Supporting Information for

A covalent organic framework-based nanoreactor for enhanced chemodynamic therapy through cascaded fenton-like reactions and nitric oxide delivery

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Content

1. Materials and instrumentations

1,3,5-Tris(4-aminophenyl)benzene and 2,5-dihydroxy-p-phenylenedicarboxaldehyde were purchased from Jilin Chinese Academy of Sciences - Yanshen Technology Co., Ltd. H_2O_2 test kits, NO assay kit, DAF-FM DA (NO fluorescent probe) were purchased from Beyotime (Shanghai, P. R. China). Azobisisobutyronitrile, acetonitrile, acetic acid, N,N-dimethylformamide and $CuCl_2$-$2H_2O$ were purchased from Sinopharm Chemical Reagent Co. Trifluorotoluene, 1,2-ethylenedithiol, tert-butyl nitrite, 1,4-dithiothreitol were purchased from Shanghai Macklin Biochemical Co., Ltd. Ultra-pure water was prepared with an Aquapro System (18 MΩ). Cell Counting Kit-8, DTNB and 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from MedChemExpress Co. Ltd. Phosphate buffered salt (PBS), Dulbecco's phosphate buffered saline (DPBS) and fetal bovine serum (FBS) were purchased from VivaCell (Shanghai, P. R. China). Dulbecco's Modified Eagle Medium (DMEM), Penicillin Streptomycin Mixtures (Pen-Strep), and Trypsin-EDTA Solution (0.25%) were purchased from HyClone Laboratories, Inc. Normocin was purchased from Invivogen (San Diego, CA, USA).

Fourier transform infrared (FT-IR) spectra were obtained in the 400-4000 cm^{-1} range using a Thermo Scientific Nicolet iS50 FT-IR Spectrometer equipped with diamond attenuated total reflection (ATR) module. Each spectrum was the average of 16 scans. Ultraviolet-visible (UV-vis) absorption spectra were recorded on a Shimadzu UV-2700 Double Beam UV-vis Spectrophotometer. Scanning electron microscopy (SEM) micrographs were recorded on a Hitachi SU8010 Scanning Electron Microscope. Transmission electron microscope (TEM) micrographs were recorded on a Hitachi HT7700 120 kV Compact-Digital Transmission Electron Microscope. 808 nm laser (FC-808-10W-MM). Powder X-ray diffraction (PXRD) patterns were obtained on a Rigaku SmartLab SE X-Ray Powder Diffractometer with Cu Kα line focused radiation (λ = 1.5405 Å) from 2θ = 2.00° up to 50.00° with 0.01° increment. Hydrodynamic particle size and zeta potential were measured using Malvern Zetasizer Nano ZS90 System. Laser scanning confocal fluorescence images were captured with a Leica TCS SP8 Confocal Laser Scanning Microscopy with an objective lens (×20). Glass bottom dishes were purchased from S3 Cellvis (Mountain View, CA, USA). Microplate assays were carried out on a Molecular Devices SpectraMax i3x Multi-Mode Microplate Detection System.

2. Cell culture and laboratory animals

The MCF-7 (human breast adenocarcinoma cell line) was provided by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, P. R. China), and cultured in DMEM supplemented with FBS (10%), Normocin (50 μg/mL), penicillin (100 U/mL) and streptomycin (100 μg/mL) in an atmosphere of CO_2 (5 vol%) and air (95 vol%) at 37°C.

Nude mice (BALB/c-nu, femina, aged 4 weeks, 9–12 g) were purchased from Spearfish (Beijing) Biotechnology Co., Ltd. Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China. All the animal experiments complied with relevant guidelines of the Chinese government and regulations for the care and use of experimental animals.

3. Snthesis of materials

3.1 Synthesis of COF (1)

A mixture of 1,3,5-tris(4-aminophenyl)benzene (23.14 mg, 0.062 mmol), 2,5-diallyloxyterephthalaldehyde (21.69 mg, 0.093 mmol), acetonitrile (25 mL) and glacial acetic acid (2.7 mL) was stirred at 25°C for 12 h. The particles were isolated by centrifugation and washed with acetonitrile three times to generate COF as a yellow powder. FT-IR (ATR, cm^{-1}): 3028 (w), 2915 (w), 1682 (w), 1615 (m), 1591 (s), 1504 (s), 1485 (m), 1418 (s), 1373 (m), 1286 (m), 1197 (s), 1144 (m), 1013 (m), 923 (m), 878 (w), 829 (s), 730 (w), 539 (w).

3.2 Synthesis of COF-SNO (2)

9 mg of **1** was dispersed in 2 mL of trifluorotoluene, 1 mg (0.00609 mmol) of azobisisobutyronitrile was added and mixed well, and 2.54 μL of 1,2-ethylenedithiol was added under vacuum, and the reaction was stirred at 90℃ for 2.5 h. **COF-SH** was separated by centrifugation and washed thoroughly with ethanol. Separated by centrifugation, the unreacted 1,2-ethylenedithiol was washed with ethanol to obtain **COF-SH**. 9 mg of **COF-SH** was dispersed in a mixture of 4 mL methanol and 1 mL toluene, and 3 μL of tert-butyl nitrite was added, and the reaction was carried out for 24 hours at 4℃ under light protection, then centrifuged, and washed with methanol and ethanol for a number of times to obtain **COF-SNO**. FT-IR (ATR,cm^{-1}): 3383 (s),2969 (s),2920 (s),1613 (m),1590 (s),1506 (s),1488 (m),1420 (s),1376 (m),1286 (m),1202 (s),1146 (m),1087 (m),1046 (s),927 (m),878 (w),829 (s),730 (w),540 (w).

3.3 Synthesis of CuO_2@COF-SNO (3)

9 mg of **2** was dispersed in water, 8 mg of $CuCl_2 \cdot 2H_2O$ was added, stirred for 30 min, centrifuged, washed twice with water, and then dispersed in 5 mL of solution containing 2 mg of NaOH, and 50 μL of 30% H_2O_2 was added and reacted for 20 min. The solution was separated by centrifugation and washed with water to obtain **CuO_2@COF-SNO**. FT-IR (ATR, cm^{-1}): 3290 (m), 2925 (m), 1660 (w), 1613 (m) 1593 (w), 1506 (m), 1479 (w), 1418 (s), 1359 (m), 1290 (w), 1195 (m), 1137 (w), 1091 (s), 1033 (w), 1010 (m), 926 (m), 896 (w), 830 (m).

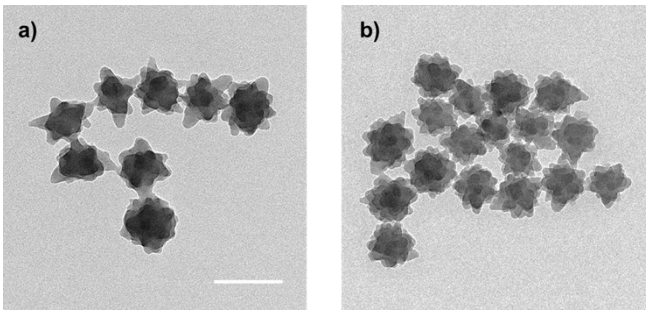

Fig. S1 TEM images of **1** and **2**. Scale bar, 200 nm.

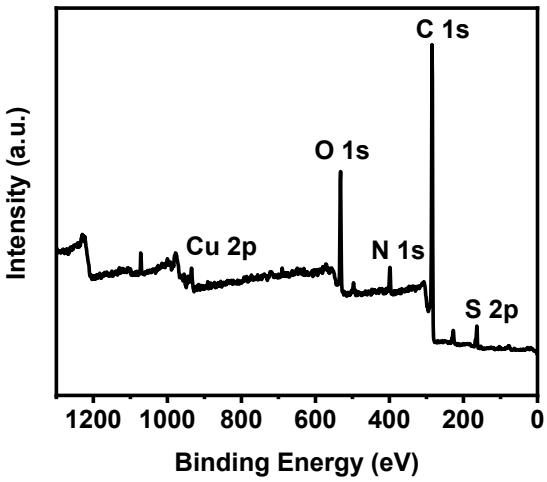

Fig. S2 X-ray photoelectron spectroscopy data of **3**.

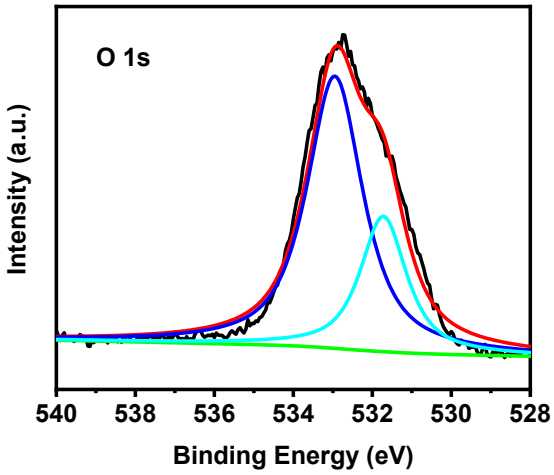

Fig. S3 High-resolution X-ray photoelectron spectroscopy data of **3** in the O 1s region.

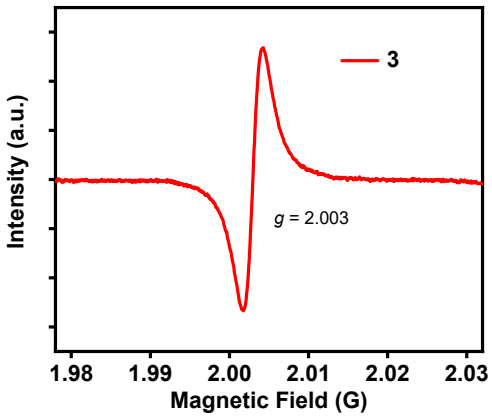

Fig. S4 Solid-state steady-state ESR spectra of **3**.

3.4 Determination of sulfhydryl groups by Ellman's method

Ellman's reagent buffer solution of DTNB, i.e. 5,5'-dithiobis(2-nitrobenzoic acid), reacts with sulfhydryl groups to displace benzoic acid, i.e. TNB. DTNB has a characteristic absorption peak at 325 nm, while TNB shows a strong absorption peak at 412 nm under weakly basic conditions, and the sulfhydryl group concentration and absorbance values are in accordance with the Lambert-Beer law. The reaction of the sulfhydryl group with DTNB proceeded quantitatively, so the sulfhydryl group content in the sample could be determined by UV-Vis spectrophotometry (1 mg/mL) at pH = 7.8.

Ellman's reagent preparation: 1 mg of DTNB was dissolved in 1 mL of a buffer solution with pH 7.8.

Establishment of standard curve: 1,4-dithiothreitol was used as the sulfhydryl standard. Take 200 μL of different concentrations of 1,4-dithiothreitol and mix with 200 μL of Ellman's reagent and 1 mL of buffer solution, incubate at 37℃ for 5 min, take 100 μL and measure the absorbance at 412 nm with an enzyme meter to establish the standard curve of concentration and absorbance.

Configuration of the test solution: 1 mg of **COF-SH** and **COF-SNO** were dispersed in 1 mL of ethanol to obtain a concentration of 1 mg/mL of dispersion.

200 μL of the dispersed solution was mixed with 200 μL of Ellman's reagent and 1 mL of buffer solution, and incubated at 37℃ for 5 min. The reaction solution was centrifuged at 13000 rpm for 10 min, and the supernatant was filtered through a 200 μm organic phase filter membrane. The supernatant was filtered through 200 μm organic phase filter membrane. 100 μL of the supernatant was taken and the absorbance at 412 nm was measured with an enzyme meter.

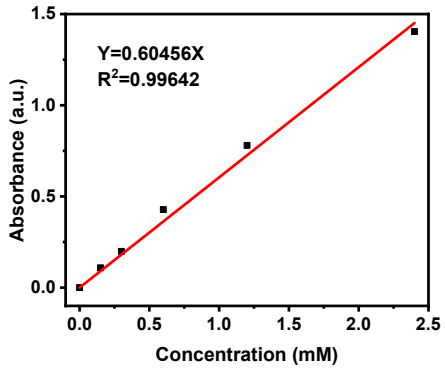

Fig. S5 Standard curve of DTNB at 412 nm based on UV-Vis absorbance.

3.5 Determination of CuO_2 loading.

3 (1 mg) was added to 2 mL of concentrated nitric acid and heated in a ventilated cooker to nitrate until the solid was completely dissolved, then it was added to water to make a volume of 10 mL, and the content of Cu in the solution was determined by inductively coupled plasma optical emission spectrometer (ICP-OES).

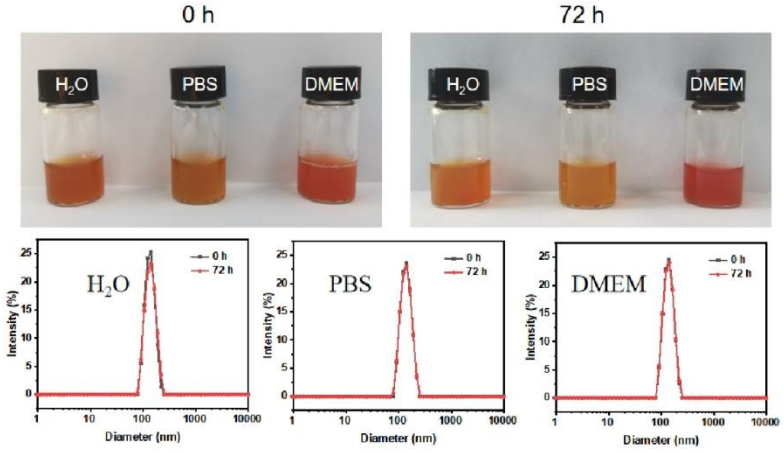

Fig. S6 The stability of **3** in different physiological solutions.

4 Detection of H_2O_2

The production of H_2O_2 induced by the decomposition of CuO_2 was assessed by a colorimetric method, which resulted in the formation of a yellow titanium peroxide complex ($Ti(IV)O_2^{2+}$) when H_2O_2 oxidized colorless titanium sulfate. 2 mg **3**, COF and 30% H_2O_2 were added to 2.0 mL of PBS ($Ti(SO_4)_2$, 0.005 mol/L) with pH = 6.5, and the UV-vis absorption was measured after 10 min.

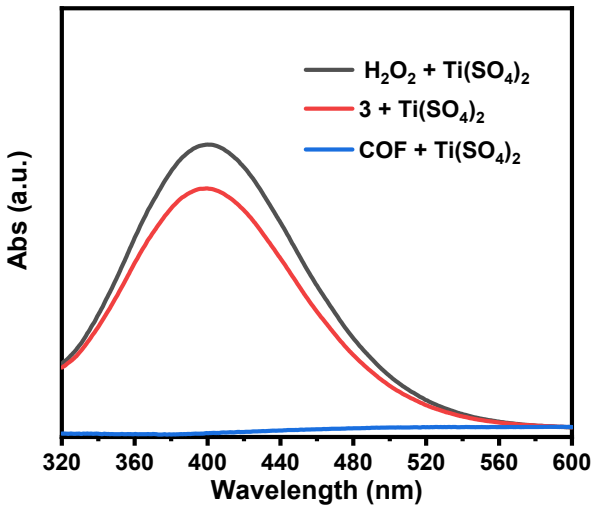

Fig. S7 UV-vis absorption spectra of titanium peroxide complexes in the presence of **3**, COF or H_2O_2.

5. Determination of glutathione consumption

A PBS buffer solution containing GSH (1 mM), **3** (200 μg/mL), and pH 6.5 was configured, and the UV-Vis absorption was measured after the supernatant was taken at 0, 5, 10, 15, and 20 min and reacted with 1 mM DTNB (pH = 7.8) for 5 min.

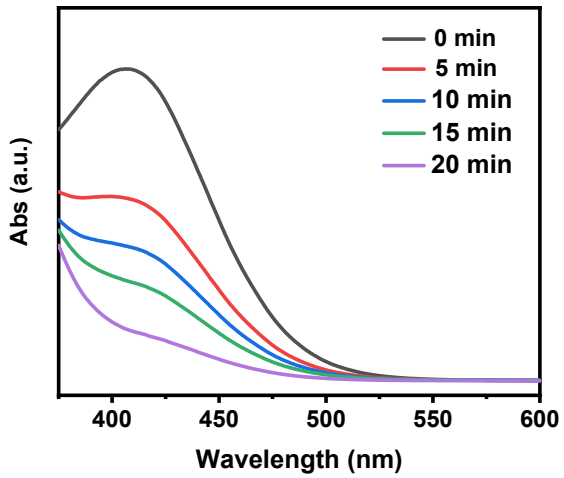

Fig. S8 The depletion of GSH.

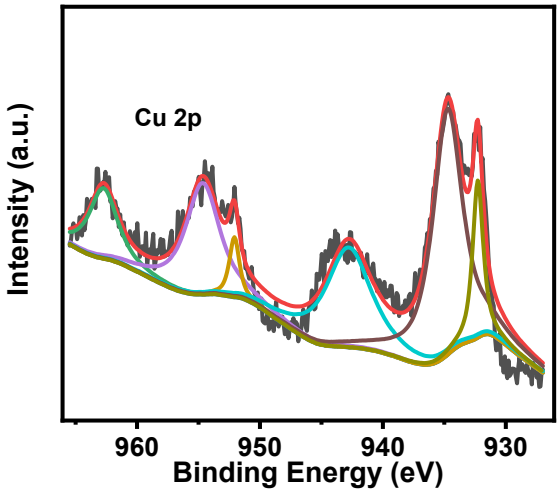

Fig. S9 High-resolution X-ray photoelectron spectroscopy data of **3** in the Cu 2p region.

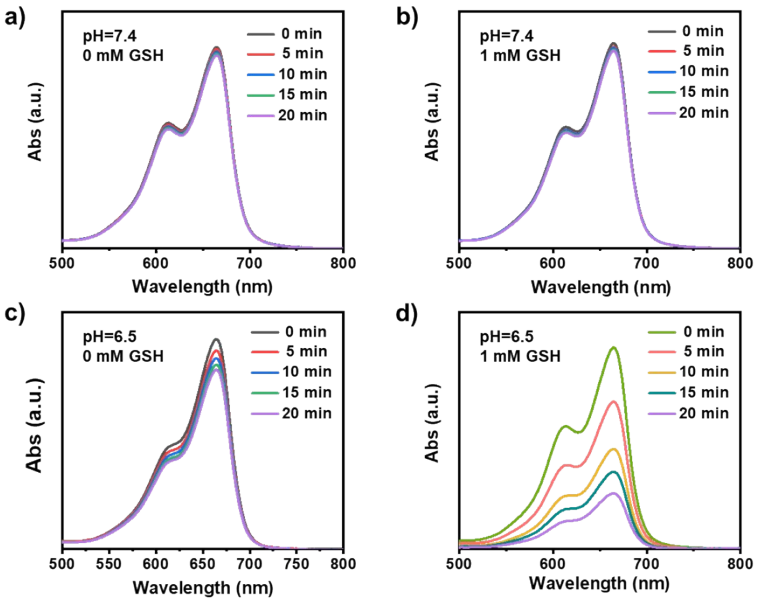

Fig. S10 UV-vis absorption spectra of methylene blue under different conditions.

6. Determination of reactive oxygen species

PBS buffer solutions containing GSH (1 mM), MB (100 μM), and **3** (200 μg/mL) at pH 6.5 and 7.4 were configured, and the supernatants were taken to measure the UV-visible absorption at 0, 5, 10, 15, and 20 min.

In the EPR experiment for trapping hydroxyl radicals, a dispersion of 1 mg/mL CuO_2@COF-SNO (1 mM GSH, pH = 6.5, 95 μL) and 5 μL 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were reacted for 5 min and then the mixture was characterized.

7. Determination of NO

Establishment of the standard curve: Remove Griess Reagent I and II and return to room temperature. Dilute the standards (1-100 μM) with the solution used for the samples to be tested. Add the standards to the 96-well plate at 50 μL/well. Griess Reagent I and Griess Reagent II were added to each well at 50 μL/well, and a standard curve of concentration versus absorbance was established by the change in UV-vis absorption spectrum at 540 nm.

3 was dispersed in buffer solutions of different pH (1 mg/mL), and the reaction was carried out at 37℃. The supernatant was taken at 0, 2, 4, 6, 8, 10, 12, and 14 h, and the absorbance at 540 nm was measured by an enzyme meter after the addition of NO detection kit reagent.

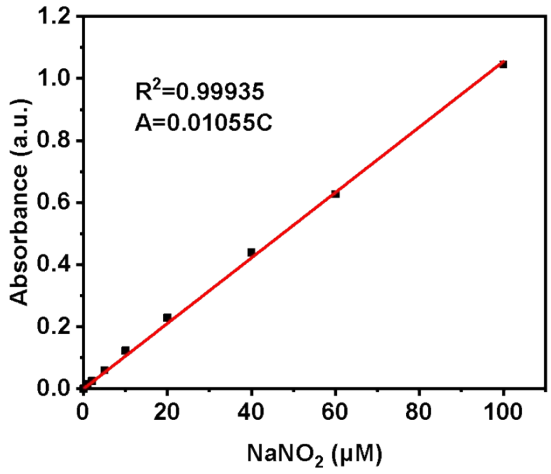

Fig. S11 Standard curve for NO.

8. Cell uptake

3-BDP was synthesized by covalently ligating BODIPY on **3**. Cells were inoculated in 35 mm dishes and cultured in DMEM medium supplemented with FBS (10%), Normocin (50 μg/mL), penicillin (100 μg/mL), and streptomycin (100 μg/mL). The cells were incubated in a CO_2 thermostat incubator with 5 vol% CO_2, 37 ℃, and saturated water vapor for 12 h. The cells were then incubated with a DPBS dispersion of **3**-BDP (100 μg/mL, 1 mL) for 4 h. The cells were washed twice with PBS and imaged using a laser confocal microscope, with the green channel being excited by a light of 488 nm, and collected at 540 ± 20 nm .

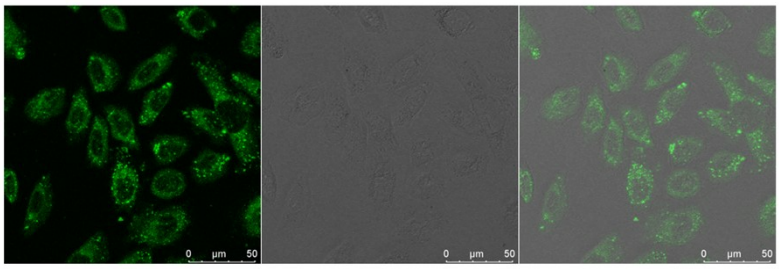

Fig. S12 Cell uptake in MCF-7 cells.

9. Detection of intracellular H_2O_2 content

MCF-7 cells were incubated with PBS, **1**, **2**, **3** (100 μg/mL) in a CO_2 incubator for 4 h. After digestion, the cells were washed twice with DPBS, centrifuged and collected into centrifuge tubes, and then homogenized sufficiently after the addition of hydrogen peroxide assay lysates in order to break up and lysed the cells, and then centrifuged to remove the supernatant and detected the absorbance at 560 nm using an enzyme marker. absorbance at 560 nm.

10. Detection of intracellular NO

Intracellular NO levels were determined by DAF-FM DA. MCF-7 cells were inoculated in tetrads and incubated at 37 ℃ for 12 h. DAF-FM DA (200 μL/well, 5 μM) was incubated for 20 min, and 500 mL of the sample (100 μg mL^{-1}) was added and incubated for 0.5, 1, and 2 h, imaging was performed using laser scanning confocal microscopy with the green channel excited by 495 nm and collected at 515 ± 20 nm.

11. Intracellular reactive oxygen species detection

Intracellular reactive oxygen species levels were determined by DCFH-DA. MCF-7 cells were inoculated in tetrads and incubated at 37 ℃ for 12 h. After incubation, the cells were divided into four groups and incubated with PBS, **1**, **2**, and **3** (50 μg mL^{-1}) for 3 h. The cells were then incubated with DCFH-HA (200 μL/well, 100 nM) for 20 min, and were imaged using laser scanning confocal focusing microscopy imaging, with the green channel excited by 488 nm and collected at 525 ± 20 nm.

12. CCK-8 cell viability assay

Cells were inoculated into 96-well plates and incubated in a CO_2 thermostat incubator with 5 vol% CO_2, 37 ℃, and saturated water vapor for 12 h. Cells were co-incubated with different concentrations of nanomaterials separately for 4 h. Cells were washed twice with PBS and reintroduced to the medium and incubated for 24 h. Finally, CCK-8 (10 μL, 5

mg/mL) was added to each well and incubated in the incubator for 2 h. The absorbance at 450 nm was recorded using an enzyme marker to record the absorbance at 450 nm.

13. Calcein-AM/PI double staining assay

MCF-7 cells were inoculated into 10 cm dishes and cultured overnight in a CO_2 thermostat incubator at 5 vol% CO_2, 37 ℃, saturated with water vapor, the cells were divided into 4 groups, and after removing the medium, the cells were incubated with **1**, **2**, **CuO_2@COF**, and **3** (120 μg/mL) for 3 h. After removing the material, the cells were continued to be Incubate for 24 h, aspirate the medium for use, scrape off the adherent cells with a scraper, then centrifuge with the medium, wash again with PBS, add 200 μL of PBS to each group for dispersion, then use AM/PI staining for 15 min, centrifuge and wash again with PBS, add 200 μL of PBS to each group, and take fluorescence imaging using a laser confocal microscope.

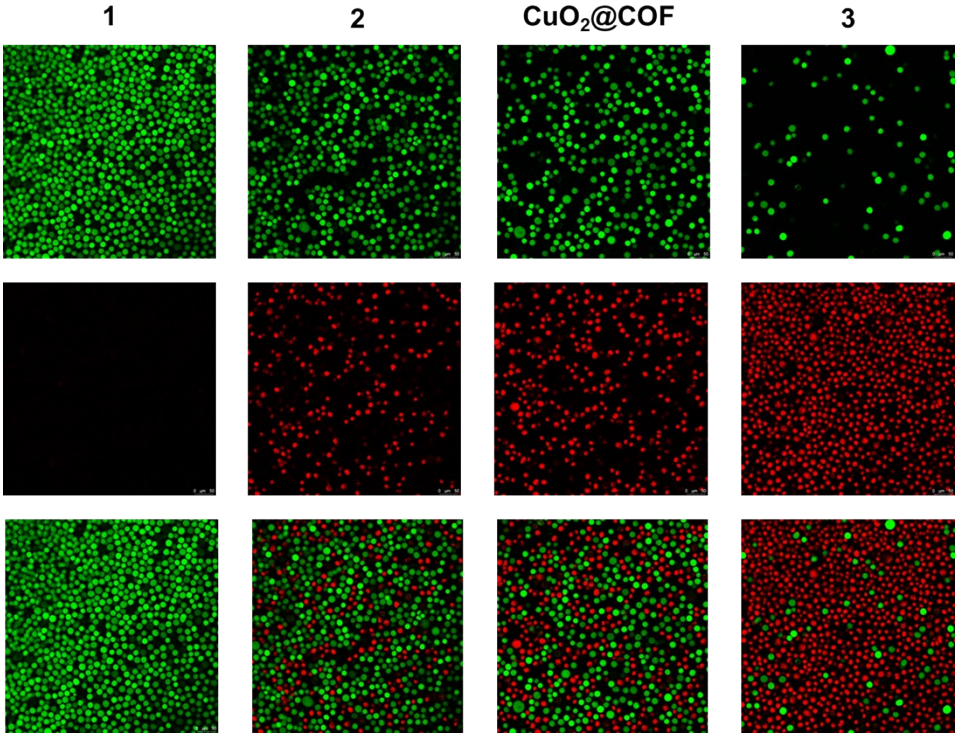

Fig. S13 Live/dead assays for different formulations.

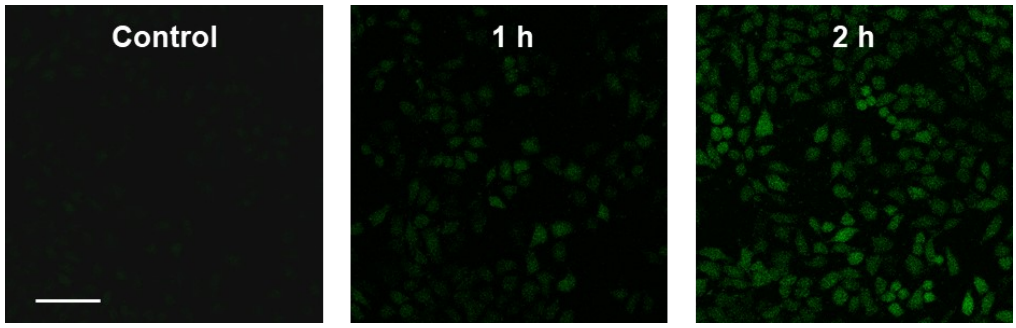

Fig. S14 NO release in MCF-7 cells.

14. Hemolysis analysis

First, fresh nude mouse blood samples (2 mL) were added to PBS solution (4 mL), and red blood cells (RBC) were separated by centrifugation at 3000 rpm for 10 minutes. After washing 5 times with PBS, dilute purified red blood cells to 20 mL with PBS. For hemolysis assay, 0.2 mL of diluted RBCs suspension was added to 1.0 mL of PBS as a negative control and 1.0 mL of deionized water as a positive control. And 1.0 mL **3** suspension at a concentration range of 30 to 150 μg/mL. All mixtures were then allowed to stand at 37 ℃ for 5 h and then centrifuged at 13000 rpm for 10 minutes. Due to the small size of **3**, it was difficult to separate **3** completely even by centrifugation at 13000 rmp for 10 minutes. Therefore, we chose the supernatant of the corresponding concentration as a control. The absorbance of 541 nm supernatant was measured by synergy SpectraMax i3x multi-mode microplate reader. The hemolytic percentage of red blood cells was calculated by the following formula: Hemolysis Rate = [(Dt – Dcc)/(Dpc – Dnc)] ×100%.

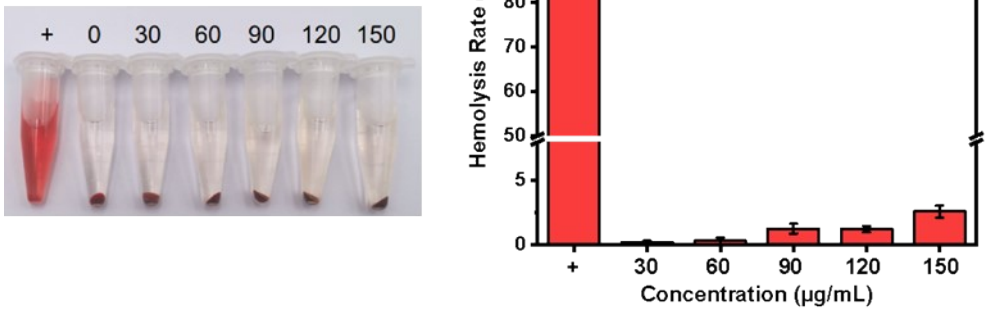

Fig. S15 Hemolysis rate of erythrocytes by different concentrations of **3**.

15. In vivo antitumor therapy

MCF-7 cancer cells (10^6 cells) suspended in DPBS (100 μL) were subcutaneously injected into the flanks of each nude mice to establish the MCF-7 xenograft model. The length (L) and width (W) of the tumor were determined by digital calipers. The tumor volume (V) was calculated by the formula $V = 1/2 \times L \times W^2$. When the tumor size reached ~100 mm^3, the nude mice bearing MCF-7 tumors (n = 25) were randomly distributed into six groups, i.e., (1) PBS, (2) **1**, (3) **2**, (4) **CuO_2@COF** and (5) **3** groups. The nanomedicine was injected intratumourally at a dose of 1 mg/mL on days 0, 3 and 5, respectively. Mice were continued to be fed for 12 days. Tumor volume and nude mouse weight were measured every 2 days.

16. Histological analysis

After 12 days of the therapy, all mice were euthanized. Choice representative tumor bearing mice from different treatment groups, the tumor tissue and major organs (heart, liver, spleen, lung and kidney) were collected for H&E histological analysis.

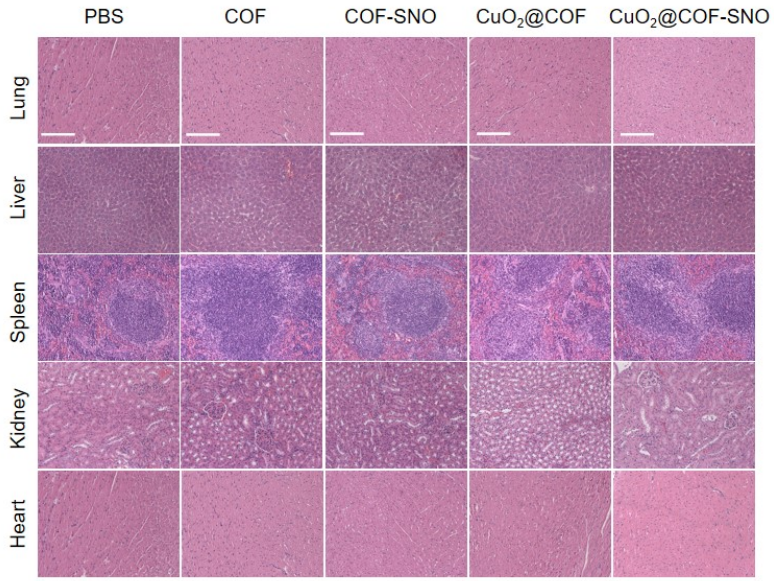

Fig. S16 H&E stained images of the major organs, including heart, liver, spleen, lung, and kidney. Scale bar, 200 μm.

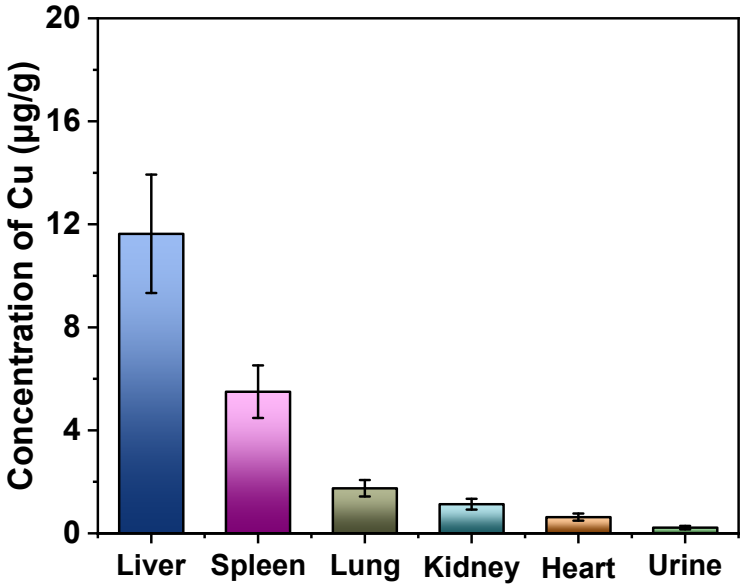

Fig. S17 The in vivo and urine biodistribution of Cu after 72 hours of intratumoural injection of **3** (n=3).