Electronic Supplementary Information

A small-molecule Fenton agent for self-augmented chemodynamic therapy by intelligently regulating intracellular acidosis

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Experimental Section

Materials and Instruments.

4-nitrophenyl carbonochloridate, and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Tianjin Heowns Biochemical Technology Co., Ltd. Chlorocarbonyl ferrocene, 4-(2-aminoethyl)benzenesulfonamide, N,N-diisopropylethylamine (DIPEA), methylene blue (MB), and general solvents were obtained from Adamas Reagent, Ltd. 2,2′-Dithiobisethanol was purchased from Alfa Aesar (Tianjin, China). Reactive Oxygen Species Assay Kit was obtained from Beyotime (Shanghai, China). Penicillin/streptomycin, Roswell Park Memorial Institute medium (RPMI 1640), Fetal bovine serum (FBS) and PBS buffer were purchased from Shanghai XP Biomed Ltd. The mouse breast cancer cell line (4T1) was purchased from Shanghai AOLU Biological Technology Co. Ltd, China. All the chemical reagents were of analytical grade and used without further purification. All aqueous solutions were prepared using ultrapure water of 18.2 MΩ cm^{-1}.

NMR spectra were recorded on a Bruker NMR spectrometer (Germany). Chemical shifts (δ) for ^{1}H NMR (400 Hz) were given in ppm. Data were reported as follows: chemical shift, integration, multiplicity (s = single, d = doublet, t = triplet, br = broad, m = multiplet) and coupling constants (Hz). High resolution mass spectra (HRMS) were recorded on a Bruker Daltonics maXis UHR-TOF MS (Germany). UV-Vis absorption spectra were measured on a TU-1901 UV-Visible spectrophotometer (China). Confocal fluorescence imaging was carried out with a confocal laser scanning microscopy (Leica TCS SP8, Germany). MTT assay was accomplished with a microplate reader (Synergy 2, Biotek, USA).

Synthesis of FSC. As shown in Fig. S1, the synthesis of Compound 1 was achieved by adding DIPEA (865 μL, 5 mmol) to a solution of 2,2'-disulfanediylbis(ethan-1-ol) (2.3 g, 14.9 mmol) in dry dichloromethane (DCM, 20 mL), which was then cooled to 0 °C before dropwise addition of 4-nitrophenyl carbonochloridate (1 g, 5 mmol) in DCM (10

mL). The solution was kept stirring for 3 hours at 0 °C and monitored by TLC. When finished, the reaction was quenched by water, and extracted with DCM for 3 times. The organic phases were combined and dried over Na_2SO_4. The pure product was obtained *via* flash chromatography using ethyl acetate/petroleum ether = 1:2 as eluent. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30-8.28 (d, J = 8 Hz, 2H), 7.41-7.39 (d, J = 8 Hz, 2H), 4.59-4.55 (t, J = 8 Hz, 2H), 3.94-3.90 (t, J = 8 Hz, 2H), 33.07-3.03 (t, J = 8 Hz, 2H), 2.94-2.90 (t, J = 8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.38, 152.37, 145.44, 125.38, 121.84, 66.91, 60.26, 41.43, 36.47; HRMS (ESI) m/z calcd for $C_{11}H_{13}NNaO_6S_2^+$ $[M+Na]^+$: 342.0076; found: 342.0090.

To a solution of 4-(2-aminoethyl)benzenesulfonamide (400 mg, 3 mmol) and triethylamine (TEA, 1 mL, 7.2 mmol) in 10 mL dry N,N-dimethylformamide (DMF) was added dropwise Compound 1 (638 mg, 2 mmol) in 5 mL DMF. The mixture was stirred intensely for 5 hours at room temperature and determined by TLC. Then, 100 mL ethyl acetate was added after removing the solvent under reduced pressure, and the mixture was washed sequentially with 1 M HCl and brine. The organic solution was dried over Na_2SO_4, filtrated, and then evaporated under reduced pressure. The pure Compound 2 was obtained *via* flash chromatography using DCM/MeOH = 20:1 as eluent. ^{1}H NMR (400 MHz, MeOD-d4) δ 7.83-7.81 (d, J = 8 Hz, 2H), 7.40-7.38 (d, J = 8 Hz, 2H), 4.27-4.23 (t, J = 8 Hz, 2H), 3.79-3.75 (t, J = 8 Hz, 2H), 3.37-3.33 (t, J = 8 Hz, 2H), 2.92-2.82 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD) δ 157.23, 144.03, 141.57, 129.16, 125.93, 62.43, 59.79, 41.51, 40.76, 37.27, 35.39. HRMS (ESI) m/z calcd for $C_{13}H_{20}N_2NaO_5S_3^+$ $[M+Na]^+$: 403.0427; found:403.0434.

Chlorocarbonyl ferrocene (248 mg, 1 mmol) was dissolved in 10 mL dry DCM, and the mixture was further dropped slowly to a solution of Compound 2 (380 mg, 1 mmol) and DIPEA (1.04 mL, 6 mmol) in 10 mL DCM. The mixture was stirred for 6 hours at room temperature. After completion, the solution was diluted with 100 mL DCM and washed with brine for 3 times. The final product FSC was obtained as an orange solid *via* flash chromatography using DCM/MeOH = 20:1 as eluent. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 -7.85 (d, J = 8 Hz, 2H), 7.35-7.33 (d, J = 8 Hz, 2H), 5.06 (br, 3H), 4.81-

4.79 (t, J = 4 Hz, 2H), 4.49-4.45 (t, J = 8 Hz, 2H), 4.43-4.41 (t, J = 4 Hz, 2H), 4.35-4.31 (t, J = 8 Hz, 2H), 4.21 (s, 5H), 3.49-3.44 (m, 2H), 3.02-2.99 (t, J = 4 Hz, 2H), 2.97-2.88 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.68, 157.19, 143.66, 141.10, 129.56, 126.67, 72.23, 70.47, 70.23, 69.93, 62.50, 62.12, 41.45, 38.49, 37.24, 35.90; HRMS (ESI) m/z calcd for $C_{24}H_{28}FeN_2NaO_6S_3^+$ $[M+Na]^+$: 615.0351; found: 615.0314.

The preparation of Fc NPs, CAI NPs, and FSC NPs. The Fc, CAI and FSC were dissolved in 8 mL DCM with Pluronic F127, respectively. The solvent was evaporated by rotary evaporation at 40 °C for about 1 h. Residual DCM was further removed by vacuum drying overnight. The obtained thin film was hydrated with 10 mL deionized water at 40 °C for 2 h to obtain the micelles. Then the mixture was filtrated through filter membrane to remove the molecule aggregates, which was further freeze-dried to obtain the final products.

Drug encapsulation efficiency (EE). The EE of drugs was determined by HPLC. Firstly, the HPLC spectra of different concentrations of Fc, CAI, FSC were recorded. Then the standard linear calibration curves were drawn according to the spectra. A certain number of micelles were dissolved in acetonitrile and the corresponding HPLC spectra were detected. The contents of drugs were calculated according to the standard linear calibration curves. The EE was obtained by the following formula.

$$EE = \frac{Mass\ of\ loaded\ drugs}{Mass\ of\ total\ drugs} \times 100\%$$

The detection of •OH. The generation of •OH was detected with MB probe. MB (10 μg/mL), H_2O_2 (500 μM) and GSH (10 mM) were added to 2-morpholinoethanesulphonic acid (MES) buffer (pH 7.4 and pH 5.5). Then FSC NPs was added to the above solution. The absorbance of MB at 664 nm was recorded at different time points. The changes of absorbance in other control groups were determined by the same protocol.

Cell culture. The 4T1 cells were incubated in a humidified atmosphere with 5% CO_2 at 37 ℃. RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin was used to culture cells.

ROS generation assay. 4T1 cells were plated in a confocal dish for 24 h. The medium containing different materials were used to incubate cells for 24 h. Then the medium was removed and PBS buffer was used to wash cells. The DCFH-DA (2 μM) probe was utilized to incubate the cells for 20 min. Finally, the cells were washed with PBS buffer and detected by confocal fluorescence imaging.

Detection of intracellular pH. 4T1 cells were plated in a confocal dish for 24 h. The medium containing different materials were used to incubate cells for 24 h. PBS buffer was used to wash cells. The BCECF-AM (5 μM) probe was used to incubate the cells for 15 min at 37 ℃. Then the cells were washed with PBS buffer and detected by confocal fluorescence imaging.

Cell viability assay. MTT assay was performed to evaluate the cell viability after the cells incubated with different materials. 4T1 cells were cultivated in 96-well microtiter plates for 24 h. The culture medium containing FSC NPs at different concentrations were used to incubate cells for 36 h. Then MTT solution (150 μL, 0.5 mg/mL) was added to incubate cells for 4 h. Finally, MTT solution was removed and 150 μL DMSO was added to dissolve the formazan. The absorbance at 490 nm was measured on microplate reader. The cytotoxicity of Fc NPs and CAI NPs was measured by the same protocol.

Cell migration assay. 4T1 cells were cultured in cell culture dish for 24 h. Then the cells were wounded by dragging a 10 μL pipette tip. PBS buffer was used to wash the cells for three times to remove the cellular debris. The cells images were recorded before incubating the materials. Then the medium with different materials were used to incubate cells for 24 h. The images of the cells were recorded at 12 h and 24 h after moving the materials.

Cell cloning assay. 4T1 cells were plated in a cell culture dish. Different materials were used to incubated cells for 24 h. After removing the material, the fresh culture was added to continuously culture cells for observing the formation of cell communities. Finally, the cells were stained with crystal violet and dried for photographs.

Detection of glutathione peroxidase 4 (GPX4) expression. 4T1 cells were plated in

a cell culture dish. Different materials were used to incubate cells for 24 h. Then the cells were collected. The expression of GPX4 was detected by western blotting experiment.

For the immunofluorescence staining assay of GPX4. 4T1 cells were plated in a confocal dish for 24 h. The cells were treated with blank medium and medium containing Fc NPs, CAI NPs, and FSC NPs for another 24 h. Then the cells were fixed with 4% PFA for 20 min at 4 ℃ and treated with 0.2% Triton X-100-PBS for exact 5 min. BAS-PBS was used to block the cells for 1 h at room temperature. Subsequently, the cells were stained with GPX4-related primary antibody, secondary antibody, and Hoechst 33342. Finally, the cells were evaluated by confocal fluorescence imaging.

Detection of the malondialdehyde (MDA) content. 4T1 cells were plated in a cell culture dish. Different materials were used to incubate cells for 24 h. Then the cells were collected. The content of malondialdehyde (MDA) was measured by the lipid peroxidation (MDA) assay kit.

Detection of mitochondrial membrane potential. 4T1 cells were plated in a confocal dish for 24 h. The medium containing different materials were used to incubate cells for 24 h. Then the medium was removed, and PBS buffer was used to wash cells. The JC-1 probe was utilized to incubate the cells for 30 min. Finally, the cells were washed with staining buffer and detected by confocal fluorescence imaging.

Animals. Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU 2023072). 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.

***In vivo* imaging.** IR808-doped F127 nanoparticle (IR808@F127 NPs) was firstly prepared. 4T1 tumor-bearing mice were intravenously injected with IR808@F127 NPs and the fluorescence images of tumor site at different times (0, 2, 4, 6, 8, 10, 12, 24, 36, 48 h) were obtained by *in vivo* imaging system.

Evaluation of *in vivo* antitumor efficacy. 4T1 cells were injected subcutaneously into the right axillary region of Balb/C mice. The mice were randomly divided into four groups: Control group, Fc NPs group, CAI NPs group, FSC NPs group. All the materials were intravenously injected to the tumor-bearing mice. The tumor sizes and body weights were measured every other day (Tumor Volume = $W^2 \times L/2$, W = width, L = length). And after 14 days, the main organs were collected for H&E staining.

Supplementary Figures

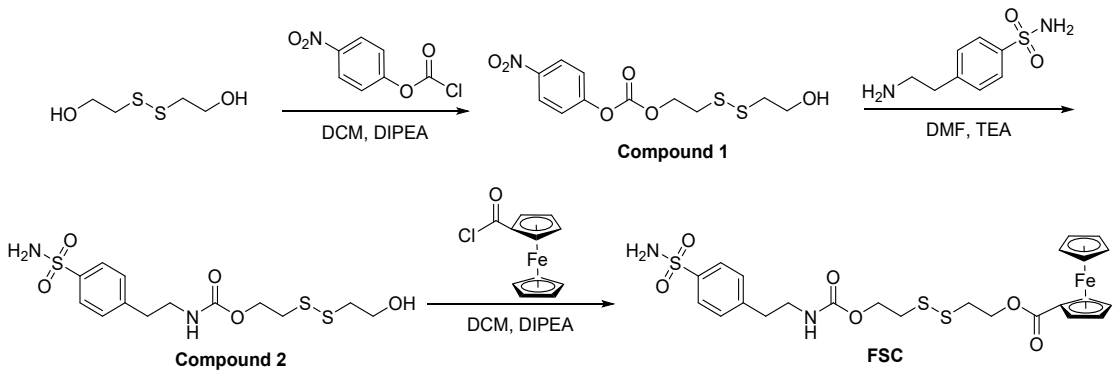
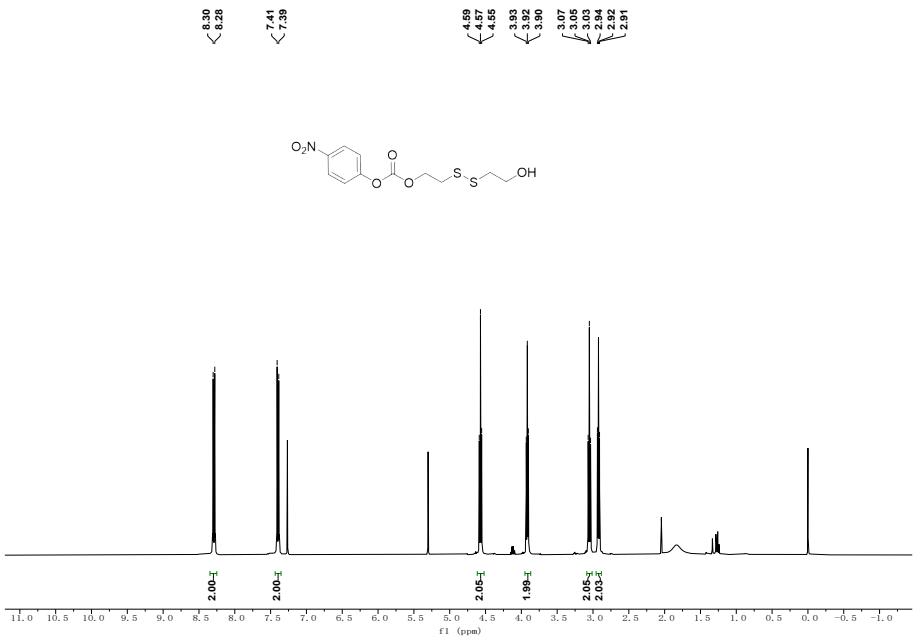
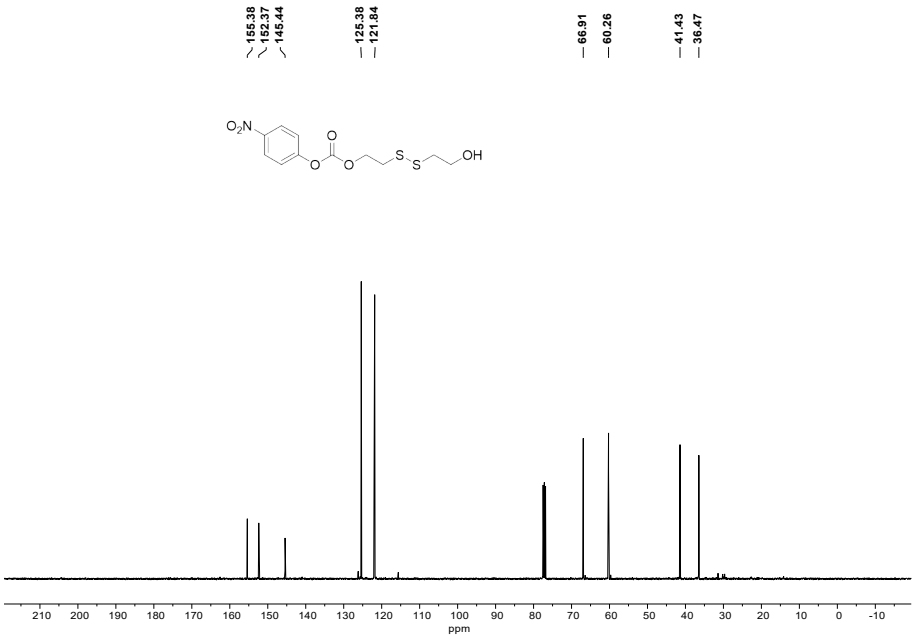


Fig. S1 The synthesis route of FSC.





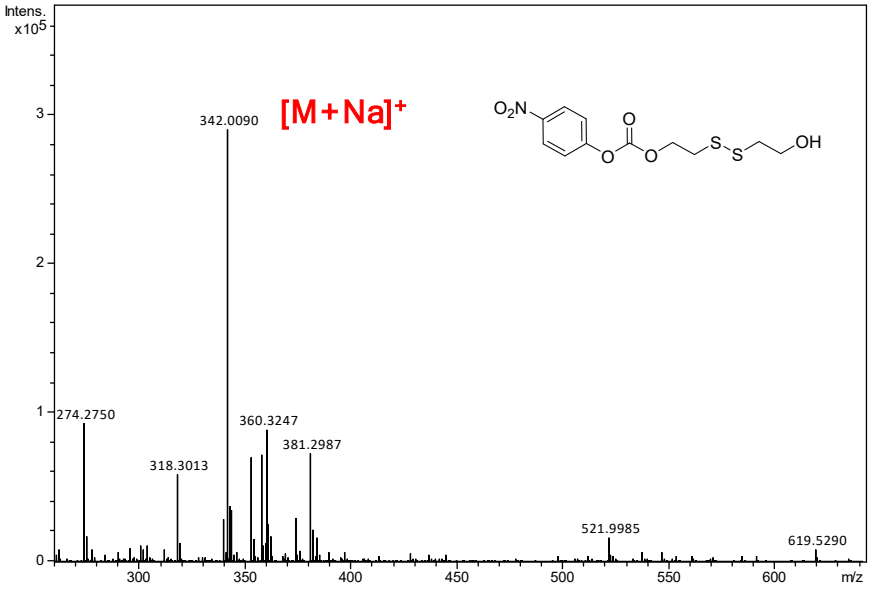
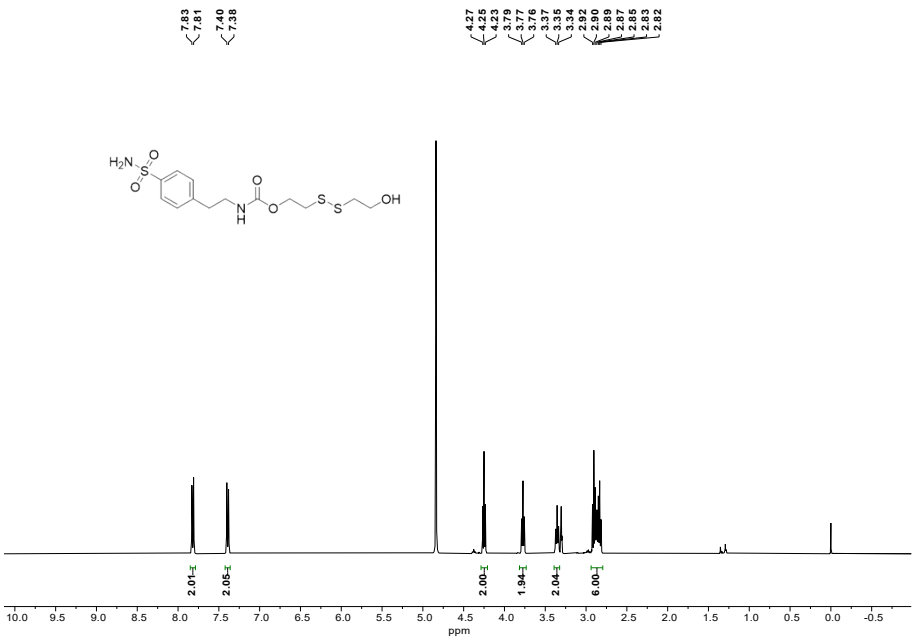


Fig. S2 ^{1}H-NMR, ^{13}C-NMR and HRMS spectra of Compound 1.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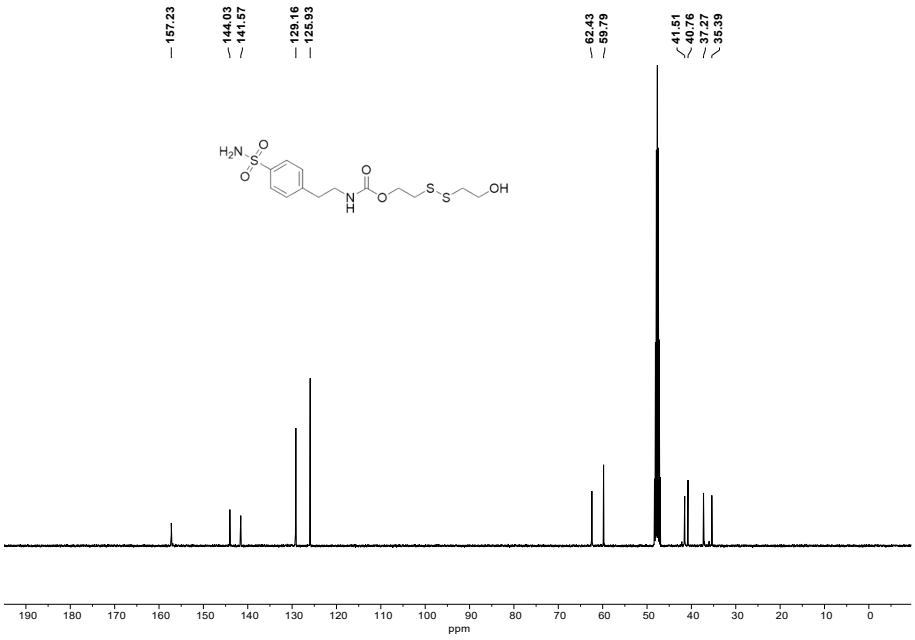
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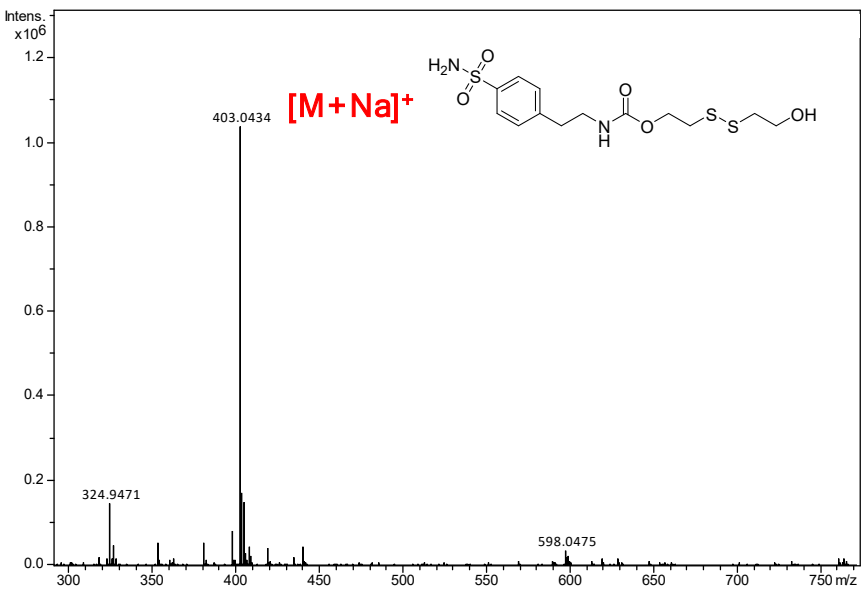
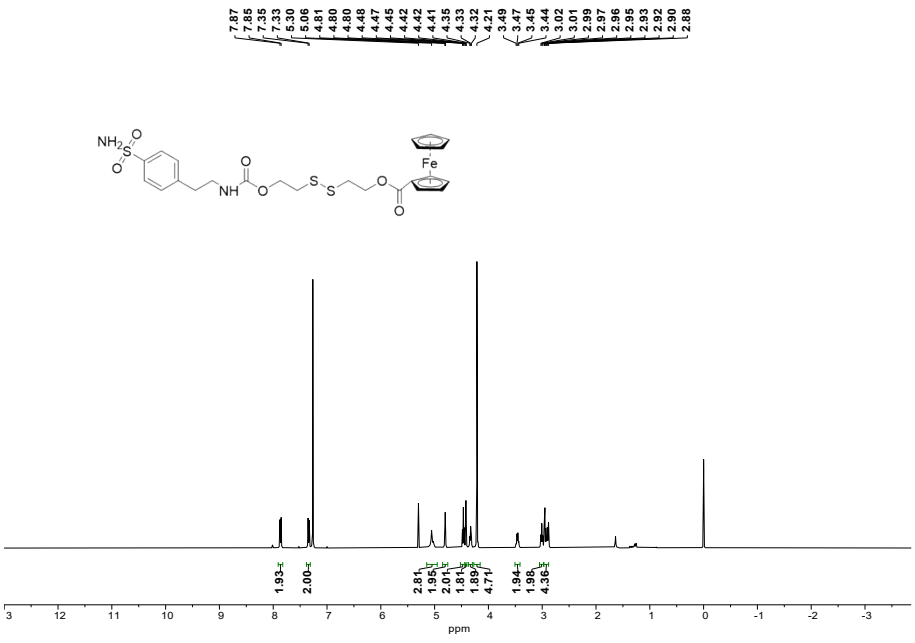
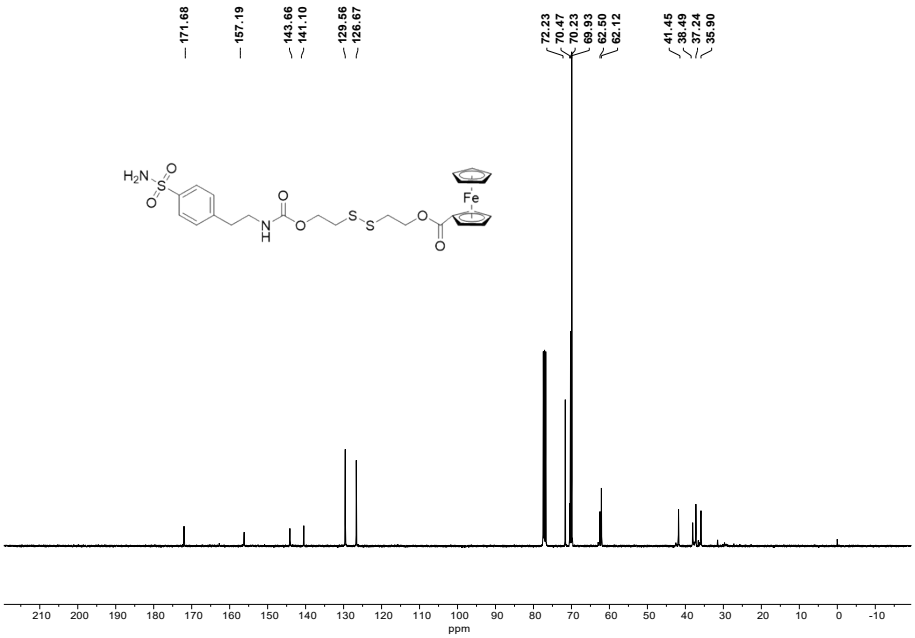


Fig. S3 ^{1}H-NMR, ^{13}C-NMR and HRMS spectra of Compound 2.





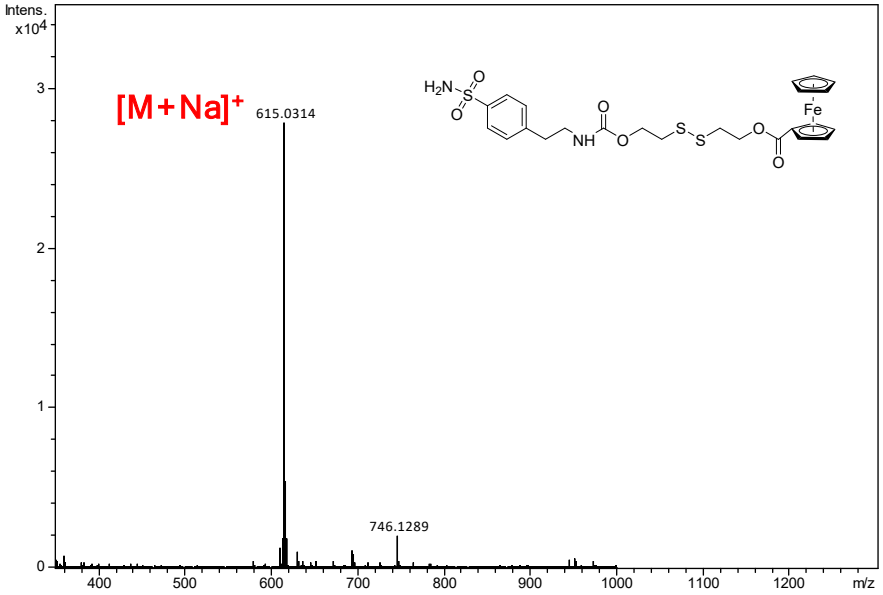


Fig. S4 ^{1}H-NMR, ^{13}C-NMR and HRMS spectra of FSC.

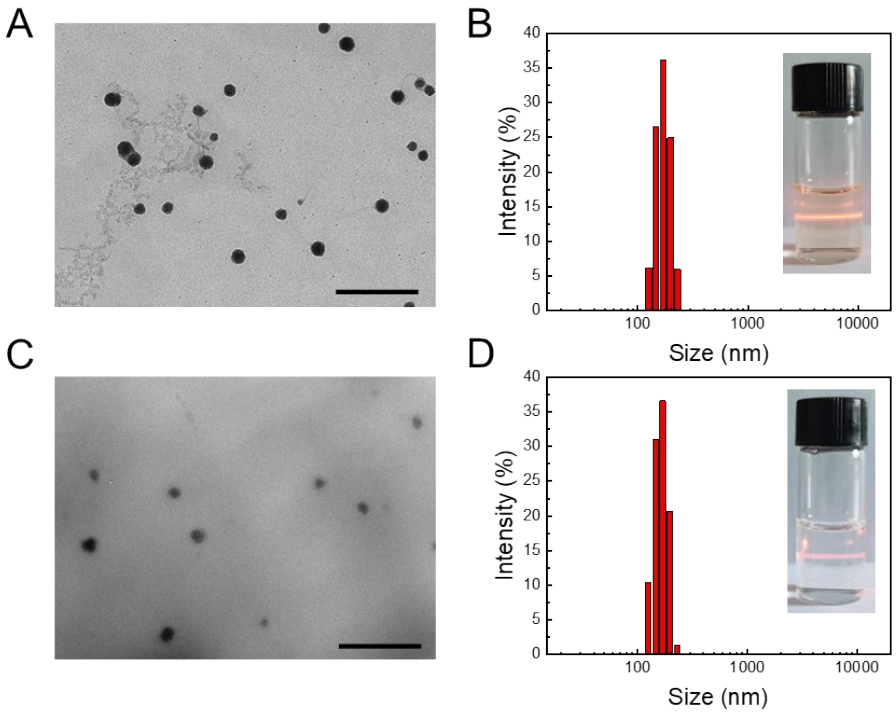


Fig. S5 TEM image (A), hydrodynamic size and the Tyndall effect photograph (the inset) of Fc NPs solution (B). TEM image (C), hydrodynamic size and the Tyndall effect photograph (the inset) of CAI NPs (D). Scale bar is 500 nm.

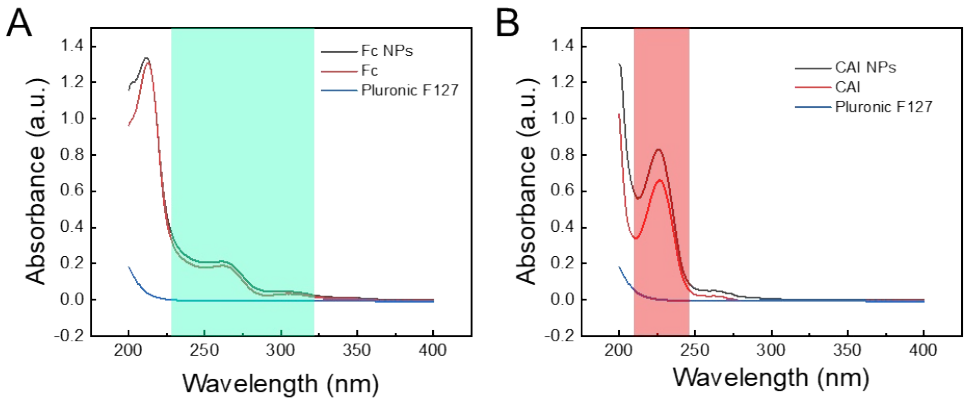


Fig. S6 The UV-Vis spectra of Pluronic F127, Fc and Fc NPs (A), CAI and CAI NPs (B).

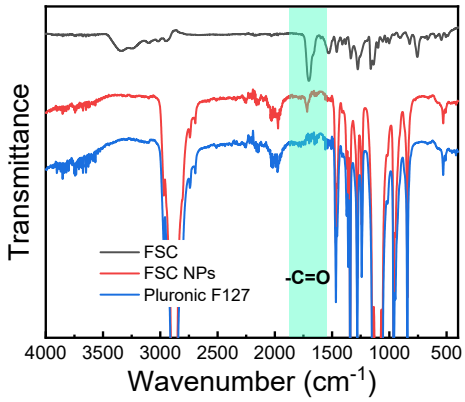


Fig. S7 The FT-IR spectra of Pluronic F127, FSC and FSC NPs.

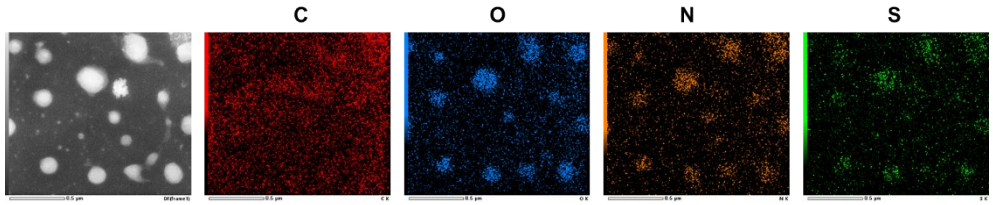


Fig. S8 Elemental analysis of FSC NPs.

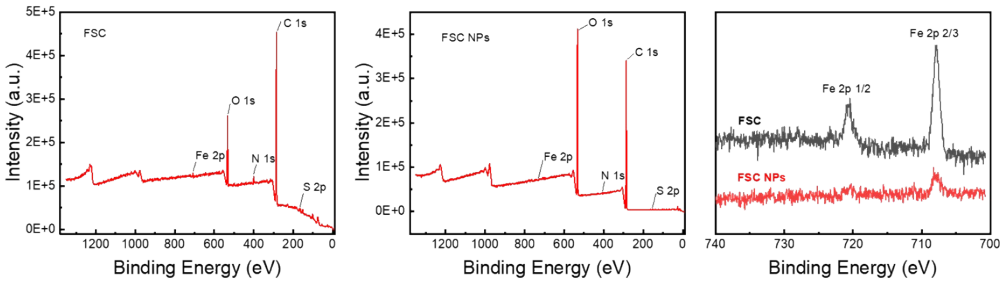


Fig. S9 XPS analysis of FSC and FSC NPs.

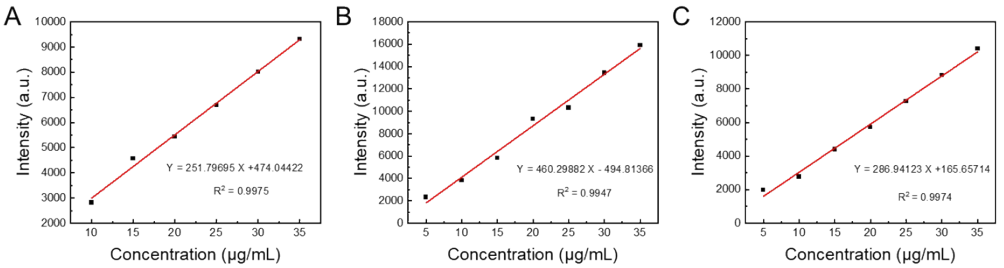


Fig. S10 The standard curve of Fc (A), CAI (B) and FSC (C). The absorbances were detected by HPLC.

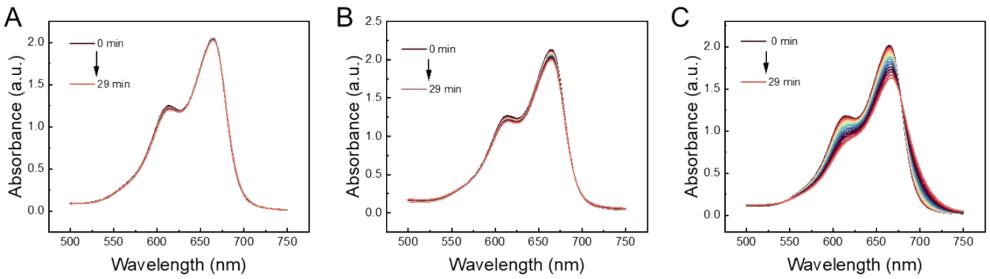


Fig. S11 The UV-Vis spectra of MB probe for the detection of •OH at pH 7.4. (A) MB + H_2O_2 + GSH. (B) MB + FSC NPs + GSH. (C) MB + H_2O_2 + FSC NPs + GSH.

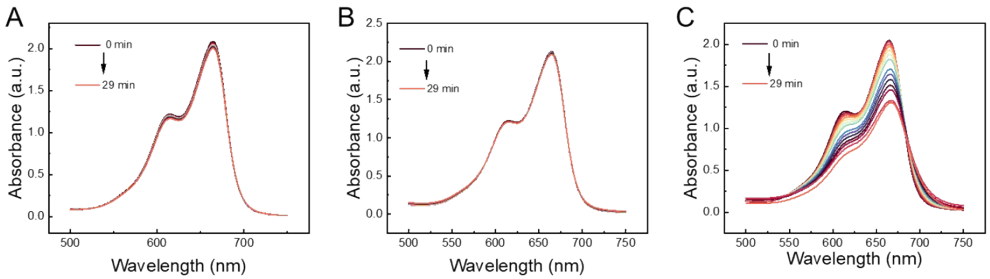


Fig. S12 The UV-Vis spectra of MB probe for the detection of •OH at pH 5.5. (A) MB + H_2O_2 + GSH. (B) MB + FSC NPs + GSH. (C) MB + H_2O_2 + FSC NPs + GSH.

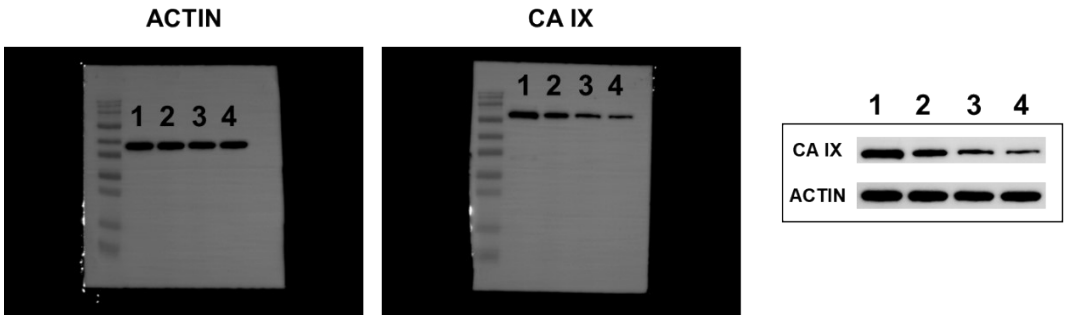


Fig. S13 Western blotting assay of the CA IX expression of 4T1 cells after different treatments. (1) Control; (2) Fc NPs; (3) CAI NPs; (4) FSC NPs.

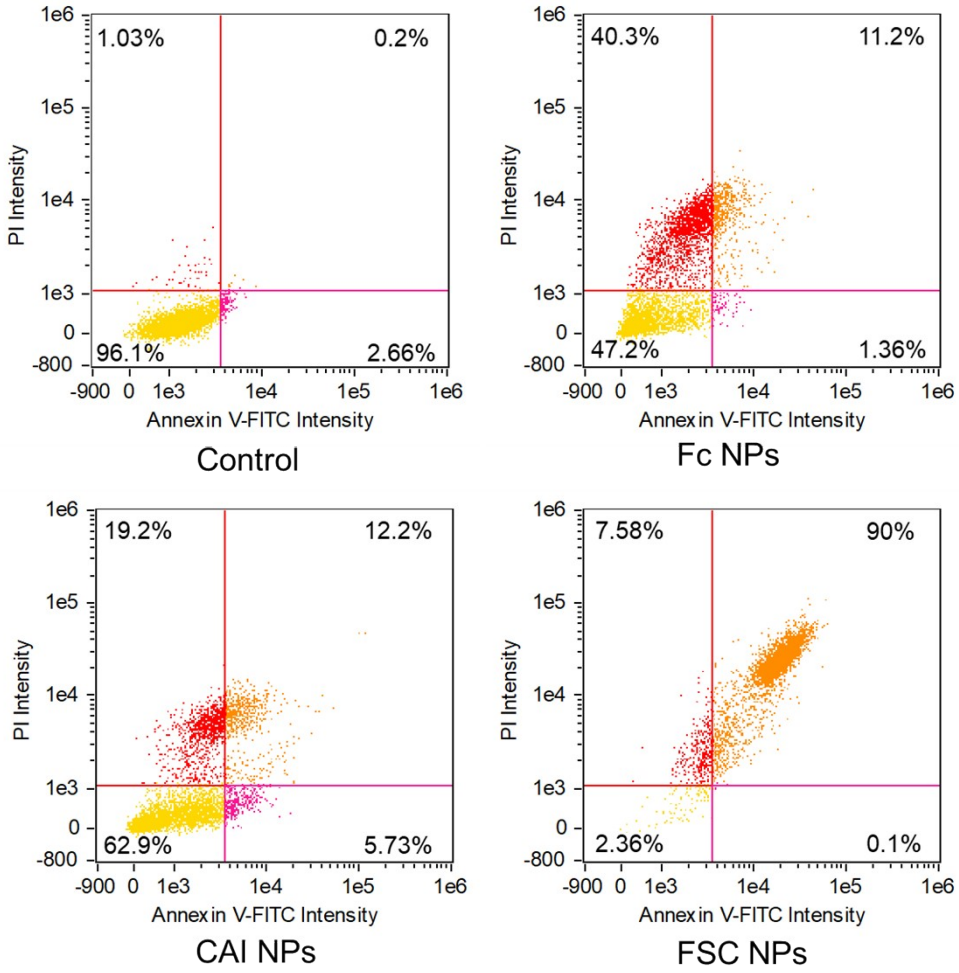


Fig. S14 Apoptosis analysis of 4T1 cells after different treatments.

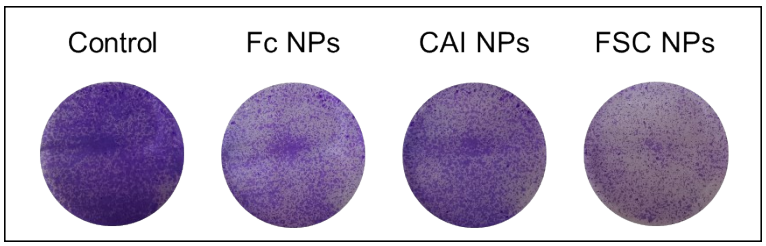


Fig. S15 Cloning experiment images of 4T1 cells after treated with different materials.

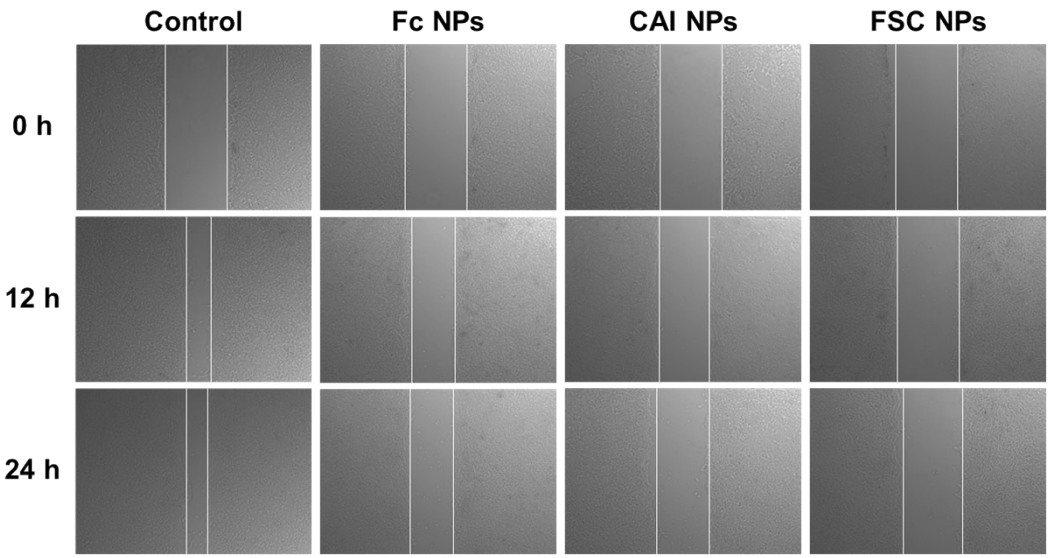


Fig. S16 Detection of cell migration after different treatments. The photographs were collected at pre-incubation (0 h), 12 h and 24 h after moving the materials.

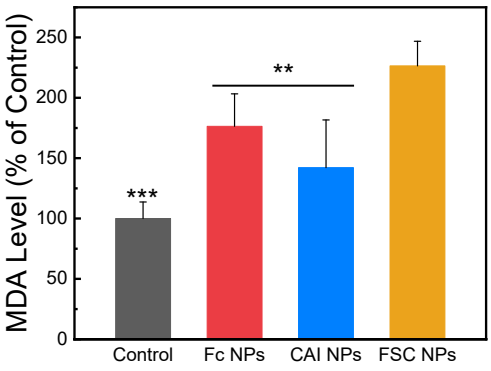


Fig. S17 Relative MDA content of 4T1 cells after different treatments. The *P* values were calculated using the t-test (***P<0.001, **P<0.01, *P<0.05).

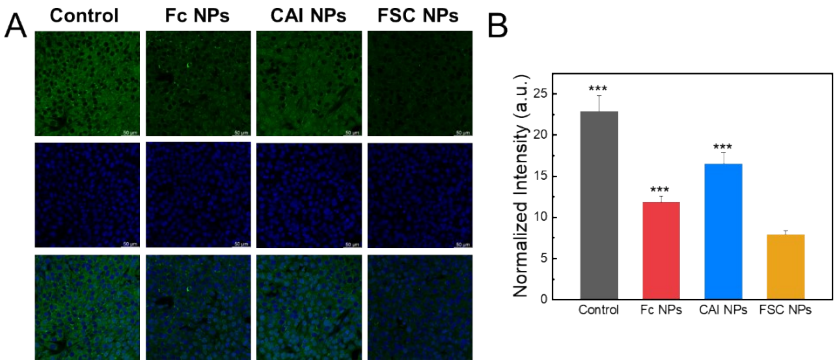


Fig. S18 Immunofluorescence staining images (A) and the quantification (B) of the GPX4 expression in different groups. The *P* values were calculated using the t-test (***P<0.001, **P<0.01, *P<0.05).

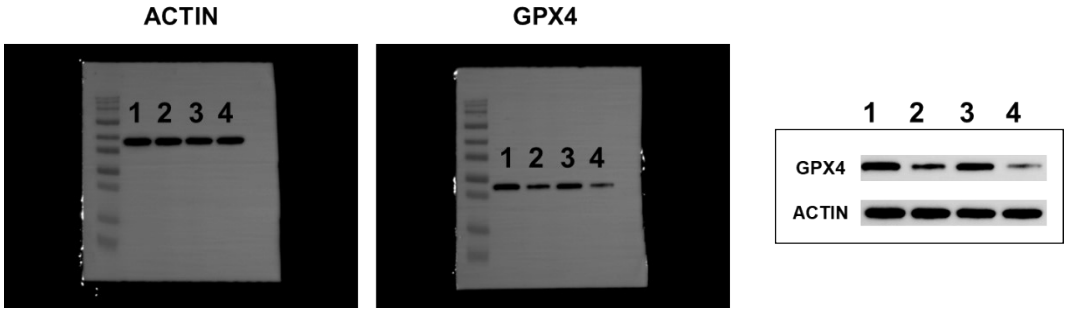


Fig. S19 Western blotting assay of the GPX4 expression of 4T1 cells after different treatments. (1) Control, (2) Fc NPs, (3) CAI NPs, (4) FSC NPs.

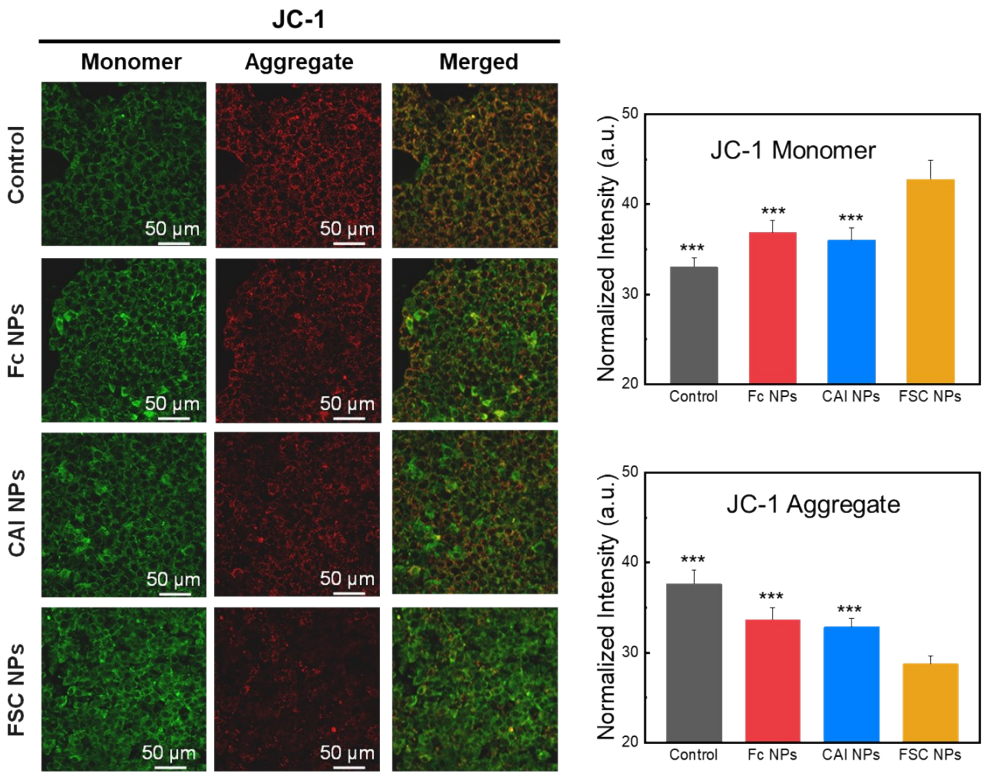


Fig. S20 Confocal fluorescence images and the quantification of the changes of mitochondrial membrane potential in 4T1 cells after incubated with various materials. The *P* values were calculated using the t-test (***P<0.001, **P<0.01, *P<0.05).

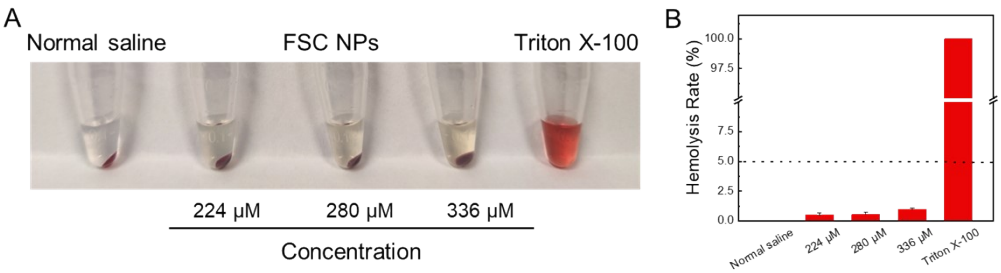


Fig. S21 Hemolysis experiment. (A) The photographs of red blood cells incubated with various concentration of FSC NPs after centrifugation. (B) The detection of hemolysis ration.

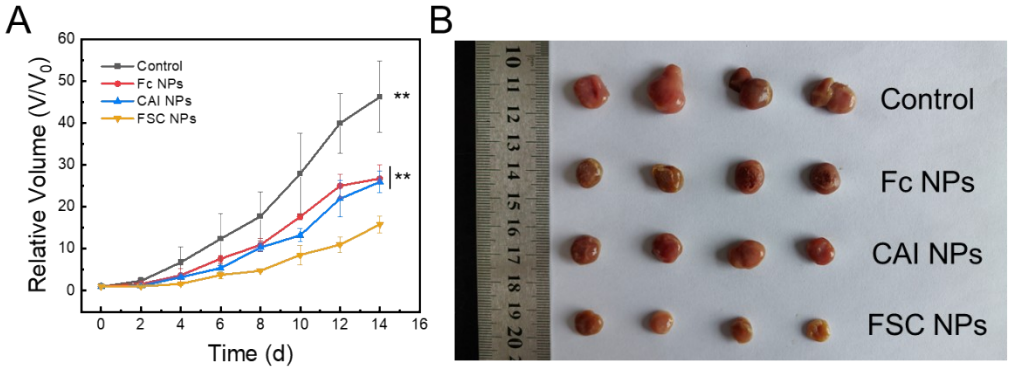


Fig. S22 Curves depicting changes in relative tumor volume (A) and tumor images (B) of the mice following various treatments. The *P* values were calculated using the t-test (***P<0.001, **P<0.01, *P<0.05).

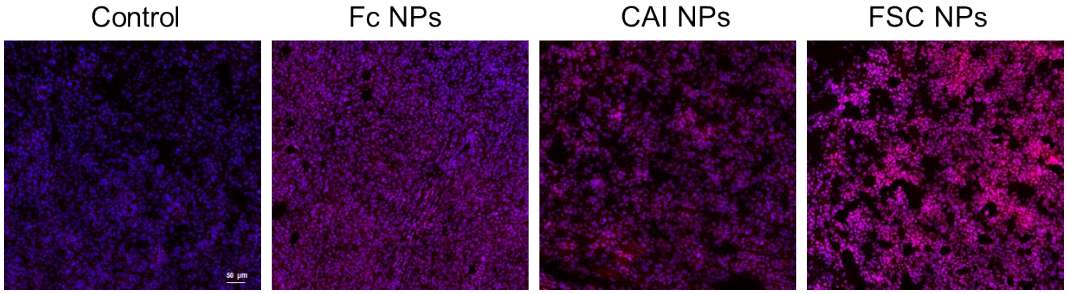


Fig. S23 ROS staining images of tumor tissue following various treatments.

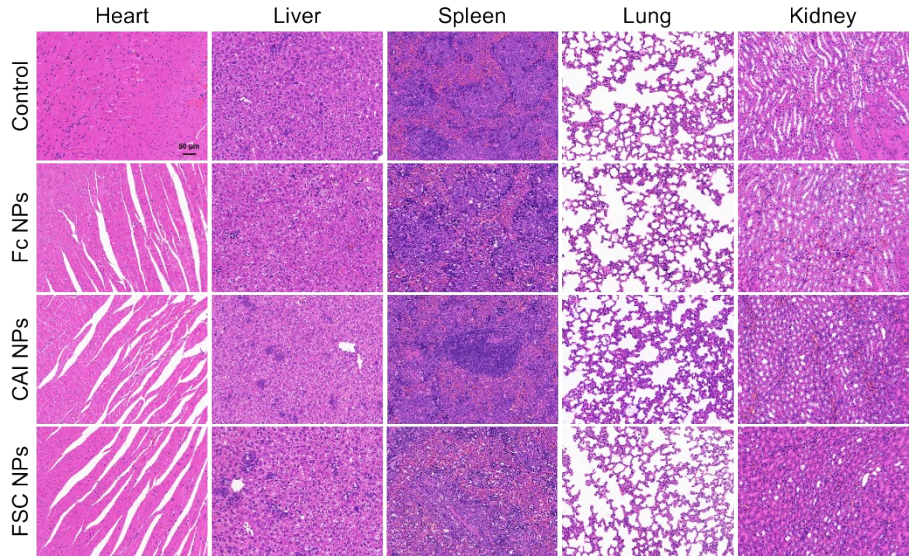


Fig. S24 The H&E staining images of main organs after different treatments.

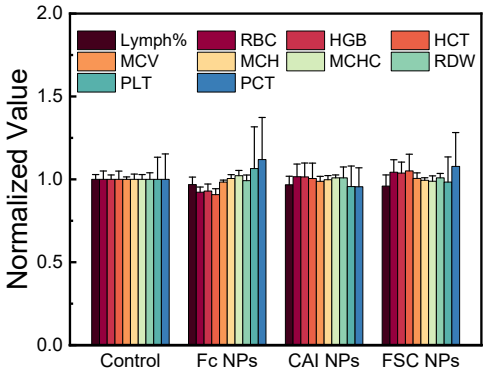


Fig. S25 The detection of routine blood.

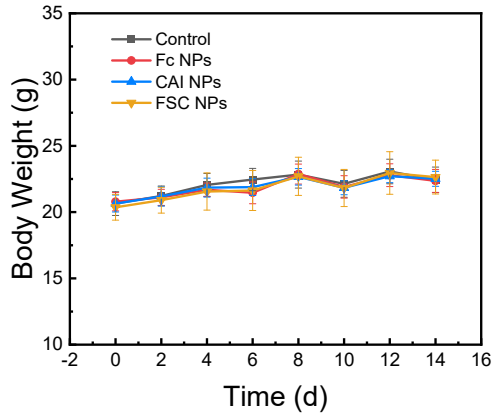


Fig. S26 Curves depicting changes in mice weight following various treatments.

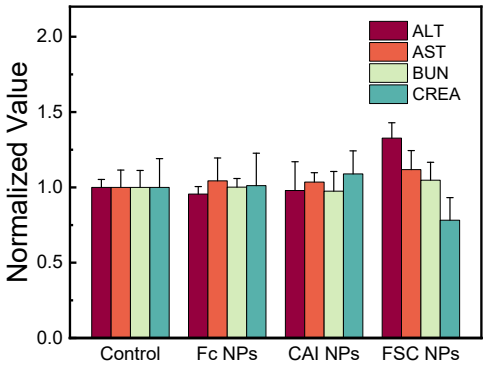


Fig. S27 The detection of several indicators related to liver and kidney function.