Electronic Supplementary Information

Intracellular self-aggregation of biomimetic Fe_3O_4 nanoparticles for enhanced ferroptosis-inducing therapy of breast cancer

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1. Materials and Methods

1.1 Materials

The $FeCl_3 \cdot 6H_2O$, Sodium acetate (NaAc), 1-ethyl-3-(3-Dimethylaminopropyl) carbodiimide HCl (EDC HCl) , Triethyla mine, Hydroxysuccinimide (NHS) , and ETH (Ethanolamine) were were purchased from Aladdin. Sulfasalazine (SAS)、1-Adamantanecarboxylic acid (ADA-COOH), and Carboxymethyl-β-cyclodextrin sodium salt (CD-COOH) were obtained from sigma-aldrich. Membrane and Cytosol Protein Extraction Kit, Phenylmethanesulfonyl fluoride (PMSF), DAPI Staining Solution, Reactive Oxygen Species Assay Kit, BCA Total Protein Concentration Assay Kit, and Reduced Glutathione Assay Kit were purchased from Beyotime. The 4T1, L02, and 293T cells were from Cell Bank, Chinese Academy of Sciences. RPMI-1640, DMEM medium, and Fetal bovine serum (FBS) was from Gibco. Inhibitor FMK, Necrostatin-1 (NEC), 3-Methyladenine (3-M), Ferrostatin-1 (Fer-1), Deferoxamine mesylate (DFO), Fluorescein Isothiocyanate (FITC), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Cyanine5 (Cy5) were obtained from MCE. GPX4 Rabbit monoclonal Antibody, GAPDH Rabbit monoclonal Antibody, and HRP-labeled Goat Anti-Rabbit Antibody were acquired from Zen-Bioscience.

1.2 Methods

The preparation of FeADA and FeCD

According to the reported method, Fe_3O_4 nanoparticals (NPs) were prepared by a simple solvothermal method[1]. Briefly, $FeCl_3 \cdot 6H_2O$ (1 g) was firstly dissolved in EG (20 mL), after which NaAc (3 g) and ETH (10 mL) were added slowly. After vigorous stirring for 30 min using a stirrer, the mixture was sealed in a teflonlined stainless-steel autoclave and the reaction was continued at 200 °C for 8 h. After cooling to room temperature, the black products were washed 3 times with deionized water and separated using a magnet. The final products Fe_3O_4 NPs were suspended in deionized water at 5 mg/mL. The preparation of ADA coated Fe_3O_4 NPs (FeADA) was referred to previous literature[2]. ADA-COOH (10 mg) was dissolved in DMSO containing

triethylamine (15 mg), then EDC HCl (15 mg) and NHS (10 mg) were added with stirring. After a sonication period of 0.5 h, Fe_3O_4 solution (2 mL) was added to the mixture and stirred overnight at room temperature, and FeADA NPs were obtained by centrifugation and water washing. For CD coated Fe_3O_4 NPs (FeCD) preparation, CD-COOH was dissolved in H_2O containing triethylamine, and the subsequent preparation process was the same as the above method.

The loading of SAS

The method of loading SAS was referred to the previous literature[3]. The Fe_3O_4 NPs (15 mg) were dispersed in 5 mL H_2O, sulfasalazine (SAS) solution in DMSO (0.1 mL) was added dropwise and stirred for 24 h at RT. Then the mixture (FeS) was obtained after magnet separation and several washing DMSO/H_2O (v/v, 1/49) solution. The above method also produced SAS-loaded FeADA NPs (FeAS) and SAS-loaded FeCD NPs (FeCS).

The preparation of FeASM and FeCSM

The method for extracting cancer cell membrane (CCM) has been referenced from previously published literature. 4T1 cells were cultured and the cell precipitate was collected by centrifugation after scraping with a cell scraper. After washing, the cell lysis buffer (containing 1% PMSF) was mixed with the cell precipitate in an ice bath for 15 min. Subsequently, the mixed solution was repeatedly freeze-thawed 3 times for complete cell lysis in liquid nitrogen and at room temperature. Afterwards, the cell precipitate was removed by centrifugation (700 g, 10 min, 4 °C), and the supernatant was centrifuged again to obtain the CCM precipitate (14000 g, 30 min, 4 °C). The resulting CCM precipitate was stored at -80 °C. To synthesize CCM-coated NPs, the prepared FeAS or FeCS (200 μg) was dispersed in 1 mL CCM suspension (obtained from 5×10^6 4T1 cells) and extruded 12 times using a micro-extruder. The resulting solution was then centrifuged (5000 g, 30 min) to obtain pure FeASM and FeCSM. Furthermore, the membrane protein was characterized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The presence of specific protein markers was

determined by western blotting analysis.

The release of SAS

The FeSM NPs (1 mg) was dispersed in PBS buffer (1 mL) at different pH values (5.5, 6.5, and 7.4) in a dialysis bag (MWCO = 8000 Da) suspended in the corresponding buffer medium (9 mL) and subjected to gentle shaking at 37 °C. At different time points, buffer medium (1 mL) was removed from each of the different samples, and UV-Vis spectra were measured at 359 nm, and then the same volume of buffer medium was replenished. The release behavior of bare FeS NPs in PBS buffer at pH 7.4 was used as a control. A standard SAS absorbance/concentration calibration curve was used to quantify cumulative SAS release.

Cell Culture

The 4T1 cells were regularly cultured in RPMI-1640 medium, while L02 and 293T were cultured in DMEM medium supplemented with 10% (v/v) FBS, and the above media were placed in an atmosphere of 5% CO_2 at 37℃.

***In vitro* cytotoxicity and biocompatibility assay**

To assess the cytotoxicity of different formulations, 5×10^3 4T1 cells per well were seeded in 96-well plates. After overnight incubation of the cells, fresh medium containing different formulations ([Fe] = 100 μg/mL for all Fe-related groups, and the concentration or free SAS corresponded to the Mix group) was added separately and incubated for 24 h. After discarding the medium, 100 μL of fresh medium containing MTT solution was added to each well of the plate. After incubation again for 4 h, the supernatant was removed and DMSO was added, and the killing effect of the different formulations on 4T1 cells was assessed by measuring the cell absorbance by a Microplate reader. Using the same method described above, we also evaluated the killing effect of samples with different concentrations in 4T1 cells. In addition, L02 and 293T cells were used to verify the toxicity of Mix group with different concentrations.

Cellular uptake and retention assays

The 4T1 cells were first inoculated at a density of 10^5/well in confocal dishes (35 mm) for 24 h. Then the Fe_3O_4, FeM, and Mix groups with an equivalent concentration of Fe (20 μg/mL) and FITC (2 μg/mL) were added to the confocal dishes and incubated for 8 h. Subsequently, the cells were washed 3 times with PBS, fixed with 4% paraformaldehyde for 15 min, and stained with DAPI for 10 min at 37 °C before CLSM observation. In order to study the intracellular retention, 4T1 cells were inoculated at a density of 1×10^5 cells in confocal dishes (35 mm) and then incubated with Fe_3O_4, FeM , and Mix groups containing an equivalent concentration of Fe_3O_4 (20 μg/mL) and FITC (2 μg/mL) for 8 h. Finally, 4T1 cells were cultured with fresh medium for another 12, 24 and 36 h. Then the cells were washed 3 times with PBS, fixed with 4% paraformaldehyde for 15 min and stained with DAPI for 10 min at 37 °C, and imaged through CLSM. In addition, the 4 T1 cells uptake versus other cell lines (MCF-7 cells, Hela cells, A549 cells) uptake of FeM (Cy5 2 μg/mL, Fe_3O_4 20 μg/mL) were studied by CLSM. The experimental protocol was consistent with the 4 T1 cell lines above.

Assessment of Ferroptosis

To investigate the cytotoxicity mechanism for cell death, 4T1 cells were treated by the Mix group (100 μg/mL) in the presence of apoptosis inhibitor FMK (10 μM), necroptosis inhibitor NEC (10 μM), autophagy inhibitor 3-M (10 μM), ferroptosis inhibitor Fer-1 (10 μM) , and iron chelator deferoxamine DFO (100 μM) for 24 h. Afterward, the cell viability was determined with MTT method.

The consumption of intracellular GSH

The 4T1 cells were cultured in 6-well plates at a density of 2×10^5 cells/well. After overnight incubation of the cells, fresh medium containing different formulations at a Fe concentration of 100 μg/mL was added separately and incubated for 24 h. Subsequently, the cells were washed 3 times with PBS. The cells were collected after digestion by trypsin and treated according to BCA Total Protein Concentration Assay Kit and Reduced Glutathione Assay Kit instructions. Glutathione and total BCA protein concentrations in each group were determined by Microplate reader.

The generation of intracellular ferrous ion

The 4T1 cells were seeded in 6-well plates at a density of 2×10^5 cells/well. After overnight incubation, fresh medium containing different formulations at a Fe concentration of 100 μg/mL was added separately and incubated for 12 h. After that, it was tested according to the ferrous ion content detection kit.

The generation of intracellular ROS

The 4T1 cells were seeded in 6-well plates at a density of 2×10^5 cells/well. After overnight incubation, 4T1 cells were treated with the different concentrations for 12 h. Subsequently, the cells were washed 3 times with PBS, and 10 μM of DCFH-DA was added to stain the cells for 20 min in the dark. Cells were then subjected to CLSM imaging after being washed 3 times with PBS.

Western Blot to detect GPX4 Expression

The 4T1 cells were cultured in 6-well plates at a density of 2×10^5 cells/well. After overnight incubation of the cells, fresh medium containing different formulations was added separately and incubated for 12 h. Afterwards, the culture medium was aspirated and the cells were scraped off with a cell scraper. The cell supernatant was collected at 12,000 rpm after being lysed with RIPA lysate, and the BCA protein assay kit was used to quantify the proteins. 10% SDS-PAGE was used to separate proteins. The gel was transferred to a PVDF membrane and closed with a sealing solution containing skimmed milk powder for 2 h, followed by incubation with anti-GPX4 antibody overnight at 4°C. The membranes were washed 3 times with TBST buffer and incubated again with HRP-labeled Goat Anti-Rabbit Antibody. The TBST buffer was clear again and the proteins were detected by an enhanced chemiluminescence system. Dose/time dependent western blot was performed using the corresponding method described above.

The generation of intracellular MDA

The 4T1 cells were seeded in 75 cm^2 dishs at a density of 2×10^6 cells/dish. After

overnight incubation, fresh medium containing different formulations at a Fe concentration of 100 μg/mL was added separately and incubated for 24 h. After that, it was tested according to the MDA content detection kit.

The generation of intracellular LPO

The 4T1 cells were cultured in confocal dishes at a density of 2×10^5 cells/dish. After overnight incubation of the cells, fresh medium containing different formulations was added separately and incubated for 12 h. The C11-BODIPY 581/591 probe diluted with serum-free medium was added and incubated for 20 min. Washed 3 times with PBS, fixed with paraformaldehyde, and finally dyed with DAPI. The contents of lipid peroxides in cells were observed by confocal laser microscopy after PBS cleaning.

Bio-distribution

Female BALB/c mice (4-6 weeks) were obtained from Hunan Slac Laboratory Animal Center (Hunan, China). All animal experiments were conducted and approved in accordance with the policies of Chongqing University Laboratory Animal Welfare Ethics Committee (No. CQU-IACUC-RE-202308-008). The 4T1 cells in the logarithmic growth phase were collected and resuspended in PBS to obtain a cell suspension with a cell concentration of approximately 2×10^7 cells/mL, and 100 μL of cell suspension was injected into the right hind leg of each mouse to establish the 4T1 tumor-bearing mice. When the tumor volume reached about 150 mm^3, the mice were randomly divided into three groups (n = 3 in each group), and the Cy5-labeled Fe_3O_4, FeM, and Mix groups were injected into tumor-bearing mice through the tail vein (Cy5 concentration: 3 mg/kg), and the biodistribution of fluorescence was measured by using an *in vivo* imaging system, as a means of tracking the distribution in each group and tumor targeting. Mice were executed after being injected for 24 h, while major organs and tumors including heart, liver, spleen, lungs, and kidneys were collected and washed with PBS. The fluorescence of all isolated organs was then measured using an imaging system. Afterwards, tumors were collected and iron content in different tumor groups was determined by ICP-MS.

In vivo anti-tumor study

The 4T1 tumor-bearing mice with a tumor volume of 100 mm^3 were randomly divided into 6 groups (n = 5 in each group) , including PBS、SAS、Fe_3O_4、FeS、FeSM, and Mix groups ([Fe] = 200 mg/mL for all Fe-related groups, and the concentration or free SAS corresponded to the Mix group). The different formulations were injected through the tail vein every 2 days, while changes in body weight and tumor size were recorded daily. Tumor-bearing mice were treated for 14 days, and tumor volume was estimated from tumor length L and tumor width W recorded by vernier caliper measurements. Tumor volume = $0.5 \times L \times W^2$. After treatment was completed at 14 days, tumor-bearing mice were sacrificed and their tumors were harvested, fixed, embedded in paraffin, and sectioned for and sectioned for hematoxylin and eosin (H&E) and TdT-mediated dUTP Nick-End Labeling (TUNEL) and Ki67 immunohistochemistr staining. GPX4 in tumors was detected by immunohistochemical staining. In addition, lipid peroxides (LPO) and reactive oxygen species (ROS) were detected by C11 BODIPY and DHE in tumor tissues.

Biosafety assessment of treatment

To evaluate the biosafety of nanomedicines during treatment, after tumor-bearing mice were treated for 14 days, the main organs (heart, liver, spleen, lungs, and kidneys) of mice in PBS、SAS、Fe_3O_4、FeS、FeSM, and Mix groups were collected, respectively. The organs were then sectioned for H&E staining to observe histological changes.

Healthy mice were randomly divided into 6 groups (n = 3 in each group) , including PBS、SAS、Fe_3O_4、FeS、FeSM, and Mix groups ([Fe] = 200 μg/mL for all Fe-related groups, and the concentration or free SAS corresponded to the Mix group). After 14 days of administration, blood samples were collected from mice by orbital blood sampling. A portion of the whole blood was collected into EDTA anticoagulated tubes for routine blood analysis, including white blood cell (WBC), red blood cell (RBC), lymphocyte (Lymph), and platelet (PLT). Another portion of the whole blood sample

were centrifuged and the supernatant was taken for blood biochemical analysis, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), uric acid (UA), and blood urea nitrogen (BUN).

Hemolysis testing

For hemolysis testing, fresh blood was taken from mice and placed in EP tubes containing sodium heparin and mixed thoroughly. The supernatant was centrifuged at 4500 rpm for 10 min to obtain the erythrocyte precipitate, which was washed 3 times and resuspended in PBS. A series of FeS, FeSM, and Mix groups suspensions with different concentrations (25 μg/mL, 50 μg/mL, 100 μg/mL, and 200 μg/mL) were prepared. The 200 μL of erythrocyte suspension was taken and mixed thoroughly with 200 μL of PBS, Triton X-100 and different concentrations of NPs suspension, respectively (n = 3 in each group). After being placed in a 37 ℃ water bath and incubated for 1 h, the supernatant was centrifuged at 10,000 rpm for 10 min, and the OD value at 570 nm was collected and determined and the hemolysis rate of each experimental group was calculated (Triton X-100 was used as a positive control, and PBS was used as a negative control).

Statistical Analysis

All experimental data were expressed as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 9. The results were statistically analyzed using t test. The data were indicated with (***) for $P < 0.05$, (**) for $P < 0.01$, and (***) for $P < 0.001$, respectively.

2. Characterization

The morphology of Fe_3O_4 NPs was observed by transmission electron microscopy (TEM, Talos F200S, Netherland) and scanning electron microscope (SEM, Quattro S, USA). The hydrodynamic radius and zeta potential were measured on a Zetasier (Malvern). Nanotrac Wave II Q Nanoparticle Size Analyzer, Microtrac. The load of SAS on Fe_3O_4 NPs was measured by the Ultraviolet and Visible spectrophotometry

(UV-Vis, Agilent Cary60, USA). The modification of ADA and CD was measured by Fourier Transform infrared spectroscopy (FT IR, Nicolet iS50, USA). The loading contents of ADA and CD were measured by Thermal-Gravimetric Analyzer (TGA, TGA2, Switzerland). MTT assays were measured using a microplate reader (SpectraMax i3x, USA). Fluorescence images were obtained by confocal laser scanning microscopy (CLSM, Leica TCS SP8, German). The distribution of nanomaterials in tumor cells was observed by Bio-Transmission Electron Microscopy (Bio-TEM, H7650, Japan). Biodistribution was obtained by *in vivo* imaging system (IVIS, Lumina III, USA). Fe content was measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, NexION 5000, Switzerland).

3. Supplementary Figures

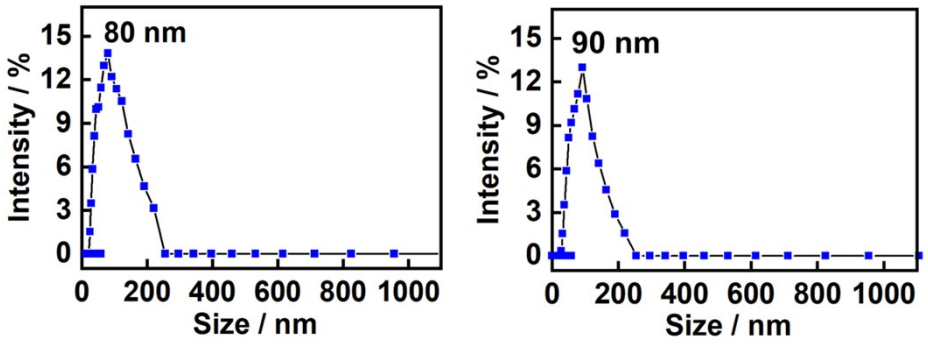

Fig. S1 Size distribution of Fe_3O_4 NPs (a) and FeM NPs (b).

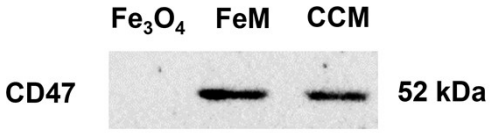

Fig. S2 Analysis of CD47 proteins in Fe_3O_4 NPs (a), FeM NPs (b), and CM (c) by western blotting.

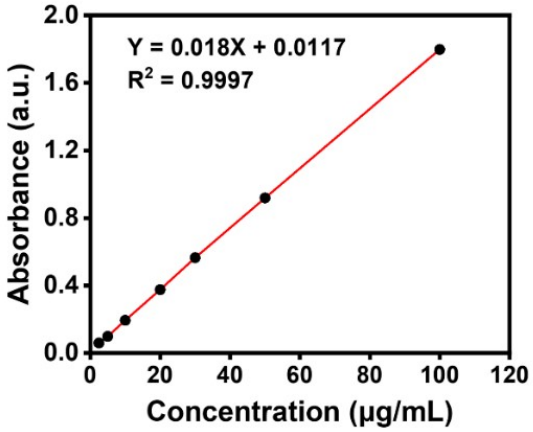

Fig. S3 The standard SAS absorption/concentration calibration curves.

Table. S1 The EE and LC of SAS in FeS, FeAS, and FeCS.

Sample	EE (%)	LC (%)
FeS	73.50	18.38
FeAS	69.43	17.36
FeCS	80.40	20.10

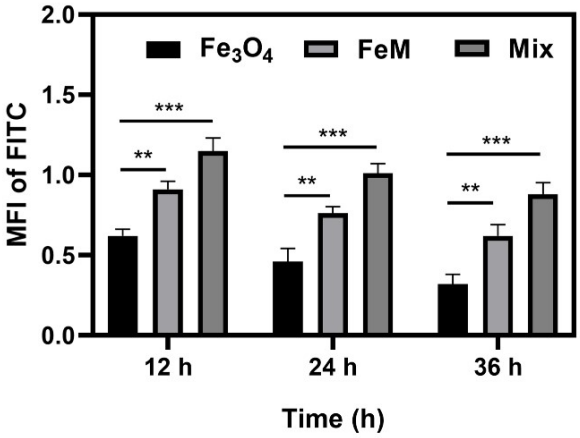

Fig. S4 The quantitative fluorescence intensities analysis of time-dependent uptake.

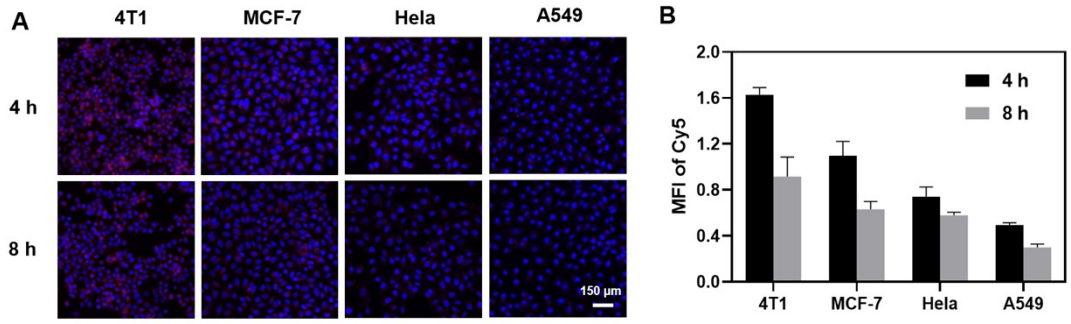

Fig. S5 (A) Cellular uptake of Cy5-labeled FeM in 4T1 cells and other three cell lines (MCF-7 cells, Hela cells and A549 cells). (B) Semi-quantitative analysis of intracellular Cy5 fluorescence intensity based on CLSM (n = 3).

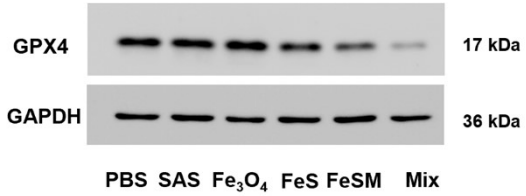

Fig. S6 Western blot analysis of GPX4 protein in 4T1 cells incubated with different treatments.

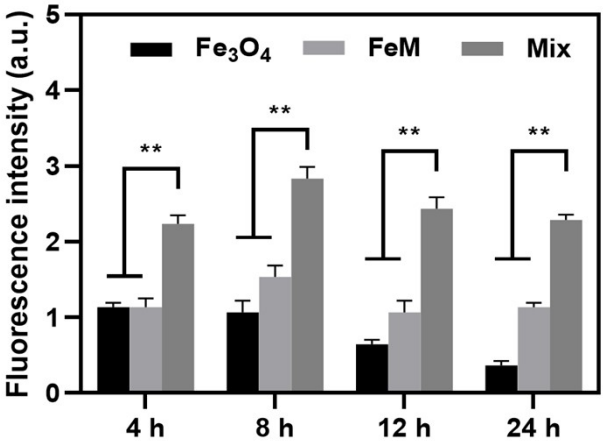

Fig. S7 Fluorescence intensity of tumor sites in 4T1 tumor-bearing mice was quantitatively analyzed at different time intervals (n = 3).

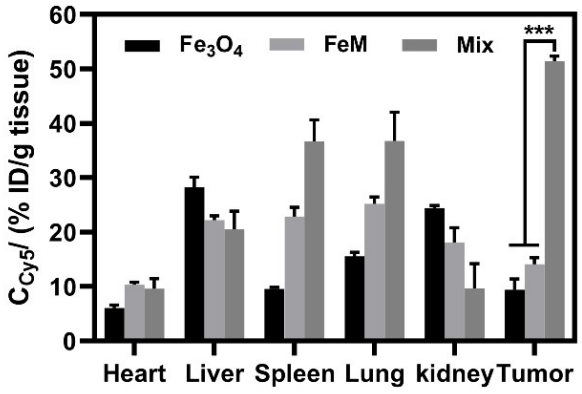

Fig. S8 The quantitative bio-distribution analysis of Cy5 in disparate organs and tumors after injection for 24 h harvested from all groups.

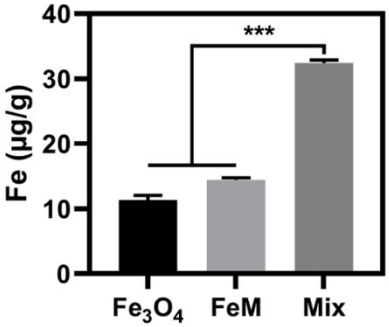

Fig. S9 The quantitative analysis of Fe content within different groups of tumors was determined by ICP-MS.

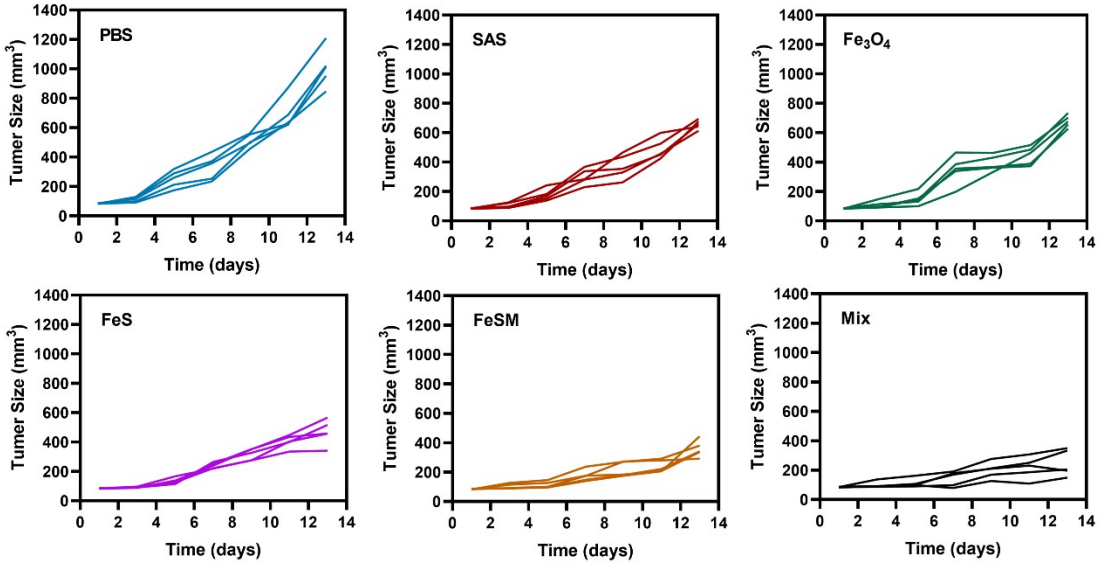

Fig. S10 The tumor volumes of mice in different groups until 14 days after treatment.

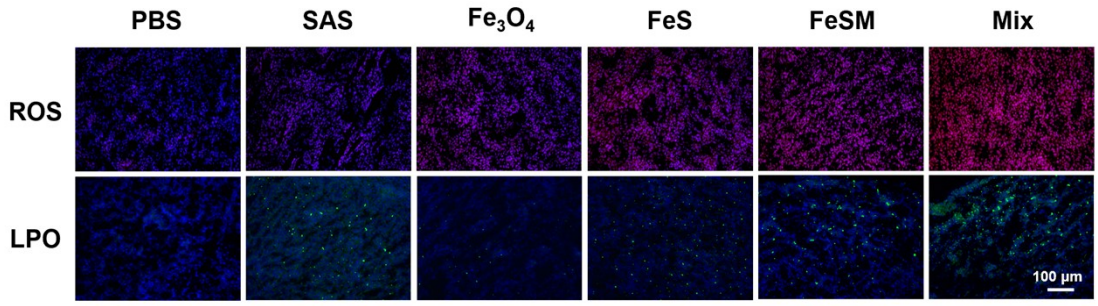

Fig. S11 Fluorescence images of tumor slices collected after different treatments and stained with ROS and LPO.

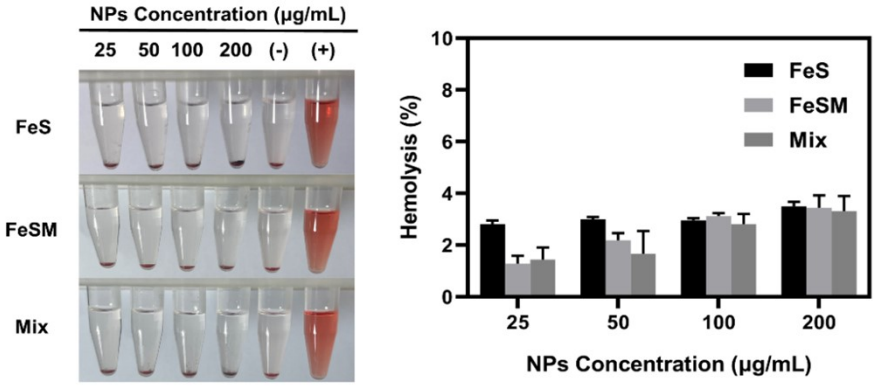

Fig. S12 The hemolysis ratio of FeS, FeSM, and Mix groups at different concentrations.

References

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