Supporting Information

for

The construction of hierarchical assemblies with in situ generation of chemotherapy drugs to enhance the efficacy of chemodynamic therapy for multi-modal anti-tumor treatments

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Experimental Section

Materials and reagents

Calcium chloride ($CaCl_2$), copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), sodium chloride (NaCl), ascorbic acid, glucose, and disulfiram (DSF) were purchased from Macleans Biochemical Co., Ltd (shanghai, China). Glucose oxidase, hydrogen peroxide (H_2O_2, 30%) and glutathione detection kit were purchased from Sigma Aldrich. Phosphate buffered saline (PBS) and sugar-free dulbecco's modified eagle medium (DMEM) were purchased from Wisent Inc. (Nanjing, China). Methylene blue (MB), 2',7'-dichlorofluorescein diacetate (DCFH-DA), H_2O_2 determination kit, cell counting kit-8 (CCK-8) were obtained from Shanghai Health Industrial Biology Company. Calcein-AM and propidium iodide (PI) were purchased from Beyotime Biotechnology Co (Haimen, China). All chemicals in this work are of analytical grade and used as received.

Instruments

The morphologies were observed by SEM (scanning electron microscope, Nano SEM-450, American FEI company) and TEM (transmission electron microscope, Tecnai G2 spirit bio, American FEI company). The element mapping analysis was recorded on a scanning transmission electron microscope (JEM-3200FS, JEOL, Japan). The X-ray diffraction (XRD) pattern was obtained on D8 Advance diffractometer (Bruck, Germany). X-ray photoelectron spectroscopy (XPS) analysis was performed on ESCALAB 250Xi (Thermo Fisher, USA). Fourier transform infrared (FTIR) spectra were recorded on Bruker Vector 22 spectrometer. pH is detected by pH meter (PHS-3C, Shanghai Lei Magnetic Instrument Factory).

The thermogravimetric analysis (TGA) was performed on the STA 449C synchronous thermal analyzer (Netzsch, Germany). An inductively coupled plasma instrument ICP-OES (iCAP RQ, Thermo Fisher, USA) was used to measure the content of Ca and Cu elements.

Synthesis of GOx-CuCaP

10 mg of GOx and 50 μL of $CuCl_2 \cdot 2H_2O$ (0.1 mol/L) were dissolved in 10 mL of sugar-free dulbecco's modified eagle medium (DMEM) and incubated for 24 h at room temperature. Then, 50 μL $CaCl_2$ (1 mol/L) was added to incubate for another 24 h. After

that, the obtained product was separated by centrifugation (15000 rpm, 10 min), washed twice with deionized water, and freeze-dried for later use.

Synthesis of GOx-CuCaP-DSF

2 mL DSF solution (40 mg/mL) in chloroform was added dropwise to the aqueous solution of GOx-CuCaP (20 mg), the resulting solution was stirred at room temperature for 12 h. DSF-loaded GOx-CuCaP (GOx-CuCaP-DSF) was collected by centrifugation (15000 rpm, 5 min) and washed three times with ultra-pure water for further use.

Catalytic ability evaluation of GOx-CuCaP-DSF

GOx-CuCaP-DSF (10 μg/mL) was incubated with glucose solutions of different concentrations (0-1000 μg/mL) for 4 h at room temperature. Then, the mixed solution of GOx-CuCaP-DSF and glucose was collected, and the H_2O_2 concentration and pH value of the mixed solution were measured be using a H_2O_2 assay kit and a pH meter, respectively.

The Ca^{2+} and Cu^{2+} release from GOx-CuCaP-DSF

The GOx-CuCaP-DSF suspension (10 $mg \cdot mL^{-1}$, 1 mL) was subjected to dialysis (MWCO = 3600) with PBS solutions of pH 5.5 or 7.5 for different duration time. To simulate the in vivo medium, the test solution was kept in a 37°C water bath with mild magnetic agitation (200 RPM). The Ca^{2+} and Cu^{2+} concentrations were determined by ICP-OES.

To study the release profile of DSF in GOx-CuCaP-DSF nanoparticles under different pH conditions, the nanoparticles were dispersed in solutions with varying pH levels (pH 5.5, 6.5, and 7.4). At specific time intervals, the solution containing the dissolved nanoparticles was subjected to centrifugation. After centrifugation, the supernatant was collected, and the amount of released DSF was quantified by referring to a standard curve. Each group was repeated three times.

In vitro detection of •OH production

Methylene blue (MB) is used as an indicator to evaluate the •OH produced by the Fenton-like reaction of copper ions in nanoparticle GOx-CuCaP-DSF in the presence of H_2O_2. The 10 mL aqueous solution with GOx-CuCaP-DSF (100 μg/mL), H_2O_2 (10 mM) and MB (10 μg/mL) were prepared and shaken at room temperature in dark. At

different time points, the mixture was centrifuged and the absorption of MB remaining in the supernatant at 664 nm was measured.

The •OH generated by Fenton-like reaction between Cu^{2+} and the H_2O_2 coming from GOx catalysis was detected by disodium terephthalate (TPA). 0.5 mM TPA was dissolved in 2 mM NaOH solution, then 100 μg/mL GOx-CuCaP-DSF and 800 μg/mL glucose were added. After 5 h, the fluorescence intensity change of the mixed solution at 422 nm was recorded (λ_{ex} = 312 nm).

The bicinchoninic acid (BCA) method[S1] was employed to measure the GOx content for determining the GOx loading and encapsulation efficiencies. ICP-OES was utilized to quantify the DSF content for assessing the DSF loading and encapsulation efficiencies. In the experimental procedure, a standard working curve was initially established using different concentrations of GOx or DSF. Following this, 10 mg of GOx and 50 μL of $CuCl_2 \cdot 2H_2O$ (0.1 mol/L) were dissolved in 10 mL of sugar-free DMEM, the mixture was incubated for 24 h at room temperature. After 24 h, 50 μL of $CaCl_2$ (1 mol/L) and DSF (5 mg) were added to the mixture and further incubated for another 24 h. Afterward, the solution underwent centrifugation at room temperature to separate the supernatant. The collected supernatant was then mixed with the BCA reagent, and the absorption of the resulting solution was measured at a wavelength of 562 nm. By referring to the previously established standard curve, the concentration of GOx in the solution could be determined. The loading efficiency of GOx was calculated by the following equation: Loading efficiency = [M (GOx added for preparation of GOx-CuCaP-DSF)- M (GOx in supernatant)]/M (GOx-CuCaP-DSF) × 100%; The encapsulation efficiency of GOx was calculated by the following equation: Encapsulation efficiency = [M (GOx added for preparation of GOx-CuCaP-DSF)- M (GOx in supernatant)]/ M (GOx added for preparation of GOx-CuCaP-DSF) × 100%.

For preparation of the GOx-CuCaP-DSF nanoparticles, 5 mg of DSF was added to a solution containing GOx-CuCaP nanoparticles at a concentration of 100 mg/mL with a total volume of 20 mL. The mixture was allowed to react for 6 h. After the reaction, the GOx-CuCaP-DSF nanoparticles were obtained by subjecting the mixture to centrifugation and subsequent drying. Any DSF that did not participate in the formation of GOx-CuCaP-DSF nanoparticles remained in the supernatant. The amount of DSF present in the supernatant was determined using the previously established standard

curve for DSF. Loading efficiency = [M (DSF added for preparation of GOx-CuCaP-DSF) – M (DSF in supernatant)]/ M (GOx-CuCaP-DSF) × 100%; Encapsulation efficiency = [M (DSF added for preparation of GOx-CuCaP-DSF) – M (DSF in supernatant)]/ M (DSF added for preparation of GOx-CuCaP-DSF) × 100%.

In vitro degradation of GOx-CuCaP-DSF and the ion release

To investigate the in vitro biodegradation behavior of GOx-CuCaP-DSF under different pH conditions, GOx-CuCaP-DSF (1 mg/mL) was dispersed in solutions with pH 7.5 and 5.5. The solutions were stirred (200 RPM) at 37°C with different deration time (5, 10 and 15 h). The morphology changes of GOx-CuCaP-DSF in these solution were observed using TEM. The responding amounts of metal ions released from GOx-CuCaP-DSF were measured using a coupled plasma instrument.

The valence distribution of Cu and •OH generation from GOx-CuCaP-DSF mediated Fenton-like Reaction

GOx-CuCaP-DSF (200 μg/mL) was incubated in 10 mL PBS solution (pH 5.5), after 6 h the resulting precipitates were obtained by centrifugation, vacuum-dried at 60°C overnight. The valence distribution of Cu after DSF loading was determined by XPS analysis. 2 mM H_2O_2 solution was added to 1 mL of PBS solution containing GOx-CuCaP-DSF for ESR analysis, in which the capture agent was 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO). GOx-CuCaP (200 μg/mL) without DSF served as a control group for comparison.

Detecting changes of intracellular glutathione levels

Glutathione depletion was detected by the Ellman method, and all experiments were performed in the dark. 1 mL of bicarbonate buffer solution (50 mM, pH= 8.7) containing GOx-CuCaP-DSF nanoparticles in a concentration of 10, 20, 40, 80 and 160 μg/mL were mixed with 20 μL of 1 mM GSH bicarbonate buffer solution (50 mM, pH = 8.7) in a 1.5 mL centrifuge tube. All centrifuge tubes were incubated at 150 rpm for 4 hours at room temperature with shaking. Positive and negative controls were 1 mM H_2O_2 + GSH and GSH solutions, respectively. Afterward, 960 μL of 0.5 M Tris-HCl (pH = 8.8) solution and 20 μL of bicarbonate buffer solution with 100 mM 5,5'-Dithiobis-(2-nitrobenzoic acid (DTNB, 50 mM, pH= 8.7) were added to the mixture and passed through at 12000 rpm for 10 min. The absorbance of the supernatant at 410

nm was measured.

To evaluate the changes in intracellular GSH concentration in HepG2 cells treated with different concentrations of nanoparticles, a probe called mono-chloro diamine (mBCl) was employed.[S2] Initially, mBCl is non-fluorescent, but it becomes fluorescent when it reacts with thiol groups, forming a blue fluorescent GSH-mono-chloro di-cyclic adduct. To conduct the experiment, HepG2 cells were seeded on specialized culture dishes for confocal laser scanning microscopy (CLSM) with a diameter of 15 mm. The cells were then treated with GOx-CuCaP-DSF nanoparticles at various concentrations ranging from 0 to 150 μg/mL for a duration of 4 h. After removing the culture medium, the cells were incubated with the mBCl probe at a temperature of 37°C for 30 min. Subsequently, the staining solution was removed, and the cells were washed three times with phosphate-buffered saline (PBS) before being observed using CLSM. The GSH depletion at the cellular level was calculated as follows:

$$Level\ of\ GSH\ depletion = \frac{I_0 - I_n}{I_0} \times 100\ \%$$

Where I_0 is the intensity of intracellular fluorescence without GOx-CuCaP-DSF nanoparticles, and I_n is the intensity of intracellular fluorescence after the addition of the GOx-CuCaP-DSF nanoparticles at a corresponding concentration.

Protein expression assay

HepG2 cells were cultured in 6-well plates for 24 h. Then, GOx-CuCaP and GOx-CuCaP-DSF (5 μg/mL) were administered to cells and incubated for 4 h, respectively. Cells were harvested by trypsinization and centrifugation, and lysed by adding a mixture of NP-40 lysis buffer containing protease inhibitors. The supernatant was collected after centrifugation at 12 000 rpm (P-gp protein was located on the cell membrane, and the pellet was collected by centrifugation after losing the cells), and then the protein was analyzed by western blotting. Primary antibodies used were as follows: GPX4 polyclonal antibody (67763-1-I; Proteintech), P-gp polyclonal antibody (22336-1-AP; Proteintech), DLAT polyclonal antibody (13426-1-AP; Proteintech), LIAS polyclonal antibody (11577-1-AP; Proteintech), NPLOC4 polyclonal antibody (11638-1-AP; Proteintech). The internal parameters were GAPDH and β-actin. Then the corresponding secondary antibodies were added. Immunoblots were imaged by the BIO-RAD imaging system (USA) and quantified with ImageJ software.

Determination of ROS generation in vitro

HepG2 cells were seeded in 35 mm Petri dishes and cultured for 24 h to make the cells adherent. Cells were then cultured for 4 h under the following four conditions (1 mL DMEM): PBS, $CuCl_2$ (20 μg/mL), GOx-CuCaP (20 μg/mL), GOx-CuCaP-DSF (20 μg/mL). Then DCFH-DA (10 μM) was added and incubated for 30 min. Finally, the treated cells were gently washed 3 times with PBS, and intracellular •OH was detected by CLSM with an excitation wavelength of 470 nm.

In vitro cytotoxicity assay

HepG2 cells in the logarithmic growth phase were digested with 0.25% trypsin, and seeded in a 96-well plate at a cell density of 1×10^4 cells/mL and 100 μL/well. After 24 h of incubation, the medium from the 96-well plate was discarded. Subsequently, different concentrations of GOx-CuCaP or GOx-CuCaP-DSF were dispersed into fresh DMEM containing different concentrations of glucose (0-1000 μg/mL), and then seeded into 96-well plates and co-cultured for 12 h. Cell viability was quantified by standard CCK-8 assay and calculated based on the following equation: cell viability = (OD490 nm of sample/OD490 nm control) × 100%. The cell viability in the control group was set to 100%.

Cell imaging with various fluorescent probes

HepG2 cells were seeded on CLSM special culture dish (φ=15 mm). After the cells were treated with different controls including PBS, $CuCl_2$, GOx-$CuCl_2$, GOx-CuCaP and GOx-CuCaP-DSF, the medium was removed and the discs were rinsed, then incubated with different probes for 30 min at 37°C. These probes including Fluo-4AM, JC-10, Mito-tracker and Hoechst 33258, were used for the detection of intracellular calcium concentration, mitochondrial membrane potential and mitochondrial number, respectively. The staining solutions were removed, and the cells were rinsed with PBS for three times and subsequently visualized by CLSM.

Apoptosis detection analysis

For quantitative analysis of apoptosis-mediated cell death, HepG2 cells were seeded in 6-well plates (1×10^5 cells per well) and cultured overnight. Subsequently, the previous medium was replaced with fresh medium containing copper chloride, GOx-CuCaP or GOx-CuCaP-DSF. The concentration of GOx-CuCaP or GOx-CuCaP-DSF

was 100 μg·mL^{-1}. The content of copper ions in different groups was comparable. After 4 h of incubation, all treated cells were trypsinized, washed twice with PBS, processed by Annexin V-FITC/PI Apoptosis Detection Kit, and apoptosis was quantified using flow cytometry.

Live/Dead Cell Staining Assay

HepG2 cells were incubated in 6-well plates with 1.0× 10^5 cells per well and incubated at 37°C for 12 h. The medium was then discarded and the cells were washed three times with PBS. Fresh medium containing copper chloride, GOx-CuCaP or GOx-CuCaP-DSF was added to each well separately. The concentration of GOx-CuCaP or GOx-CuCaP-DSF was 100 μg·mL^{-1}. The content of copper ions in different groups was comparable. After an additional 4 h of co-culture, cells were stained with calcein-AM (2 μM) and PI (4 μM) and then imaged using CLSM.

In vivo anti-tumor experiments

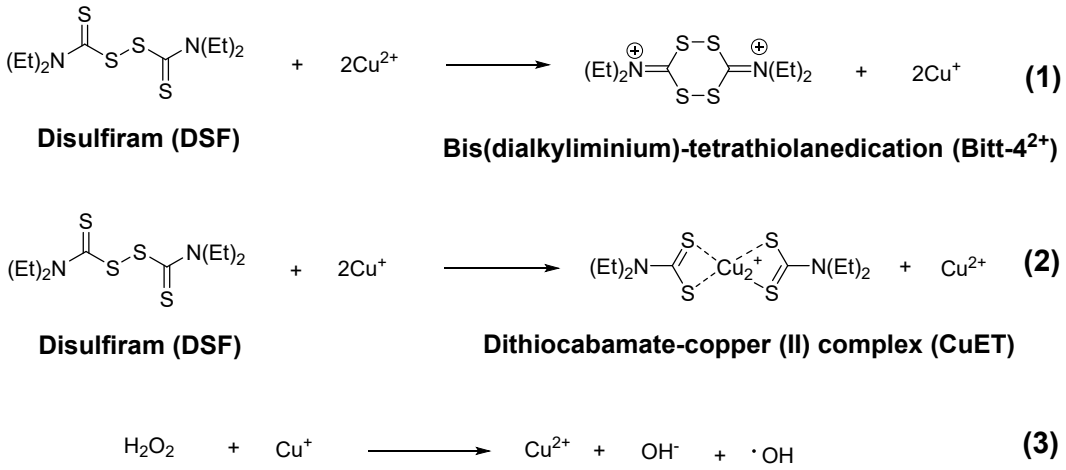
A HepG2 tumor model was established by injecting 0.2 mL of HepG2 cells (approximately 1×10^7/mL) into the right axilla of healthy female nude mice. When the tumor volume reached approximately 70 mm^3, the tumor-bearing mice were divided into four groups consisting of five mice each. These groups received different intravenous injections: (1) physiological saline (control group); (2) $CuCl_2$; (3) GOx-CuCaP; and (4) GOx-CuCaP-DSF. The injections were administered every two days for a total of 16 days. The mice received 100 μL of the respective substances based on the following dosages: Physiological saline: 100 μL, $CuCl_2$: 1.59 mg/kg, GOx-CuCaP: 16.35 mg/kg, GOx-CuCaP-DSF: 20 mg/kg. Before each treatment, the tumor volume and body weight of the mice were recorded. The tumor volume was calculated using the formula V = (length×width^2) / 2.

The animal experiments in this study were conducted in accordance with the guidelines approved by the Model Animal Research Center (MARC) of Nanjing University of Chinese Medicine. The experiments were performed under the Laboratory Animal Ethics Approval Certificate No. 202308A049. To ensure the welfare of the animals, certain criteria were followed during the experiment. The maximum diameter of the mouse tumors was limited to 20 mm, and the maximum tumor volume was restricted to 2000 cubic millimeters. These limits help to ensure that the tumor size does not excessively burden the animals. Additionally, according to the

Guidelines for Assessment of Humane Endpoints in Animal Experiments (RB/T 173-2018), the tumor weight should not exceed 10% of the body weight of the mice. After the 16-day treatment period, the mice were euthanized, and major organs were collected for further analysis. Histological analysis was performed on these organs 16 days after euthanasia, likely to study the effects of the treatments on the organs.

Statistical analysis

Data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) and LSD post hoc tests were performed using GraphPad Prism 8 software for multiple group comparisons. Statistical significance is indicated by an asterisk. (ns: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).



Scheme S1 Mechanisms of DSF/Cu^{2+} reactions including DSF reduction by Cu^{2+} (1), DSF-Cu^{+} chelation (2), and the generation of hydroxyl radicals via a Fenton-like reaction (3).

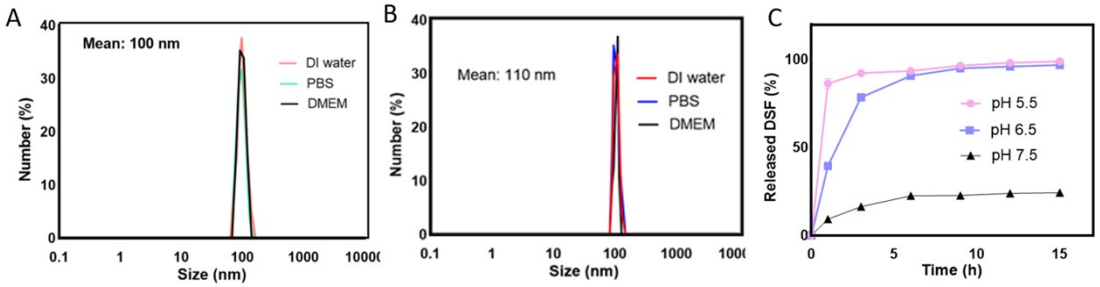


Figure S1. Hydrodynamic size distribution of GOx-CuCaP (A) and GOx-CuCaP-DSF (B) after keep in different media (DI water, PBS and DMEM) for 7 d. (C) Time-dependent DSF release from the GOx-CuCaP-DSF NPs dispersed in buffer solutions with different pH values.

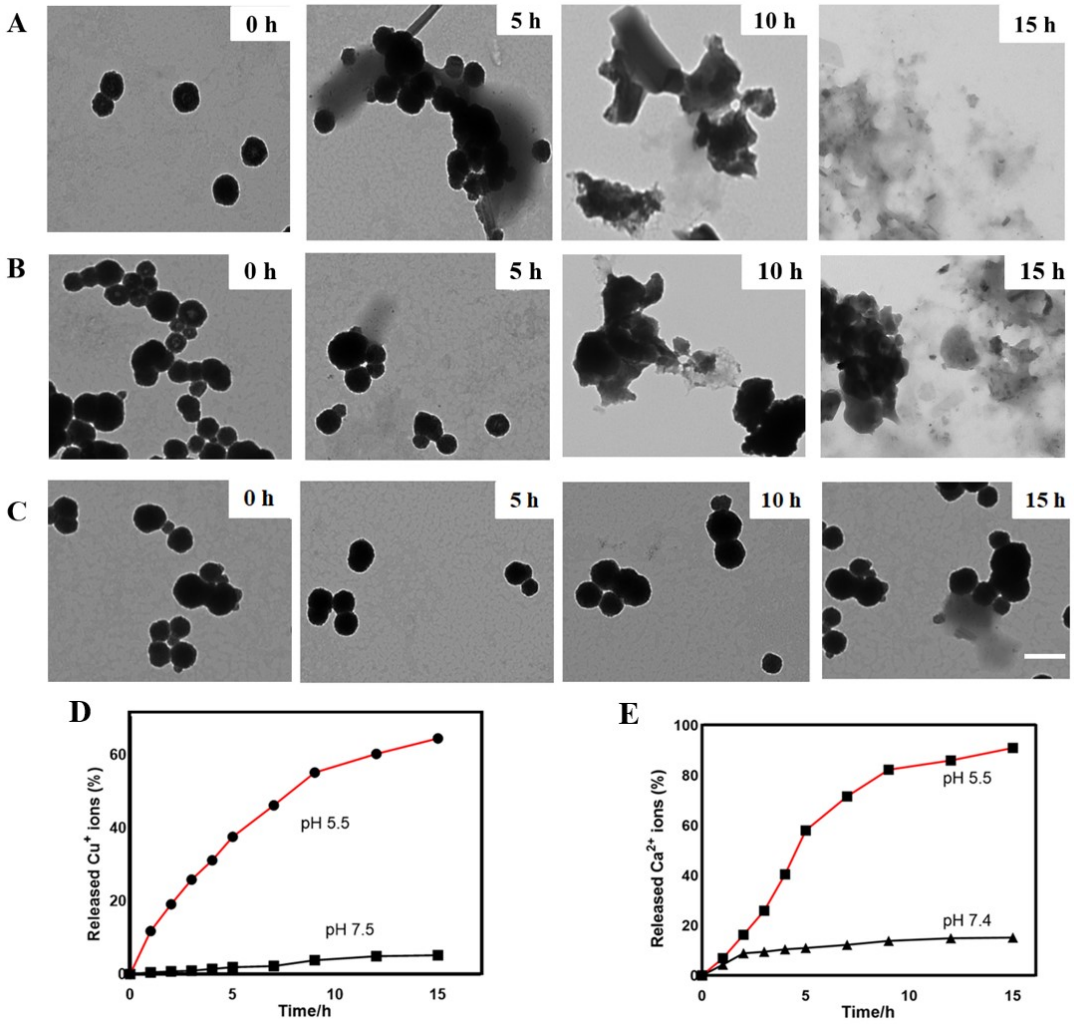


Figure S2. TEM images of GOx-CuCaP-DSF in pH 5.5 (A), pH 6.5 (B) and pH 7.5 (C) for different time points. Scale bar: 100 nm. (D) The percentage changes of Cu^{+} and (E) Ca^{2+} release from GOx-CuCaP-DSF in pH 5.5 and 7.5 solution with different time points.

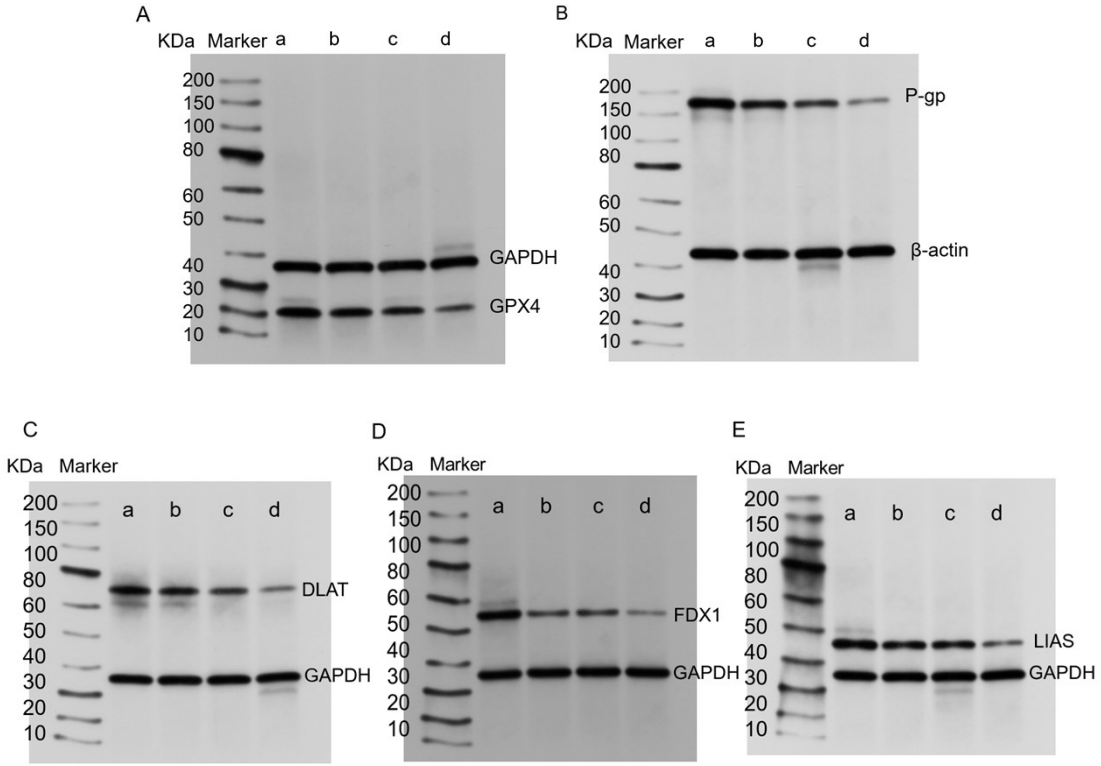


Figure S3. Original western blot images for GXP4 (A) and P-gp (B). here a) control, b) $CuCl_2$, c) GOx-CuCaP, and d) GOx-CuCaP-DSF. Original western blot analysis of DLAT (C), FDX1 (D), LIAS expression levels (E) in HepG2 cells after different treatments. Internal reference is GAPDH and the cells were treated with a) control, b) $CuCl_2$, c) GOx-CuCaP, and d) GOx-CuCaP-DSF.

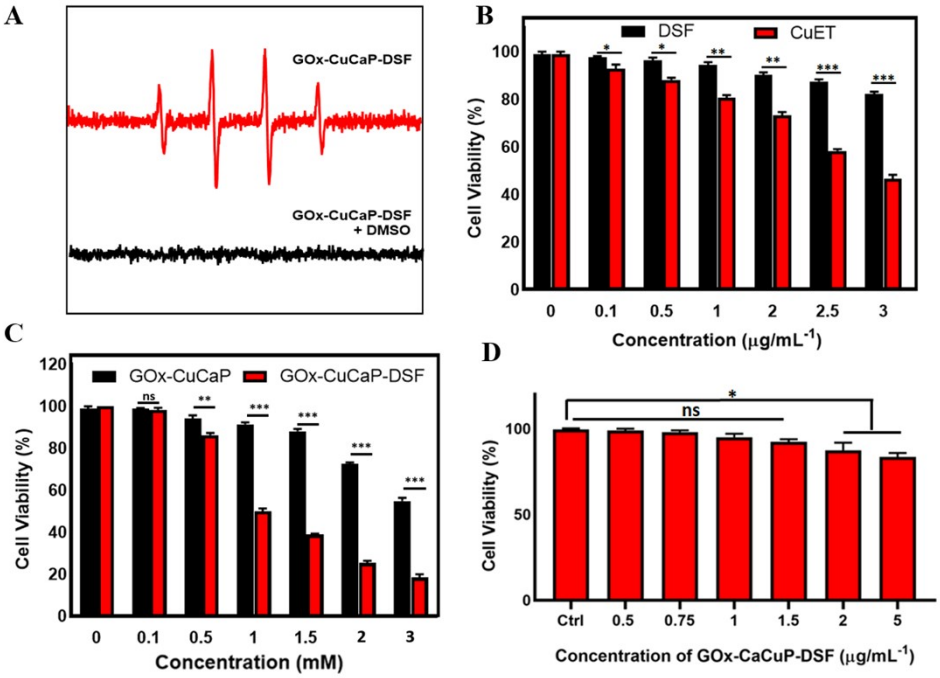


Figure S4. (A) ESR spectra of GOx-CuCaP-DSF in the absence and presence of DMSO after incubation with H_2O_2 at pH 5.5. [GOx-CuCaP-DSF] = 100 μg·mL^{-1}, [DMSO] = 100 μM, [H_2O_2] = 100 μM. (B) Viability of HepG2 cells incubated with different concentration of DSF and CuET for 24 h (mean ± SD, n = 3). (C) Viability of HepG2 cells incubated with different concentration of GOx-CuCaP-DSF and GOx-CuCaP for 24 h (mean ± SD, n = 3). (D) Viability of HL7702 cells incubated with different concentration of GOx-CuCaP-DSF for 24 h (mean ± SD, n = 3). (ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).

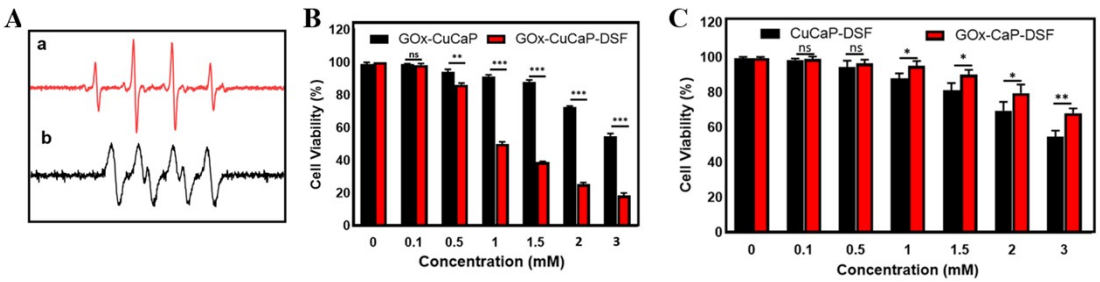


Figure S5. (A) ESR detection of nanoparticles. a: hydroxyl radical was detected in PBS solution. b: superoxide anion was detected in methanol solution. Viability of HepG2 cells incubated with different concentration of GOx-CuCaP-DSF and GOx-CuCaP (B), CuCaP-DSF and GOx-CaP-DSF (C) for 24 h (mean ± SD, n = 3). (ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).

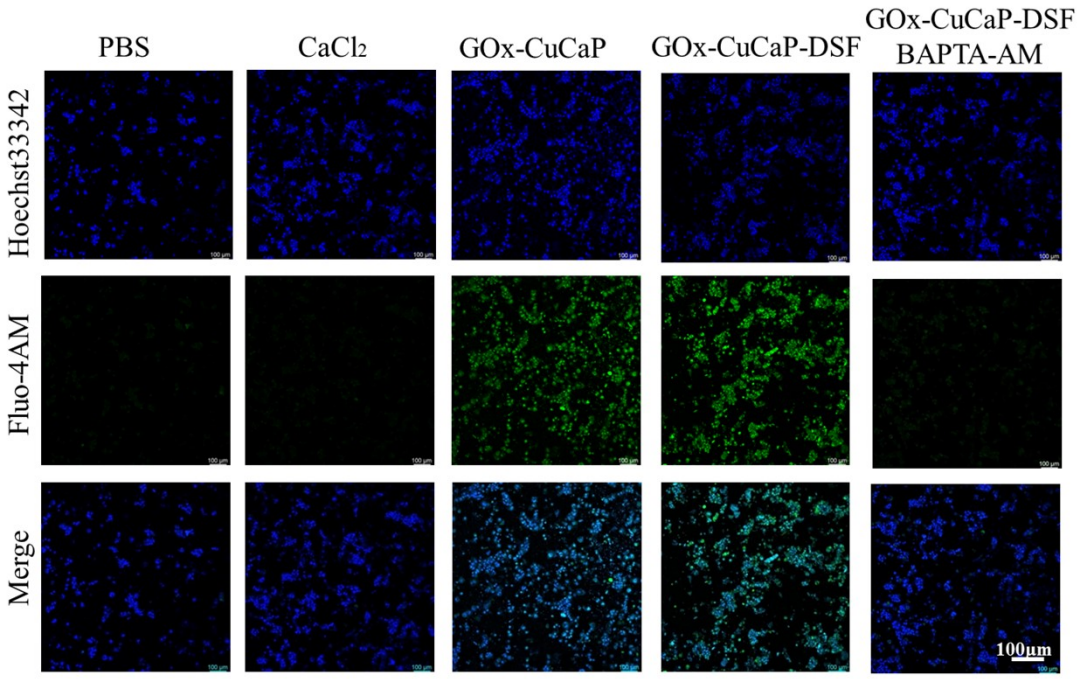


Figure S6. CLSM images of HepG2 cells treated with different substances including PBS, $CaCl_2$, GOx-CuCaP and GOx-CuCaP-DSF for 4 h, followed by staining with Hoechst 33342 (blue fluorescence) and Fluo-4AM (green fluorescence). Scale bar: 100 μm.

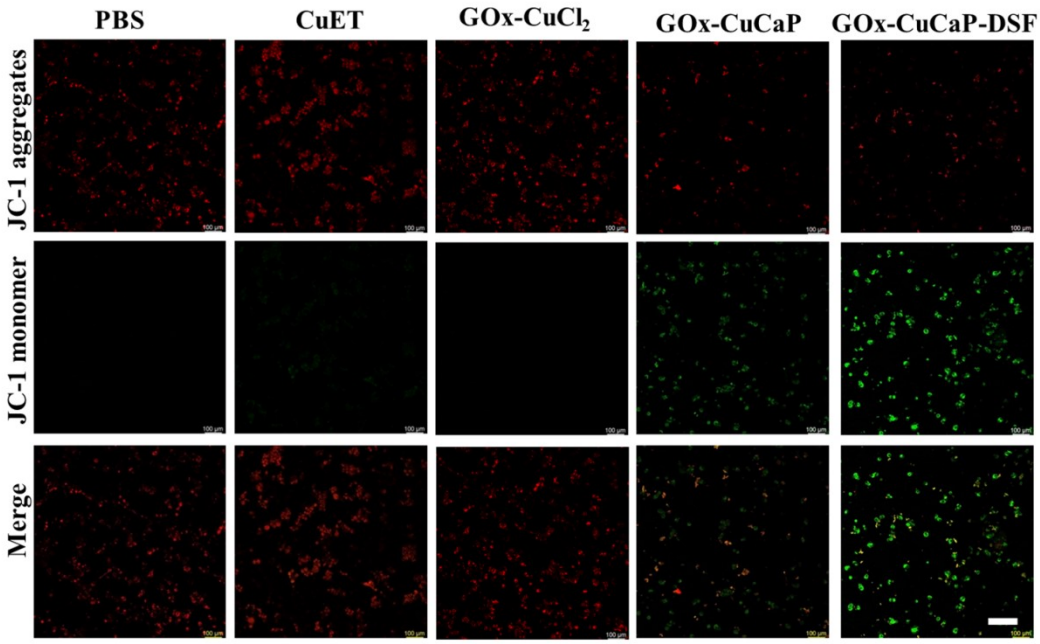


Figure S7. CLSM images of HepG2 cells treated with different substances including PBS, CuET, GOx-$CuCl_2$, GOx-CuCaP and GOx-CuCaP-DSF for 4 h, followed by staining with JC-1 aggregates (red fluorescence) and JC-1 monomer (green fluorescence). Scale bar: 100 μm.

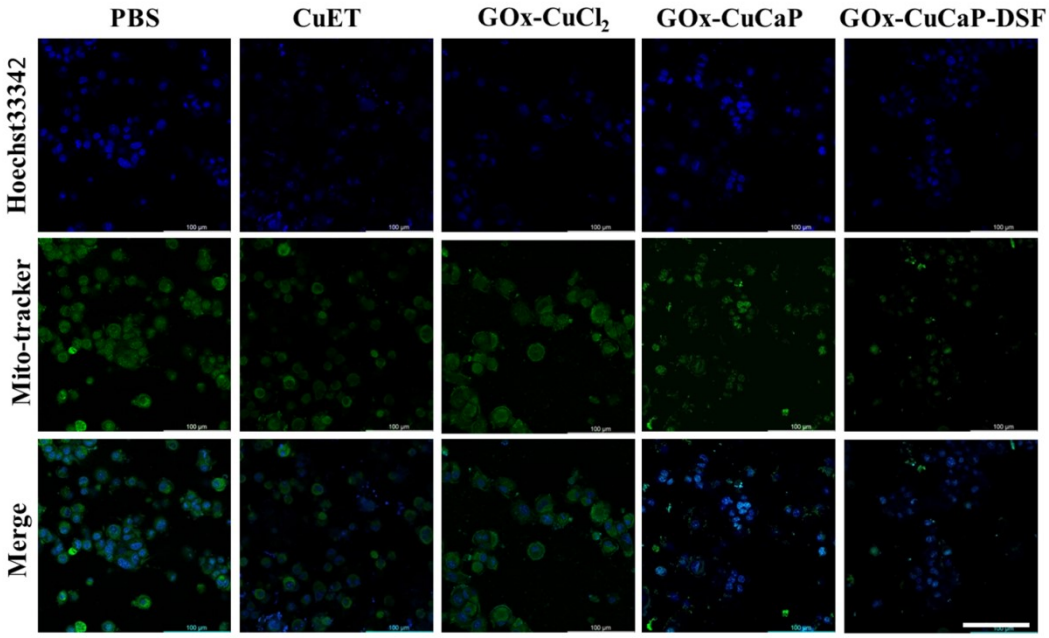


Figure S8. CLSM images of HepG2 cells treated with different substances including PBS, CuET, GOx-$CuCl_2$, GOx-CuCaP and GOx-CuCaP-DSF for 4 h, followed by staining with Hoechst 33342 (blue fluorescence) and Mito-tracker (green fluorescence). Scale bar: 100 μm.

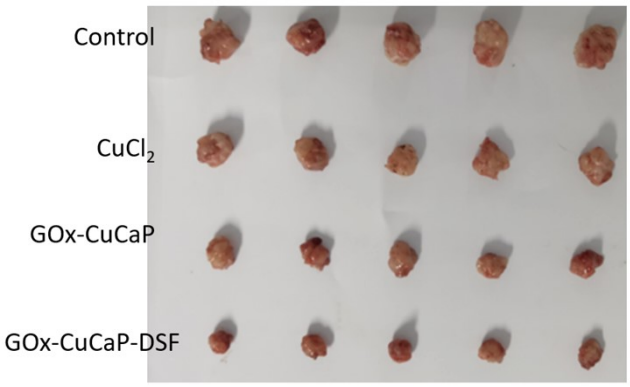


Figure S9. Digital photographs of the tumor dissection.

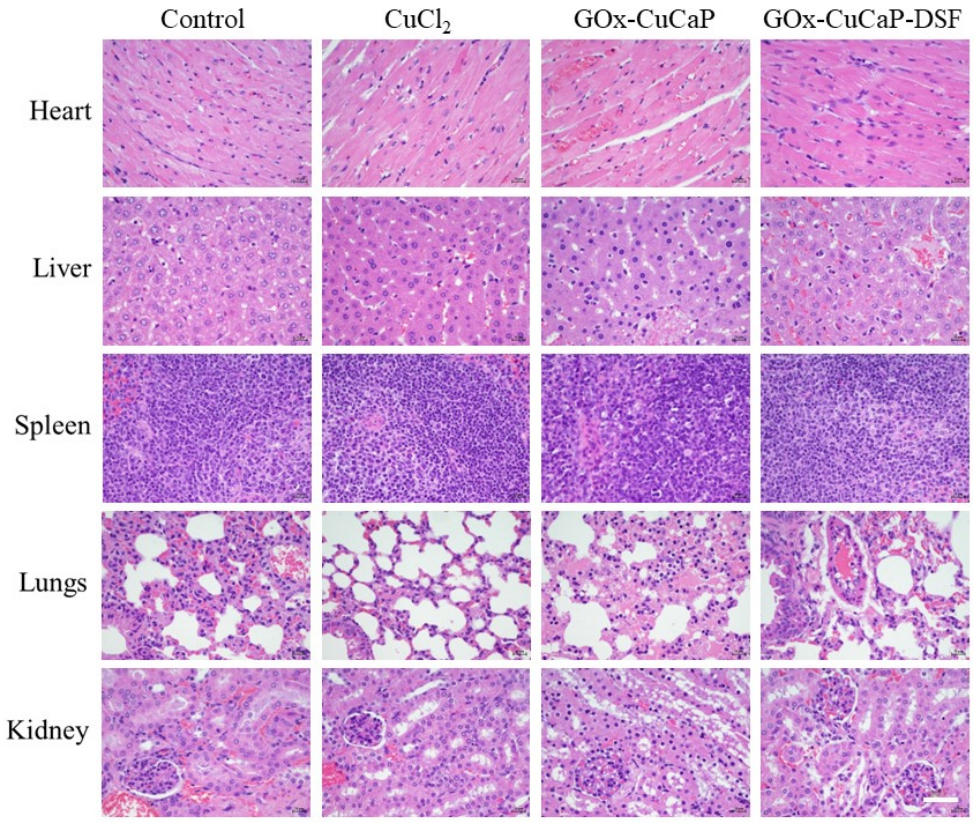


Figure S10. Representative H&E stained images of the major organs of mice in different groups after 16-day treatment. Scale bar: 50 μm.

Table S1. Element content of GOx-CuCaP and GOx-CuCaP-DSF.

	GOx-CuCaP	GOx-CuCaP-DSF
O 1s	34.57	36.88
C 1s	45.35	42.19
Ca 2p	6.11	7.29
N 1s	9.56	8.68
Cu 2p	1.24	0.68
S 2p	1.57	2.15
P 2s	1.6	2.6

Table S2. The component content of GOx-CuCaP and GOx-CuCaP-DSF detected by TGA.

	GOx-CuCaP (%)	GOx-CuCaP-DSF (%)
moisture	0.43	2.21
organic	13.11	24.66
inorganic	86.46	73.13

S1 J. Ming, T. B. Zhu, W. H. Yang, Y. R. Shi, D. D. Huang, J. C. Li, S. J. Xiang, J. J. Wang, X. L. Chen and N. F. Zheng, *ACS Appl. Mater. Inter.*, 2020, **12**, 51249-51262.
S2 V. R. Perera, G. L. Newton, J. M. Parnell, E. A. Komives and K. Pogliano, *Bba-Gen Subjects*, 2014, **1840**, 2851-2861.