Supplementary Information

Endoperoxide-containing covalent organic framework as a singlet oxygen reservoir for cancer therapy

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Reagents

1,4-Dimethylnaphthalene, *N*-bromosuccinimide (NBS), benzoyl peroxide, ethylene glycol, methacrylic anhydride were purchased from China National Pharmaceutical (Shanghai, China). 1,3-diphenylisobenzofuran (DPBF), 2,5-dihydroxyterephthalaldehyde (DHa) and tetra-(p-amino-phenyl)-porphyrin (TAPP) were obtained from Tianjin Heowns Biochemical Technology Co. Ltd. 2,2'-Azobis(2-methylpropionitrile) (AIBN) was purchased from Damao Chemical Reagent Factory (Tianjin, China). Annexin V-Alexa Fluor 488/PI, Fetal bovine serum (FBS), RPMI 1640 and PBS were purchased from Biological Industries (Beit Haemek, Israel). Antibiotics (penicillin/streptomycin) and 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl Tetrazolium Bromide (MTT) were purchased from Sigma-Aldrich. Calcein-AM/PI double staining kit was obtained from Yeasen Biotech Co. Ltd. (Shanghai, China). 2′, 7′-Dichlorofluorescein diacetate (DCFH-DA) were purchased from Beyotime Biotechnology (China).The experimental water used was Mill-Q secondary ultrapure water (18.2 $M\Omega \cdot cm^{-1}$). All chemicals were of analytical grade and were used without further purification. Mouse breast cancer cell line (4T1) was purchased from Jiangsu KeyGEN BioTECH Co., Ltd. Balb/C mice (4-6 weeks old, female) were used in the experiment.

Instruments

The nuclear magnetic resonance spectroscopy (^1H NMR) was analyzed by 400 MHz Bruker NMR spectrometer. Powder X-ray diffraction (PXRD) pattern was measured on a Rigaku SmartLab SE X-Ray Powder Diffractometer. Transmission electron microscopy (TEM) was applied on a JEM-100CX II electron microscope. Fourier infrared spectrometer (Nicolet iS50 FT-IR) was used to characterize the infrared spectrum. Zeta potential and dynamic light scattering (DLS) was performed on a Malvern Zeta Sizer Nano (Malvern Instruments). UV-Vis absorption spectra were measured on pharmaspec UV-1700 UV Visible spectrophotometer (Shimadzu, Japan). Confocal fluorescence imaging studies were performed using a TCS SP8 confocal laser scanning microscope (Leica, Germany). MTT assay was performed in a microplate

reader (Synergy 2, Biotek, USA). In vivo fluorescence imaging experiments were conducted using Bioluminescent Living Imager (IVIS Lumina III, USA).

Synthesis of the dimethylnaphthalene derivative DN

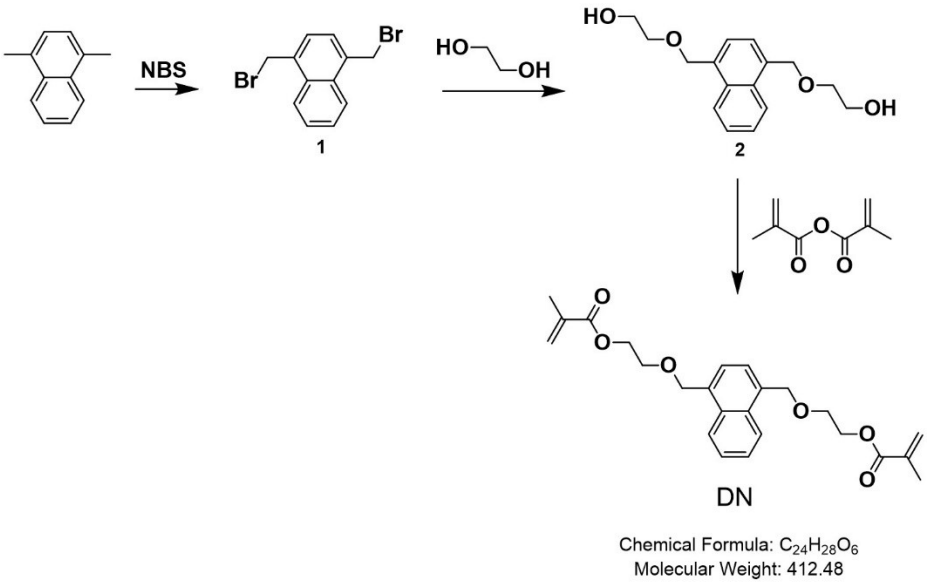

Fig. S1 Synthesis process of DN.

Synthesis of 1: 1,4-dimethylnaphthalene (7.0 mmol) was dissolved in anhydrous dichloromethane (40.0 mL) and degassed with nitrogen. Then *N*-bromosuccinimide (21.0 mmol) and benzoyl peroxide (0.7 mmol) were added into the above solution. The mixture was stirred at 55 ^{o}C for 6 h. After cooling to room temperature, the reaction mixture was washed with 2.0 M HCl (2×15 mL), 2.0 M NaOH (2×20 mL), brine, and dried with $MgSO_4$. The crude product was obtained after evaporating the solvent, which was further purified by column chromatography with 1 : 9 (vol/vol) dichloromethane/hexane.

Synthesis of 2: Ethylene glycol (45.0 mmol) was firstly dissolved in anhydrous THF (10 mL) and then added dropwise to a solution of NaH (16 mmol) in anhydrous THF (10 mL). After stirring for 30 min, **1** (7.0 mmol) was added into the mixture and reacted under reflux overnight. After removal of the solvent, the residue was purified by column chromatography with petroleum ether/EtOAc (3:7→1:9, vol/vol).

Synthesis of DN: **2** (15.0 mmol) was melted at the desired temperature under N_2 atmosphere and then methacrylic anhydride (37.5 mmol) was added. The mixture was stirred at 120 °C for 3 h. Excess methacrylic anhydride was removed under reduced pressure. The final methacrylated precursor was collected as liquid and dried under vacuum.

Preparation of COF

According to the reported method, the porphyrin-based COF was synthesized by DHa (19.9 mg, 0.12 mmol) and TAPP (40.5 mg, 0.06 mmol) in 3.3 mL of mixed solution (dichlorobenzene: butanol: 6 M acetic acid = 5:5:1). Firstly, the aforementioned mixture was sonicated for 10 min, and then placed in a 20 mL Pyrex tube to degass for 3 times through a freezing pump-thawing cycle, followed by sealed and heated at 120 °C for 3 days. After cooling to temperature, the products were collected and washed with tetrahydrofuran and acetone. The as-synthesized COF was mechanical exfoliation in a mortar and then dispersed in water and treated with a 1500 W ultrasound for 2 h. Subsequently, the dispersion was bathed in a 360 W ultrasound for 6 h. The obtained COF nanoparticles were collected through centrifugation (13000 rpm, 10 min) for subsequent experiments.

Preparation of COF@DN

Firstly, porphyrin-based COF nanoparticles (1 mg) and DN (1 mg) were dispersed into 1 mL of acetonitrile. The mixture was stirred at room temperature for 12 hours, followed by centrifugation and washing with acetonitrile and water to remove the unloaded DN. Finally the obtained COF@DN was stored in water for further use.

Preparation of COF@polyDN

Firstly, COF (1 mg), DN (1 mg) and AIBN (0.015 mg) were dispersed into 1 mL acetonitrile and stirred at room temperature for 12 h. Then the suspension was heated to 70 ℃ and stirred for another 12 h. After the reaction, COF@polyDN was collected by centrifugation and washed with acetonitrile and water to remove the monomers. The final product was dispersed in water for further use.

Calculation of loading capacity and loading efficiency of DN

DN solutions with different concentrations (1.0, 1.5, 2.0, 2.5, 3.0 μg/mL) were prepared

and the UV-vis spectrum from 220 to 300 nm of each solution was recorded. In the preparation process of COF@DN, the UV-vis spectrum of pristine DN solution was first recorded. Then COF nanoparticle were added into DN solutions and stirred for 12 h. After the encapsulation, the dispersion was centrifuged and the supernatant and all the washing solutions were collected for UV-vis analysis. Considering the measurement range, pristine DN solution and final supernatant were diluted 100 times for UV-vis analysis. The loading capacity and loading efficiency of DN were calculated via the equation and standard curve of DN.

$$Loading\ capacity = \frac{M_{initial\ \ DN} - M_{DN\ in\ supernatant}}{M_{COF}}$$

$$Loading\ efficiency = \frac{M_{initial\ DN} - M_{DN\ in\ supernatant}}{M_{initial\ DN}} \times 100\%$$

$^1O_2$ capture ability of DN

A DMF solution of DN (0.1 mg/mL) and TAPP were irradiated under 635 nm laser (0.625 W/cm^2) for 20 min with O_2 bubbling. The changes of UV-vis absorption at 227 nm were recorded to monitor the structure of DN. The experimental results were deducted the TAPP absorption value and other external effects.

$^1O_2$ release ability of COF@EPO and COF@polyEPO

Firstly, COF, COF@DN or COF@polyDN (0.1 mg) were dispersed in 1.8 mL water and subjected to 635 nm laser irradiation (0.625 W/cm^2) for 20 min. Then each solution was separated into two parts. One of them was used as the baseline and the other was added 0.2 mL of DPBF (0.5 mM) immediately. The UV-vis absorbance of DPBF at 417 nm was monitored along with time.

Cell culture

4T1 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS and 100 U/mL of 1% antibiotics (penicillin/streptomycin) at 37 °C in a 95% air/5% CO_2 humidified incubator.

MTT assay

4T1 cells were first planted into 96-well plates and incubated for 24 h. Then COF, COF@DN and COF@polyDN with different concentrations in RPMI 1640 medium containing 10% PBS were added in the well and incubated for another 24 h. Subsequently, 200 μL of MTT solution (0.5 mg/mL) was added into the 96-well plate and incubated for 4 h. Then DMSO was used to dissolve formazan crystal. The absorbance at 490 nm was measured with the RT 6000 microplate reader. The data were collected as mean values +/─ S.D. (n = 4) form four independent experiments.

The in vitro therapeutic effect was also evaluated by MTT assay. 4T1 cells were first planted into 96-well plates for 24 h and then COF, COF@EPO and COF@polyEPO with different concentrations in RPMI 1640 medium containing 10% PBS were added in the well. For COF@EPO and COF@polyEPO groups, the materials were freshly prepared by the laser irradiation of COF@DN and COF@polyDN under 635 nm laser (0.625 W/cm^2) for 20 min, respectively. After incubation for 24 h, the nanoparticles were washed with PBS. Subsequently, 200 μL of MTT solution (0.5 mg/mL) was added into 96-well plate for 4 h incubation and DMSO was then used to dissolve formazan crystal. The absorbance at 490 nm was measured with the RT 6000 microplate reader. The data were collected as mean values +/─ S.D. (n = 4) form four independent experiments.

CLSM imaging

To explore the intracellular $^1O_2$ release, 4T1 cells were first cultured in a glass-bottom dishes (20 mm) for 24 h and then incubated with freshly prepared 100 μg/mL COF@polyEPO (COF@polyDN irradiated under 635 nm for 20 min) for different time (1, 2, 3, 6, 9, 12, 24 h). After washed by PBS for 3 times, DCFH-DA (1 μg/mL) was added into the media for staining. The free dyes was then removed and the fluorescence intensity was assessed with CLSM.

Live/dead cell staining assay was applied to evaluate the therapeutic effect in vitro. 4T1 cells were first cultured in a glass-bottom dishes (20 mm) for 24 h, and then divided into 5 groups (PBS, polyDN, COF@ polyDN, polyDN+L and COF@polyEPO) and incubated with 100 μg/mL corresponding nanoparticles for 12 h. The groups which

need laser irradiation were irradiated under 635 nm laser (0.625 W/cm^2) for 20 minutes before incubation. Subsequently, calcein-AM/PI probe was added to the dishes and incubated for another 15 min. Finally, the cells were washed three times with PBS and imaged with CLSM (Ex: 488 nm, Em: 500-560 nm).

Establishment of tumor model

Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU 2021077). All animal experiments were conducted and obeyed the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, Biology Institute of Shandong Academy of Science, China. Balb/C female mice (4-6 weeks old) were raised under normal conditions of 12 h light and dark cycles and given access to food and water optionally. To establish the tumor models, 4T1 cells (approximately 1×10^6) were dispersed in 80 μL PBS and injected subcutaneously into the flanks of the Balb/C mice. After the tumor volume reached approximately 75~100 mm^3, the mice were used for the subsequent experiments.

In vivo therapeutic effect

The Balb/c tumor-bearing mice were randomly divided into five groups: PBS, polyDN, COF@polyDN, polyDN+L and COF@polyEPO. The corresponding nanoparticles (10 mg/kg) were intratumorally injected to the mice. The groups which need laser irradiation were irradiated under 635 nm laser (0.625 W/cm^2) for 20 minutes before injection. The body weight and volume of tumors were recorded during the three-weeks treatment. The data were collected as mean values +/─ S.D. (n = 3) form three independent experiments. After treatment course, the tumors and major organs (heart, liver, spleen, lung, and kidney) were harvested H&E staining.

The calculation formula of tumor volume: v = (tumor width)^2 × (tumor length)/2

Ex vivo fluorescent imaging

To investigate the biological distribution of nanoparticles in tumors and organs, Balb/c mice were intratumorally injected with 0.3 mg of COF@polyEPO dispersed in 50 μL

saline. Then the mice were sacrificed at different time after injection and the tumor and organs were removed for fluorescent imaging.

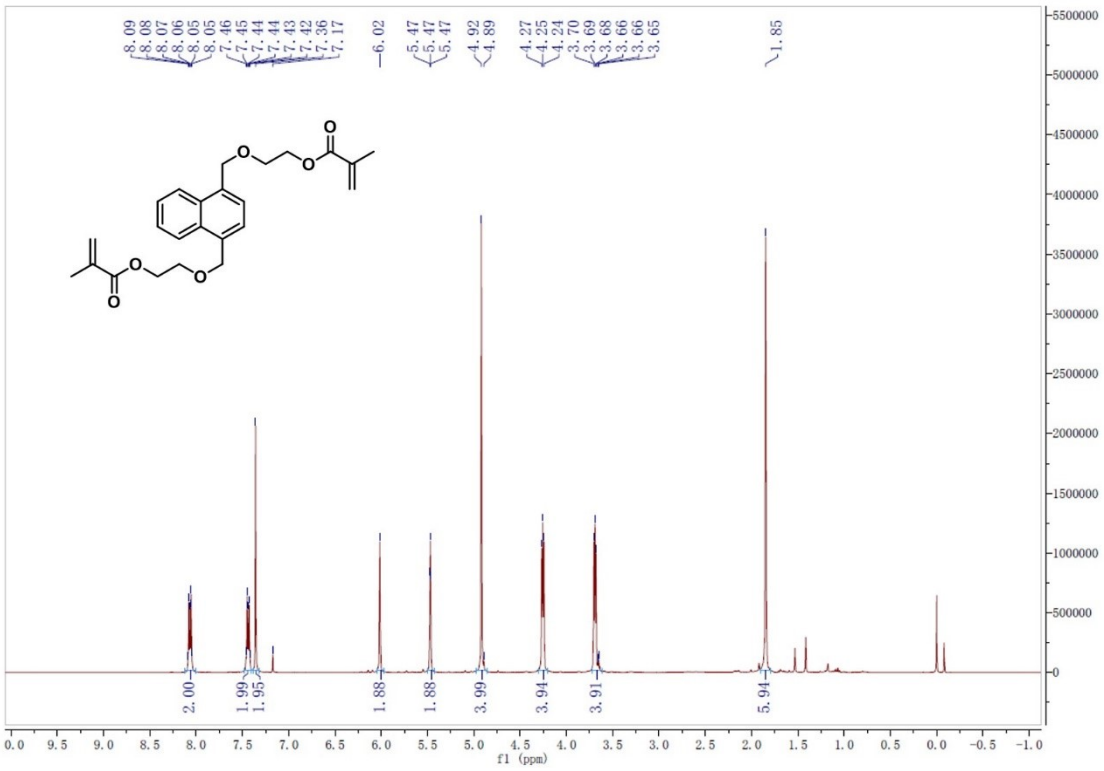

Fig. S2 ^{1}H NMR of DN.

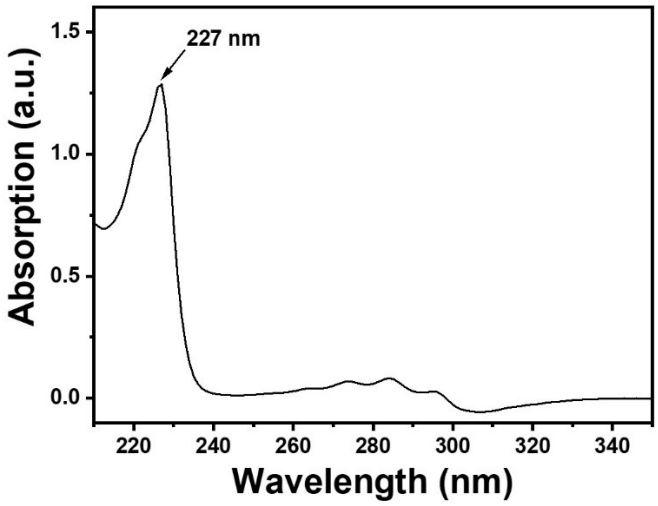

Fig. S3 UV-vis spectrum of DN in acetonitrile.

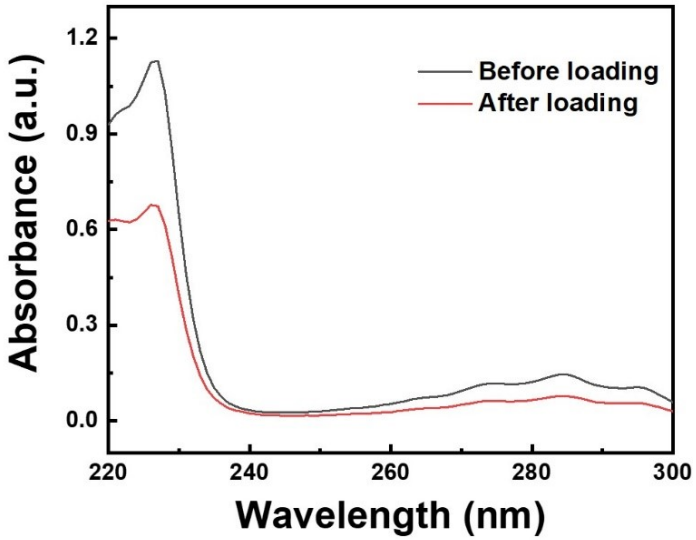

Fig. S4 UV-vis spectra of supernatant before and after DN loading.

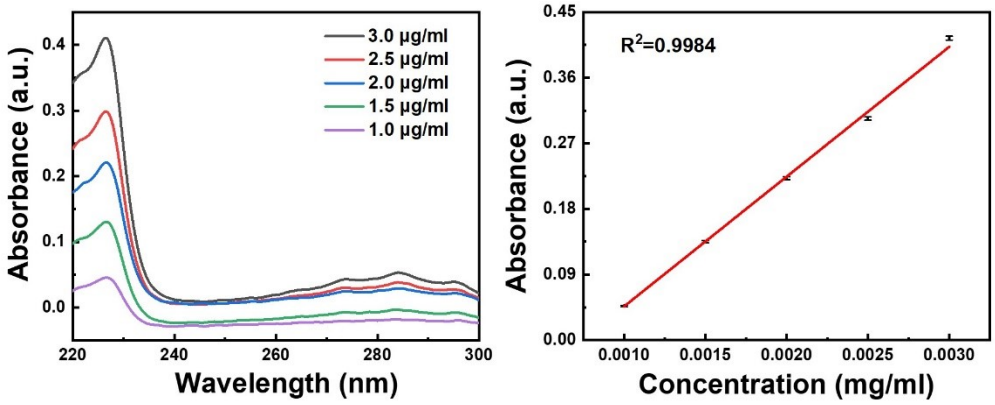

Fig. S5 UV-vis spectra of DN at different concentration (left) and standardization curve of absorption for DN (right).

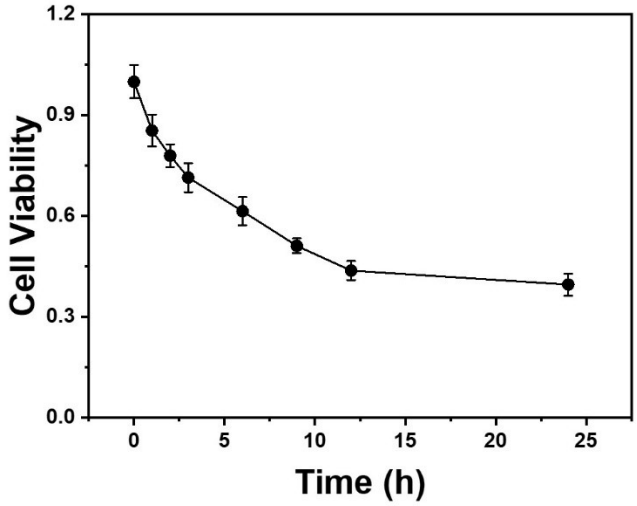

Fig. S6 The time-viability relationship of COF@polyEPO at the concentration of 100 μg/mL. Data are presented as mean values ± SD (n = 4).

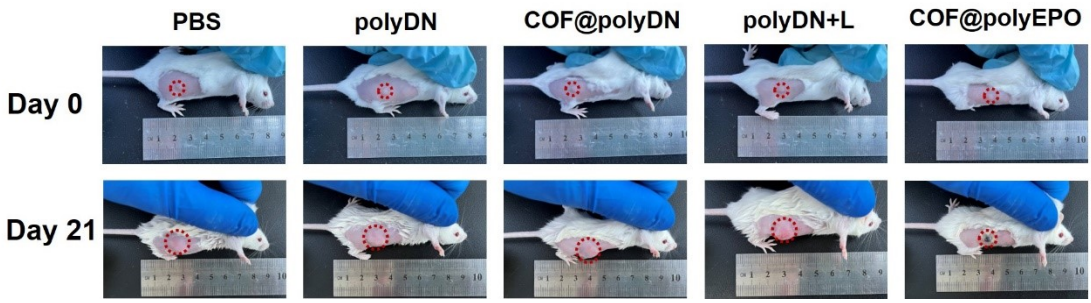

Fig. S7 Photographs of 4T1 tumor-bearing mice taken at Day 0 and Day 21 in different groups.

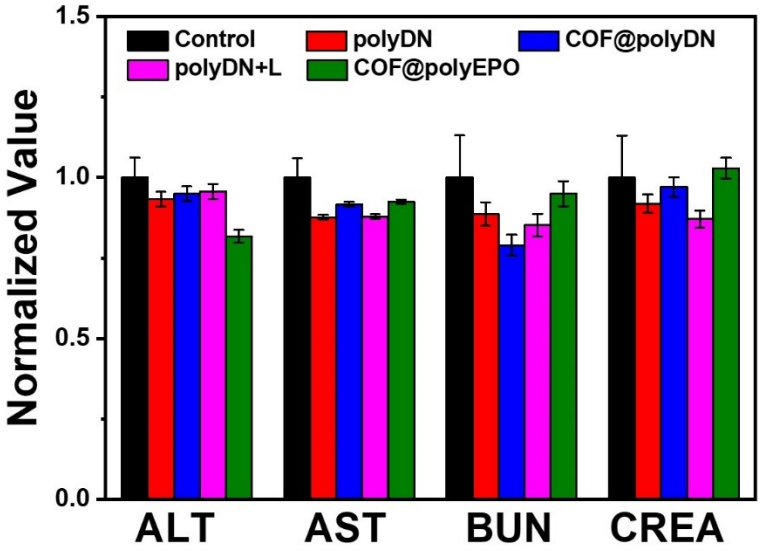

Fig. S8 Blood routine examination of the mice with different treatments. The test items are alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (CREA). Data are presented as mean values ± SD (n = 3).

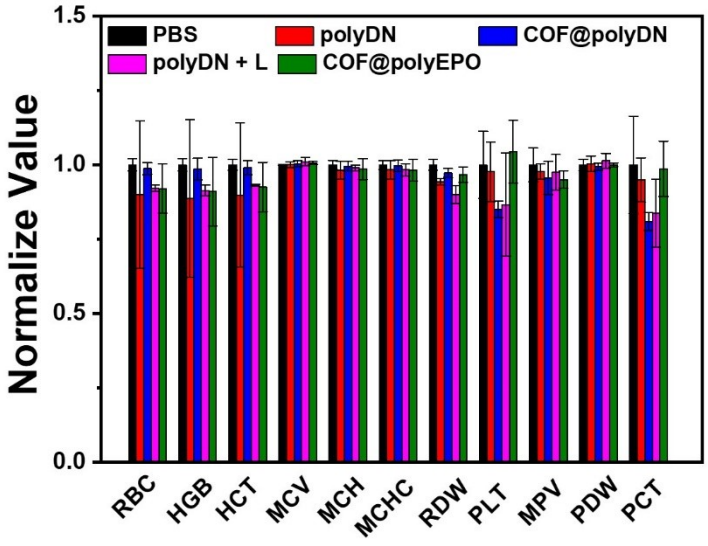

Fig. S9 Hematological data of the mice with different treatments. The test items are red blood corpuscle (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets (PLT), mean

platelet volume (MPV), platelet distribution width (PDW) and plateletcrit (PCT). Data are presented as mean values ± SD (n = 3).

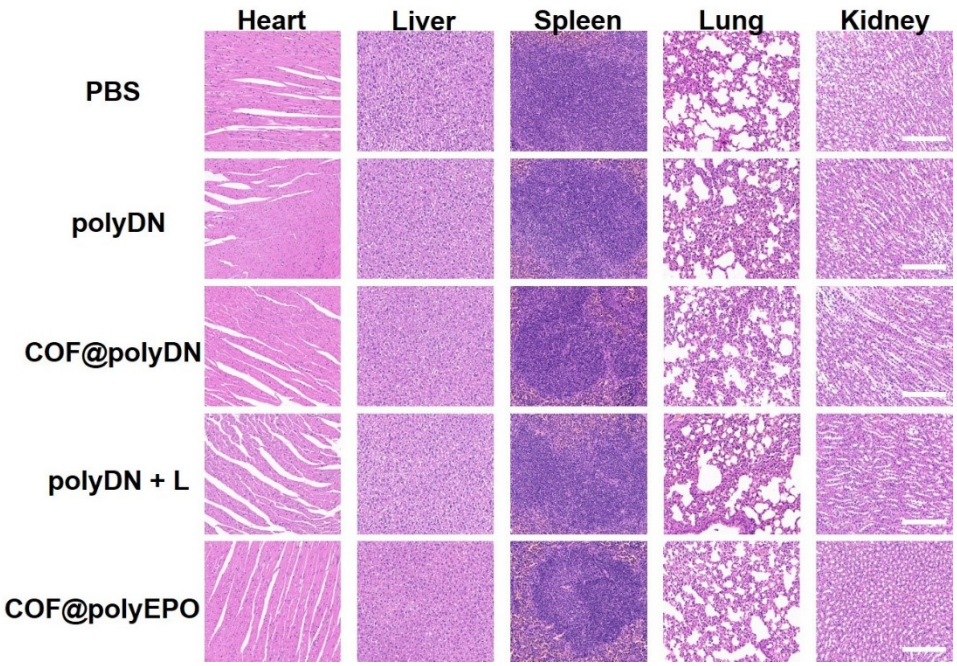

Fig. S10 H&E staining of major organs (heart, liver, spleen, lung and kidney) with different treatments (scale bar = 200 μm).

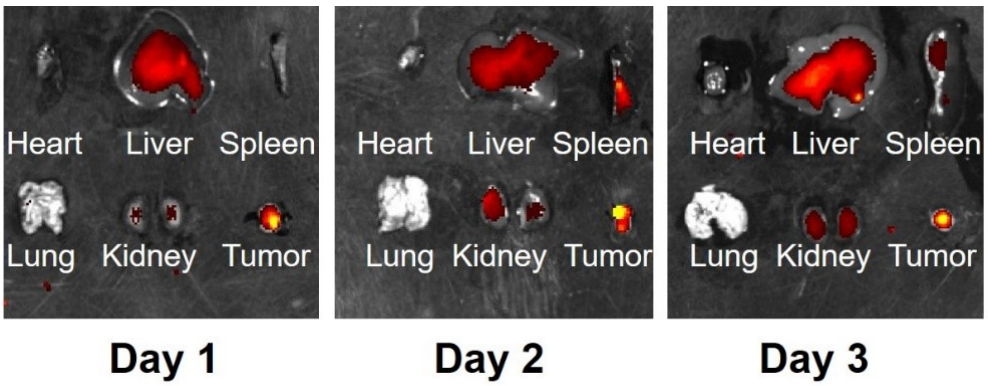

Fig. S11 The fluorescence images of collected tissues from 4T1 tumor-bearing BALB/c mice after intratumoral injection of COF@polyEPO.