Supporting Information

Sustained-Release Nanocapsule Based on a 3D COF for Long-Term Enzyme Prodrug Therapy of Cancer

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EXPERIMENTAL SECTION

Materials and reagents. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and hydrogen peroxide (H_2O_2, 30%) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Energy Chemical Technology (Shanghai, China) Co., Ltd. Horseradish peroxidase (HRP), 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Mito-Tracker Green was purchased from Shanghai Beyotime Biotechnology Co., LTD. LysoTracker Green and ER-Green[DiOC6(3)] were purchased from Jiangsu KeyGEN BioTECH Co., Ltd. Hoechst 33342 was purchased from Beijing Solarbio Technology Co., LTD. 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), propidium iodide (PI), and calcein acetoxymethyl ester (Calcein-AM) were purchased from Sigma Chemical Company. Confocal dish was purchased from Cellvis, Mountain View, CA. The mouse mammary carcinoma cells (4T1 cell) were purchased from AOLU Biological Technology Co. Ltd. (Shanghai, China). Female Balb/c mice (6-8 weeks, 20 g) were obtained from SiPeiFu Beijing Biotechnology Co., Ltd. Fetal bovine serum (FBS) in cell culture medium was purchased from Gibco. The experimental water used was Mill-Q secondary ultrapure water (18.2 MΩ·cm-1). All the other chemical reagents were of analytical grade and used without further purification.

Instruments. Transmission electron microscopy (TEM) imaging was carried out on a HT7700 electron microscope (HITACHI, Japan). High resolution transmission electron microscopy (HRTEM) imaging was carried out on a JEM-2100 electron microscope (JEOL, Japan). X-ray photoelectron spectroscopy (XPS) were carried out using an Escalab 250Xi instrument (Thermo Scientific, USA). The crystal structure of the samples was determined by powder X-ray diffraction (PXRD) patterns (Bruker D8, Germany). Zeta potential was performed on a Malvern Zeta Sizer Nano (Malvern Instruments). Absorption spectra were measured on a U-4100 UV-visible spectrophotometer (HITACHI, Japan). All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glass-calomel electrode. Absorbance in MTT assay was measured in a

microplate reader (RT 6000, Rayto, USA). Confocal fluorescence imaging experiments were performed with TCS SP8 confocal laser scanning microscopy (Leica Co., Ltd. Germany) with a 20× objective. Live animal imaging system (IVIS Lumina III, US) was applied *in vivo* imaging. Fluorescence spectra were obtained with FLS-980 Edinburgh. Blood glucose level was tested on a commercial glucometer (Yuwell, China).

Synthesis of 3D COF. 3D COF was synthesized under solvothermal method by preparing in a 1:1 molar ratio solution of 1,3,5,7-tetrakis(4-aminophenyl)adamantane (TAPA, 10.1mg, 0.02mmol) and 2,3,6,7-Tetra(4-formylphenyl)tetrathiafulvalene (TTF-4CHO, 12.4mg, 0.02mmol) in a mixture of o-dichlorobenzene (1.0mL) and 6M aqueous acetic acid (0.1mL) into a 10 × 8 mm (o.d × i.d) Pyrex tube. The tube was flash frozen at 77 K (liquid N_2 bath), evacuated to an internal pressure of below 8 Pa and flame sealed. The reaction was heated at 140 ℃ for 120 h, yielding a crimson solid, which was isolated by filtration and washed with anhydrous N,N-dimethylacetamide (DMF) and anhydrous tetrahydrofuran (THF), the resulting powder was immersed in anhydrous THF for 72 h and dried at 120 ℃ and under vacuum overnight. Yield: 16 mg, 73%. (From weighing raw materials to post-reaction treatment, even the drying process must be protected from light.)

Comparison of COF dispersion before and after grinding. 1 mg of 3D COF before and after grinding was respectively dissolved in 1 mL of water and ultrasonic for 30 seconds. The samples were placed on the test tube rack and observed at 0min and 5min to compare their dispersity.

Synthesis of HRP@COF and HRP_{IR808}@COF. First of all, horseradish peroxidase (HRP, 2mg) and covalent-organic framework (COF, 2mg) were dissolved in 2 mL of doubly distilled water. Then, the solution was stirred overnight at low temperature (more than 12 hours). Finally, the as-prepared HRP@COF nanoparticles were obtained by centrifugation at 14500 rpm for 10 minutes and washed with water for twice to remove the residual reactants. Fluorescence dye IR808 was linked to HRP, and HRP-IR808@COF was prepared in a similar way to HRP@COF.

Nanoparticles Characterization. The COF and HRP@COF were analyzed by transmission electron microscope (TEM), scanning electron microscope (SEM), dynamic light scattering (DLS), X-ray diffraction (XRD) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The free radicals produced by IAA + HRP were detected by electron spin resonance (ESR) with 2,2,6,6-tetramethylpiperidine (TEMPO) as free radical capture. In addition, Zeta potential, ultraviolet-visible spectra (UV-vis), and infrared spectra (IR) of IAA, HRP, COF and HRP@COF were obtained.

HRP Loading Rate Detection. The HRP loading rate was obtained by BCA kit.

HRP Release Rate Experiment. 2.5 mg of HRP@COF were dispersed in 5 mL water, and then the solution was divided into 5 parts evenly and allowed to let stand for a while. The supernatant was centrifuged at 14500 rpm for 10 min at 0, 2, 4, 6 and 8 h, respectively. Next, 20 μL of TMB and 3 μL of H_2O_2 (0.01 mol/L) were added into the supernatant, and the absorbance was measured at 403 nm by UV spectrophotometer.

Determination of the Effect of IAA on HRP Activity. 1 μg of HRP was dispersed in aqueous solutions (1 mL) with different concentrations of IAA (0, 20, 40, 60 and 80 μg/mL, respectively). All solutions were incubated at 37℃ for 90 minutes. Add 100 μL of each of the above solution into 5 mL PBS (pH=6.5), and then add 200 μL of TMB and 100 μL of H_2O_2 (0.01 mol/L) immediately. The absorbance was measured at 370 nm by UV spectrophotometer. For verifying the effect of incubation time, 1 μg of HRP and 20 μg of IAA were dispersed in aqueous solutions (1 mL) and allowed to let stand for 0, 0.5, 1, 1.5, 3, 6, 9 and 12 h, respectively. The next experiment steps are the same as the above.

Determination of the Effect of IAA on HRP@COF Activity. To test HRP activity in HRP@COF, 300 μg of HRP@COF was dispersed in aqueous solutions (1 mL) with different concentrations of IAA (0, 50, 100 and 150 μg/mL, respectively) and all solutions were incubated at 37℃ for 2 h. Then add 100 μL of each of the above solution into 5 mL PBS (pH=6.5), and then add 200 μL of TMB and 100 μL of H_2O_2 (0.01 mol/L) immediately. The absorbance was measured at 370 nm by UV spectrophotometer. For

verifying the effect of incubation time, 300 μg of HRP@COF and 200 μg of IAA were dispersed in aqueous solutions (1 mL) and allowed to let stand for a while. The supernatant was centrifuged at 14500 rpm for 10 min at 0, 0.5, 1, 1.5, 3, 6, 9 and 12 h, respectively. The next experiment steps are the same as the above **Determination of The Effect of ROS on HRP Activity.** In order to detect the effect of ROS on HRP activity, porphyrin was selected as the ROS generator, because that under 635 nm laser irradiation, porphyrins can produce ROS effectively. Add 300 μL of porphyrin (1 mg/mL) and dilute to 1mL with water, and then add 1 μL of HRP (1 mg/mL). The laser group was irradiated with 635 nm laser for 10 min and the control group was placed in a dark environment without any treatment. The two groups were all placed in a dark environment for another several hours and then take out 100 μL of each group solution and add it into 2 mL PBS (pH=6.4). After adding 200 μL of TMB and 100 μL of H_2O_2 (0.01 mol/L), the absorbance was measured at 370 nm by UV spectrophotometer.

Cell Culture. Mouse breast cancer cell line (4T1 cells) used in the experiments were cultured in Roswell Park Memorial Institute (RPMI) 1640 with 10% fetal bovine serum (FBS) and 100 U/mL of 1% penicillin/ streptomycin in a humidified environment containing 5% CO_2. The medium was changed every 2 days, and cells were digested by trypsin and resuspended in fresh complete medium before plating.

Cell Colocalization. 4T1 cells cultured in a 20 mm confocal microscope dish (2 × 104 cells) were incubated with 110 μg/mL of HRP_{IR808}@COF for 4h. Then, the cells were washed by PBS for three times and incubated with fresh media containing LysoTracker Green, Mito-Tracker Green, ER-Green[DiOC6(3)] and Hoechst 33342 (1.0 μM) for 30 min. After wash by PBS for three times, the cells were observed by TCS SP8 confocal laser scanning microscopy (CLSM).

Cellular uptake of HRP_{IR808}@3D COF. 4T1 cells cultured in a 20 mm confocal microscope dish (2 × 104 cells) were incubated with CPZ (inhibitor of clathrin-mediated endocytsis), EIPA (inhibitor of macropinocytosis) for 1 h at 37°C or 4°C (inhibition of energy dependent uptake). Then, he cells were washed by PBS for three times and incubated with fresh media containing 110 μg/mL of HRP_{IR808}@COF for

another 4h. Finally, after wash by PBS for three times, the cells were observed by TCS SP8 confocal laser scanning microscopy (CLSM).

***In Vitro* Detection of ROS Generation.** 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect intracellular ROS. The excitation wavelength of DCFH-DA was 488 nm and the fluorescence was collected in the range of 500-550 nm. Flow Cytometry Analysis: The 4T1 cells were incubated overnight and treated with different conditions (PBS, IAA, HRP, HRP@COF, IAA+HRP and IAA+HRP@COF, respectively). After washed with PBS for three times, the cells were stained with DCFH-DA at 37 °C for 20 min. Then the cells were resuspended in PBS and analyzed by flow cytometry with 488 nm excitation. CLSM Analysis: The 4T1 cells were incubated in confocal dishes overnight to adherent to the wall, and then treating the cells for 10 h with different conditions (PBS, IAA, HRP, HRP@COF, IAA+HRP and IAA+HRP@COF, respectively). The cells were washed with PBS and stained with DCFH-DA at 37°C for 15 min. After washing cells with PBS for three times, the images were obtained using CLSM.

***In Vitro* Cytotoxicity Detection.** The in vitro cytotoxicity of IAA+HRP@COF was evaluated by methyl thiazolyl tetrazolium (MTT) assay and live/dead cell staining assay.

MTT Assay. Briefly, 4T1 cells were inoculated into 96-well plates and cultured overnight to adherent to the wall. Six groups of cells were performed as follows: PBS, IAA, HRP, HRP@COF, IAA+HRP and IAA+HRP@COF. Each tested agent was diluted with culture medium to different concentrations (27.5, 55, 82.5, and 110 μg/mL, respectively) and added to the 96-well plates for 10 h incubation. Then the medium was removed from the wells, and 150 μL of MTT solution (0.5 mg/mL) was added for incubation for another 4 h incubation. Finally, the MTT medium was removed, and 150 μL of DMSO was added under slight shake in the dark. The absorbance of each group at 490 nm was measured by a microplate reader. MTT assay was also performed after 4T1 cells were incubated with different concentrations of COF (50, 100, 150, 200, 250 μg/mL, respectively). Five groups of cells were performed as follows: PBS, IAA+HRP (4h), IAA+HRP@COF (4h), IAA+HRP (8h) and IAA+HRP@COF (8h). Each tested

agent was diluted with culture medium to equal concentrations (55 μg/mL IAA and 55 μg/mL HRP@COF) and added to the 96-well plates for different incubation time (4 or 8 h). The next experiment steps are the same as the above. All the cell viability was expressed as relative cell viability by normalizing to PBS treated cells.

Live/Dead Cell Staining Assay. 4T1 cells were inoculated into confocal dishes and divided into 6 groups: PBS, IAA, HRP, HRP@COF, IAA+HRP and IAA+HRP@COF. The cells were cultured overnight to adherent to the wall before adding the materials. Then, 6 groups of cells were incubated with the above materials respectively for 10 h. After removing the culture medium containing different materials and washing dishes twice with PBS, the cells were stained with Calcein-AM (20 nM) and PI (4μM) in PBS buffer solution for 20 min. Finally, the cells were washed twice with PBS, and confocal imaging was performed using a TCS SP8 confocal laser scanning microscopy. Green fluorescence of Calcein-AM was excited at 488 nm and detected with a 500–550 nm bandpass filter. Red fluorescence of PI was excited at 633 nm and detected with a 660–710 nm bandpass filter.

Flow Cytometry Analysis. The cells were divided into 6 groups: PBS, IAA, HRP, HRP@COF, IAA+HRP and IAA+HRP@COF. After 24 h incubation, the cells were labeled with annexin V−FITC and PI and analyzed by flow cytometry.

Tumor Model Establishment. Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU2021083). All the animal experiments complied with relevant guidelines of the Chinese government and regulations for the care and use of experimental animals. Female BALB/c nude mice (4-6 weeks old, ~20 g) were housed under normal conditions with 12 h light and dark cycles and given access to food and water ad libitum. The 4T1 cells were suspended in 50 μL of serum-free 1640 medium and subcutaneously injected into the alar of the mice. The tumor volume = (length× width^2) /2 and the mice were treated when the tumor volume was about 70 mm^3.

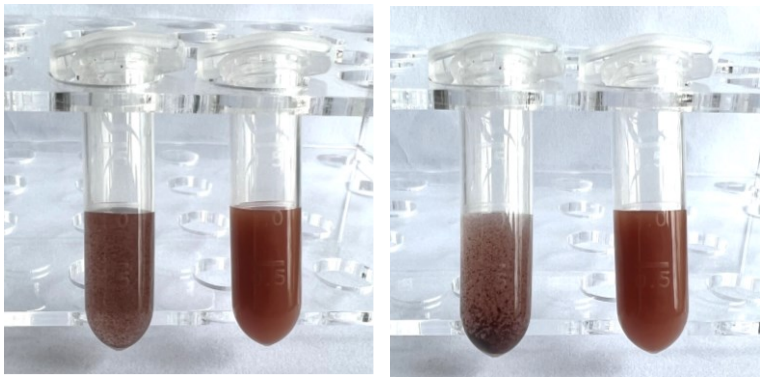
***In vivo* fluorescent imaging.** The tumor-bearing mice were randomly divided into 3 groups, and intratumorally injected with PBS, HRP_{IR808}, HRP_{IR808}@COF. At 1, 5, 11,

24 hours post-injection, *in vivo* fluorescent imaging of the tumor-bearing mice were obtained by using live animal imaging system. After 24h and 48h, the mice were dissected to observe the fluorescence distribution in each organ.

***In vivo* Antitumor Therapy.** The tumor-bearing mice were randomly divided into 6 groups, and intratumorally injected with PBS, IAA, HRP, HRP@COF, IAA+HRP and IAA+HRP@COF (10 mg/kg), respectively, one injection every other day and three times in total. Tumor volume and body weights were recorded every other day for 14 days. The H&E staining of the organs (heart, liver, spleen, lung, and kidney) and tumors were tested at 14 days post-injection and the volumes of tumors were measured after 12 h of each treatment.

Blood Biochemical Parameters Analysis. The blood of each group of mice was collected for subsequent analysis at post 14 days intratumor injection. The blood sample of mice was collected from the retro-orbital plexus into a coagulation-promoting tube, and then centrifuged at 3000 rpm 4 °C for 20 min to obtain plasma samples. The routine blood test parameters including platelet count (PLC), red blood cell count (RBC) and hemoglobin (HGB), and the serum biochemical parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea (UREA) and creatinine (CREA).

SUPPORTING FIGURES



0 min **5 min**

Figure S1. Photographs of suspensions of bulk 3D COF (left) and nanoscale ground 3D COF (right) before and after 5 min standing.

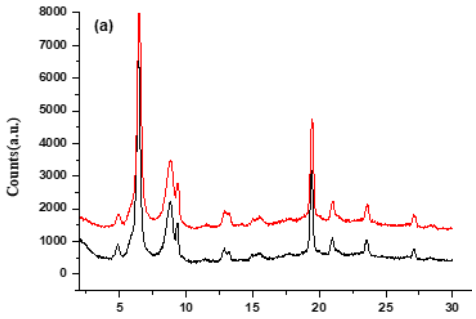


Figure S2. Comparison of the PXRD of 3D COF before (black) and after grinding (red).

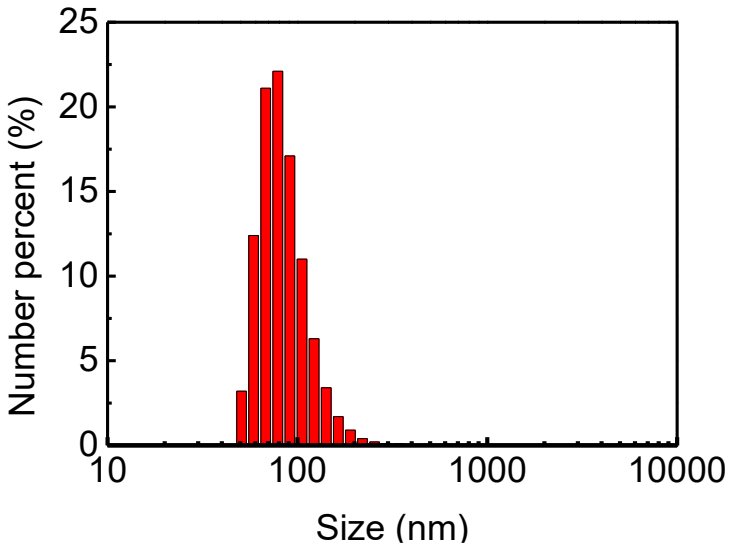


Figure S3. DLS size distribution of 3D COF.

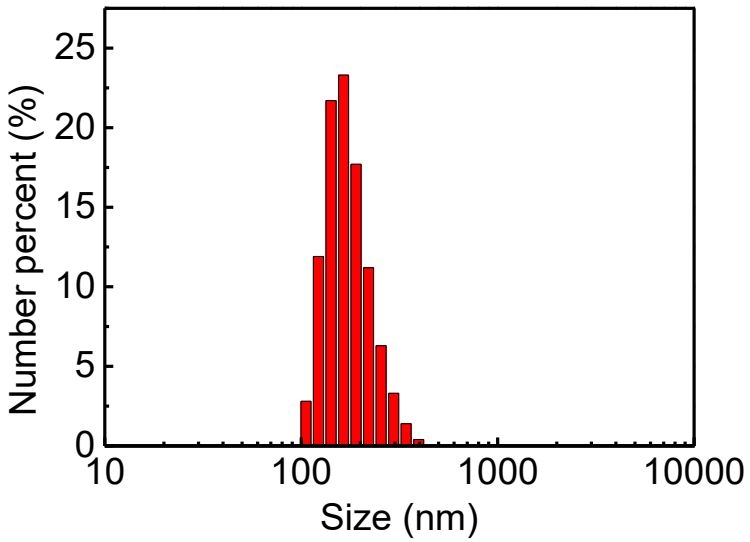


Figure S4. DLS size distribution of HRP@3D COF.

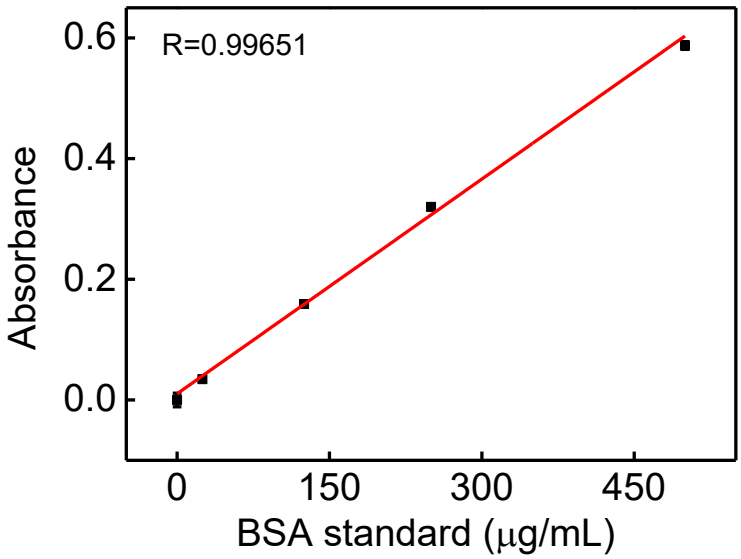


Figure S5. Linear relationships between the absorbance intensity of HRP and the HRP concentration. The error bar is the standard deviation from the mean (n = 3).

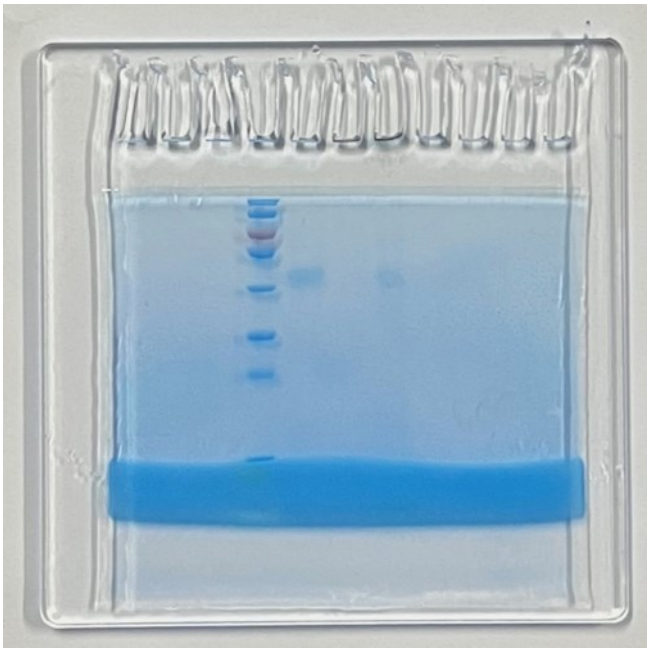


Figure S6. The uncropped and unprocessed image of the full gel and blot.

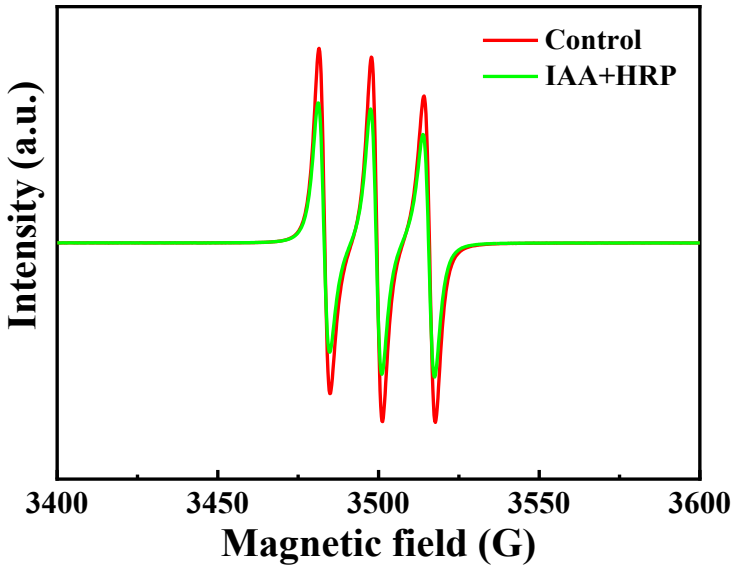


Figure S7. ESR analysis of free radicals production using TEMPO as the spin trapping agent.

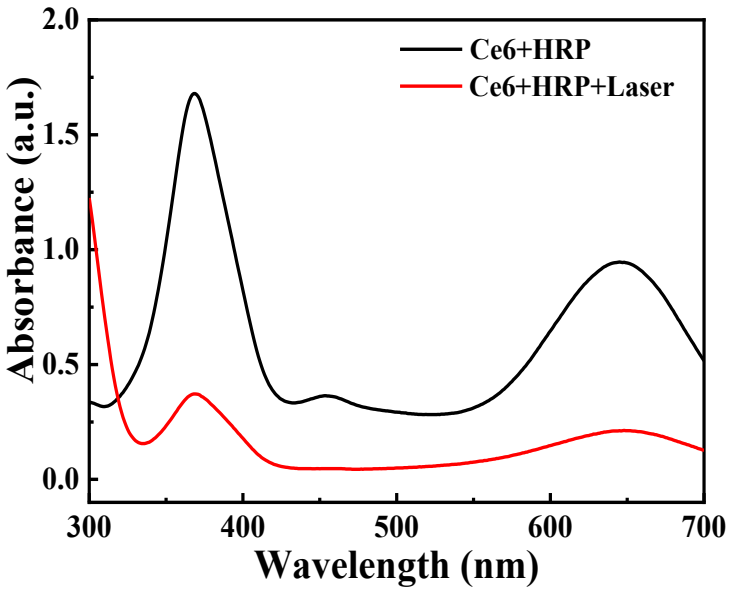


Figure S8. The absorbance spectra of TMB solution with different treatments.

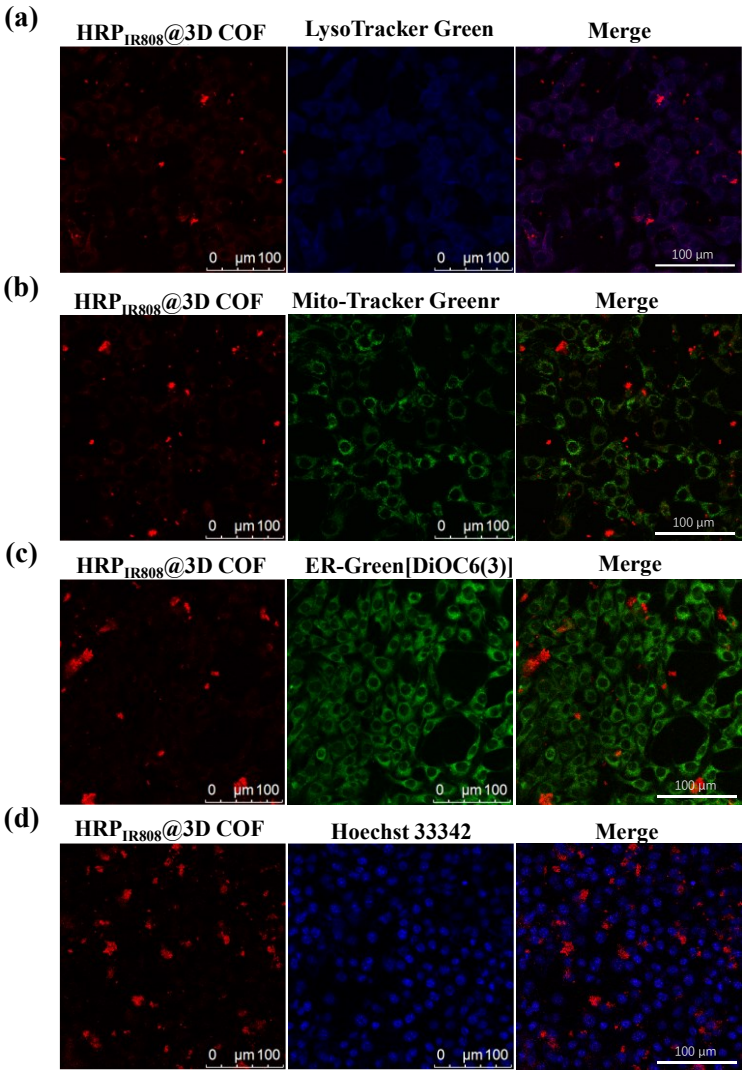


Figure S9. CLSM images of 4T1 cells after treatment with HRP@3D COF and stained by a) LysoTracker Green b) Mito-Tracker Green, c) ER-Green[DiOC6(3)] and d) Hoechst 33342. The error bar is the standard deviation from the mean (n = 3).

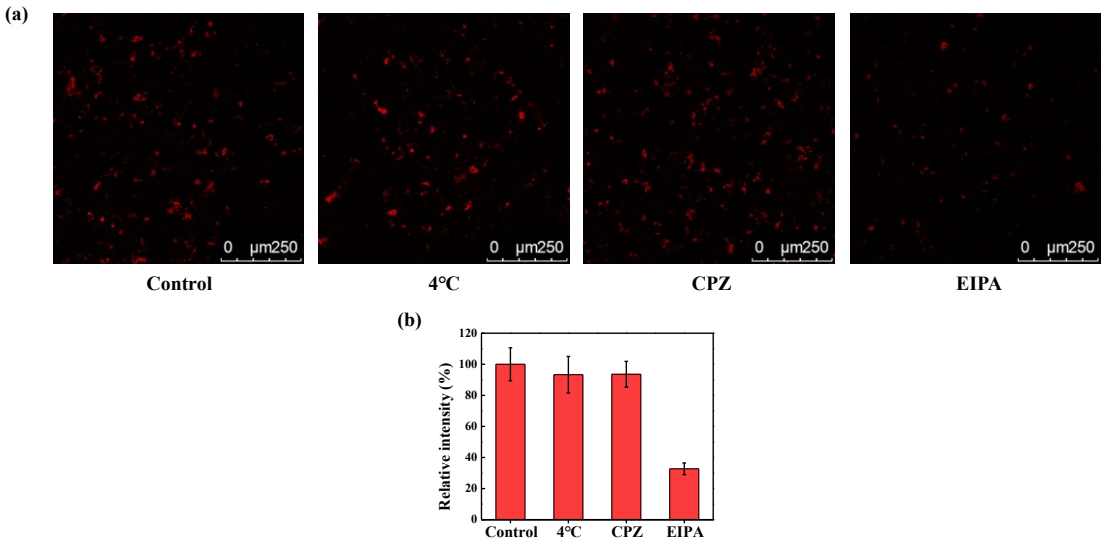


Figure S10. (a) Cellular uptake of HRP_{IR808}@3D COF by 4T1 cells treated by CPZ (inhibitor of clathrin-mediated endocytsis), EIPA (inhibitor of macropinocytosis) for 1 h at 37°C or 4°C (inhibition of energy dependent uptake). Scale bars are 100 μm. (b) Relative fluorescence intensities of IR808 from HRP_{IR808}@3D COF internalized by 4T1 cells in Figure S7a. The error bar is the standard deviation from the mean (n = 3).

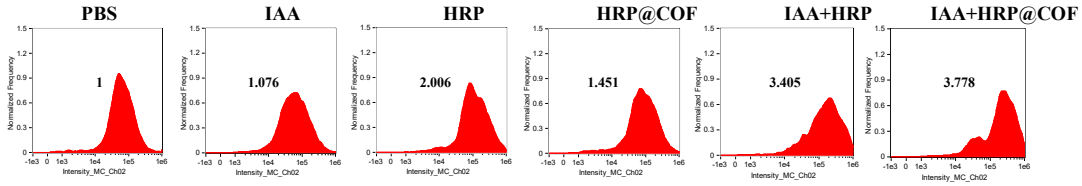


Figure S11. ROS production analysis of 4T1 cells treated under different conditions using flow cytometry and based on DCF-DA fluorescence intensity.

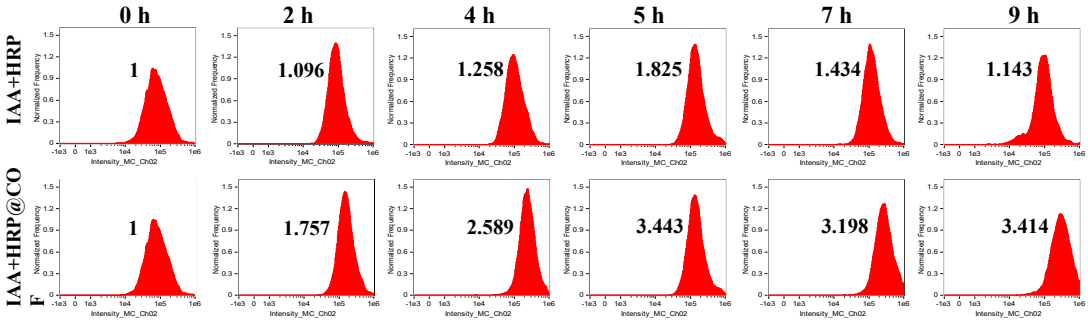


Figure S12. Real-time ROS generation analysis of 4T1 cells subjected to various treatments for different durations using flow cytometry and based on DCF-DA fluorescence intensity.

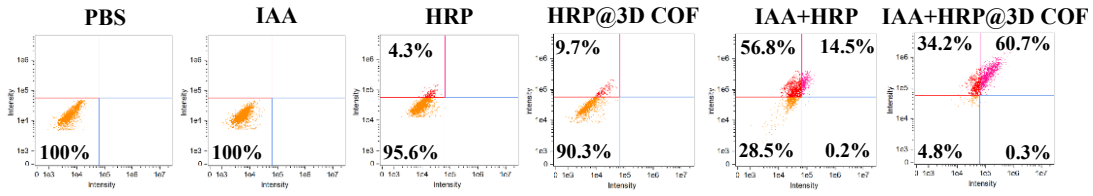


Figure S13. Flow cytometric analysis of apoptosis in cells subjected to different treatments.

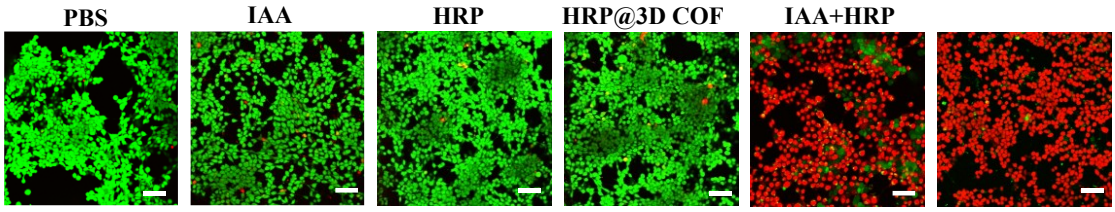


Figure S14. Live/dead cell staining assay of cells subjected to different treatments. Scale bars = 50 μm.

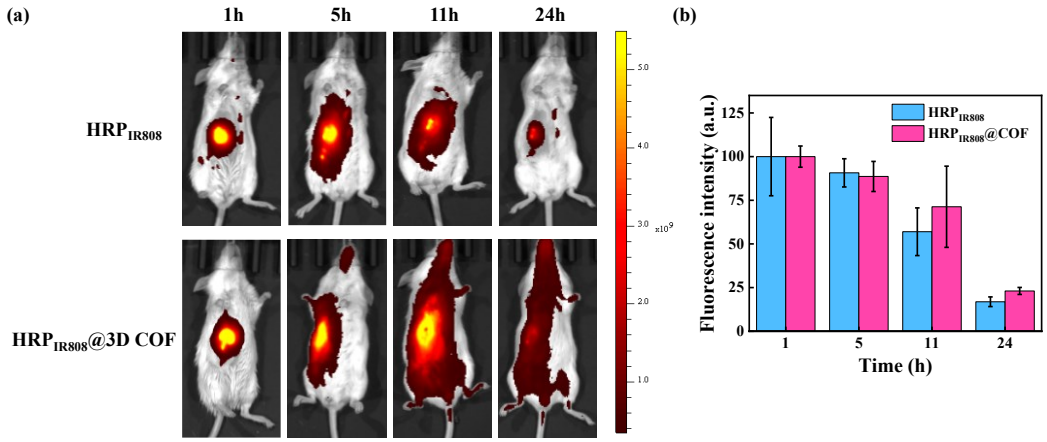


Figure S15. (a) *In vivo* fluorescent imaging of the tumor-bearing mice at different time postinjection; (b) Relative fluorescence intensity in tumor region. The error bar is the standard deviation from the mean (n = 3).

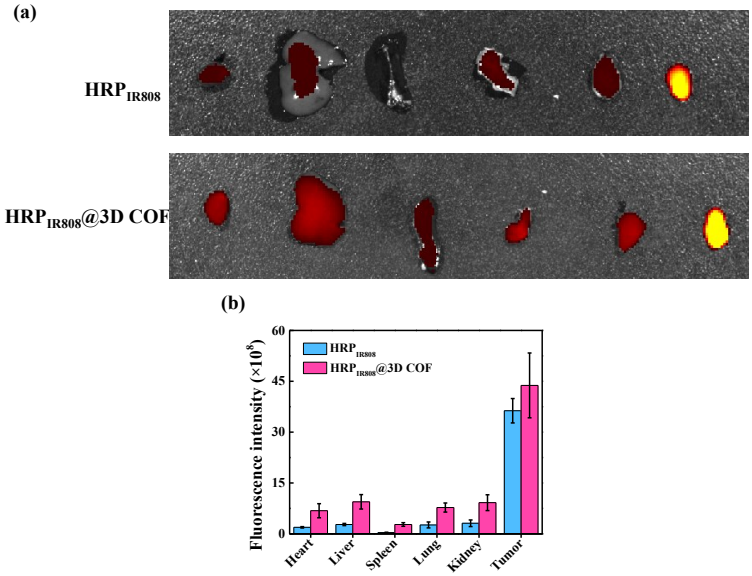


Figure S16. (a) *Ex vivo* fluorescence images of major organs and tumor at 24 h post-injection; (b) Relative fluorescence intensity in major organs and tumor. The error bar is the standard deviation from the mean (n = 3).

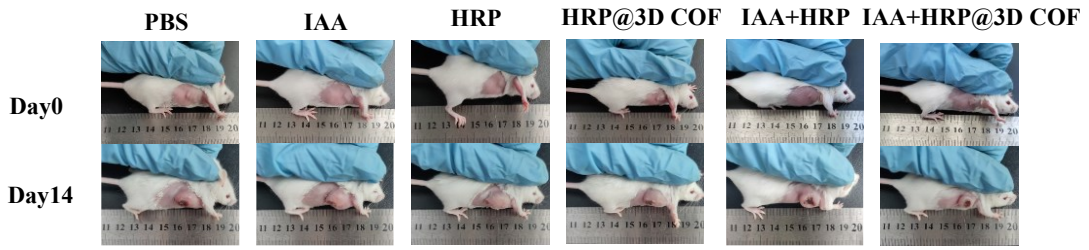


Figure S17. Photographs of tumors in mice with different treatments at day 0 and day 14.

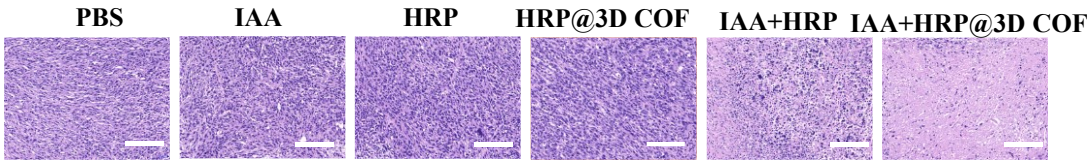


Figure S18. H&E-stained images of tumor slices collected from tumor-bearing mice after the different treatments. All scale bars are 100 μm.

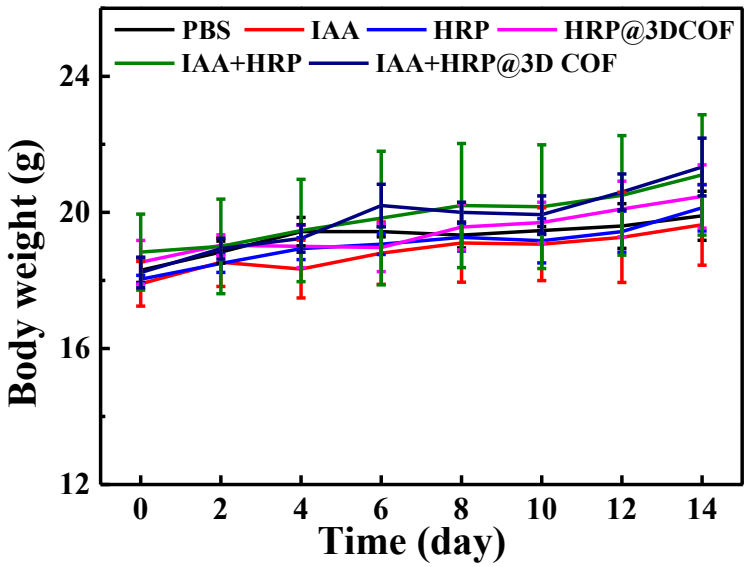


Figure S19. Body weight changes of mice within 14 days during treatment. The error bar is the standard deviation from the mean (n = 3).

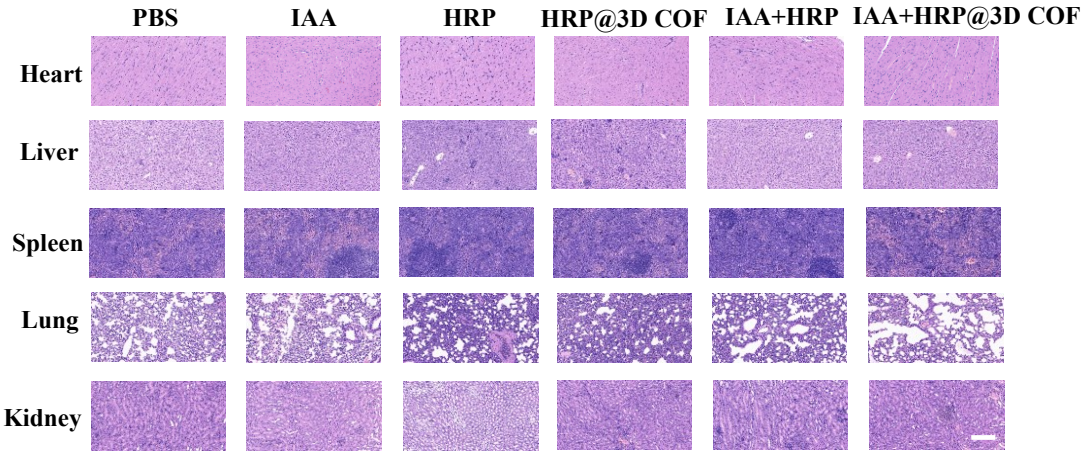


Figure S20. H&E staining of the five major organs (heart, liver, spleen, lung and kidney) with different treatments after 14 days. All scale bars are 150 μm.

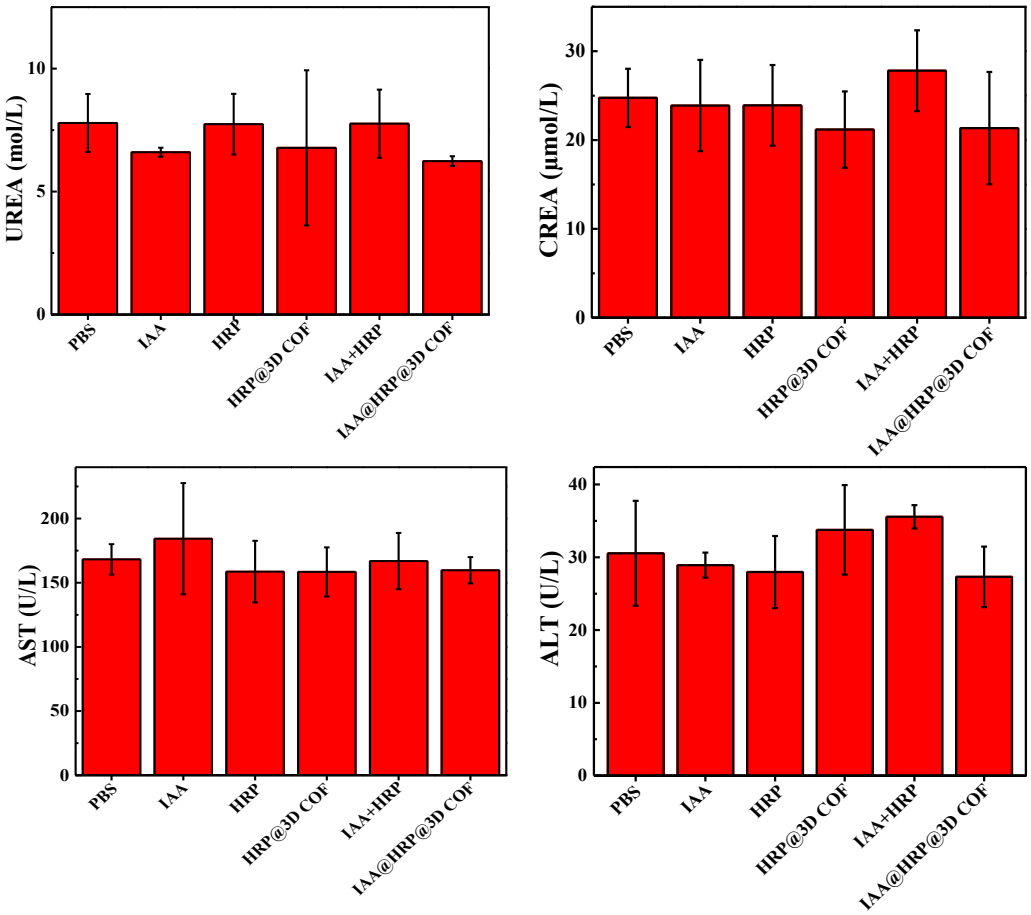


Figure S21. Blood biochemical parameters after different treatments for 7 days. The blood was collected for detection of the levels of UREA, CREA, ALT, and AST. The error bar is the standard deviation from the mean (n = 3).

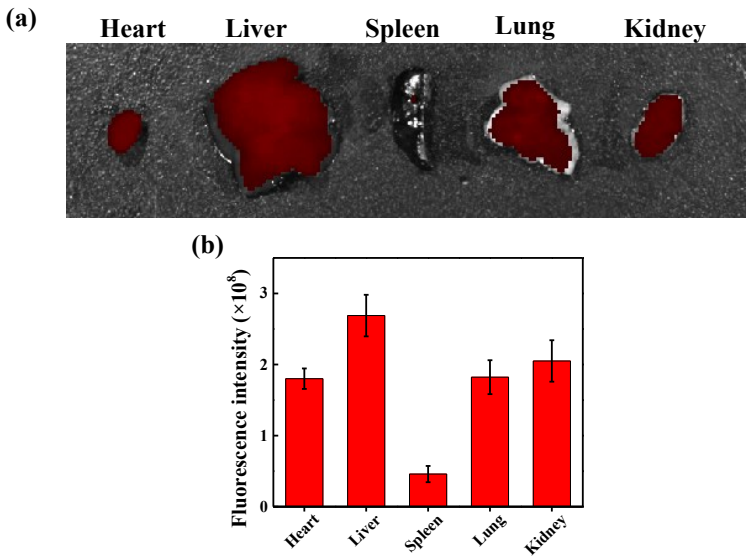


Figure S22. (a) *Ex vivo* fluorescence images of major organs at 48 h post-injection of HRP_{IR808}@ 3D COF; (b) Relative fluorescence intensity in different organs. The error bar is the standard deviation from the mean (n = 3).