Electronic Supplementary Material (ESI) for Inorganic Chemistry Frontiers.

Copper-based inorganic nanozyme enhances the electrical conductivity to synergistically induce the pyroptosis, ferroptosis, and apoptosis of tumor

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Materials and methods

Materials.

Copper chloride ($CuCl_2$), potassium oleate, 1,2-Tetradecanediol, oleylamine (OAM), 3,3',5,5'-Tetramethylbenzidine (TMB), methylene blue (MB) , MQAE (chloride ion probe), fluorescein isothiocyanat (FITC) were purchased from Shanghai Aladdin Co. Ltd. The new indocyanine green (ICG) was purchased from Macklin Co., Ltd. Hydrogenated soy lecithin (HSPC), cholesterol, and DSPE-MPEG 2000 were purchased from A. V. T. (Shanghai) Pharmaceutical Co., Ltd. Calcein-AM/PI staining kit and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Solarbio Co., Ltd. (Beijing). JC-1 staining, DCFH-DA probe, TUNEL apoptosis kit, and 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) were purchased from Beyotime Biotechnology Co Ltd. (Shanghai, China). Annexin V-FITC/PI Apoptosis Detection Kit (Keygen Biotech., China) were obtained from Sinopharm Group Chemical Reagent Co., Ltd.

Synthesis of LCuPC NPs.

CuPC nanoparticles were prepared by reverse-phase microemulsion method. Briefly, 1 mL of oleylamine was added into the mixed solution of ethanol (10 mL) and n-hexane (10 mL). Subsequently, 500 μL of deionized water was further added into the resulting solution under stirring. After 10 min, 1,2-tetradecanediol (50 mg) and potassium oleate (40 mg) were dissolved into the mixture. After that, $CuCl_2$ (1 mL, 20 mg/mL) was dropised into the mixed solution and then reacted at 60 ^oC for 24 h. Finally, the product (CuPC) was collected with centrifugation, washed with ethanol, and dried via freeze drying. Afterwards, cholesterol (30 μL, 10 mg/mL), hydrogenated soy lecithin (HSPC, 500 μL, 10 mg/mL), and DSPE-MPEG 2000 (100 μL, 10 mg/mL) were added to n-hexane solution containing CuPC (1mg/mL, 10 mL), The n-hexane solvent was removed by rotary evaporation to form thin film. Subsequently, the deionized water was poured into rotary flask, and then the solution was sonicated for 30 min. Finally, the product (LCuPC) was collected via centrifugation.

Fenton catalytic activity of LCuPC.

Fenton catalytic activity of CuPC was evaluated by TMB color test. Firstly, LCuPC solution (0 μL、5μL 、10 μL 、20 μL 、50μL 、100 μL 1 mg/mL) were added into phosphate buffer solution (940 μL、935μL、930μL、920 μL、890 μL、840 μL) containing H_2O_2 (30%, 10 μL) and TMB (dissolved in DMSO, 1 mg/mL, 50 μL). About 2min, the different concentrations of LCuPC solutions were centrifuged, and then the absorbance of supernatant was measured using Uv-Vis spectrum. In addition, the catalytic activity of LCuPC at different pH conditions and reaction time was also investigated using the similar method.

LCuPC enhanced electrocatalytic activity Investigation.

The electrocatalytic performance of CuPC was investigated via the MB degradation assay. Firstly, CuPC solution (1 mg/mL) was added to a 24-well plate containing MB solution (1 mM). Subsequently, the square wave electric field was applied at reacted system, and the parameters of square wave electric field was set as follows: the reaction voltage (10-20 V), the frequency (0-1000 mHz), the applied time (0-30 min). The residual amount of MB in the solution was quantitatively analyzed by UV-Vis spectrum.

GSH consumption of LCuPC.

DTNB as a GSH probe was used to detect the change of GSH in the solution. Briefly, different concentrations of CuPC solution (1 mg/mL, 20 μL or 50 μL) were added into GSH solution (10 mM, 100 μL) at room temperature under magnetic stirring. At different time points, the resulting solution was taken into tube (100 μL), and then fresh PBS containing DTNB (900 μL, 0.1 mM) was mixed into tube. About 2 min, the absorbance of the mixture was detected by UV-Vis spectrum.

Cu ion release of LCuPC under different pH conditions.

LCuPC solution (4 mg/mL, 100 μL) was added into fresh MES solutions (10 mL) with different pH conditions (pH=5.5, pH=6.5, pH=7.4). Subsequently, the mixed solutions were sharken at 37° using a shaker. Afterwards, 100 μL of the mixed solution was taken, and then centrifuged at 14000 rpm for 10 min. Finally, the supernatant was diluted using nitric acid solution (0.1 mM), and then measured using ICP-OES.

Cytotoxicity of LCuPC.

L929 cells or 4T1 cells were seeded in 96-well plates, and then the density of cells were adjusted to 1×10^4 cells/well. The L929 cells were treated with LCuPC for 24 h. After that, 100 μL of fresh medium containing MTT (10%) solution was added to each well, and the plate was incubated at 37 °C for 30 min. The viability of L929 cells were measured via a microplate reader at the wavelength of 450 nm. In addition, 4T1 cells were treated with LCuPC and LCuPC+E for 24 h and 48 h, respectively. The parameters of applied electric field were as follows: the voltage (5V), the frequency (10 mHz), and applied time (5 min). After that, the viability of 4T1 cells was mesured using the similar method.

Internalization and clorichloridion levels of LCuPC in cells.

The fluorescein isothiocyanate (FITC) was rationally labeled on LCuPC to study cell uptake behavior. Firstly, 4T1 cells were cultured with LCuPC (50 μg/mL) for different time. Subsequently, the media were removed, and the cells were washed with fresh PBS, fixed with paraformaldehyde. After that, the nuclei were stained with DAPI for 10min. The cells was observed by CLSM or analyzed quantitatively by flow cytometry. In addition, 4T1 cells were also treated with different concentrations of LCuPC for 6 h. Afterwards, the media were removed, the cells were further incubated with MQAE (5 mM) for 30 min. Finally, the green fluorescence of 4T1 cells was measured by CLSM observation and flow cytometry analysis.

Intracellular ROS production and LPO Detection.

4T1 cells were incubated with different samples for 6 h. Subsequently, the media were removed, the cells were washed with fresh PBS and then treated with DCFH-DA probe for 30min. The ROS production of 4T1 cells was analyzed by CLSM observation. In addition, the lipid peroxidation of 4T1 cells was detected using the C11-BODIPY$^{581/591}$ probe. Briefly, the cells were treated with E (10 mHz, 5V, 5 min), LCuPC NPs (50 μg/mL), LCuPC (50 μg/mL) + E (10 mHz 5V 5 min) for 12 h. Afterwards, the media were removed, the cells were washed, then stained with C11-BODIPY$^{581/591}$ probe for 30 min. After that, the excessive C11-BODIPY$^{581/591}$ was removed via fresh PBS washing, and then the cells were observed by CLSM.

Western blot assay.

4T1 cells were seeded in 6-well plates (3×10^5 cells per well) and incubated overnight. After that, the media were removed, and then the cells were treated with Saline, E (10 mHz, 5V, 5 min), LCuPC, and LCuPC+E (10 mHz, 5V, 5 min), and then incubated for 12 h. The cells were collected, lysed, and treated with total protein extraction solution (200 μL) and then the total protein contents were quantified via Bradford protein assay kit. Subsequently, the protein loading buffer was heated to 99 ℃ for 10 min. Afterwards, 15% SDS-PAGE was used to separate the samples. Subsequently, the protein bands were transferred to polyvinylidene difluoride (PVDF, 0.22 μm) membranes and incubated with primary antibodies against cleaved caspase 3 (CC3), p-JNK, GPX4 and cleaved caspase 1, GSDMD overnight at 4 ℃. Next, the PVDF membranes containing protein bands were incubated with the corresponding secondary antibodies.Finally, the protein bands were visualized with the ECL imaging system.

In vivo antitumor activity studies.

Female BALB/c mice (4 weeks old) were purchased from Speifu Biotechnology Co., Ltd. (Beijing, China). All animal experiments were performed in accordance with the protocol approved by the Biomedical Ethics Committee of Binzhou Medical University. Injecting 2×10^5 4T1 cells were subcutaneously injected into the right hind leg of 4-week-old balb/c mice to establish 4T1 tumor-bearing mice model. When the tumor volume reached 100 mm^3, the mice were divided into four groups: (1) Saline, (2) Square wave electric field (E), (3) LCuPC (4 mg/mL 100 μL), (4) LCuPC+E. The injection dosage of LCuPC was 4 mg/kg, and the parameters of applied electric field were as follows: frequency (10 mHz), voltage (5V), and time (5 min). Tumor size and body weight of mice were recorded every 2 days. Tumor volume was calculated by the following equation: $Volume=width^2\times length/2$. After treatment for 14 days, the mice were sacrificed, the tumor and major organs were excised, fixed with 4% paraformaldehyde, cut into slices, and then stained for H&E, immunohistochemical anlysis, and TUNEL analysis.

Biodistribution and pharmacokinetics of LCuPC in vivo.

4T1 tumor-bearing mice were injected with ICG-coated LCuPC at the dosage of 3 mg/kg. Subsequently, at different time points, the fluorescence of the mice were observed through a NIR-II imager. After 72h, the mice were sacrificed, and the main organs (heart, liver, spleen, lung, kidney) and tumors were excised, and observed via NIR-II imager, meanwhile the fluorescence intensity of tissue were collected. In addition, pharmacokinetic analysis of LCuPC was performed at post-injection of LCuPC at the dosage of 5 mg/kg. At different time points, the blood of rats was collected, and then digested with concentrated nitric acid. Finally, the copper contents of blood were analyzed using ICP-OES.

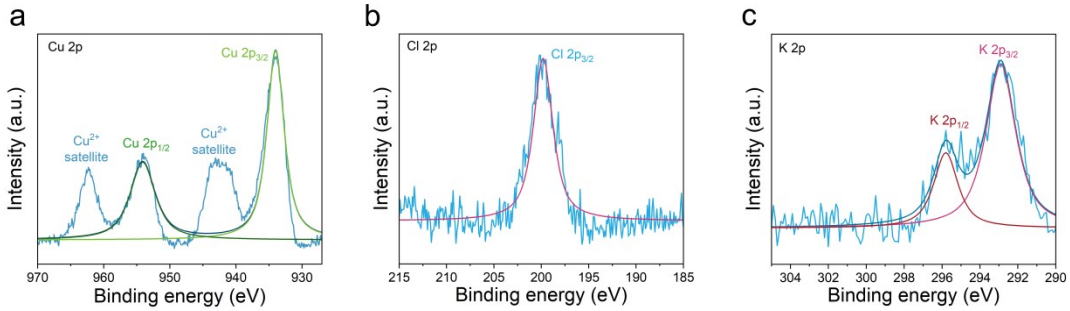

Figure S1. (a) Cu2p, (b) Cl2p, and (c) K2p XPS spectrum of CuPC.

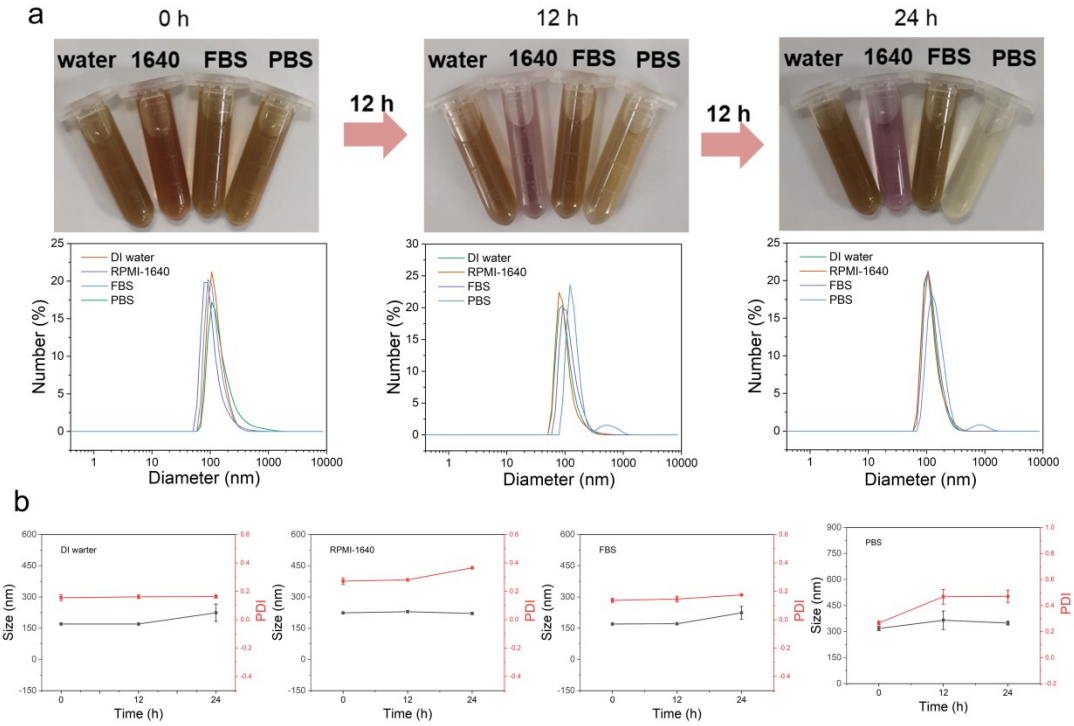

Figure S2. (a) Photographs of LCuPC dispersed in water, phosphate buffer solution (PBS), RPMI-1640 and 10% fetal bovine serum (FBS) with different time. (b) Hydrodynamic particle size and PDI variation of LCuPC dispersed in water, phosphate buffer solution (PBS), RPMI-1640 and 10% fetal bovine serum (FBS) with different time.

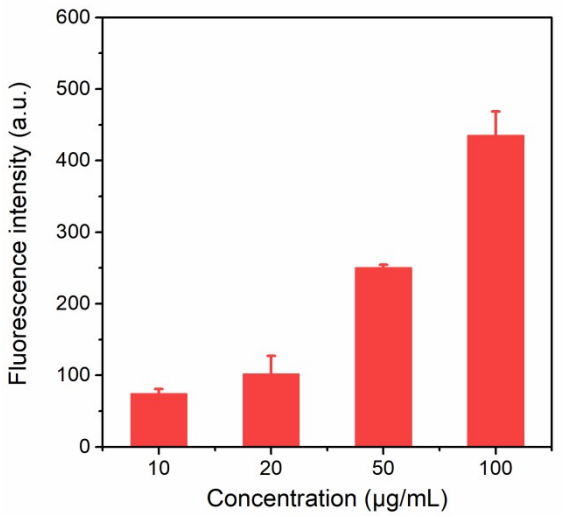

Figure S3. The fluorescence intensity of LCuPC-catalyzed H_2O_2 solution in the prescence of AFP probe.

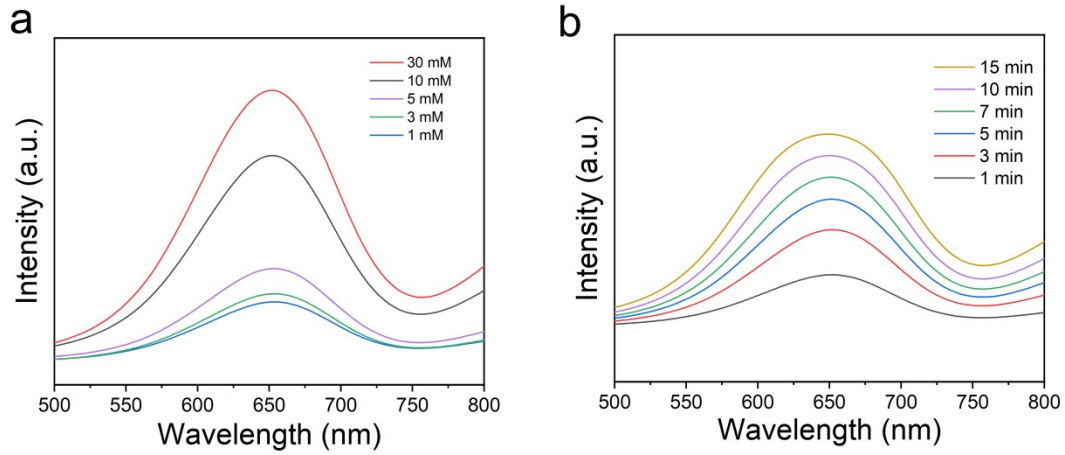

Figure S4. (a) UV absorption spectra of TMB solution catalyzed by CuPC under different concentrations of H_2O_2 buffer solution (pH=4.5, 10%). (b) UV absorption spectra of TMB and H_2O_2 solution (pH=4.5) catalyzed by CuPC after different reaction time.

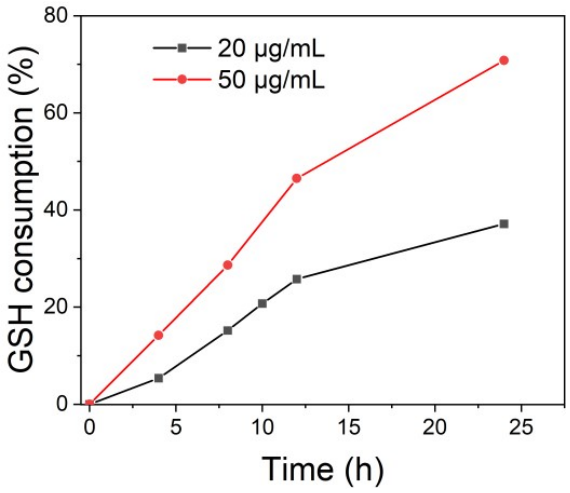

Figure S5. The ratio of GSH depletion with the increase of time at different concentrations of CuPC.

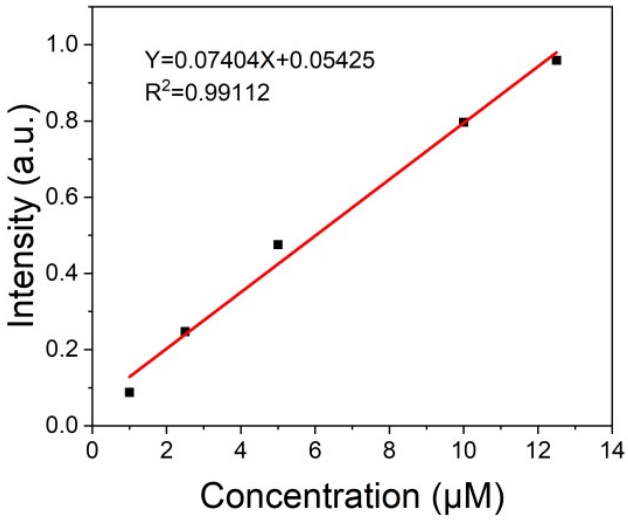

Figure S6. Standard curves of MB with different concentrations.

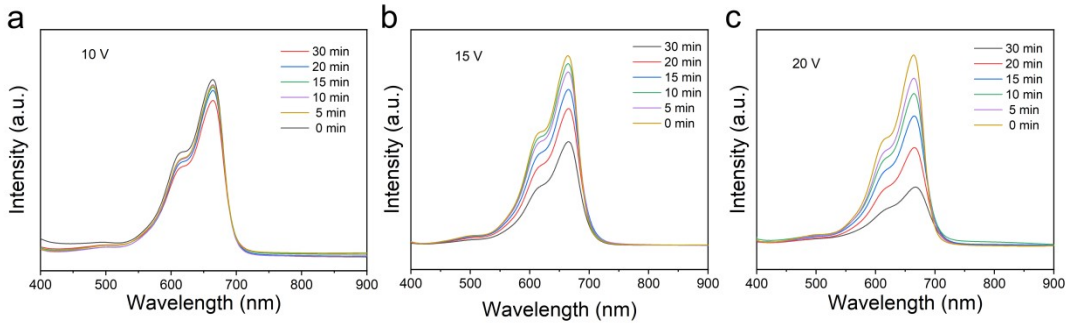

Figure S7. UV-vis spectra of MB solution treated with CuPC (100 μg/mL) plus electric field (10 mHz) for different time: (a) 10 V, (b) 15V, and (c) 20V.

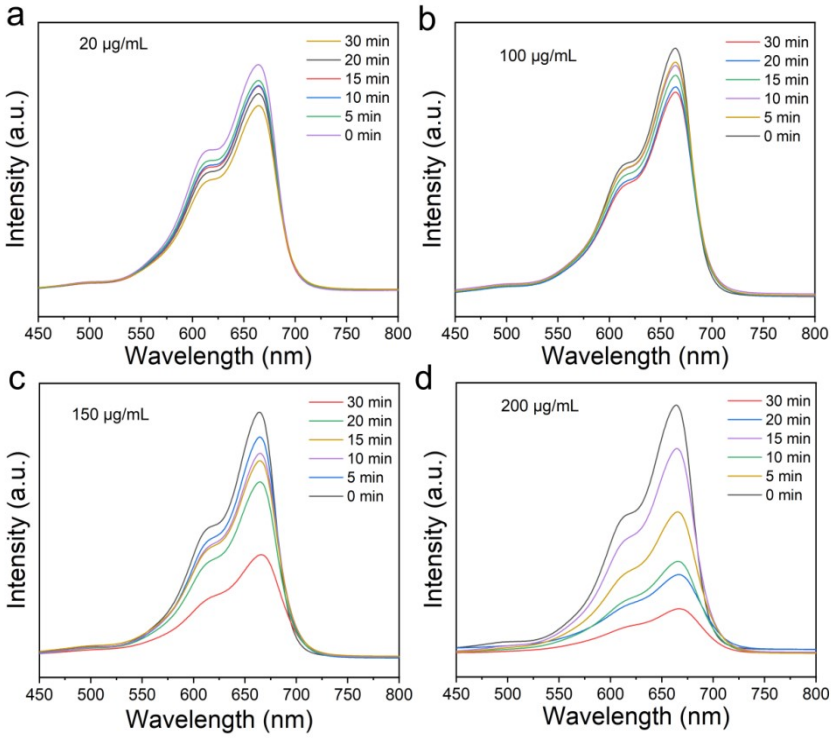

Figure S8. UV-vis spectra of MB solution treated with different concentrations of CuPC plus electric field (10mHz) for different time: (a) 20 μg/mL, (b) 100 μg/mL, (c) 150 μg/mL, (d) 200 μg/mL.

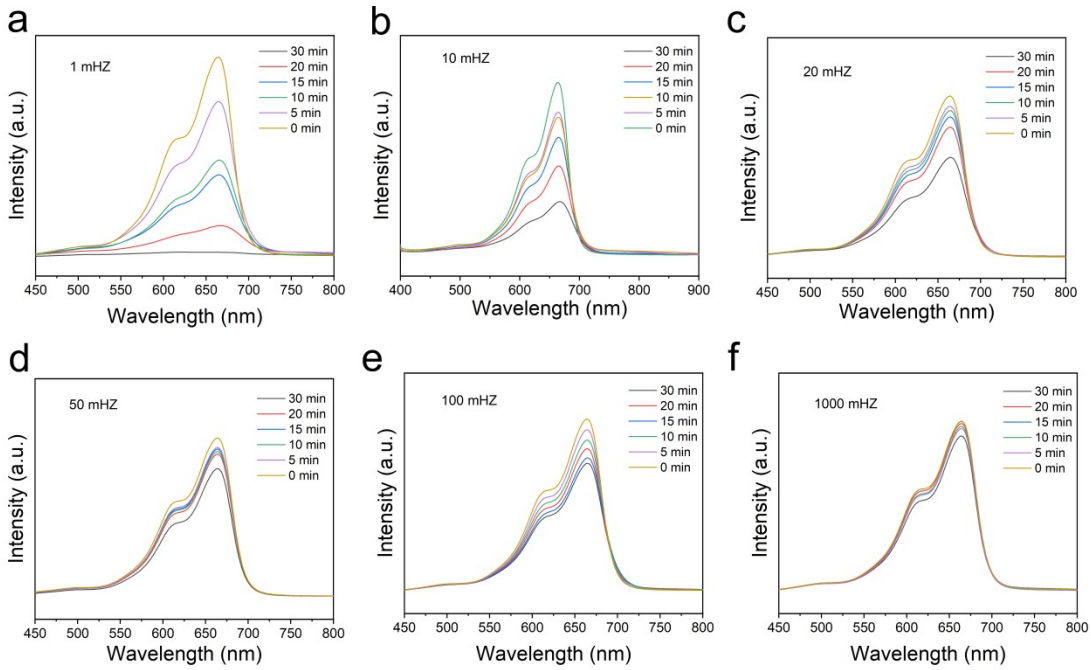

Figure S9. UV-vis spectra of MB solution treated with CuPC plus different frequencies of electric field for different time: (a) 1mHZ, (b) 10 mHZ, (c) 20 mHZ, (d) 50 mHZ, (e) 100 mHZ, (f) 1000 mHZ.

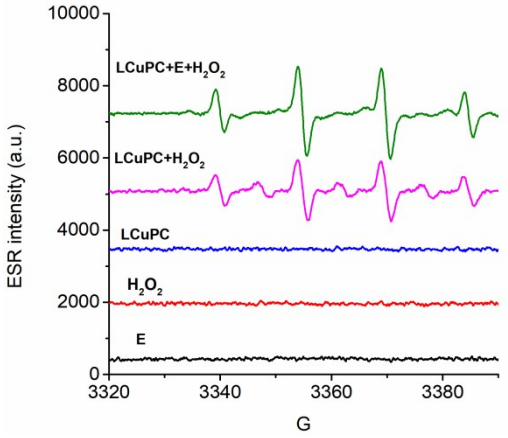

Figure S10. Electron spin resonance (ESR) spectra of H_2O_2 solution treated with different samples.

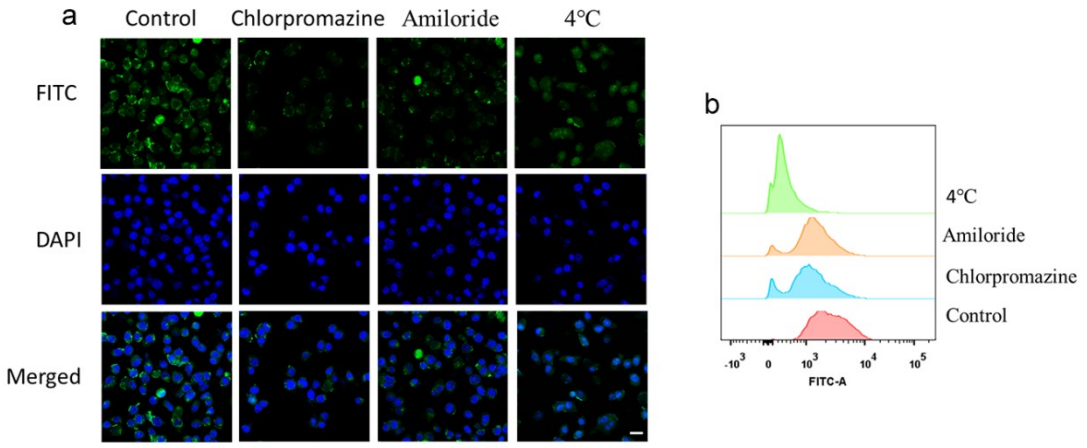

Figure S11. (a) CLSM images and (b) flow analysis of 4T1 cells treated with LCuPC plus chlorpromazine, amiloride or 4°C. Scale bar: 20 μm

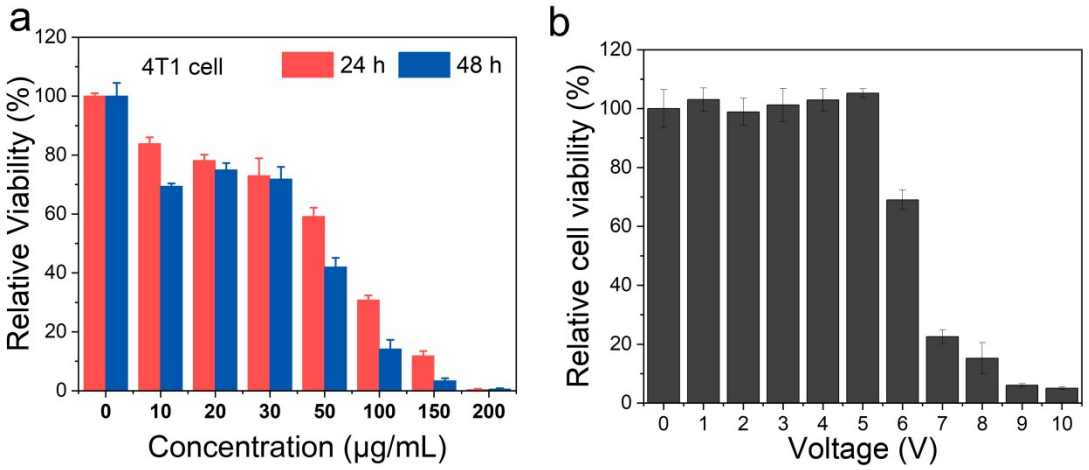

Figure S12. (a) The viability of 4T1 cells incubated with different concentrations of LCuPC. (b) The viability of 4T1 cells treated with different voltages of electric field.

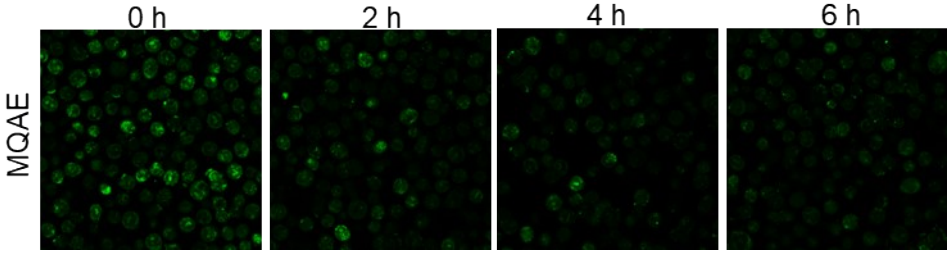

Figure S13. CLSM observation for chloridion levels of 4T1 cells treated with LCuPC (50 μg/mL) for different time.

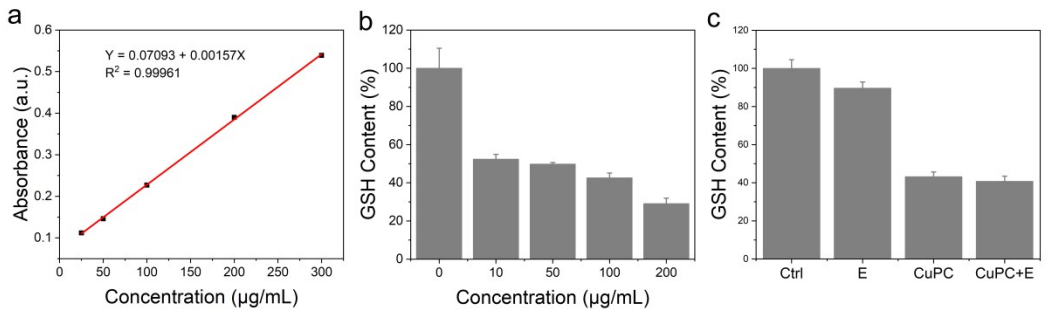

Figure S14. (a) Absorbance standard curves of GSH. (b) GSH content of 4T1 cells treated with different concentrations of LCuPC. (c) GSH content of 4T1 cells treated with different samples.

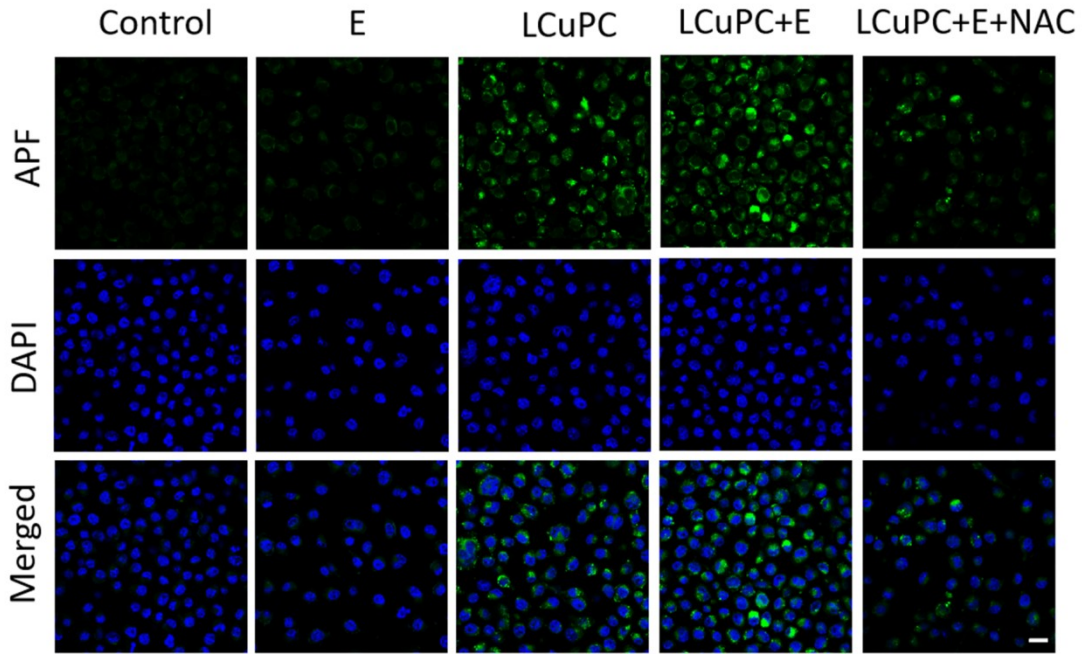

Figure S15. CLSM observation for ·OH production in 4T1 cells treated with different samples.

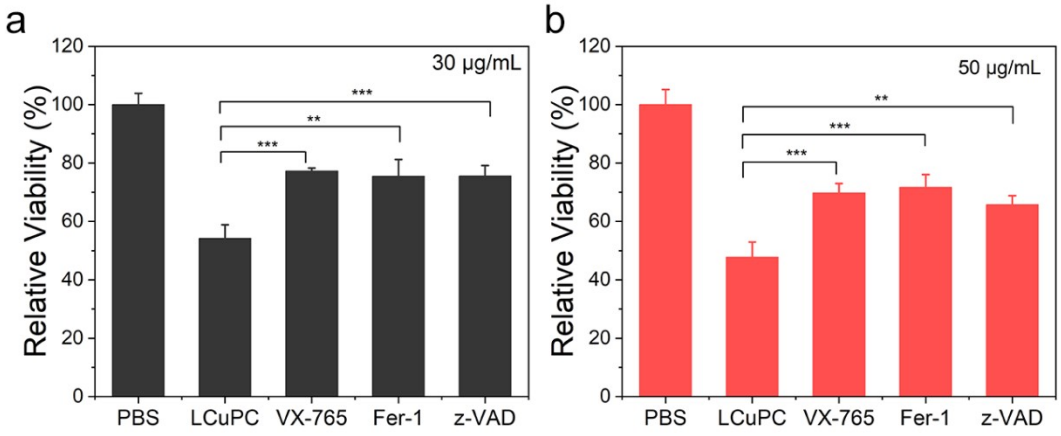

Figure S16. The viability of 4T1 cells treated with LCuPC, LCuPC+pyroptosis inhibitor (VX-765), LCuPC+ferroptosis inhibitor (Fer-1), and LCuPC+apoptosis inhibitor (z-VAD).

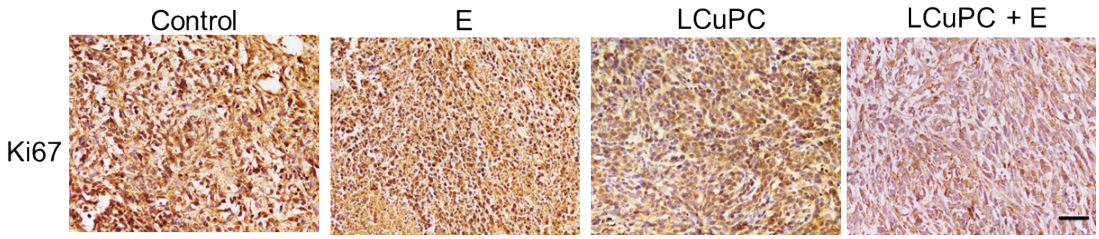

Figure S17. Ki67 staining of tumor tissue after different treatments. Scale bar: 50 μm

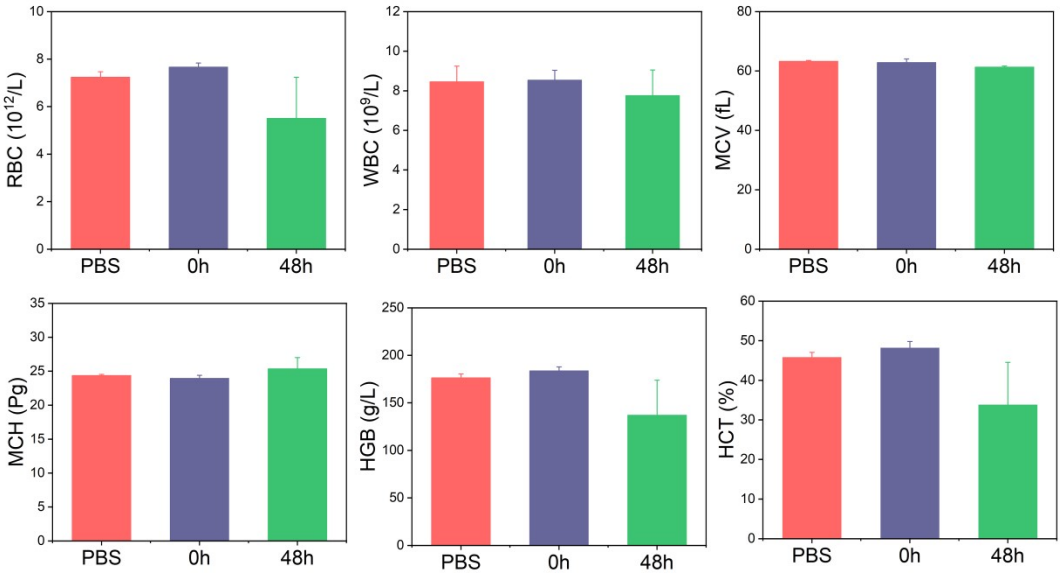

Figure S18. Blood routine analysis of mice treated with PBS and LCuPC.