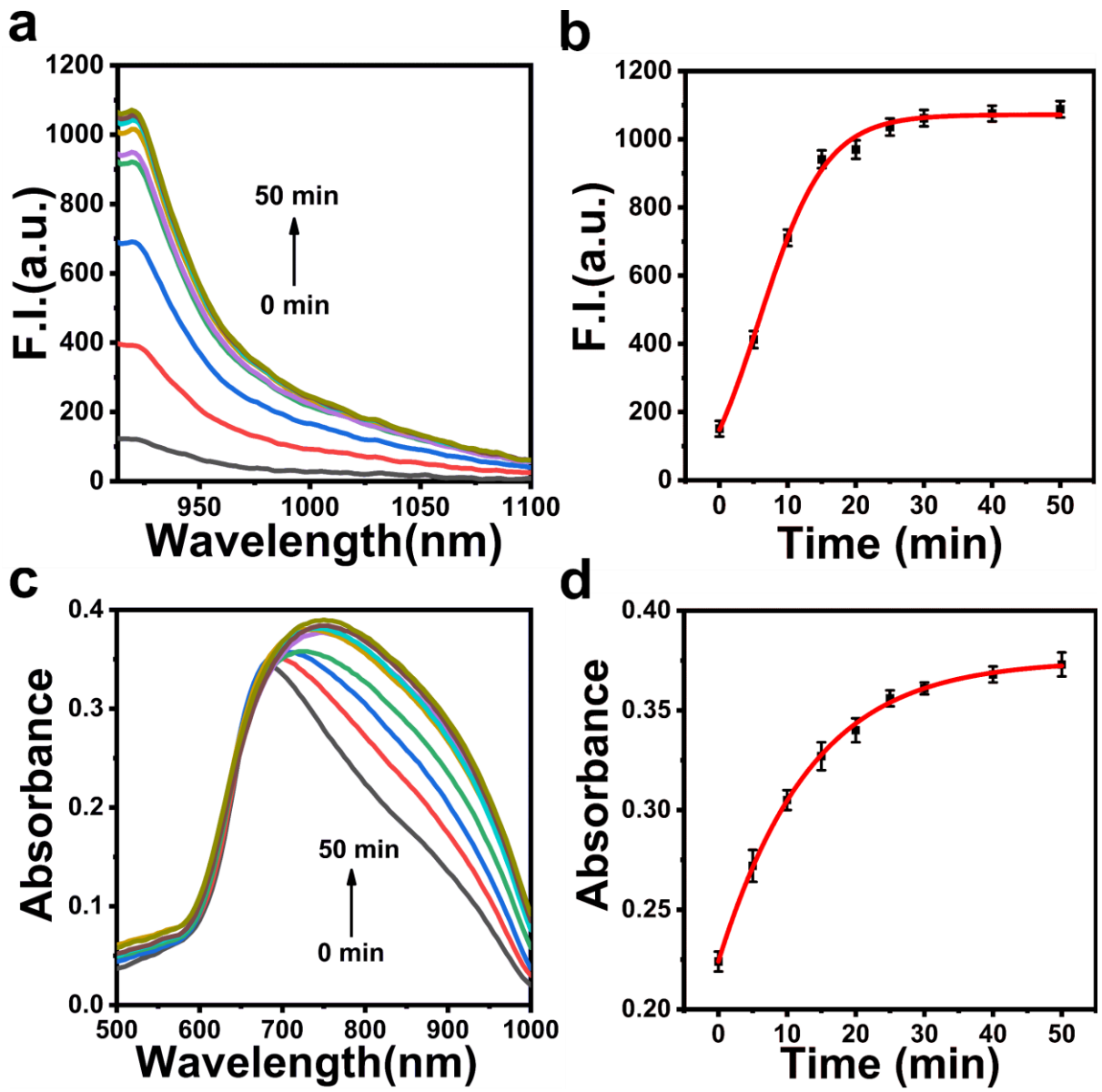


Fig. S5 (a) Time-dependent NIR-II fluorescence spectra of probe TC-H_2O_2 (10 μM) in PBS (pH= 7.4, 10% DMSO) after incubation with 50 μM H_2O_2 at 37 ℃ for varied time. (b) The plot of fluorescent intensity at 920 nm after incubation with 50 μM H_2O_2 at 37 ℃ for varied time. (c) Time-dependent absorption spectra of of probe TC-H_2O_2 (10 μM) in PBS (pH= 7.4, 10% DMSO) after incubation with 50 μM H_2O_2 at 37 ℃ for varied time. (d) The plot of absorbance at 800 nm at different time points after incubation with 50 μM H_2O_2 at 37 ℃.

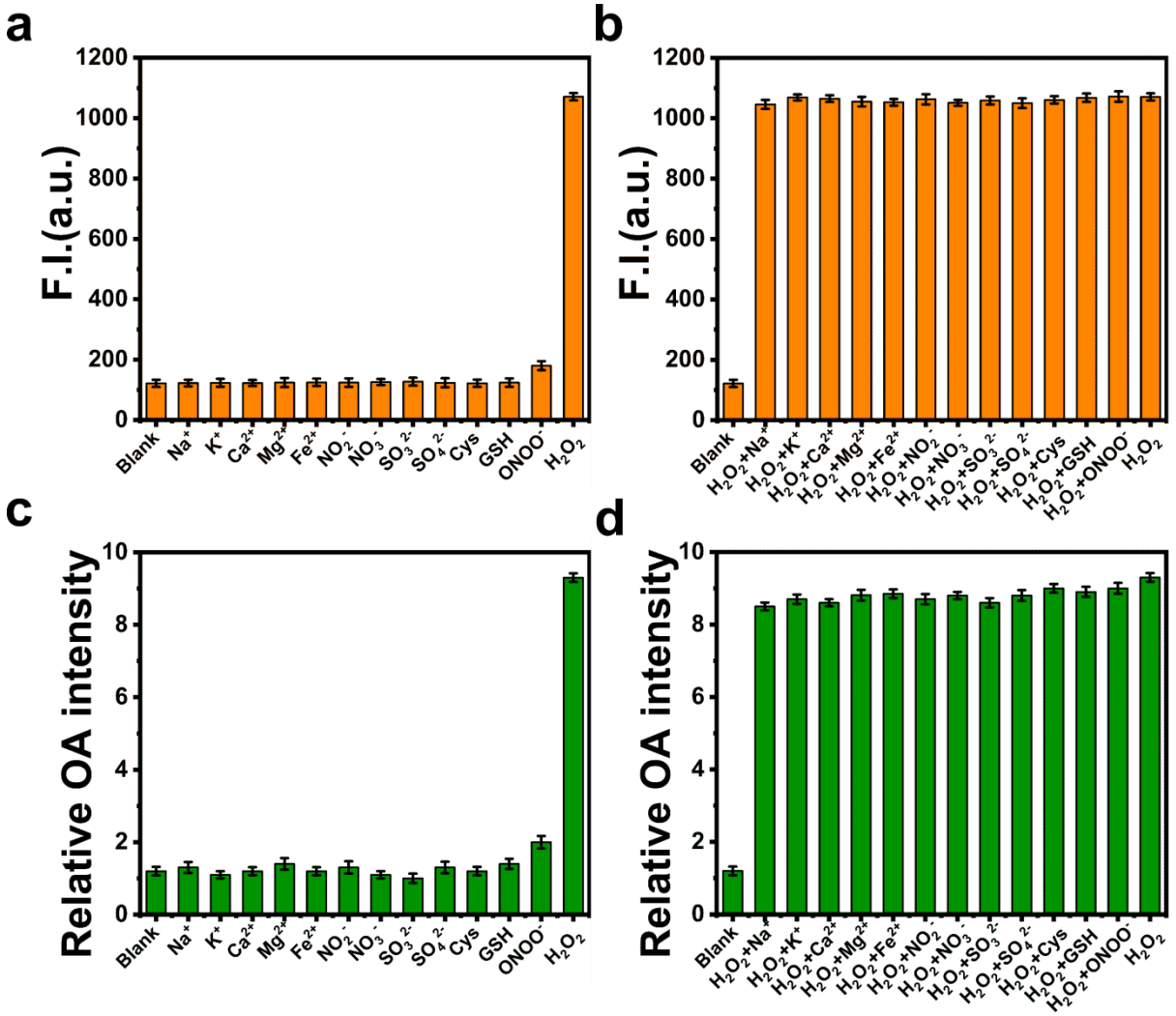


Fig. S6 (a) Fluorescent intensity at 920 nm of the probe TC-H_2O_2 (10 μM) upon incubation with different substances respectively in PBS (pH= 7.4, 10% DMSO) at 37 ℃ for 50 min. (Cations and anions: 1 mM; Cys: 200 μM; GSH: 10 mM; $ONOO^-$: 50 μM; H_2O_2: 50 μM) (b) Fluorescent intensity at 920 nm of the probe TC-H_2O_2 (10 μM) upon treatment with 50 μM H_2O_2 and simultaneously in the presence of individual potential interferent respectively for 50 min in PBS (pH= 7.4, 10% DMSO) at 37 ℃. (Cations and anions: 1 mM; Cys: 200 μM; GSH: 10 mM; $ONOO^-$: 50 μM; H_2O_2: 50 μM) (c) Relative optoacoustic intensity of the probe TC-H_2O_2 (10 μM) in the presence of different substances respectively for 50 min in PBS (pH= 7.4, 10% DMSO) at 37 ℃. (Cations and anions: 1 mM; Cys: 200 μM; GSH: 10 mM; $ONOO^-$: 50 μM; H_2O_2: 50 μM) (d) Relative optoacoustic intensity of the probe TC-H_2O_2 (10 μM) in the presence of 50 μM H_2O_2 and simultaneously in the presence of individual potential interferent respectively for 50 min in PBS (pH= 7.4, 10% DMSO) at 37 ℃. (Cations and anions: 1 mM; Cys: 200 μM; GSH: 10 mM; $ONOO^-$: 50 μM; H_2O_2: 50 μM).

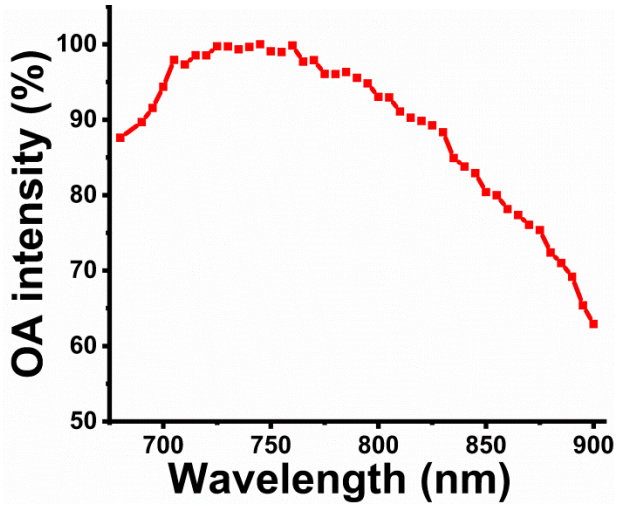


Fig. S7 Normalized optoacoustic intensities for the probe TC-H_2O_2 (10 μM) upon incubation with 50 μM H_2O_2 for 50 min as a function of excitation wavelength.

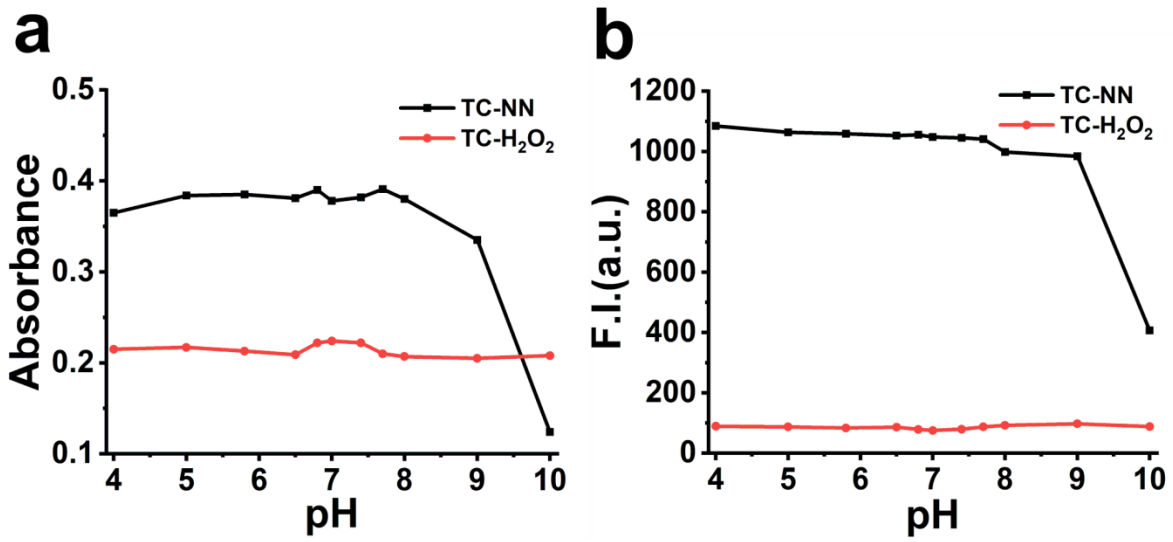


Fig. S8 (a) Effect of pH on the absorbance at 800 nm for TC-NN (10 μM, 10%DMSO) and TC-H_2O_2 (10 μM, 10%DMSO). (b) Effect of pH on the fluorescence intensity at 920 nm for TC-NN (10 μM, 10%DMSO) and TC-H_2O_2 (10 μM, 10%DMSO).

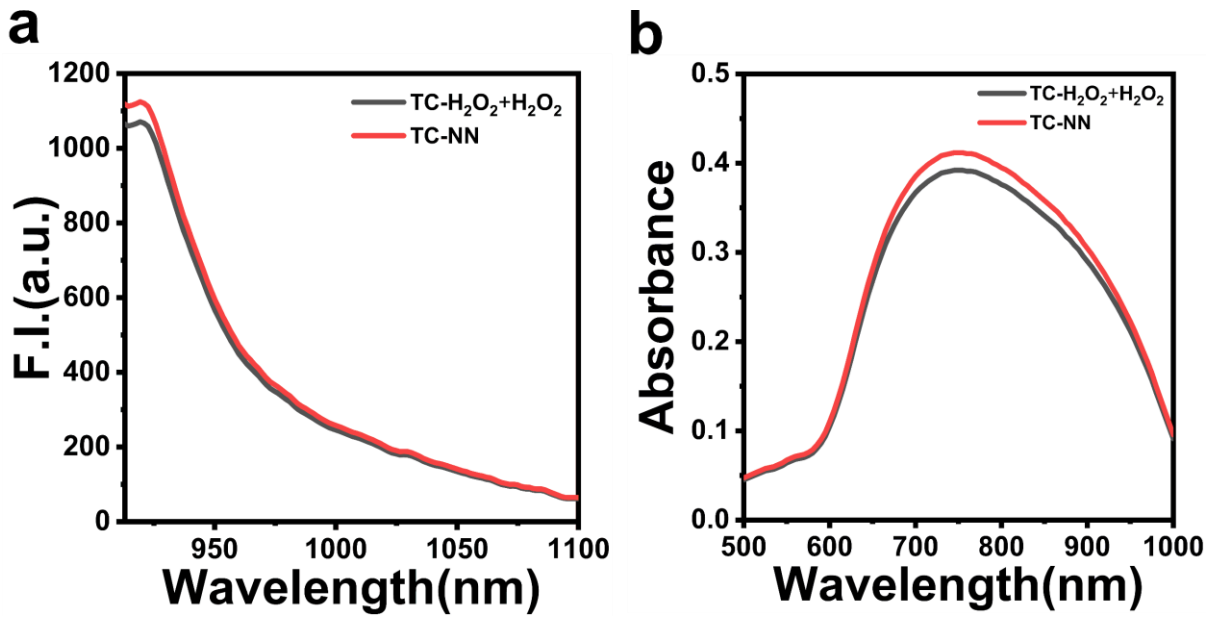


Fig. S9 (a) NIR-II fluorescence spectra for the probe TC-H_2O_2 (10 μM, 10% DMSO in PBS) with incubation of 50 μM H_2O_2 and for the TC-NN (10 μM, 10% DMSO in PBS). (b) Absorption spectra for the probe TC-H_2O_2 (10 μM, 10% DMSO in PBS) with incubation of 50 μM H_2O_2 and for the TC-NN (10 μM, 10% DMSO in PBS).

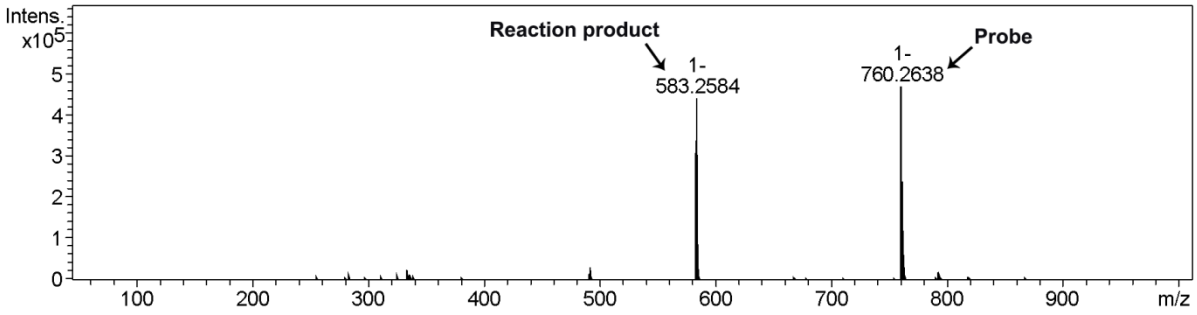


Fig. S10 HR mass spectrum of the probe TC-H_2O_2 after being incubated with 50 μM H_2O_2 for 15 min (incomplete reaction) in PBS (pH= 7.4, 10% DMSO) at 37 ℃.

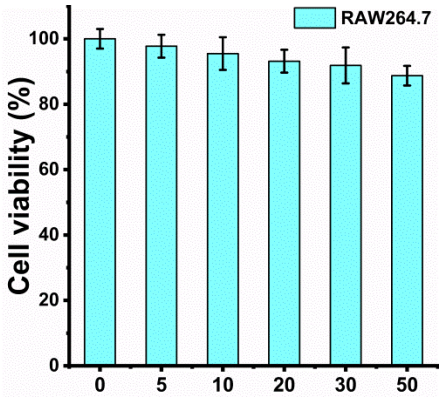


Fig. S11 Cell viability for RAW264.7 cells in the presence of the probe TC-H_2O_2 at varied concentrations. Three independent experiments were conducted; and for each independent experiment, the assays were conducted in eight replicates. Data represent mean ± SD. Error bars represent the standard deviation (SD).

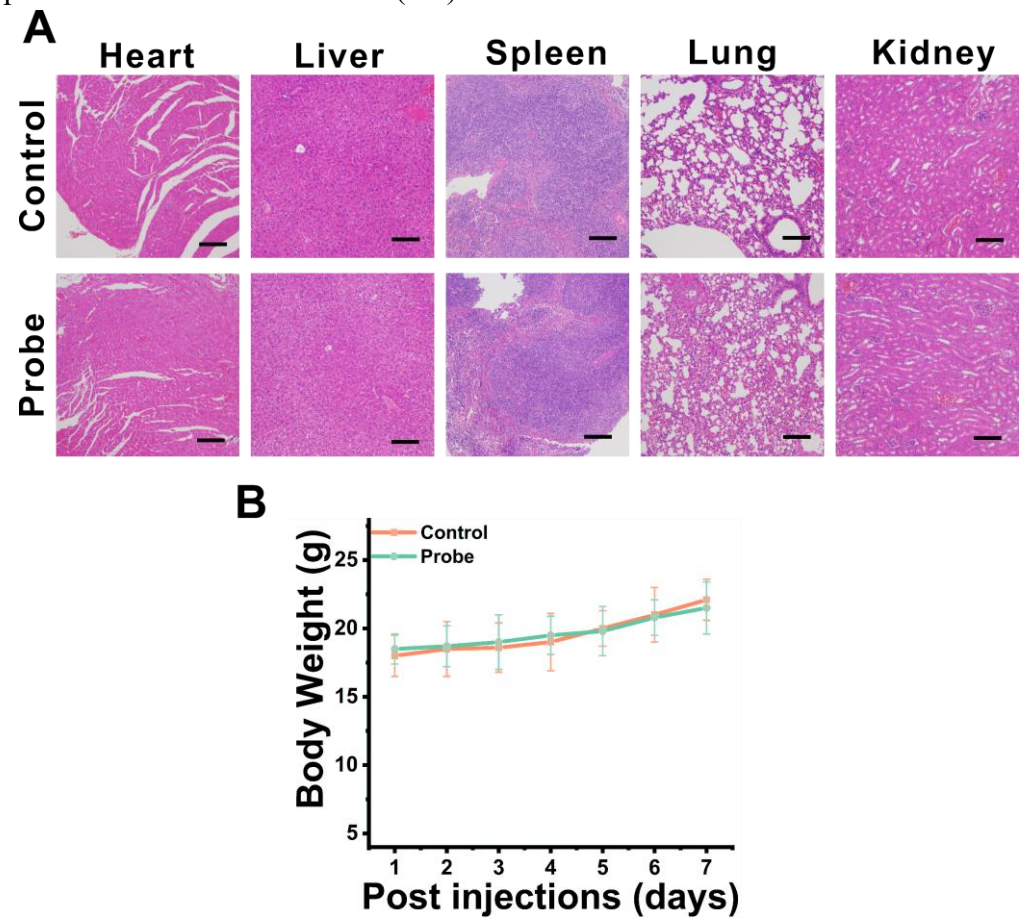


Fig. S12 (A) Histological sections (H&E staining) of heart, liver, spleen, lung and kidney for the healthy mice upon intravenously injected with saline (the control) or the probe TC-H_2O_2 (3.45 mg·kg^{-1}) daily for 7d. Scale bar: 100 μm. (B) The body weights changes of healthy mice within 7d upon intravenously injected with saline (the control) or the probe TC-H_2O_2 (3.45 mg·kg^{-1}) daily.

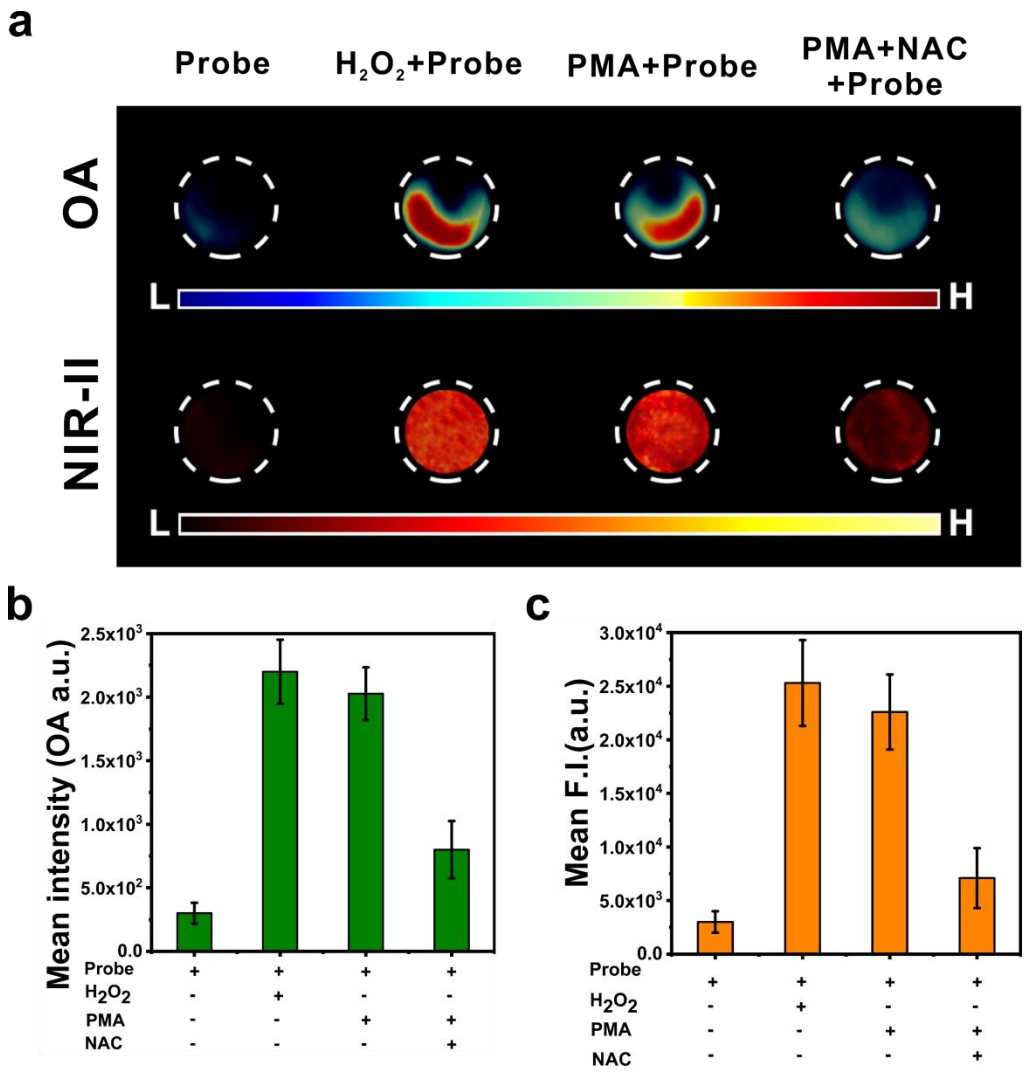


Fig. S13 (a) Optoacoustic (OA) images and NIR-II fluorescent images for RAW264.7 cells incubated with different substances and subsequently with the probe TC-H_2O_2 (10 μM) at 37 °C. (b) Mean optoacoustic intensities corresponding to the optoacoustic images in (a) for RAW264.7 cells. (c) Mean NIR-II fluorescence intensities corresponding to the fluorescent images in (a) for RAW264.7 cells.

PMA: a stimulant for generating endogenous H_2O_2; NAC: a H_2O_2 scavenger.
For optoacoustic imaging: excitation at 800 nm.
For NIR-II fluorescence imaging: excitation: 808 nm, emission filter: 900-1700 nm.

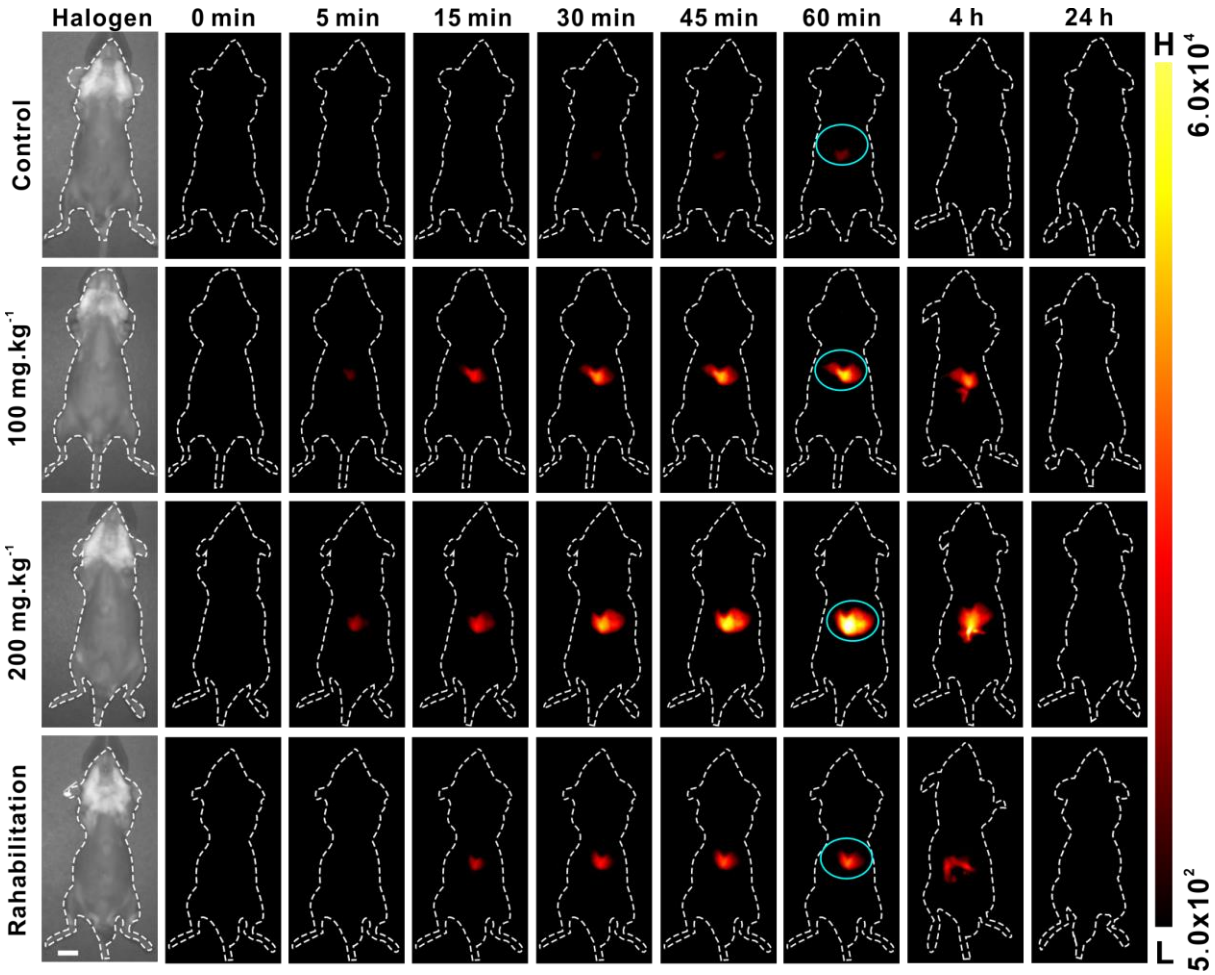


Fig. S14 The NIR-II fluorescence images of the mice treated with the saline (control), 100 mg kg^{-1} INH, 200 mg kg^{-1} INH or 200 mg kg^{-1} INH+100 mg kg^{-1} NAC (Rehabilitation), followed by tail intravenous injection of 3.45 mg kg^{-1} TC-H_2O_2 in saline (containing 5% DMSO). Cyan circle: liver region. The images of the mice under halogen light serve as the bright-field images. Scale bar: 1 cm.

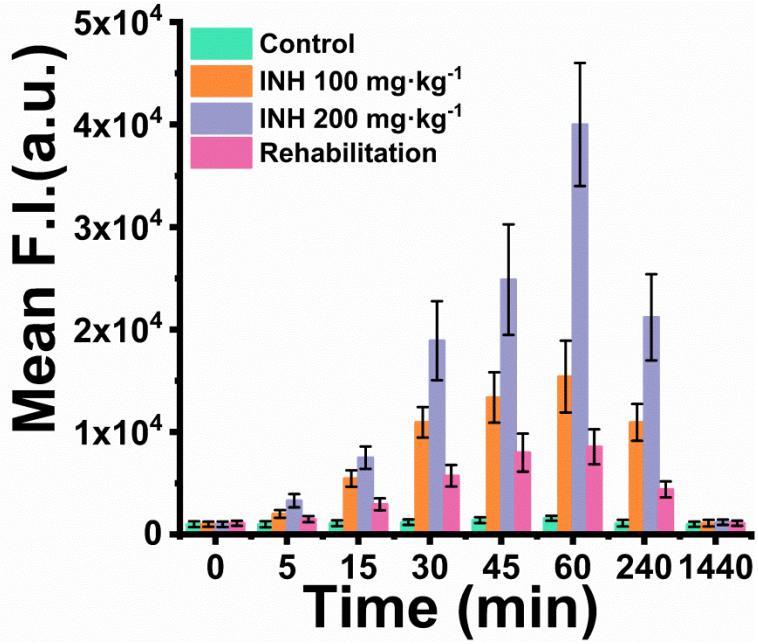


Fig. S15 Mean fluorescence intensities at ROI (cyan circle) of the liver region in mice of Fig. S14.

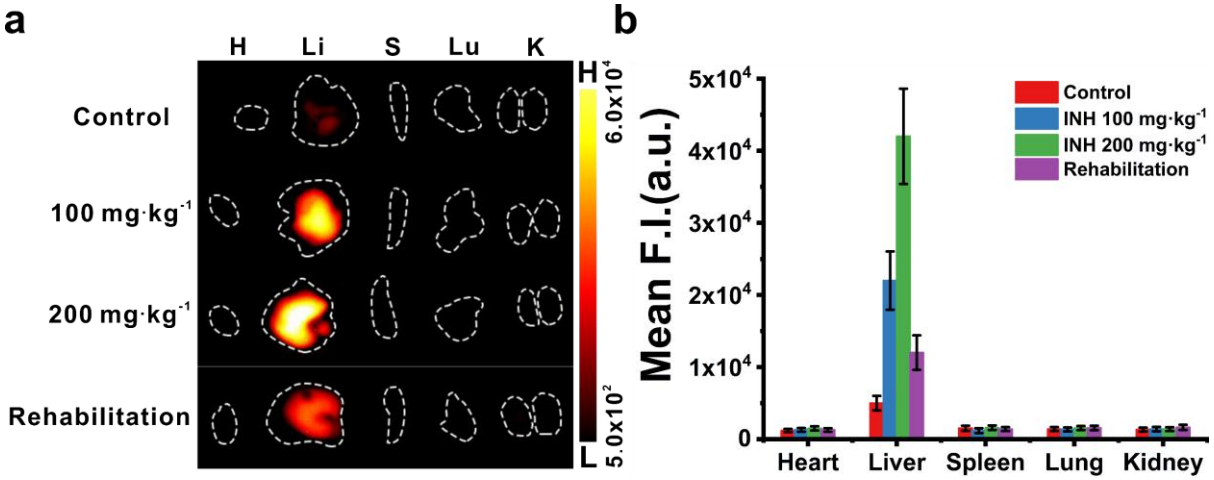


Fig. S16 (a) NIR-II Fluorescence images for major organs harvested from the mice in different groups. (b) Mean NIR-II fluorescent intensities for major organs corresponding to (a).

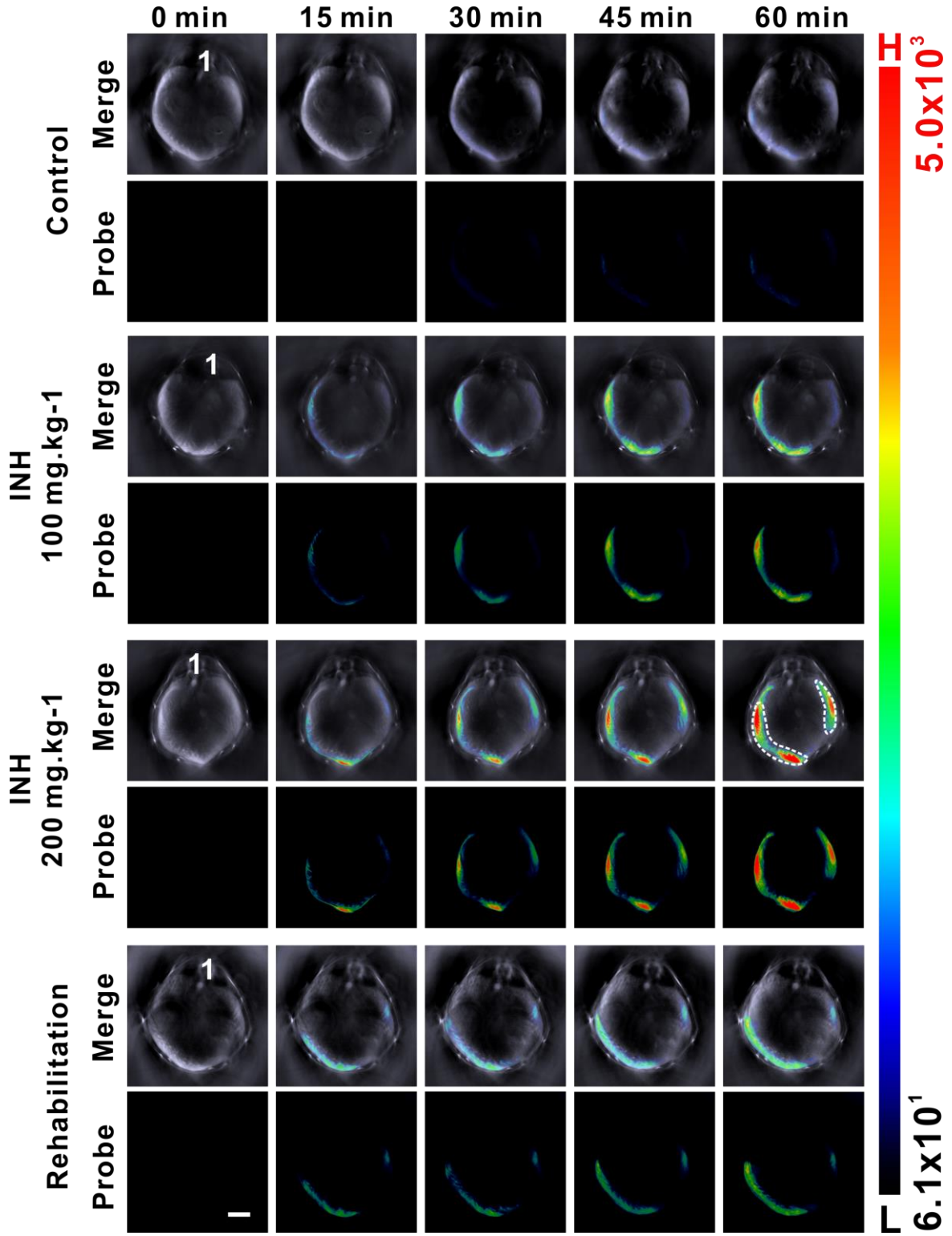


Fig. S17 Cross-sectional MSOT images of the mice from different groups at varied time points upon i.v. injection of TC-H_2O_2. The mice groups were treated with saline, 100 mg·kg^{-1} INH, 200 mg·kg^{-1} INH, 200 mg·kg^{-1} INH followed by 100 mg·kg^{-1} NAC. Upper panel: Overlay of the activated probe's signal with the grayscale single-wavelength (900 nm) background image. Organ labelling: 1. Spinal cord; White dotted circles: region of interest (ROI) at liver region. Scale bar: 3 mm.

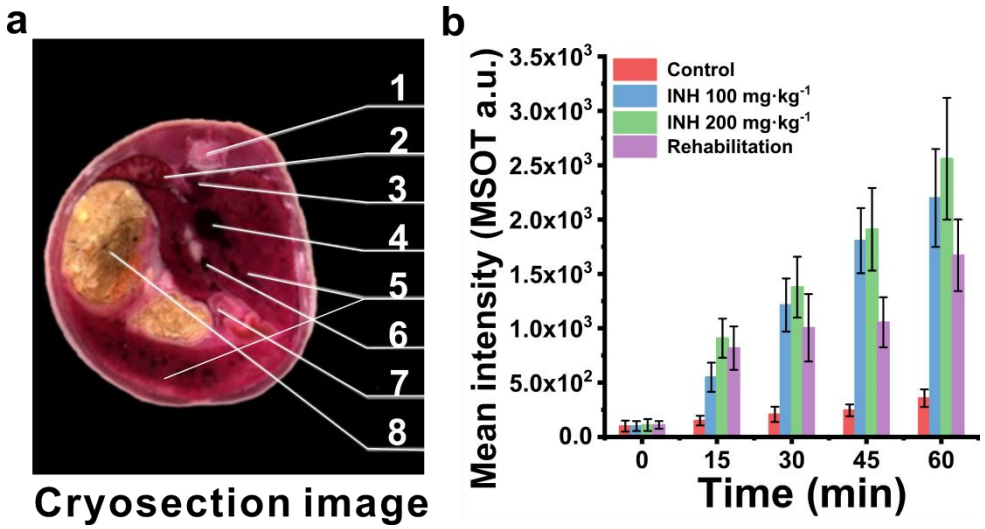


Fig. S18 (a) A cryosection image of a male mouse with the cross-section's location comparable to those shown in Fig. S17. 1. Spinal cord; 2. Spleen; 3. Thoracic aorta; 4. C. vena cava; 5. Liver; 6. Portal vein; 7. Intestines; 8. Stomach. (b) Mean MSOT intensities in the liver regions of different groups for varied time upon probe TC-H_2O_2 injection. (corresponding to Fig. S17).

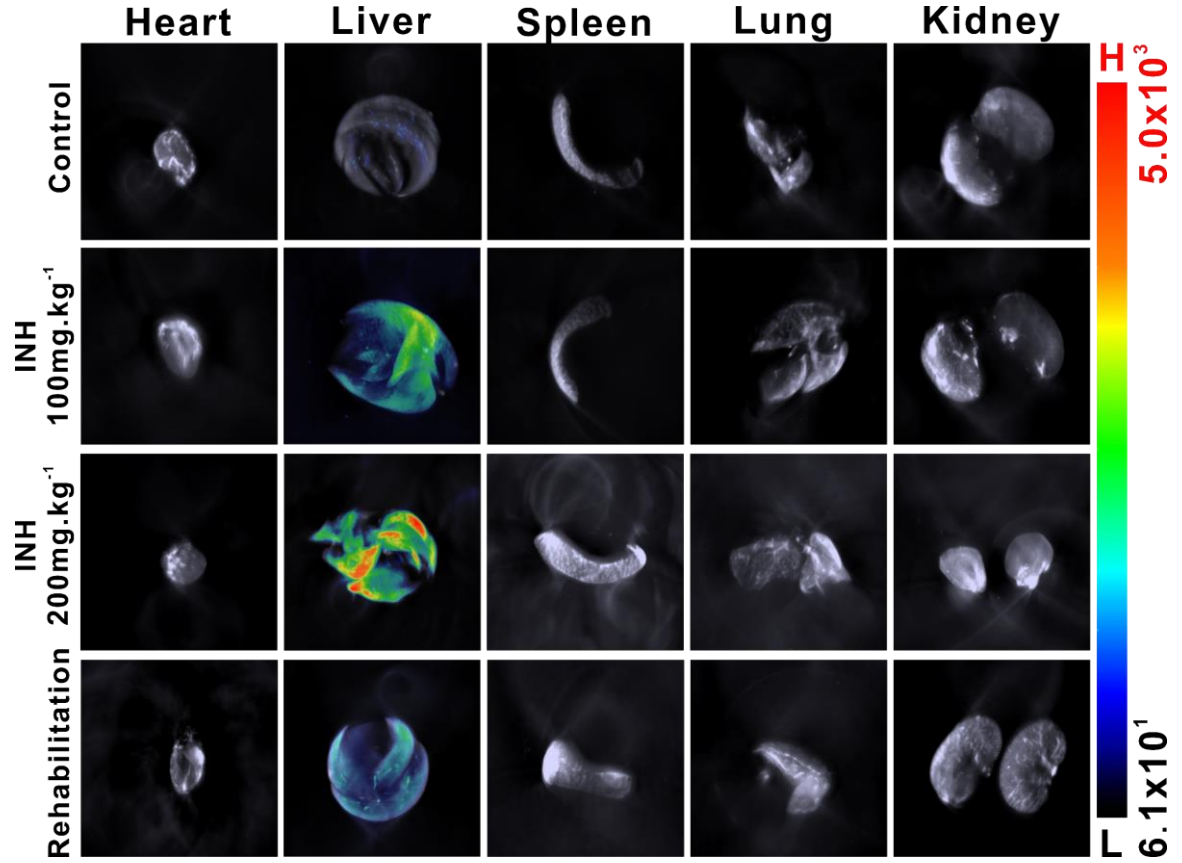


Fig. S19 MSOT images for excised organs (heart, liver, spleen, lung, kidney) of the mice from different groups after pretreatment with saline, 100 mg·kg^{-1} INH, 200 mg·kg^{-1} INH, 200 mg·kg^{-1} INH followed by 100 mg·kg^{-1} NAC (rehabilitation group), at 60 min after i.v. injection of the probe TC-H_2O_2. The excised organs underwent immediate embedment in phantom. Scale bar: 5 mm

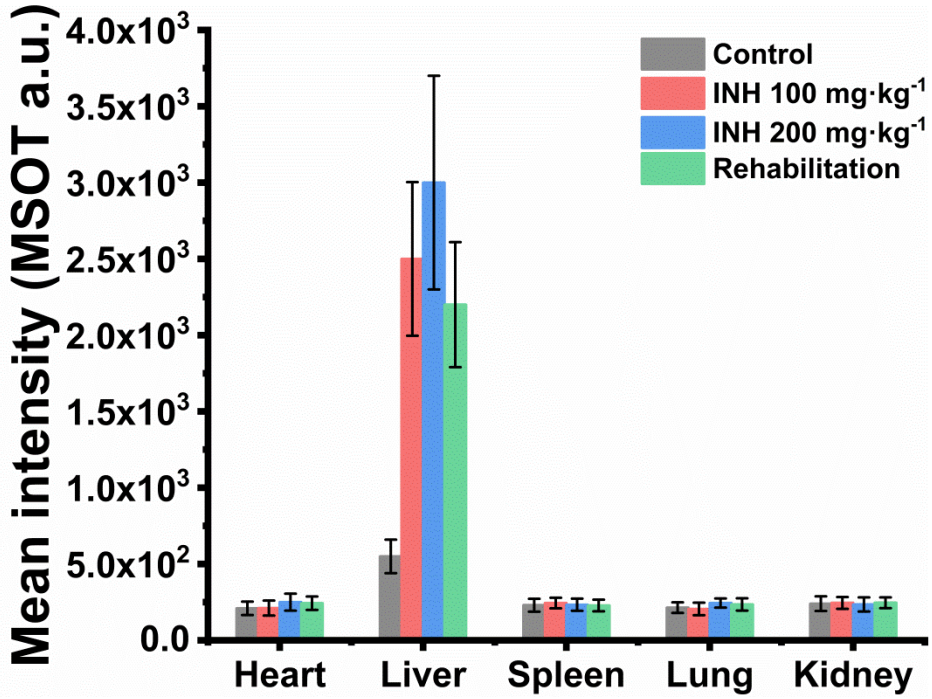


Fig. S20 Mean MSOT intensities in major organs corresponding to Fig. S19.

References

[1] Y. Wu, J. Chen, L. Sun, F. Zeng and S. Wu, *Adv. Funct. Mater.*, 2019, **29**, 1807960.