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An Enzyme Nanopocket Based on Covalent Organic Frameworks for Long-Termed Starvation Therapy and Enhanced Photodynamic Therapy of Cancer

Xiuyan Wan,[†] Huiwen Zhang,[†] Wei Pan, Na Li, and Bo Tang**

College of Chemistry, Chemical Engineering and Materials Science, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Institute of Molecular and Nano Science, Shandong Normal University, Jinan 250014, P. R. China

*lina@sdnu.edu.cn (N. Li)

*tangb@sdnu.edu.cn (B. Tang)

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EXPERIMENTAL SECTION

Materials and reagents. 2,5-dihydroxyterephthalaldehyde (DHa) and tetra (p-amino-phenyl) porphyrin (TAPP) were obtained from Changchun Third Party Pharmaceutical Technology Co. Ltd. Catalase (CAT) (from bovine liver) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Sulfo-Cyanine3 (Cy3) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Fluorescein isothiocyanate (FITC), 2′,7′-dichlorofluorescin (DCFH) and D-glucose were all of analytical purity and were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Glucose oxidase (GOx) (from aspergillus toxin), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMD) were purchased from Sigma Chemical Company. Hoechst 33342 was purchased from Beijing Solarbio Science & Technology Co., Ltd, China. Calcein-AM/PI Double Stain Kit and Reactive Oxygen Species Assay Kit (2',7,-dichlorofluorescin diacetate, DCFH-DA) were purchased from Beyotime (Nantong, China). The mouse mammary carcinoma cells (4T1 cell) were purchased from Procell Life Science Co., Ltd. (Wuhan, China). All the other chemical reagents were of analytical grade and used without further purification.

Instruments. Transmission electron microscopy (TEM, HT7700, Japan) was employed to characterize the morphologies of the nanoreactors. Fourier infrared spectrometer (Nicolet iS50 FT-IR) was used to characterize the infrared spectrum. Powder X-ray diffraction (XRD) pattern was obtained on a Rigaku SmartLab SE X-Ray Powder Diffractometer with Cu Kα line focused radiation (λ = 1.5405 Å). Scanning electron microscopy (SEM) micrographs were recorded on a Hitachi SU8010 Scanning Electron Microscope. Zeta potential and DLS size were performed on a Malvern Zeta Sizer Nano (Malvern Instruments). Fluorescence spectra were obtained using a FLS-980 Edinburgh Fluorescence Spectrometer with a Xenon lamp. UV-vis spectroscopy was

achieved with UV-1700 (Shimadzu, Japan). The absorbance was measured in a microplate reader (Synergy 2, Biotek, USA) for the MTT assay. Confocal fluorescence imaging studies were performed using a TCS SP8 confocal laser scanning microscope (Leica, Germany). All pH measurements were performed with a digital pH-meter (pH-3e, LeiCi, China). In vivo fluorescence images were captured using live animal imaging system (IVIS Lumina III, US).

Preparation of COF nanoparticles. The porphyrin-based COF was prepared in according to the reported method, which is produced by the reaction of DHa (19.9 mg, 0.12 mmol) and TAPP (40.5 mg, 0.06 mmol) in dichlorobenzene/butyl alcohol/6 M acetic acid (5/5/1, v/v/v, 3.3 mL).[1] The aforementioned mixture was firstly sonicated for 10 min, then degassed in a Pyrex tube (20 mL) through freeze-pump-thaw cycles for three times and sealed off. The tube was heated for 3 days at 120 °C. After cooling to room temperature, the product was collected and washed with THF and acetone for three times, respectively. The as-synthesized COF, which is dispersed in water, were ground and then treated with ultrasound for 2 hours at the power of 1500 W. Next the aqueous dispersion was sonicated in the ultrasonic bath for 6 hours at the power of 360 W. The obtained COF nanoparticles were collected *via* centrifugation (13000 rpm, 10 min) for subsequent use.

Preparation of COF@GOx&CAT. The isoelectric points of GOx and CAT are about 4.9 and 5.4, respectively. As revealed by the ζ-potentials of COF under different pH values, the COF remained negatively charged beyond 2.2. Therefore, the pH value 4.5 was chose to be the condition for COF@GOx&CAT preparation *via* ionic interactions. The pH of PBS used was adjusted to 4.5. The as-synthesized COF nanoparticles (1 mg) was dissolved in 500 μL of PBS (pH = 4.5), which was then added with 500 μL of PBS solution (pH = 4.5) of GOx (0.05 mg) and 500 μL of PBS solution (pH = 4.5) of CAT (0.397 mg). The mixture was stirred for 24 hours at 4

°C, followed by centrifugation (13000 rpm, 10 min) and washing three times with PBS buffer to remove the unabsorbed GOx and CAT to obtain the resultant COF@GOx&CAT.

Detection of DLS size. The DLS size data was given based on intensity. COF (2 mg) and COF@GOx&CAT (2 mg) were dissolved in 2 mL of water at pH 4.5 and then sonicated in the ultrasonic bath for 0.5 hours at the power of 360 W, respectively. Then the DLS size of COF and COF@GOx&CAT were detected based on intensity *via* a Malvern Zeta Sizer Nano instruments. To detect the DLS size of COF@GOx&CAT with different treatments, COF@GOx&CAT (1 mg/mL) in PBS, normal saline, DMEM, MEM, RPMI 1640 were prepared and kept for 24 h. Then these dispersions are centrifuged (13000 rpm, 10 min) and dissolved in water. The DLS size of COF@GOx&CAT with different treatments were detected based on intensity after sonication for 30 min.

Preparation of FITC labelled GOx. GOx-FITC was prepared *via* mixing 10 mg GOx and 1 mg fluorescein isothiocyanate (FITC) in 5 mL of PBS solution and stirring for 24 hours. Excessive FITC was removed by dialysis in PBS solution. Then the powder of GOx-FITC was collected through freezing-drying.

Preparation of Cy3 labelled CAT. To activate the carboxyl groups in CAT, EDC (18 mg) and NHS (10 mg) were added to 2 mL PBS solution of CAT (10 mg) and kept stirring in dark place for 30 min. Then 3 mL of PBS solution of Sulfo-Cyanine3 (Cy3, 0.5 mg) was added in above-mentioned solution and stirred for 24 hours. After that, excessive Cy3 was removed by dialysis in PBS solution. The powder of CAT-Cy3 was collected by freezing-drying.

Calculation of the loading capacity and loading efficiency of GOx. GOx-FITC solutions with different concentrations (2.5, 5.0, 7.5, 10.0, 12.5, 15.0 μg/mL) were prepared, then the

fluorescence spectra (excitation at 488 nm) from 500-550 nm were recorded by a fluorescence spectrometer. The COF@GOx-FITC&CAT was synthesized using the method of preparing COF@GOx&CAT. After centrifuged, the supernatant and all the washing solutions were collected to measure the loading capacity and loading efficiency. The loading capacity was calculated according to the following equations: $(M_{initial\ drug} - M_{drug\ in\ supernatant})/M_{nanoparticles}$. The loading efficiency was according to the following equations: $(M_{initial\ drug} - M_{drug\ in\ supernatant})/M_{initial\ drug} \times 100\%$. The loading capacity and loading efficiency were calculated to be 46.1 μg/mg and 92.2%, respectively.

Calculation of the loading capacity and loading efficiency of CAT. CAT-Cy3 solutions with different concentrations (2, 3, 4, 5, 6, 7 μg/mL) were prepared, then the fluorescence spectra (excitation at 554 nm) from 562-600 nm were recorded. The COF@GOx&CAT-Cy3 was synthesized using the method of preparing COF@GOx&CAT-Cy3. After centrifuged, the supernatant and all the washing solutions were collected to measure the loading capacity and loading efficiency. The loading capacity was calculated according to the following equations: $(M_{initial\ drug} - M_{drug\ in\ supernatant})/M_{nanoparticles}$. The loading efficiency was according to the following equations: $(M_{initial\ drug} - M_{drug\ in\ supernatant})/M_{initial\ drug} \times 100\%$. The loading capacity and loading efficiency were calculated to be 392.58 μg/mg and 98.9%, respectively.

SDS-PAGE gel electrophoresis. To detect GOx and CAT associated with COF, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE gel) (NuPage 4-12% Bis-Tris Gel) electrophoresis was conducted at 120 V for 1.5 hours. GOx only and CAT only and COF only were used as controls. After SDS-PAGE gel electrophoresis, the gel was washed twice with

deionized water and then stained with Coomassie blue for 30 min. After staining, the gel was washed three times with deionized water before taking pictures.

Detection of pH value. The GOx solution (0.393 mg/mL), COF@GOx&CAT (1 mg/mL) solution which stored in PBS for different times (0, 1, 6, 12 months) and COF@GOx&CAT (1 mg/mL) with different treatments (acidic solution with pH value of 4.0, BSA solution, diluted fetal bovine serum and cell lysis solution) for 24 hours were prepared, then the COF@GOx&CAT with different treatments was centrifuged and re-dispersed in PBS. After added glucose (10 mmol/L) for incubation at 37 °C with 30 min, the pH value was measured with a digital pH meter.

Detection of H_2O_2. (1) A H_2O_2 molecular probe (ER-H_2O_2) was utilized for H_2O_2 detection. Glucose (10 mmol/L) was added to the COF@GOx (1 mg/mL) solution and COF@GOx&CAT (1 mg/mL) solution containing ER-H_2O_2 probe at 37 °C to a total volume of 1 mL, and incubated for 2 hours respectively. Next the fluorescence of supernatant was detected after centrifugation (Figure 2b). (2) Potassium permanganate ($KMnO_4$) was also used for H_2O_2 detection based on the reaction between $KMnO_4$ and H_2O_2, which will cause the decrease of absorbance of $KMnO_4$ at 525 nm. The CAT solution (0.393 mg/mL), COF@GOx&CAT (1 mg/mL) solution that stored in PBS for different times (0, 1, 6, 12 months) and COF@GOx&CAT (1 mg/mL) with different treatments (acidic solution with pH value of 4.0, BSA solution, diluted fetal bovine serum and cell lysis solution) for 24 hours were prepared, then the COF@GOx&CAT with different treatments was centrifuged and re-dispersed into PBS. After adding H_2O_2 (10 mmol/L), these solutions were incubated at 37 °C with 30 min. Then 50 μL of Potassium permanganate (120 mmol/L) was added drop by drop and fully oscillated, respectively. The absorption spectra of the solutions were detected by a UV-visible spectrophotometer (Figure S7, S9 and S10).

Detection of O_2 concentration. To compare the O_2 content of different groups, COF@GOx&CAT (1 mg/mL), COF@GOx (1 mg/mL), COF@CAT (1 mg/mL), glucose (10 mmol/L) and H_2O_2 (10 mmol/L) were prepared. Glucose was added to the COF@GOx solution and COF@GOx&CAT solution, as well as H_2O_2 was added to COF@CAT solution. The O_2 content of these solutions at 37 °C was detectable by a portable dissolved oxygen meter.

Detection of ROS. ABMD and DCFH was utilized for $^1O_2$ and ROS detection. After COF@GOx&CAT (1 mg/mL) was dispersed in solutions with or without the addition of glucose (10 mmol/L) to a total volume of 1 mL and incubated for 2 hours at 37 °C, ABMD or DCFH was added into the solutions and irradiated or unirradiated with 635 nm laser for 10 min. And the absorption spectra of the solutions containing ABMD at 403 nm were detected by a UV-vis spectrophotometer after centrifugation. The fluorescence of the solutions containing DCFH were determined via a fluorescence spectrometer after centrifugation (λ_{ex}= 488 nm, λ_{em}= 525 nm).

Cell culture. 4T1 cells were incubated in cell culture dishes with a diameter of 10 cm containing RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 1% penicillin, and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

Detection of intracellular ROS generation level. 4T1 cells were cultured in confocal dishes overnight at 37 °C and divided into 6 groups: PBS, COF, COF+Laser, COF@CAT+Laser, COF@GOx&CAT+Laser, COF@GOx&CAT+Laser+Glucose. For the glucose-containing group, glucose (1 mg/mL) was added to the confocal dishes. And the different nanoparticles (200 μg/mL, in terms of COF) in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) were added to the confocal dishes as well as incubated with 4 hours, respectively. Then all 4T1 cells further treated with fresh culture media containing DCFH-DA (1 μg/mL) for

20 min before irradiation. For irradiation groups, 635 nm laser (0.2 W/cm^2) was utilized to irradiate 4T1 cells with 10 min. Subsequently, the cells were stained with Hoechst 33342 (1 μg/mL) for another 15 min and washed for three times as well as analyzed with CLSM (λ_{ex} = 488 nm, λ_{em} = 515-560 nm).

***In vitro* therapeutic effect.** 4T1 cells were seeded in 96-well plate and cultured for 24 h at 37 °C. And all 4T1 cells were divided into 7 groups: PBS, COF, COF+Laser, COF@CAT+Laser, GOx, COF@GOx+Laser, COF@GOx&CAT+Laser. The nanoparticles of different concentrations (6.25, 12.5, 25, 50, 100, 200 μg/mL, in terms of COF@GOx) in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) were injected into the well. Then all 4T1 cells were co-incubated for 4 hours and treated with fresh culture media. For irradiation groups, 4T1 cells were irradiated with 635 nm laser (0.2 W/cm^2) for 10 min. All groups were incubated for 12 hours before adding 150 μL of MTT solution (0.5 mg/mL) and then incubated for another 4 hours. After that, MTT solution was removed and 150 μL of DMSO was injected to each well. Then a microplate reader was employed to measure the absorbance at 490 nm.

***In vitro* starvation therapy.** 4T1 cells were cultured in 96-well plate and incubated for 24 h at 37 °C. COF@GOx (100 μg/mL) in RPMI 1640 media supplemented with 10% (v/v) fetal bovine serum (FBS) was injected into the well and incubated for 24 hours after the glucose of different concentrations (0, 40, 80, 100, 200, 400, 800, 1000 μg/ml) were added. Then the media were removed and 150 μL of MTT solution (0.5 mg/mL) was added to the well. After 4 hours, MTT solution was discarded and 150 μL of DMSO were added, then the absorbance was monitored at 490 nm *via* a microplate reader.

Live/dead cell staining assay. To detect the viable and dead cells, 4T1 cells were cultured in confocal dishes overnight and divided into 7 groups: PBS, COF, COF+Laser, COF+CAT+Laser, GOx, COF+GOx+Laser, COF+CAT+GOx+Laser. The different nanoparticles (200 μg/mL, in terms of COF@GOx) in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) were added to the confocal dishes and incubated with 4 hours, respectively. For laser irradiation groups, 635 nm laser (0.2 W/cm^2) was utilized to irradiate the cells for 10 min. All the groups were further cultured for 12 hours. Finally, the cells were stained with Calcein AM (λ_{ex}=490nm, λ_{em}=515nm) / Propidium Iodide (PI) (λ_{ex}=535nm, λ_{em}=617nm) for 15 min and analyzed with CLSM.

Tumor model establishment. Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU 2019032). 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 mice (6-8 weeks) were fed under normal conditions. The 4T1 breast cancer model was employed as an example to evaluate the therapeutic effect. 1×10^7 4T1 cells in 100 μL of serum-free RPMI 1640 medium were injected subcutaneously into the right axillary region of Balb/c mice. The mice were utilized in subsequent experiments after the tumor size had reached approximately 75-100 mm^3.

***In vivo* fluorescence imaging.** IR808 dye was employed to accomplish in vivo fluorescence imaging. 50 μL of COF@GOx-IR808 (0.15 mg/mL, in terms of GOx-IR808) and 50 μL of GOx-IR808 (0.15 mg/mL) were administrated into the 4T1 tumor bearing Balb/C mice, respectively. At different time points of 0, 4, 8 hours and 5 days post injection, the fluorescence in the mice was recorded with a live body imaging system.

***In vivo* therapeutic effect.** 4T1 breast cancer bearing mice were divided into 7 groups: PBS, COF, COF+Laser, COF@GOx, GOx, COF@GOx+Laser, COF@CAT&GOx+Laser. The different nanoparticles (7.5 mg/kg, in terms of the COF@GOx) were injected to the cancer position of each mouse. After 4 hours, 635 nm laser (0.2 W/cm^2) was employed to treat with each mouse of irradiation groups for 10 min. The body weight and tumor size of each mouse were registered every two days for 14 days during treatment (tumor volume = width^2 × length/2). And the relative tumor volume was calculated using the formula V/V_0 (V_0 was the tumor volume at the beginning of the treatment).

Immunohistochemical analysis. 4T1 breast cancer bearing mice were divided into 7 groups: PBS, COF, COF+Laser, COF@GOx, GOx, COF@GOx+Laser, COF@GOx&CAT+Laser. The different materials (7.5 mg/kg, in terms of the COF@GOx) were injected to the cancer position of each mouse. After 4 hours, 635 nm laser (0.2 W/cm^2) was employed to treat with each mouse of irradiation groups for 10 min. 12 hours after treatment, the representative tumor-bearing mice from different treatment groups were sacrificed. Then tumors were collected for immunohistochemical analysis. K_i-67 detection was employed for measuring the proliferation of tumor by automatic multispectral imaging system.

Histopathological analysis. 4T1 breast cancer bearing mice were divided into 7 groups: PBS, COF, COF+Laser, COF@GOx, GOx, COF@GOx+Laser, COF@GOx&CAT+Laser. The different materials (7.5 mg/kg, in terms of the COF@GOx) were injected to the cancer position of each mouse. After 4 hours, 635 nm laser (0.2 W/cm^2) was employed to treat with each mouse of irradiation groups for 10 min. 12 hours after treatment, the representative tumor-bearing mice from different treatment groups were sacrificed and the tumors were harvested to use for hematoxylin and eosin (H&E) staining. 14 days after treatment, the other representative tumor-

bearing mice from different treatment groups were sacrificed and five major organs (liver, lung, spleen, kidney, and heart) were harvested to use for H&E staining.

Blood biochemical parameters and hematological parameters analysis. 4T1 breast cancer bearing mice were intratumor injected with PBS or COF@GOx&CAT (7.5 mg/kg). After 4 hours, 635 nm laser (0.2 W/cm^2) was employed to treat with each mouse of COF@GOx&CAT groups for 10 min. 7 days after treatment, the blood sample of mice was collected from the retro-orbital plexus into an anticoagulant tube for blood routine analysis as well as into a coagulation-promoting tube and centrifuged at 3000 rpm for 5 min to obtain plasma samples. The serum biochemical parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (CREA) were tested using the automatic biochemical analyzer. And the hematological parameters including red blood cell (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), Platelets (PLT), hematocrit (HCT), mean platelet volume (MPV), platelet distribution width (PDW) and procalcitonin (PCT) were tested.

SUPPORTING FIGURES

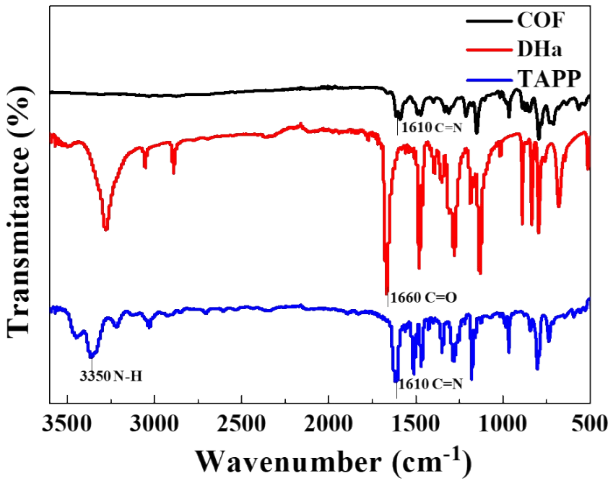

Figure S1. The FT-IR spectra of COF, DHa and TAPP.

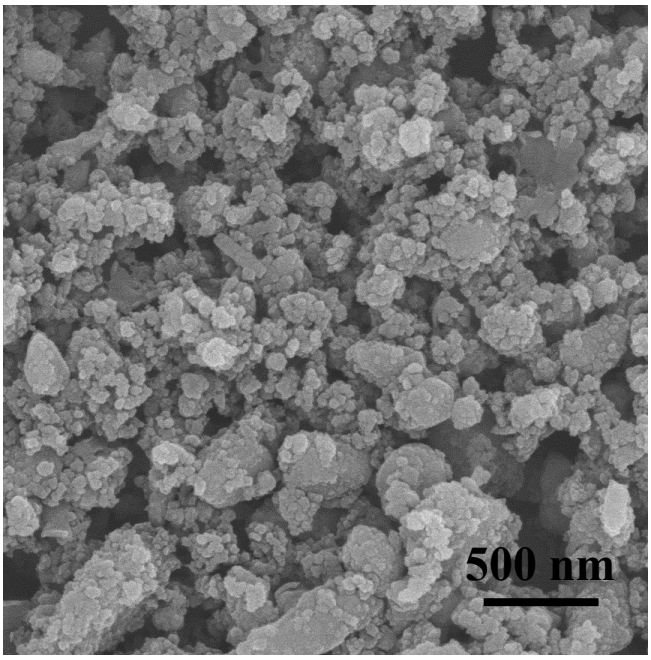

Figure S2. SEM image of COF.

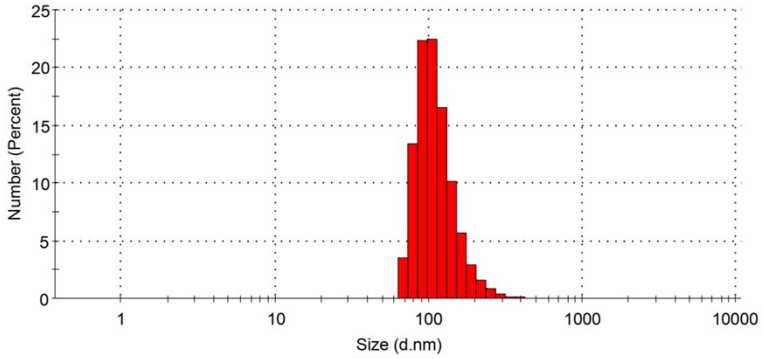

Figure S3. DLS size distribution of COF.

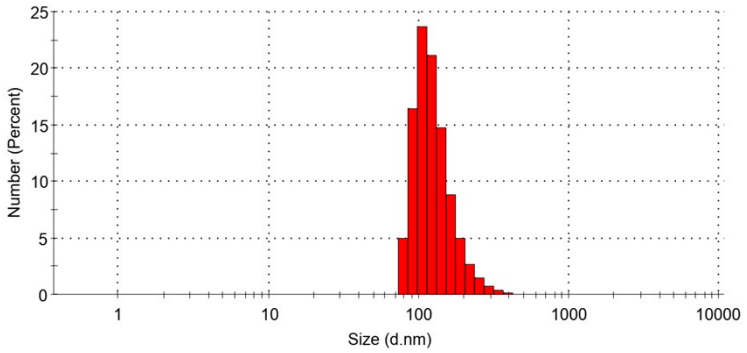

Figure S4. DLS size distribution of COF@GOx&CAT.

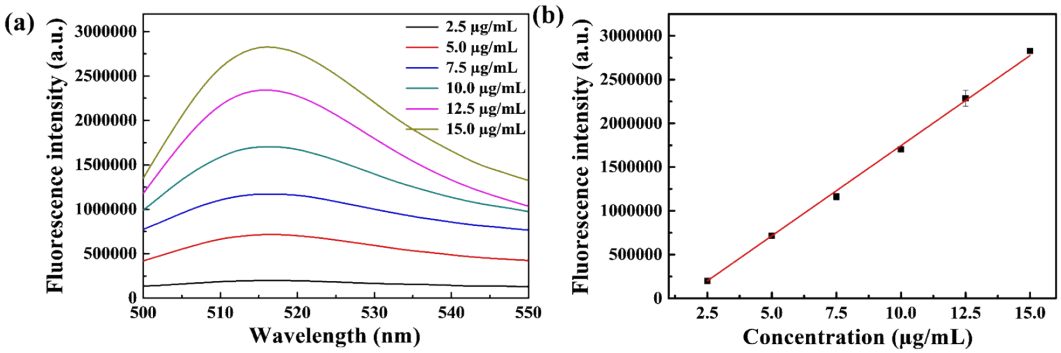

Figure S5. (a) Fluorescence spectra of GOx-FITC with different concertrations. (b) Linear relationships between the fluorescence intensity of GOx-FITC at 525 nm and the GOx concentration. The error bar is the standard deviation from the mean (n = 3).

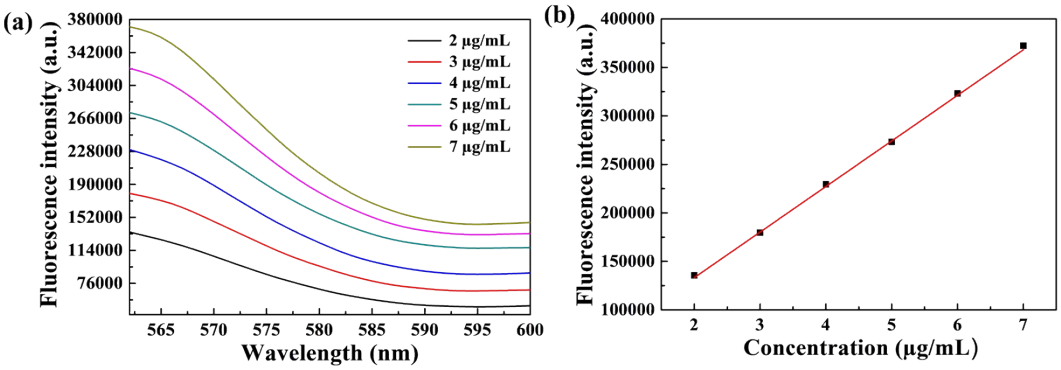

Figure S6. (a) Fluorescence spectra of CAT-Cy3 with different concertrations. (b) Linear relationships between the fluorescence intensity of CAT-Cy3 at 562 nm and the CAT concentration. The error bar is the standard deviation from the mean (n = 3).

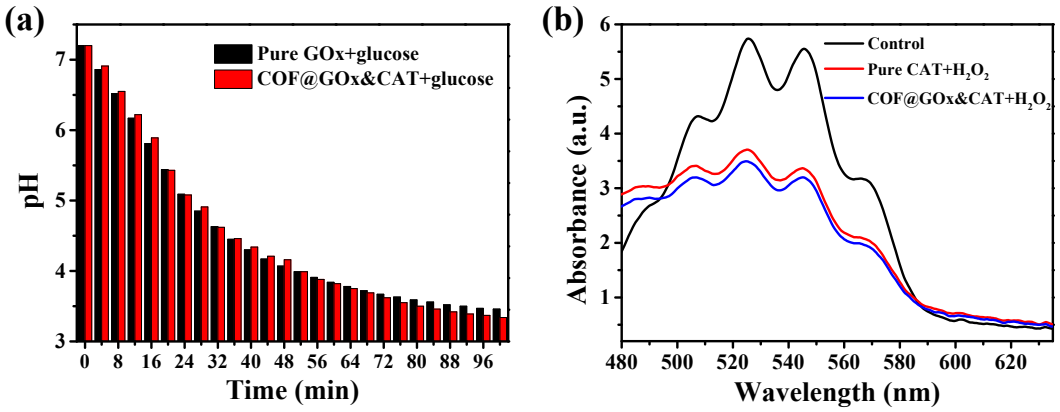

Figure S7. (a) The pH values of GOx and COF@GOx&CAT solution with glucose at different time; (b) The UV-Vis spectra of $KMnO_4$ solution with different treatments after 30 min.

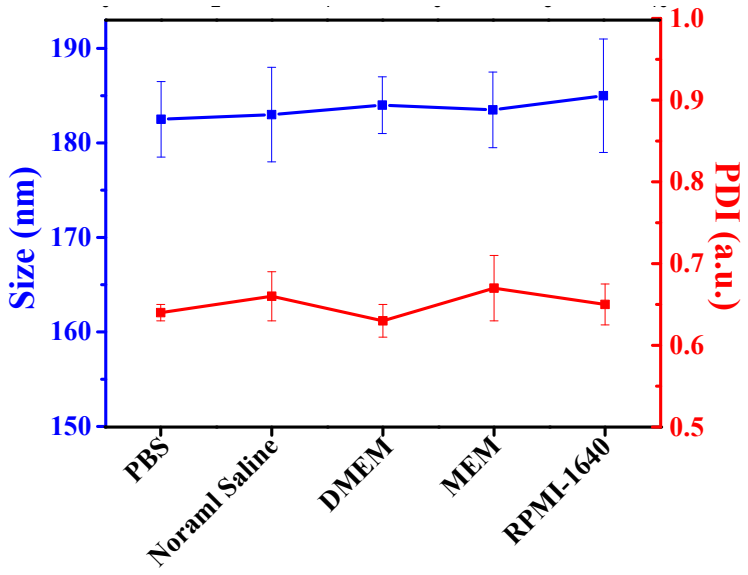

Figure S8. DLS measured size and PDI of COF@GOx&CAT with different treatments for 24 h. The error bar is the standard deviation from the mean (n = 3).

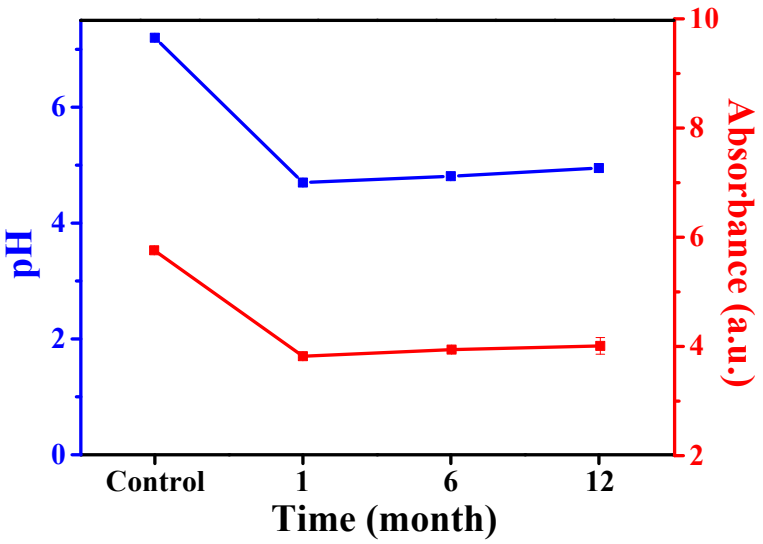

Figure S9. The pH values of solution containing COF@GOx&CAT stored for different times after added glucose for 30 min. The absorbance of $KMnO_4$ at 525 nm in solution containing COF@GOx&CAT stored for different times after added 10 mM H_2O_2 for 30 min. The error bar is the standard deviation from the mean (n = 3).

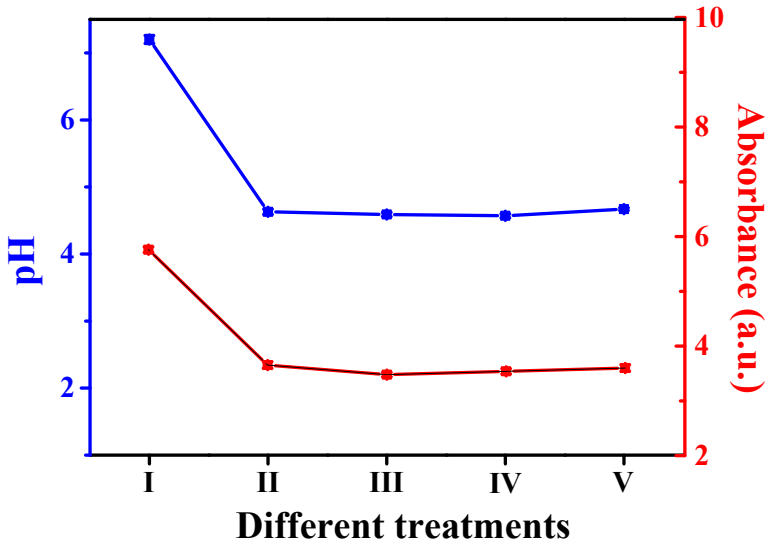

Figure S10. The pH values of solution containing COF@GOx&CAT with different treatments (I control, II acidic solution with pH value of 4.0, III BSA solution, IV diluted fetal bovine serum, and V cell lysis solution) after added glucose for 30 min. The absorbance of $KMnO_4$ at 525 nm in solution containing COF@GOx&CAT with different treatments after added 10 mM H_2O_2 for 30 min. The error bar is the standard deviation from the mean (n = 3).

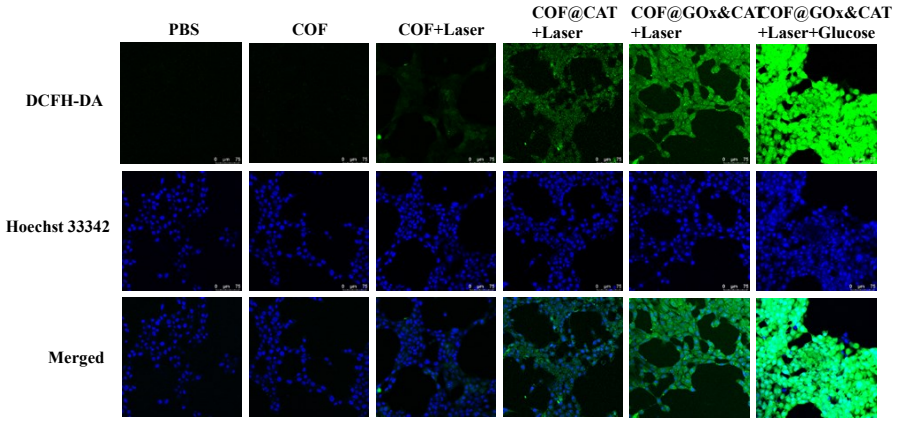

Figure S11. Confocal laser scanning microscopy (CLSM) images of 4T1 cells with different treatments stained with DCFH-DA (green) and Hoechst 33342 (blue) to estimate ROS generation based on DCFH-DA fluorescence intensity. All scale bars are 75 μm.

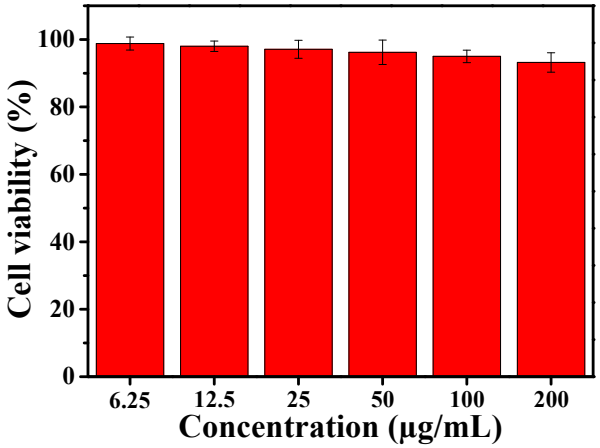

Figure S12. Cell viabilities of 4T1 cells after incubated with various concentrations of COF.

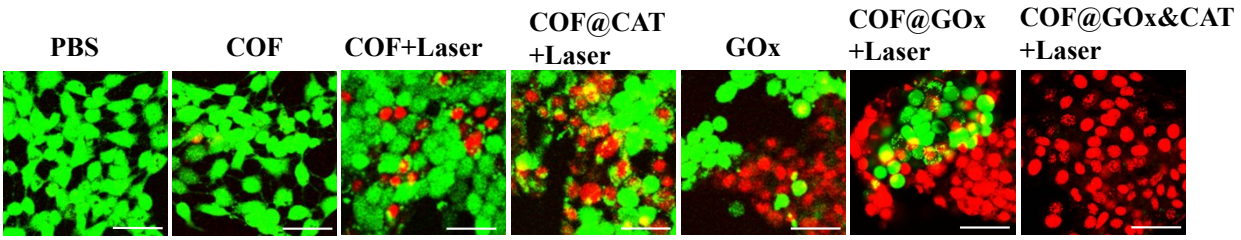

Figure S13. Live/dead cell staining assay to visualize the cell viability of 4T1 cells under different treatments. All scale bars are 50 μm.

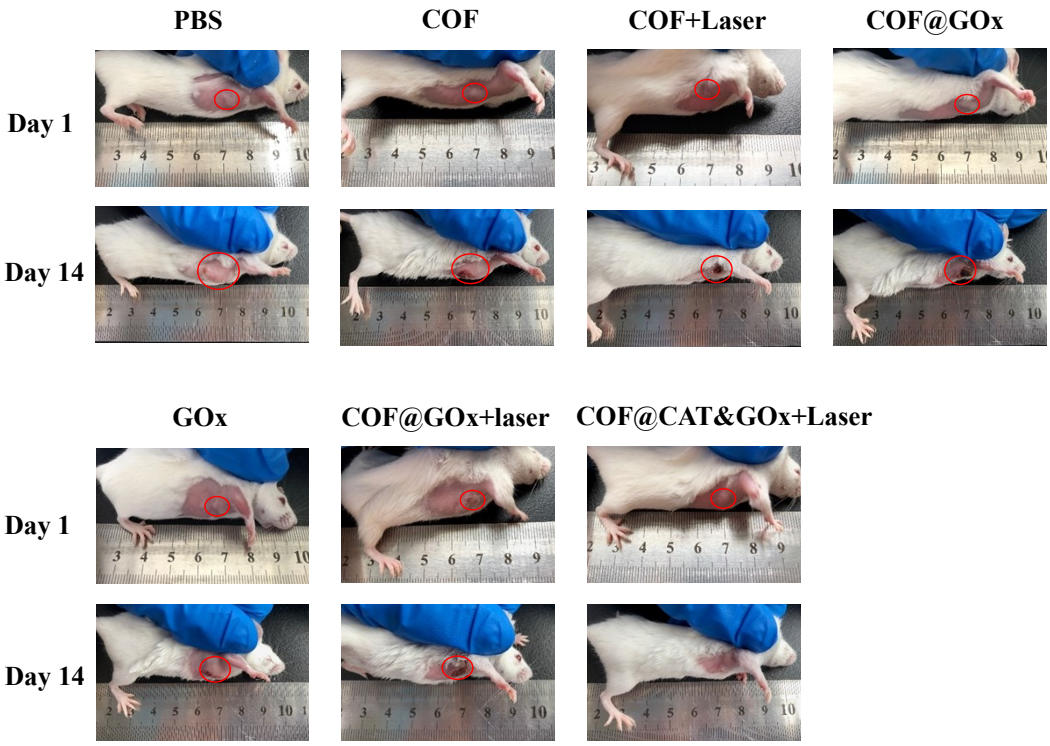

Figure S14. Photographs of tumors in mice at day 0 and day 14.

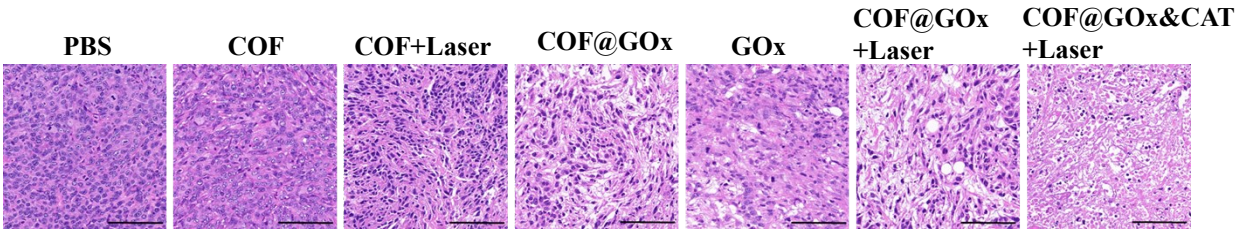

Figure S15. H&E-stained images of tumor slices collected from tumor bearing mice after different treatments. All scale bars are 100 μm.

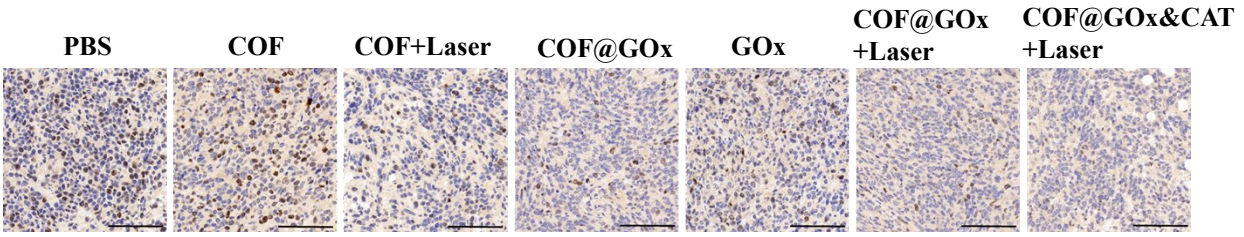

Figure S16. Ki-67 analysis of tumor slices collected from tumor-bearing mice after different treatments. All scale bars are 100 μm.

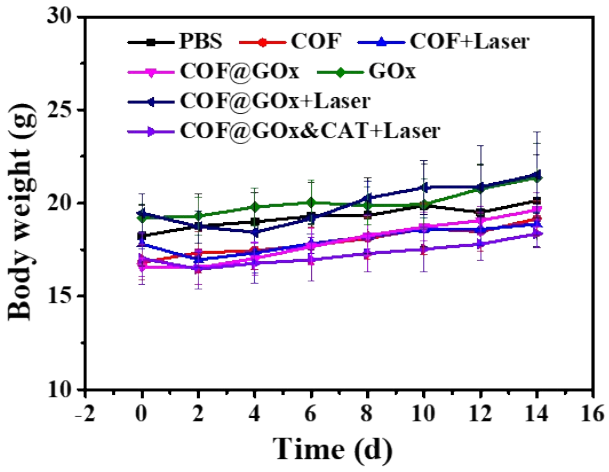

Figure S17. Body weight changes of mice within 14 days during treatment. The error bar is the standard deviation from the mean (n = 3).

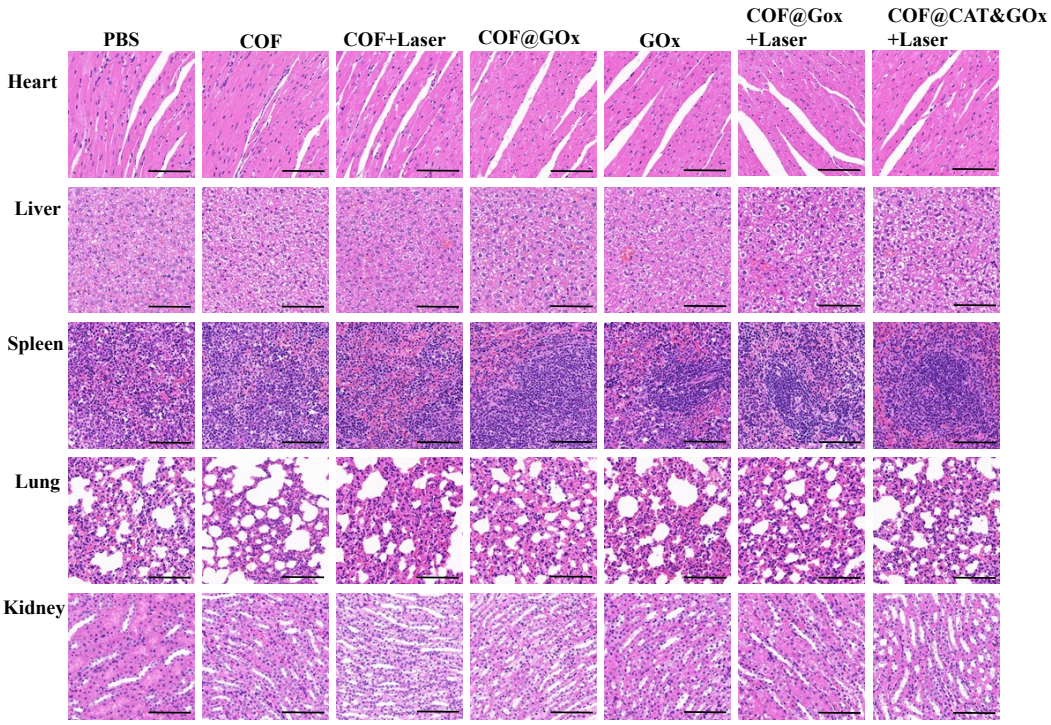

Figure S18. H&E-stained images of the five major organs (heart, liver, spleen, lung and kidney) with different treatments after 14 days. Scale bars are 100 μm.

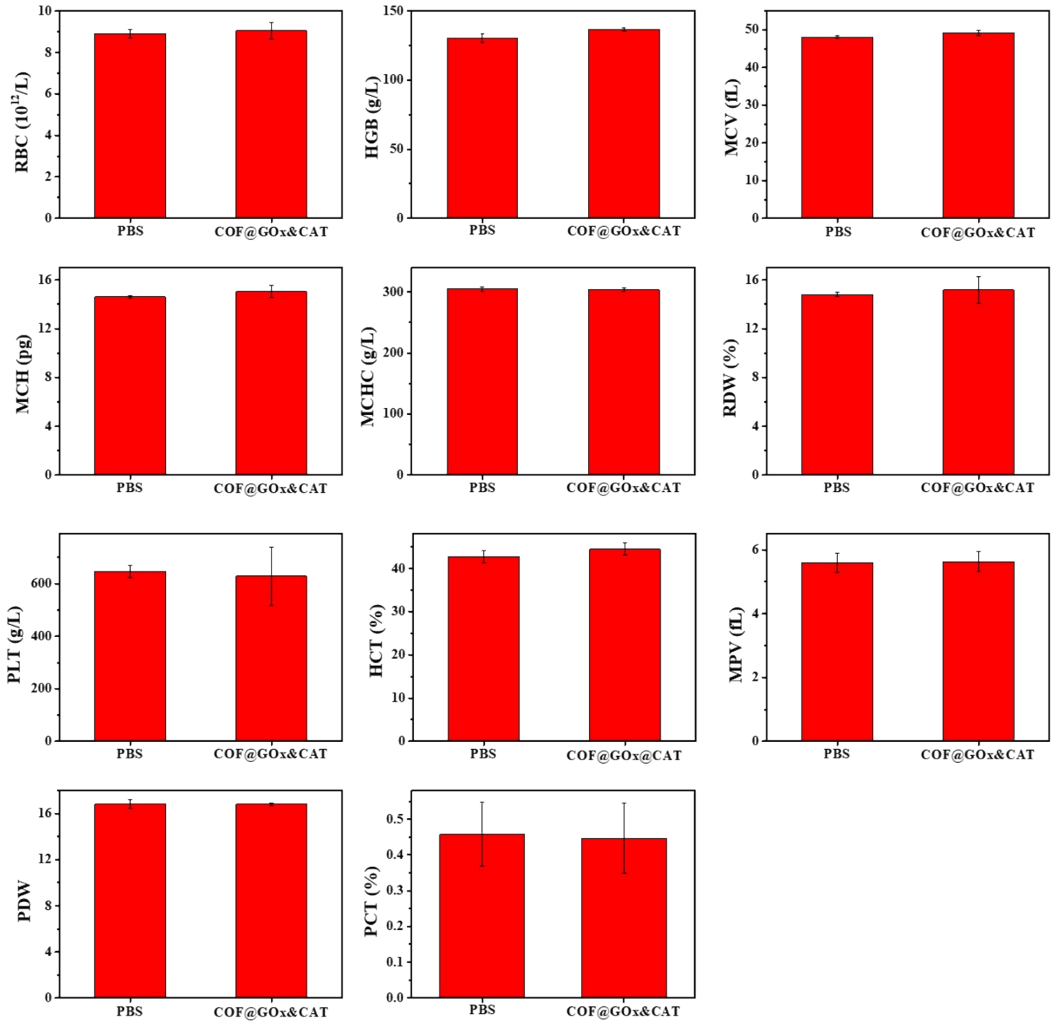

Figure S19. Hematological parameters after different treatments for 7 days. The blood was collected for detection of the levels of RBC, HGB, MCV, MCH, MCHC, RDW, PLT, HCT, MPV, PDW and PCT. The error bar is the standard deviation from the mean (n = 3).

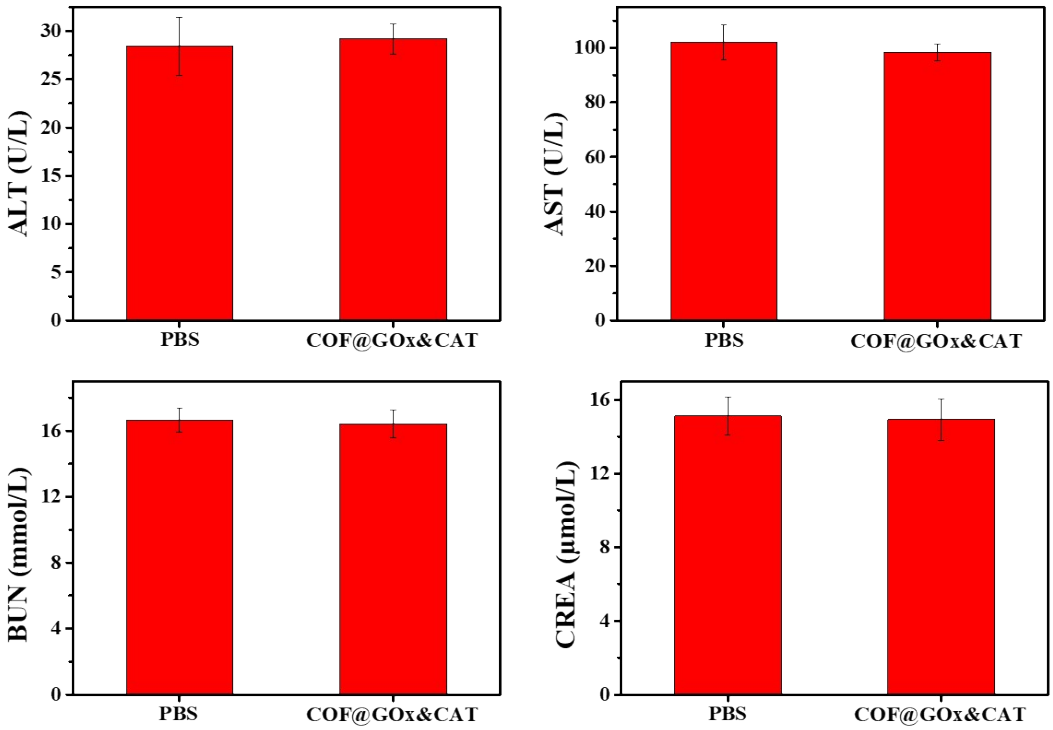

Figure S20. Blood biochemical parameters after different treatments for 7 days. The blood was collected for detection of the levels of ALT, AST, BUN and CREA. The error bar is the standard deviation from the mean (n = 3).

References

[1] Y. Zhang, L. Zhang, Z. Wang, F. Wang, L. Kang, F. Cao, K. Dong, J. Ren, X. Qu, *Biomaterials* **2019**, *223*, 119462.