Supporting information

All-in-One $CoFe_2O_4$@Tf Nanoagent with GSH Depletion and Tumor-Targeted Ability for Mutually Enhanced Chemodynamic/Photothermal Synergistic Therapy

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1. **Materials and methods**

1.1 Materials

All of the chemical reagents were utilized in this work without any further purification. Allyl glycidyl ether (AGE), 3-mercaptopropionic acid (MPA), Sodium hydride (NaH), Ferric chloride ($FeCl_3 \cdot 6H_2O$), Cobalt chloride hydrate ($CoCl_2 \cdot H_2O$), 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB), Methylene blue (MB), 3,3',5,5'-Tetramethylbenzidine (TMB), Glutathione (GSH), and 3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were supported by Aladdin (Shanghai, China). Polyethylene glycol monomethyl ether ($mPEG_{113}$) was obtained from Shanghai Macklin Biochemical Co., Ltd. 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), propidium iodide (PI), Calcein acetoxymethyl ester (Calcein-AM), Cell Mitochondria Isolation Kit (JC-1) were purchased from Beyotime (Shanghai, China). Annexin V-FITC/PI apoptosis detection kit was purchased from Biosharp (Beijing, China). High glucose Dulbecco's modified Eagles medium (DMEM), penicillin/streptomycin, 0.25% trypsin-EDTA, and Fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA).

1.2 Preparation of CFOT

Synthesis of mPEG-P(AGE-MPA): poly (ethylene glycol)-poly (allyl glycidyl ether-3-mercaptopropionic acid) (mPEG-P(AGE-MPA)) was synthesized by the previous method in our Labs.[1-3] Briefly, mPEG (10.00 g), AGE (4.20 mL), and NaH (0.24 g) were dissolved in a 100 mL Schlenk flask filled with N_2, and reacted at 100 ℃ for 24 h. After terminating the reaction with ethanol, the mPEG-PAGE was obtained by precipitation with ether. Subsequently, mPEG-PAGE (11.48 g), MPA (3.48 mL), and 2,2-bimethoxy-2-phenylacetophenone (0.25 g) were dissolved in 100 mL THF and filled with N_2. After reaction at UV light for 8 h, the mPEG-P(AGE-MPA) was obtained by precipitation with ether and freeze-dried.

Preparation of CFO: $CoCl_2 \cdot H_2O$ (0.2 mM), $FeCl_3 \cdot 6H_2O$ (0.4 mM), mPEG-P(AGE-MPA) (150 mg), and CH_3COONa (500 mg) were dissolved in Ethylene glycol and magnetically stirred at room temperature for 60 min. Following, the mixture was transferred to the Teflon reactor and reacted at 180 ℃ for 24 h. After cooling, the reaction solution was dialyzed with deionized water, and freeze-dried to obtain brown-black solid CFO.

Modification of transferrin: the holo-form (ferric) transferrin (0.05 g) was incubated in 1

mM EDTA, 3% (v/v) acetic acid for 5 min at 25 ℃ and then dialyzed in 50 mM HEPES buffer pH 7.4 to obtain apo-form (apo-Tf). Next, apo-Tf was incubated at room temperature for 2-4 h at pH 7.4 in the presence of 27 mM NH_4HCO_3 with a 100-fold molar excess of CFO to obtain CFOT [4].

1.3 GSH depletion measurements

To investigate time-dependent GSH consumption, CFOT NPs (200 μg mL^{-1}) was incubated with GSH (1 mM) at the varied time (0, 10, 30, 60, 90, 120,150, 180, and 240 min). Similarly, to confirm the concentration-dependent GSH consumption, CFOT NPs (200, 100, 50 25, 0 μg mL^{-1}) were shaken with GSH (1 mM) for 4 h at 37 ℃. After centrifugation, DTNB was added to the supernatant with incubation for another 10 min, and then the absorbance of DTNB has immediately detected via UV–vis absorption spectroscopy (Lambda 950, Perkinelmer Management (Shanghai) Company Limited).

1.4 •OH generation detection

The indicator MB was used to measure the •OH produced by the reaction of CFOT NPs with H_2O_2. The solutions of PBS (pH 6.5), GSH (1 mM), H_2O_2 (10 mM) and CFOT NPs (400 μg mL^{-1}) were added into MB solution (10 μg mL^{-1}) according to the reaction groups (groups: PBS, GSH, H_2O_2, CFOT, CFOT+H_2O_2+GSH, CFOT+H_2O_2+GSH+ L (Laser: 808 nm, 1.2 W cm^{-2}). Following, the mixtures were incubated for 2 h and then the absorbance of the supernatant was measured at 400~800 nm after centrifugation. The degradation effect of CFOT NPs (400 μg mL^{-1}) on MB (10 μg mL^{-1}) was studied by measuring the absorbance at different reaction time (0~300 min). In TMB assay, CFOT with different concentration (200, 100, 50, 25, 12.5, 6.25, 0 μg mL^{-1}) H_2O_2 (10 mM) and GSH (1mM) were severally added into the acetate buffer solution (pH 6.5) containing TMB before measurement of the UV–vis absorption spectra (400~800 nm) to detect the generation of •OH. In addition, the application of EPR technology further proves the production of •OH. Reaction groups include: (1) CFOT at pH 6.5, (2) CFOT+ GSH at pH 6.5, (3) CFOT+ H_2O_2 at pH 6.5, (4) CFOT+ H_2O_2 +GSH at pH 6.5, (5) CFOT+ H_2O_2+GSH+L at pH 6.5, (6) CFOT+ H_2O_2 +GSH at pH 5.5, (7) CFOT+ H_2O_2 +GSH at pH 7.4.

1.5 In Vitro Photothermal Effect

The photothermal ability of the CFOT NPs was characterized using an IR thermal camera (Testo883, FOTRIC INC). The aqueous dispersions of CFOT NPs with different concentrations

were irradiated under 808 nm laser (1.2 W cm^{-2}) for 10 min. Then, 0.25 mL of aqueous solution (400 μg mL^{-1}) was irradiated with an 808 nm laser at different power densities (0.6, 0.9, and 1.2 W cm^{-2}). Moreover, five on/off cycles of irradiation were used to investigate the photothermal stability of CFOT NPs.

Evaluation of Photothermal of CFOT in aqueous solution, following the previous report[5, 6], the photothermal conversion efficiency (η) was evaluated using the following formula:

$$\eta = \frac{hS(T_{max} - T_{min}) - Q_{dis}}{I(1 - 10^{-A\lambda})}$$

Where h is the heat transfer coefficient of the system, S means the surface area of the container, T_{max} and T_{surr} are the maximum steady-state temperature of the sample solution and surrounding temperature, respectively. I is the power of 808 nm laser, and A_λ represents the absorbance of the CFOT aqueous solution at 808 nm. Additionally, Q_{dis} represents heat associated with the light absorbance of the solvent, and it was calculated to be Q_{dis} = (5.4 × 10^{-4}) I. The unknown hS can be calculated as follows:

$$hS = mC_{water}/\tau_s$$

where τ_s was measured to be 132.40 from the results in Fig. 2e. Additionally, m and C_{water} are the weight (0.25 g) and heat capacity (4.2 J g^{-1} k^{-1}) of water, respectively. According to the formula, the hS was measured to be 7.93 mW $°C^{-1}$. By substituting these values into the above formulas, the 808 nm laser photothermal conversion efficiency of CFOT was calculated to be 46.5%.

1.6 Cell Culture

HUVEC, LO-2 and HepG2 cells were cultured in DMEM medium with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2. H22 cells were cultured in 1640 medium with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2.

1.7 Cellular Uptake

To explore the targeting of transferrin, HepG2 cell phagocytosis of CFO and CFOT was conducted employing a confocal laser scanning microscope (CLSM, LSM 800 With Airscan, Carl Zeiss). FITC labeled CFO and CFOT were acquired by the following method. CFO

modified by BSA and CFOT were respectively dispersed into PBS (pH 9.0), followed by slowly adding FITC (1 mg mL^{-1}). The mixtures were stirred vigorously at 4 ℃ for 12 h to achieve chemically linking, and then FITC-modified CFO and CFOT were acquired. HepG2 cells were seeded in Confocal Dish (1 mL, 1 × 10^5 cells/well), incubating for 24 h. The FITC labeled CFO and CFOT were incubated with cells for 2 h at 37 ℃. Subsequently, the cells were washed thrice with PBS and the nuclei were stained with Hoest33342 for 20 min. After PBS washing, the fluorescence images were captured on a CLSM.

To further investigate the uptake of CFOT by different cells (HUVEC, LO-2, HepG2, H22), the Co element concentrations in cells were measured by inductively coupled plasma-mass spectrometry (ICP-MS, EMXplus-10/12, Agilent Technologies Inc.). HUVEC, LO-2, HepG2, and H22 cells were seeded in 6-well plate (1 ×10^5 cells/well) with incubation of 24 h, and then treated with CFOT for 2 h. After several washes with PBS, the Co element concentrations in cells were measured by ICP-MS.

1.8 Detection of intracellular GSH

Briefly, HepG2 cells were seeded in 6-well plate (1 ×10^5 cells/well) and cultured for 24 h. Then, the culture medium was substituted with the fresh one containing different concentrations of CFOT. After 12 h of treatments, the amounts of GSH in cells were measured using GSH Assay Kit (Jiancheng, Nanjing, China) according to the manufacturer's instruction.

1.9 Chemodynamic activity in vitro

The intracellular production of •OH was detected by a fluorescent probe (DCFH-DA). HepG2 cancer cells were seeded in a Confocal Dish (100 μL, 1 × 10^5 cells/well) for 24 h (5% CO_2, 37 ℃). and then treated with different treatments: (1) PBS; (2) CFO; (3) CFOT; (4) PBS+L;(5) CFO+L; (6) CFOT+L. After incubation for 12 h, the cells were washed with PBS several times. Then, DCFH-DA solution (0.2 μM) was added to the cell medium for another 30 min. The fluorescence images were acquired by CLSM.

1.10 In vitro toxicity assay and synergistic therapy performance

HUVEC, LO-2, H22, and HepG2 cells were inoculated in 96-well plates (5×10^3cells/well) and incubated at 37 °C for 24 h. Subsequently, the CFO and CFOT (0, 25, 50, 100, 200, and 400 mg mL^{-1}, respectively) were added. After the coincubation for 24 h, the culture medium was substituted by MTT (0.5 mg mL^{-1}) culture solution and incubation for 4 h. Ultimately,

dimethyl sulfoxide (150 μL) was added to each well after removing the medium. Cell viability was calculated by measuring the absorbance at λ = 490 nm to the control via a microplate analyzer.

In order to explore the synergistic therapy performance, HepG2 and H22 cancer cells were cultivated into 96-well plates and incubated for 24 h, respectively. Next these cells were treated with PBS, CFO, CFOT, PBS+L, CFO+L and CFOT+L (L: laser, 808 nm,1.2 W cm^{-2}, 10 min) for 24 h. The relative cell viability after synergistic treatment was determined by performing the standard MTT assay. To observe the cell viability using CLSM, Calcein AM, and PI solutions were utilized to distinguish live and dead cells, respectively. In order to further detect the apoptosis induced by synergistic therapy, A total of 1×10^5 HepG2 cells were inoculated in a 6-well plate and cultured for 24 h, then were treated with PBS, CFO, CFOT, PBS+L, CFO+L, and CFOT+L (L: laser, 808 nm,1.2 W cm^{-2}, 10 min) for 24 h. The cells were digested by trypsin and centrifuged. Annexin V-FITC/PI staining was performed for 20 min and then detected by flow cytometry (FACS Calibur, Becton, Dickinson and Company).

1.11 Lipid peroxidation initiated by CFOT

HepG2 cells were incubated in Confocal Dish (1×10^5 cells/well) for 24 h, and then co-cultured with the medium of different groups (PBS, CFOT, PBS+L, and CFOT+L, L: laser, 808 nm,1.2 W cm^{-2}, 10 min) for 8 h. After washing twice with PBS, the cells were stained with C11-BODIPY$^{581/591}$ dye (10 μM) for 30 min and observed by CLSM.

1.12 Changes of cell mitochondrial membrane potential

The JC-1 fluorescent probe was used to evaluate the effect on the mitochondrial membrane potential. In short, HepG2 cells were incubated in Confocal Dish (1×10^5 cells/well) for 24 h, and then co-cultured with the medium of different groups (PBS, CFOT, PBS+L and CFOT+L, L: laser, 808 nm,1.2 W cm^{-2}, 10 min) for 12 h. Finally, the cells were stained with JC-1 for 20 min, washed with PBS, and the fluorescence changes were observed by CLSM.

1.13 Lysosomal Membrane Permeabilization

HepG2 cells were incubated in Confocal Dish (1×10^5 cells/well) for 24 h. After 12 h co-cultured with the medium of different groups (PBS, CFOT, PBS+L, and CFOT+L, L: laser, 808 nm,1.2 W cm^{-2}, 10 min), the cells were stained with AO (5 μM) for 30 min and then imaged

with CLSM. Excitation: 488 nm; Emission: 515~545 nm for the green channel, 610~640 nm for the red channel.

1.14 Animal tumor model.

Balb/c mice (4~5 weeks) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China), and all animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Guangdong University of Technology and approved by the Animal Ethics Committee of Guangdong University of Technology. The tumor cell model of H22 xenograft was established by subcutaneously injecting a density of ~1×10^6 cells into the back. Balb/c mice can be used for in vivo treatment analysis, biological distribution, and safety evaluation when the tumor volume reaches ~100 mm^3.

1.15 In vivo optimal irradiation time study and biodistribution study

CFO and CFOT were intravenously injected into tumor-bearing mice (200 μL, 5 mg kg^{-1}). The tumor site was irradiated for 10 min, and the temperature of the tumor site was recorded at different time points (0, 2, 5, 8, 12, 24 h) post-administration. The mice's heart, liver, spleen, lung, kidney, and tumor were dissected after irradiation. Then the Co content of the tissue samples was measured by ICP-MS.

1.16 In vivo anticancer therapy

H22 tumor-bearing mice were randomly divided into 6 groups (5 mice in each group), and different treatment methods were used: (1) injection of PBS (control group), (2) injection of CFO, (3) injection of CFOT NPs, (4) injection of PBS and laser irradiation for 10 min, (5) injection of CFO and laser irradiation for 10 min, (6) injection of CFOT and laser irradiation for 10 min. Among them, the laser irradiation power of 808 nm is 1.2 W cm^{-2}. Tumor volume and body weight of the mice were recorded every two days for 14 days. The volume of the tumor was obtained by $V = length\times width^2/2$. On day 14, the tumors were collected and weighed, and the collected organs were used for histological analysis. After intravenous injection, the mice in groups (4), (5), and (6) were anesthetized, and the temperature changes in the tumor region within 10 min were recorded by infrared thermal imager (326Pro, FOTRIC, China), and the mice were photographed at different time points (0, 2, 4, 6, 8, 10 min).

1.17 Histology analysis

On day 14, the mice were killed, and the tumor and main organs were collected and fixed

with 10% neutral buffered formalin, cut into a series of slices with a thickness of 3~5 μm. Then, they were stained with H&E and observed under Leica inversed fluorescent microscope. All the statistics were based on standard protocols.

1.18 Statistical Analysis.

All assays were performed in three independent experiments (technical triplicates). Statistical calculations for bioassays were performed using GraphPad Prism 5.0 software. All results are expressed as mean values ± SD. A value of $p < 0.05$ was considered statistically significant.

2. Figures and tables

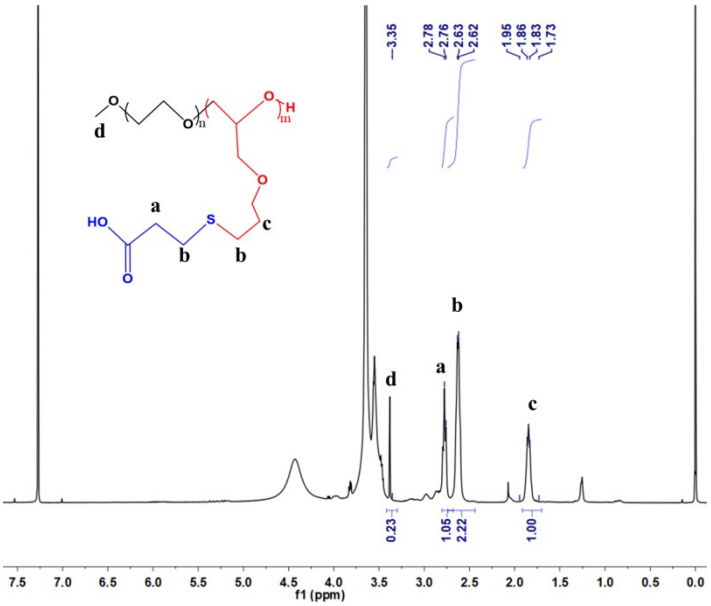

Fig. S1. ^{1}H NMR spectrum of mPEG-P(AGE-MPA).

Table S1. The content of Co and Fe elements in CFO and CFOT.

Simple	Co content	Fe content	$CoFe_2O_4$ content	Molar ratio (Fe: Co)
CFO	3.73±0.18%	6.04±0.96%	14.85%	2: 1.06
CFOT	3.40±0.31%	6.92±0.56%	13.55%	2: 1.02

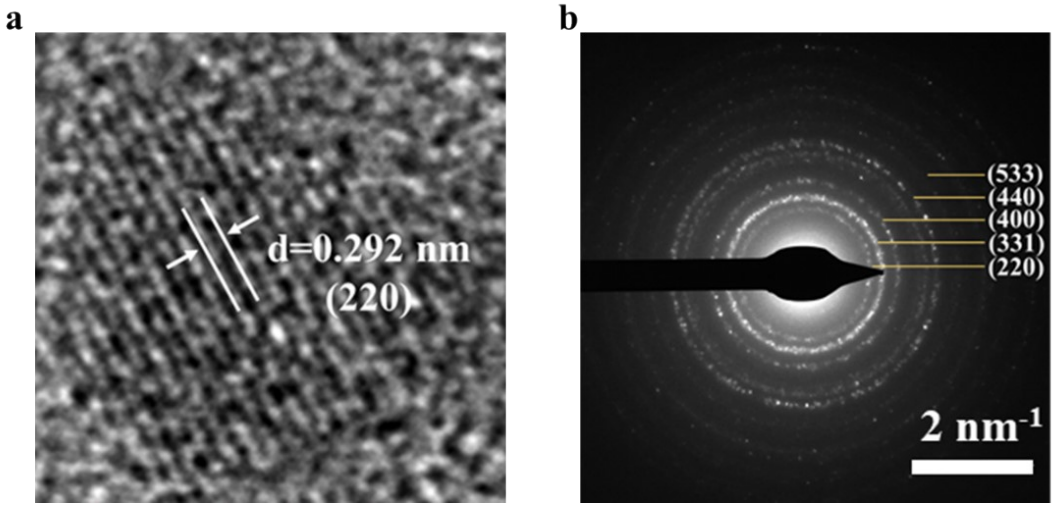

Fig. S2. (a) High-resolution TEM image of as-synthesized CFOT. (b) SEAD pattern of CFOT.

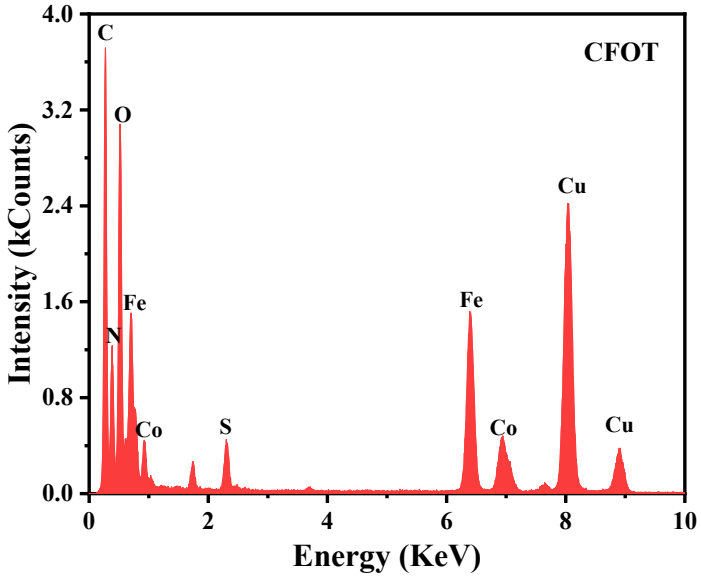

Fig. S3. TEM-EDS spectrum of CFOT.

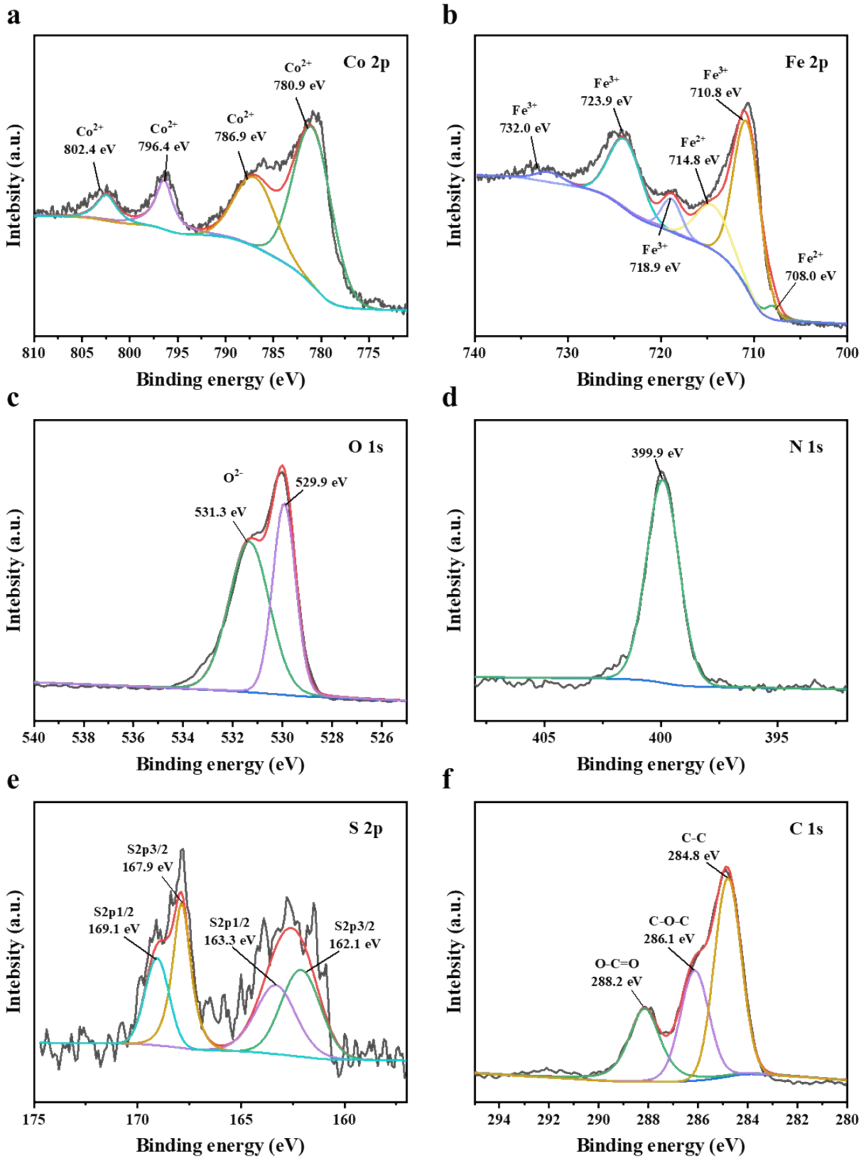

Fig. S4. (a) Co 2p, (b) Fe 2p, (c) O 1s, (d) N 1s, (e) S 2p and (f) C 1s orbital XPS spectra of CFOT, respectively.

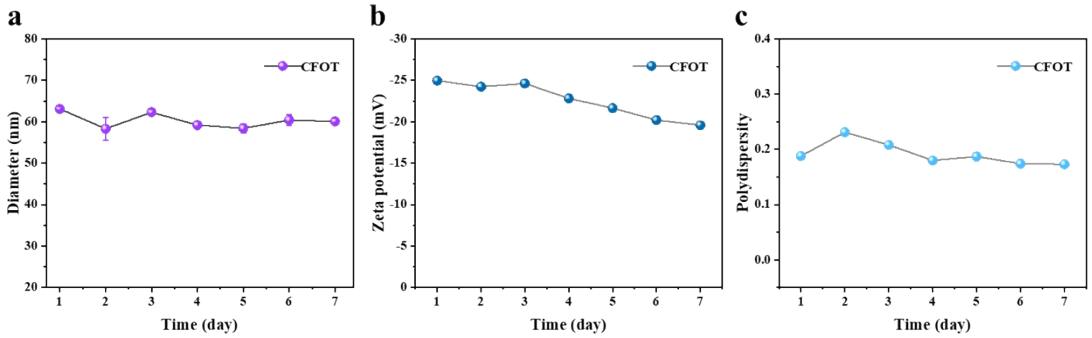

Fig. S5. (a) Size, (b) zeta potential and (c) polydispersity of CFOT in 7days.

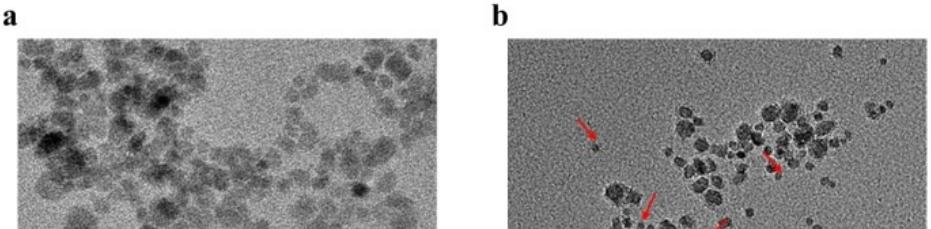

Fig. S6. (a) the TEM image of CFOT, and (b) the TEM image of CFOT after reacting with GSH (1 mM) and H_2O_2 (10 mM) for 6 h. Scale bar: 50 nm.

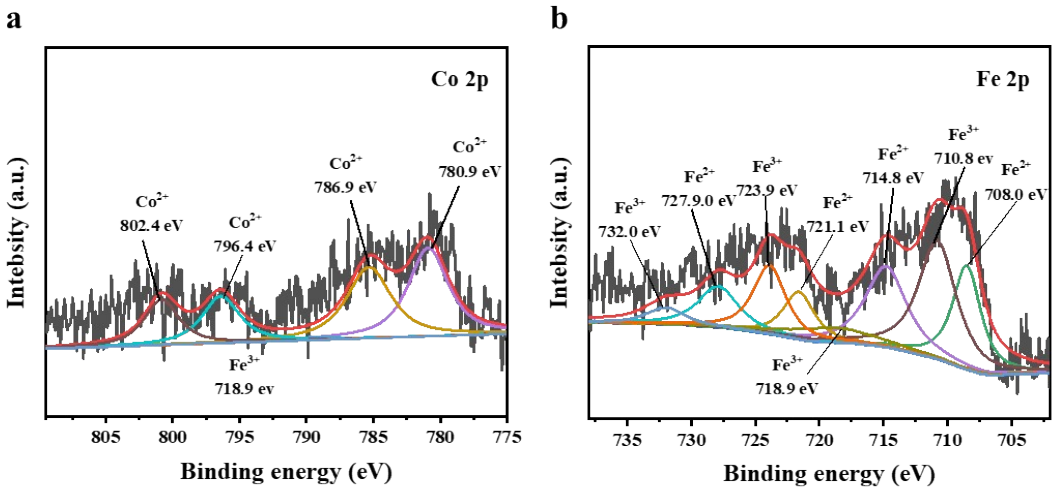

Fig. S7. (a) Co 2p and (b) Fe 2p orbital XPS spectra of CFOT after reacting with GSH, respectively.

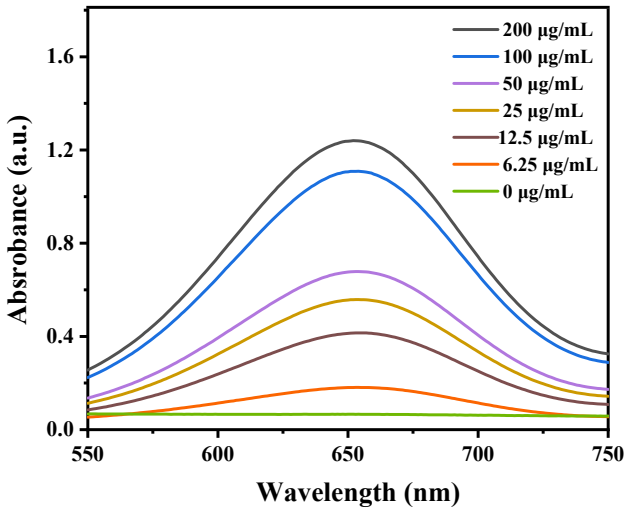

Fig. S8. UV–vis absorbance spectra of TMB solutions with the different concentrations of CFOT.

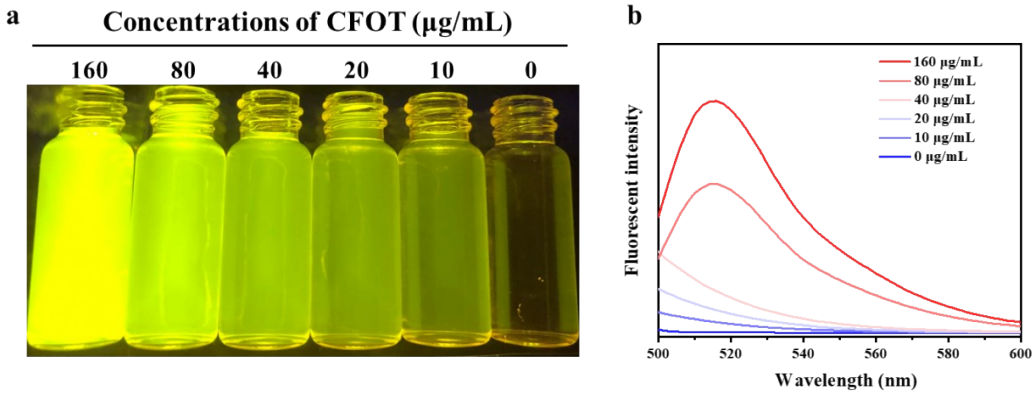

Fig. S9. (a) Naked-eye observable fluorescent signal of FITC-labeled CFOT under UV illumination at 302 nm. (b) Corresponding fluorescence intensity of FITC-labeled CFOT.

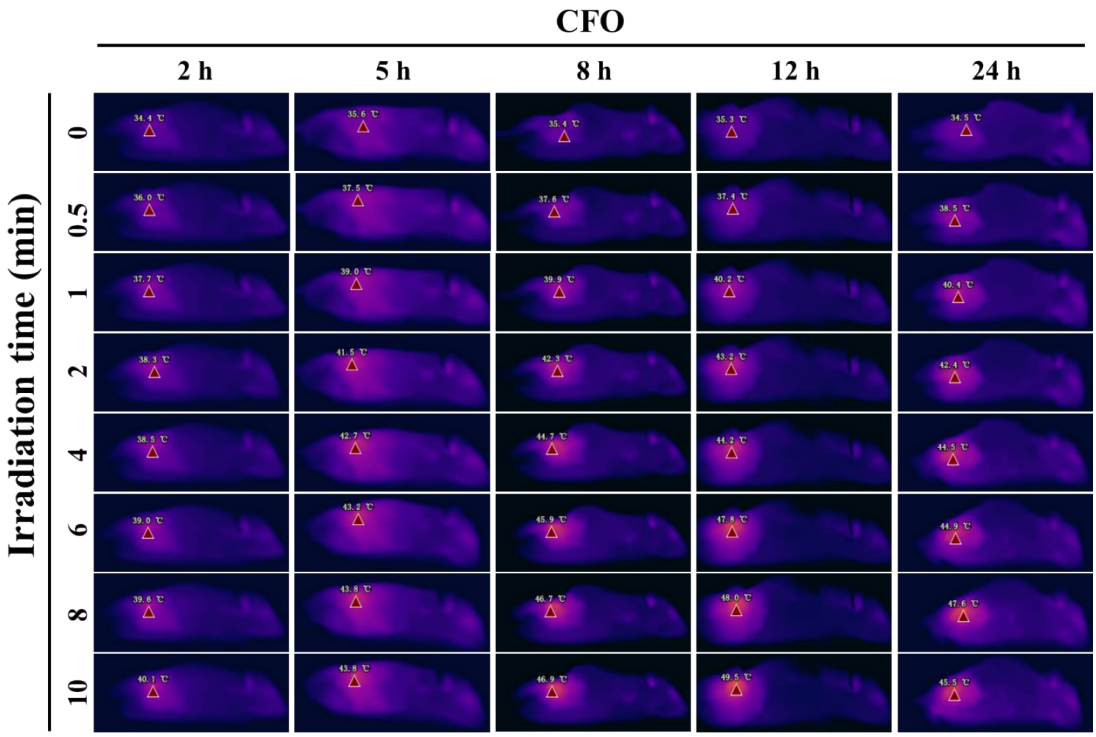

Fig. S10. Thermal imaging pictures of CFO.

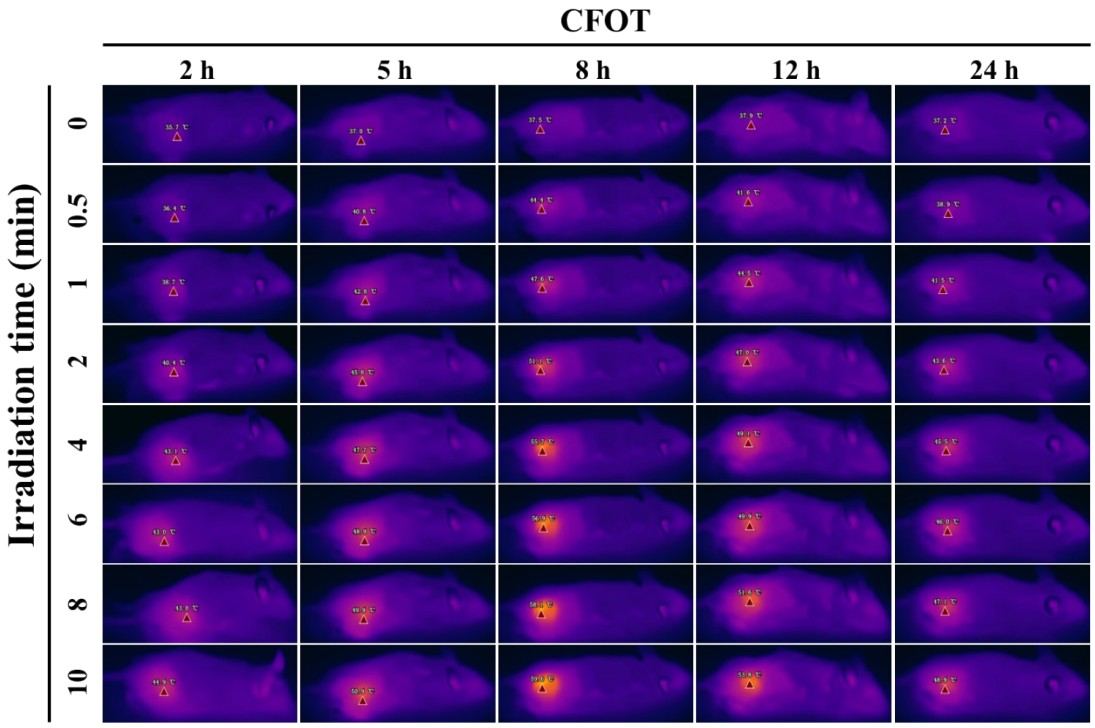

Fig. S11. Thermal imaging pictures of CFOT.

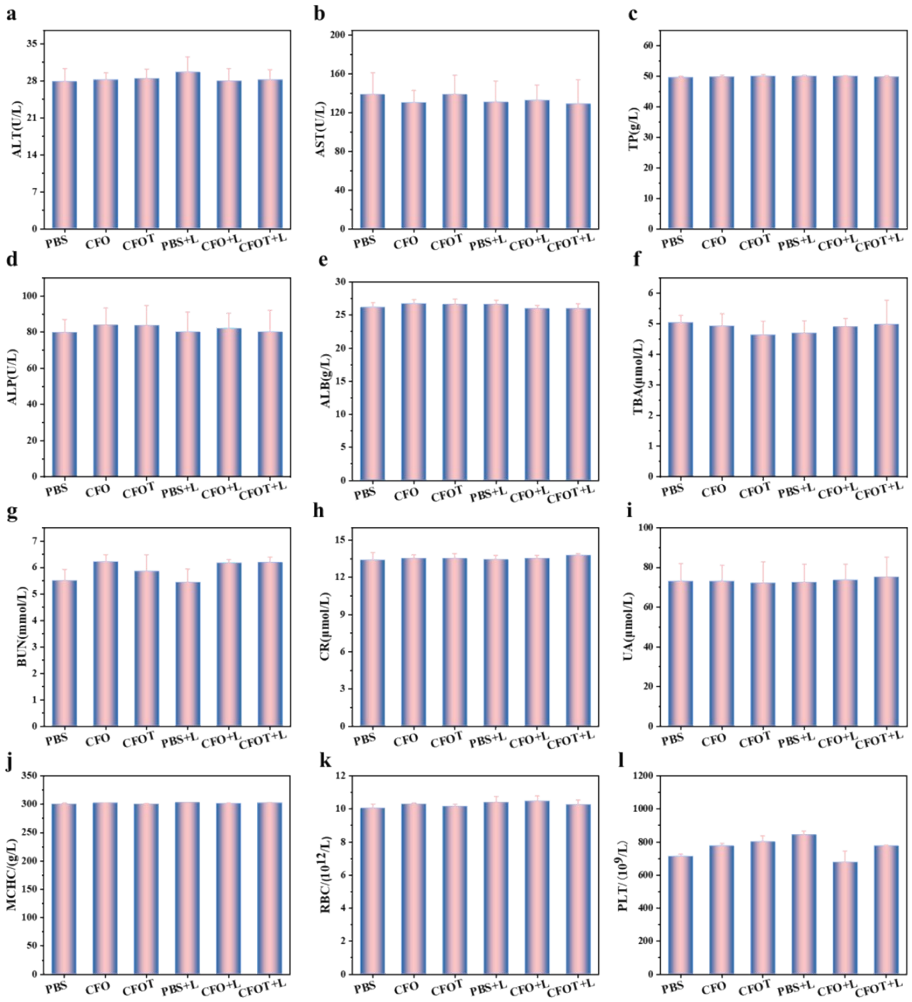

Fig. S12. Blood analysis. Error bars stand for ± SD (n = 5).

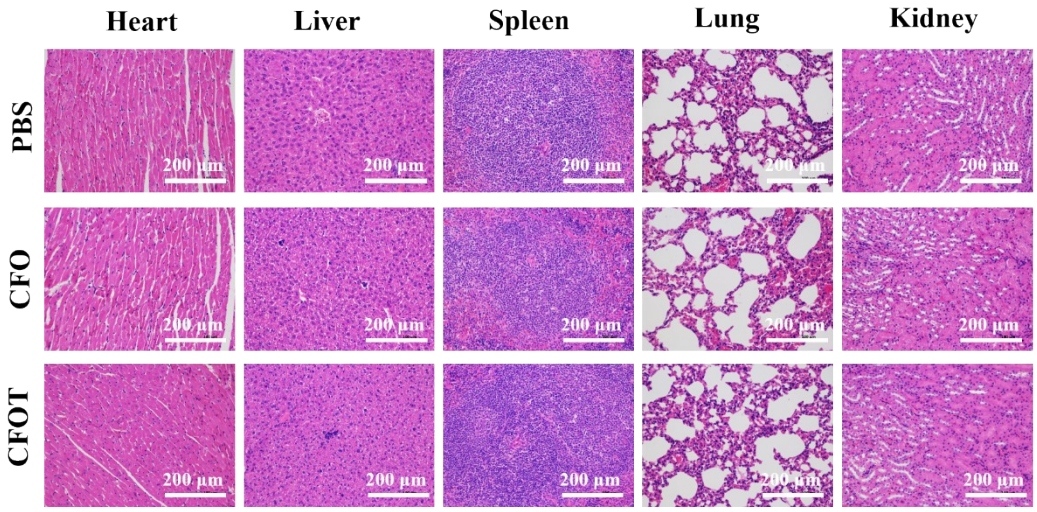

Fig. S13. Representative histological images of major organs treated with PBS, CFO, and CFOT, were collected on day 14. Scale bar: 200 μm.

References

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