Electronic Supplementary Information

Zwitterionic polymer-coated magnetic nanoparticles induced chemotherapy and ferroptosis for triple-negative breast cancer therapy

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Supplementary Experimental Section

Materials

Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ethylene glycol (EG), ammonium acetate (NH_4OAc), dimethyl sulfoxide (DMSO), sodium dihydngen phosphate anhydrous (NaH_2PO_4), ammonium hydroxide aqueous solution, sodium phosphate dibasic anhydrous (Na_2HPO_4), acetonitrile, and anhydrous ethanol were purchased from Shanghai Chemical Reagents Company (China). 3-(Trimethoxysilyl)propyl methacrylate (MPS) was purchased from Sigma-Aldrich (China). 2,2′-Azobis(2-methylpropionitrile) (AIBN), polyglutamic acid (PGA, $M_W = 1 \times 10^6$ g/mol), and reduced glutathione (GSH) were purchased from Shanghai Aladdin Chemistry Company (China). N,N′-Bis(acryloyl)cystamine (BAC) was purchased from Alfa Aesar (China). N-(3-Sulfopropyl)-N-methacryloxyethyl-N,N-dimethylammonium betaine (SBMA) was purchased from Adamas-beta (China). Gambogenic acid (GNA) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (China). Ferrostatin-1 (Fer-1) and 3-methyladenine (3-MA) were purchased from Selleck Chemicals (USA). Necrostatin-1 (Nec-1), carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK), Annexin V-FITC/PI apoptosis kit, and antibody for Bcl-2 were purchased from Beyotime Institute of Biotechnology (China). Deferoxamine (DFO) mesylate was purchased from Target Molecule Corp (USA). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, trypsin-ethylene diamine tetra acetic acid (Trypsin-EDTA, 0.05%), Cell Counting Kit-8 (CCK-8), and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from

Keygen Biotech Company (Nanjing, China). FerroOrange was purchased from Dojindo Molecular Technologies Company (Japan). Fetal bovine serum (FBS) was purchased from Life Science (Gibco^{TM}, Pittsburg, USA). C11-BODIPY lipid peroxidation sensor was purchased from Life Science (Invitrogen^{TM}, Pittsburg, USA). Anti-glutathione peroxidase 4 antibody, anti-vinculin, and anti-GAPDH were purchased from Abcam (Cambridge, UK). Antibodies for Bax, AKT, p-AKT, mTOR, and p-mTOR were purchased from Cell Signaling Technology, Inc (MA, USA). Antibody for PI3K was purchased from Proteintech Group, Inc (USA). Deionized water was utilized in the experiments.

Characterization

The morphology of nanoparticles was attained by transmission electron microscope (TEM, Tecnai G2 20 TWIN). The hydrodynamic diameter was measured by dynamic light scattering (DLS, Zetasizer Nano ZS90 analyzer). Fourier transform infrared (FT-IR) spectra were recorded *via* FT-IR spectrometer (Thermofisher Nicolet 6700). Magnetic characterization curves were tested by Quantum Vibrating Sample Magnetometer (VSM) at 300 K. Ultraviolet spectrophotometer spectra were obtained *via* PerkinElmer Lambda 750 spectrophotometer. The absorbance of cells was measured by microplate reader (Epoch 2 Microplate Spectrophotometer, BioTek Instruments, Inc.). Flow cytometry analysis was attained by flow cytometer (Beckman Coulter Gallios). Confocal laser scanning microscopy (CLSM) images were obtained by Nikon C2+ laser scanning confocal microscope. The metal ion concentration was recorded by P-4010 inductively

coupled plasma-atomic emission spectrometry (ICP-AES).

Preparation of Fe_3O_4 nanoparticles

Fe_3O_4 nanoparticles were attained *via* improved solvothermal method. $FeCl_3 \cdot 6H_2O$ (1.08 g, 4 mmol), PGA (0.5 g), and NH_4OAc (3.85 g, 50 mmol) were dispersed in 70 mL of ethylene glycol and stirred at 160 ℃ for 60 min. Then the solution was transferred into a Teflon lined stainless-steel autoclave with a continuous reaction at 200 ℃ for 12 h. After cooling to room temperature, the final product was washed with ethanol and deionized water alternatively for three times and separated by magnet. The obtained Fe_3O_4 nanoparticles were redispersed in deionized water at 4 ℃ for further use.

Degradation of Fe_3O_4

To explore the degradation of Fe_3O_4, 2 mL of Fe_3O_4 dispersion was suspended in the 1.4×10^4 Da dialysis bag in 200 mL of phosphate buffered saline (PBS) (pH 5.0) with 10 mmol/L of GSH using an incubator shaker at 37 ℃. 2 mL of release medium was collected at predetermined time points, and 2 mL of fresh buffer solution with corresponding pH value and GSH concentration was added. The concentration of Fe in the supernatant taken out was measured by ICP-AES. Each experiment was repeated in three times.

Drug release behavior of Fe_3O_4@PSBMA-GNA

The drug release behavior of Fe_3O_4@PSBMA-GNA was assessed *in vitro* using an

incubator shaker at 37 ℃. 2 mL of Fe_3O_4@PSBMA-GNA dispersion was immersed in the molecular weight cutoff 1.4×10^4 Da dialysis bags in 200 mL of PBS (pH 5.0, 7.4) with different concentrations of GSH (0 mM, 10 mM), respectively. After extracting 2 mL of supernatant at the scheduled time points, the same volume of fresh buffer solution was replenished. The cumulative release of GNA was calculated based on the concentration of the supernatant measured by UV-vis spectrometry. Each experiment was repeated three times.

Cell culture

Mouse fibroblast cell line (L929 cells, normal cells), human triple-negative breast cancer cell lines (MDA-MB-231 cells and MDA-MB-453 cells), and mouse triple-negative breast cancer cell line (4T1 cells) were purchased from the Cell Bank of the Chinese Academy of Sciences. L929 and MDA-MB-231 cells were cultured in DMEM containing 10% (v/v) FBS and 1% antibiotics (penicillin/streptomycin, 100 U/mL). MDA-MB-453 and 4T1 cells were cultured in RPMI-1640 medium containing 10% (v/v) FBS and 1% antibiotics (penicillin/streptomycin, 100 U/mL). All cells were incubated at 37 ℃ in 5% CO_2 atmosphere.

Cell uptake assay

MDA-MB-231 cells were cultured on the confocal dishes for 12 h. Subsequently, Fe_3O_4@PSBMA (50 μg/mL), GNA (5 μg/mL), Fe_3O_4-GNA (50 μg/mL), and Fe_3O_4@PSBMA-GNA (50 μg/mL) were added into dishes, respectively. Untreated cells

were considered as the control group. After 6 h of incubation and washing with PBS for three times, FerroOrange fluorescent probe (1 μM), as a ferrous ion fluorescence probe, was co-incubated for 30 min at 37 ℃. Ultimately, confocal laser scanning microscopy (CLSM) was performed for the fluorescence imaging of cells (Ex: 543 nm, Em: 580 nm).

Transcriptomic analyses

RNA-sequencing (RNA-seq)-based transcriptomic analyses were performed on MDA-MB-231 cells. Firstly, MDA-MB-231 cells were cultured on 6-well plates for 12 h. Fe_3O_4@PSBMA-GNA (50 μg/mL) was then incubated with cells for 12 h. Untreated cells were considered as the control group. The cells were harvested, and total RNA was extracted by Trizol reagent (Life Technologies, USA) and purified. Each group included three biological replicates. The quality control, library construction, RNA-seq, and bioinformatics analysis were performed in BGI (Beijing Genomics Institute). RNA-seq was performed using the MGISEQ-2000 sequencer. The raw data were filtered through a SOAP-nuke filter. All the analyses were conducted with the online bioinformatics platform Dr. Tom (http://biosys.bgi.com) provided by BGI.

Western blotting assay

The effect of nanoparticles on the expression of ferroptosis-related and apoptosis-related proteins was investigated by western blotting assay. First of all, MDA-MB-231 cells were treated with Fe_3O_4@PSBMA (50 μg/mL), GNA (5 μg/mL), Fe_3O_4-GNA (50 μg/mL), and Fe_3O_4@PSBMA-GNA (50 μg/mL) for 12 h, respectively. Subsequently,

cells were washed with PBS, lysed on cell lysis buffer supplemented with phenylmethanesulfonylfluoride (PMSF) on the ice bath, collected, and centrifuged for 15 min (4 ℃, 12000 rpm). The protein concentration in the supernatant of each group was determined by a protein quantification kit (BCA Assay). Next, the protein samples were loaded to the polyacrylamide gels for separation, and then transferred to the polyvinylidene difluoride (PVDF) membranes. The membranes were blocked, incubated with the anti-GPX4 antibody (1:1000) at 4 ℃ overnight, washed three times with tris buffered saline with Tween (TBST), and incubated with the horseradish peroxidase-coupled secondary antibody for 1 h subsequently. After washing, the enhanced chemiluminescence system was used to detect the protein band. Similarly, the expression of XcT, Bcl-2, Bax, p-mTOR, m-TOR, PI3K, p-AKT, and AKT proteins was detected by the same steps as described above.

Animal model

BALB/c female nude mice (4-5 weeks old) and ICR female mice (5-6 weeks old) were purchased from GemPharmatech (Nanjing, China). The animal raising conditions and experiments were strictly in accordance with the Guide for the Care and Use of Laboratory Animals of Fudan University, and complied with the protocols approved by the Animal Ethics Committee of Fudan University.

Safety estimation

To assess the safety of nanoparticles *in vivo*, ICR mice were randomly divided into

six groups (n = 4) and injected intravenously with PBS, Fe_3O_4, and Fe_3O_4@PSBMA (2 mg/mL). After the 7-day and 16-day treatments, the blood biochemical and blood routine indexes of blood samples collected from each mouse were evaluated.

Statistical analysis

The data were presented as mean ± standard deviation. Unpaired student's t-test was used to evaluate statistically significant differences between two groups. Statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

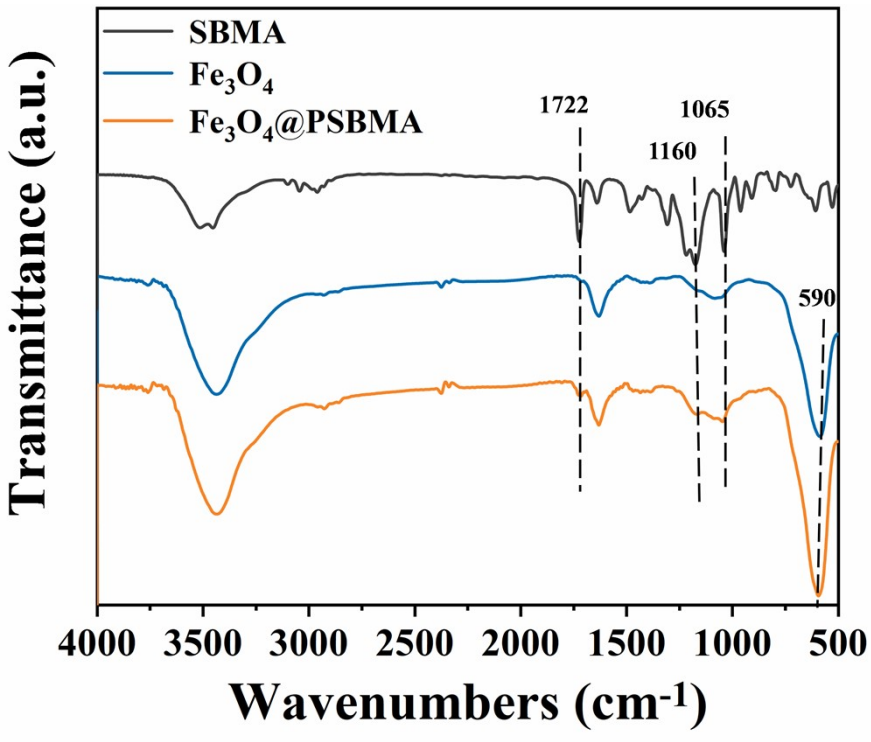

Fig. S1 FT-IR spectra of SBMA, Fe_3O_4, and Fe_3O_4@PSBMA.

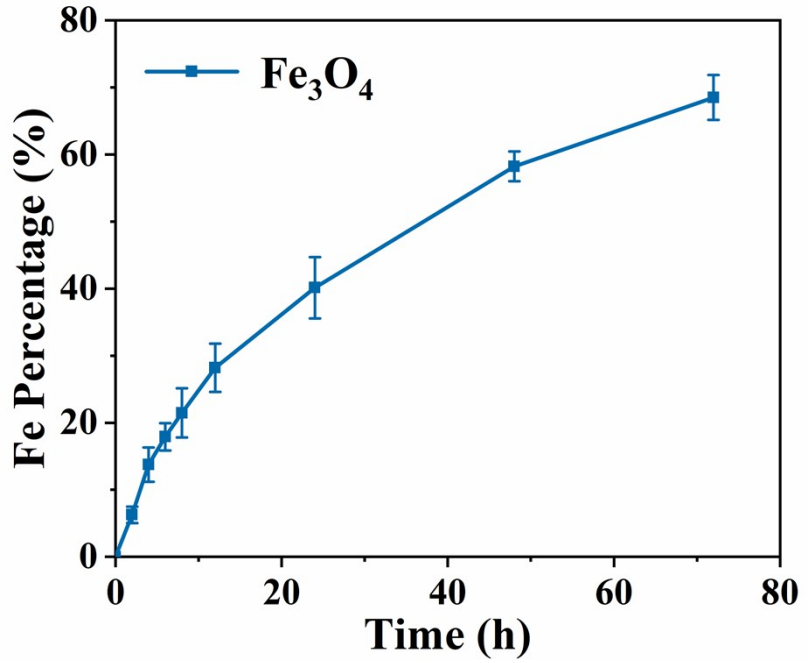

Fig. S2 The accumulated release of Fe from Fe_3O_4 in pH 5.0 PBS with 10 mmol/L of GSH; n = 3.

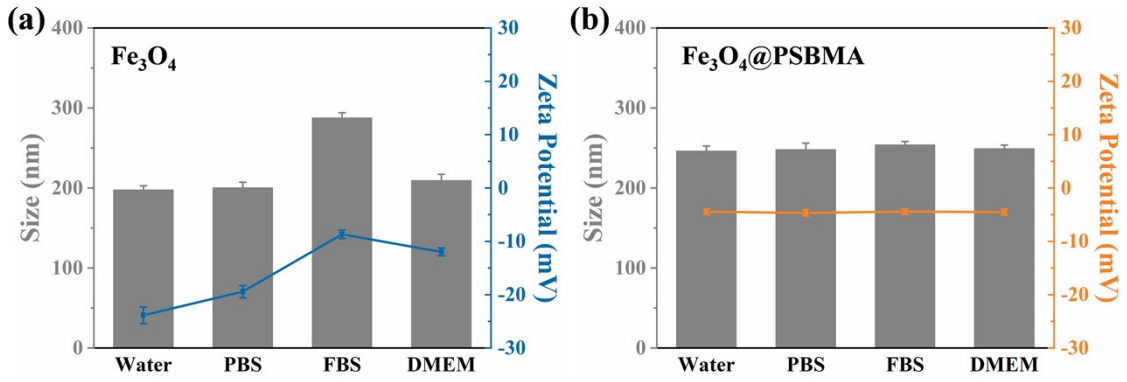

Fig. S3 The size and zeta potential of (a) Fe_3O_4 and (b) Fe_3O_4@PSBMA dispersed in water, PBS, FBS, and DMEM, respectively; n = 3.

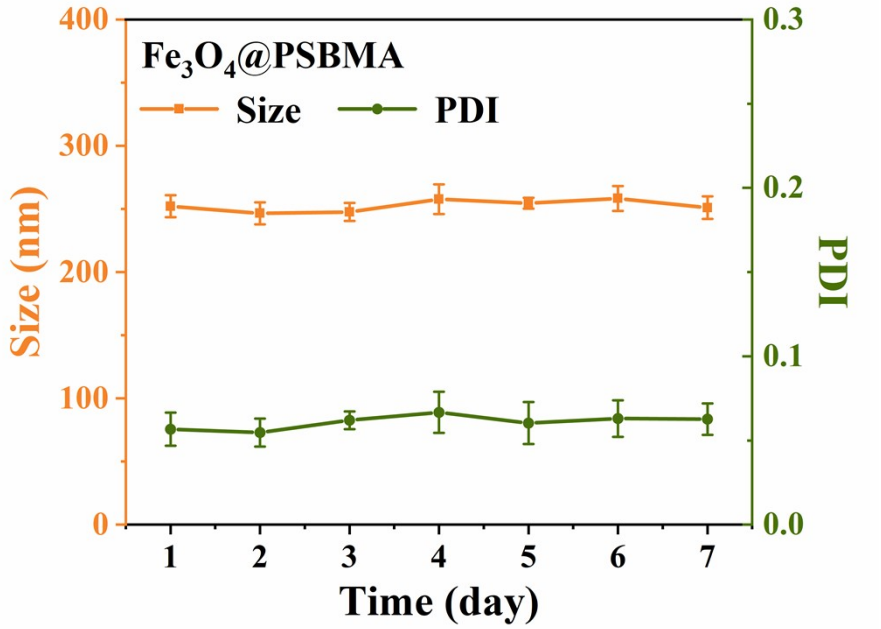

Fig. S4 Changes of size of Fe_3O_4@PSBMA within 7 days; n = 3.

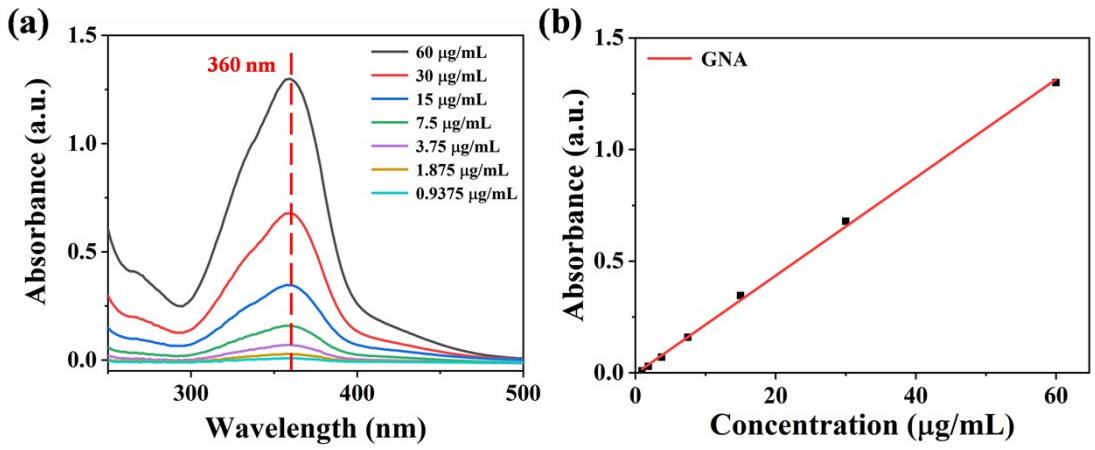

Fig. S5 (a) UV-vis spectra of GNA at various concentrations. (b) Calibration curve of GNA at 360 nm.

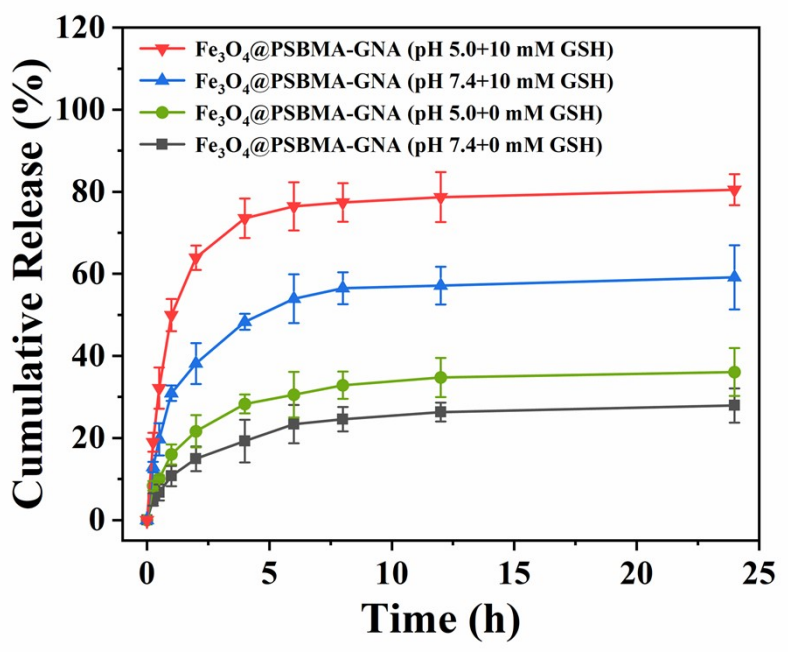

Fig. S6 Drug release of GNA from Fe_3O_4@PSBMA-GNA under different conditions, respectively; n = 3.

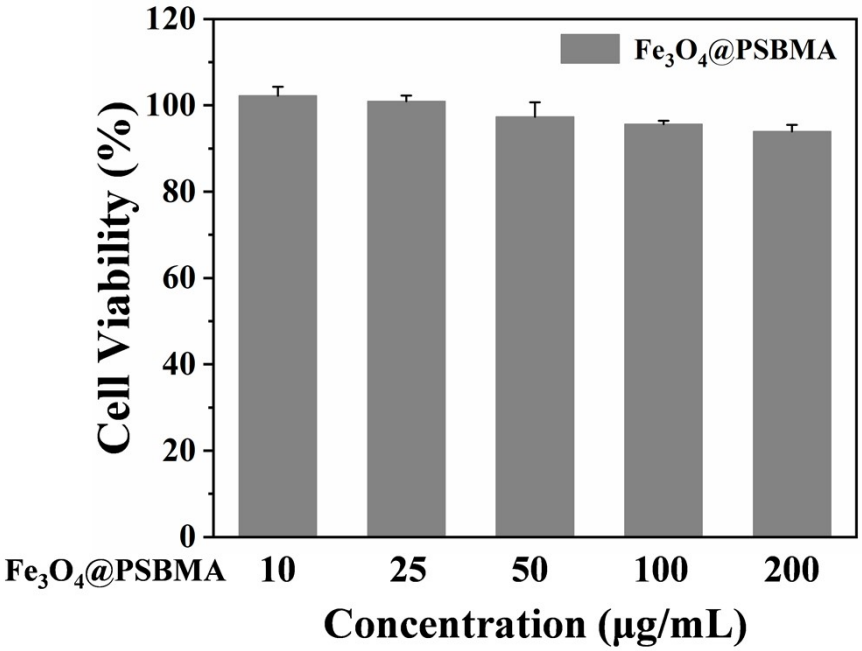

Fig. S7 Cell viability of L929 cells treated with different concentrations of Fe_3O_4@PSBMA; n = 6.

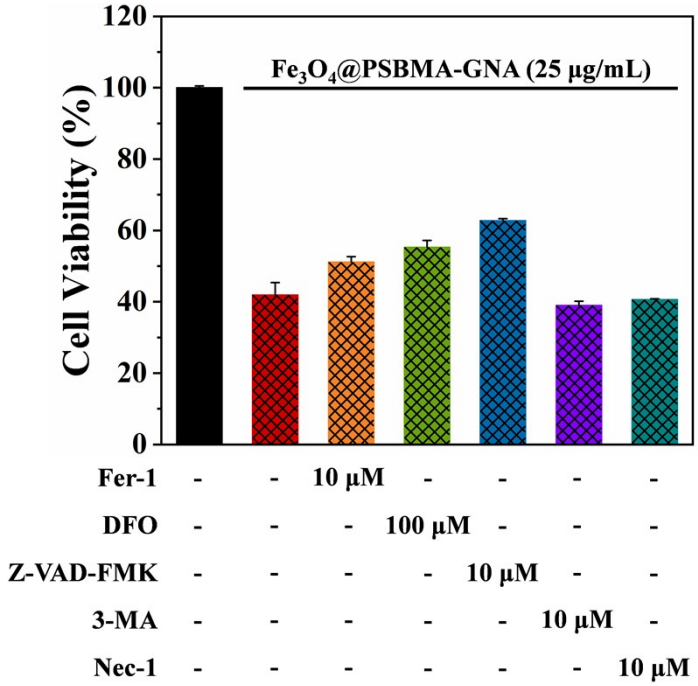

Fig. S8 Cell viability of MDA-MB-231 cells treated with Fe_3O_4@PSBMA-GNA (25 μg/mL) in the presence of Fer-1, DFO, Z-VAD-FMK, 3-MA, and Nec-1, respectively; n = 6.

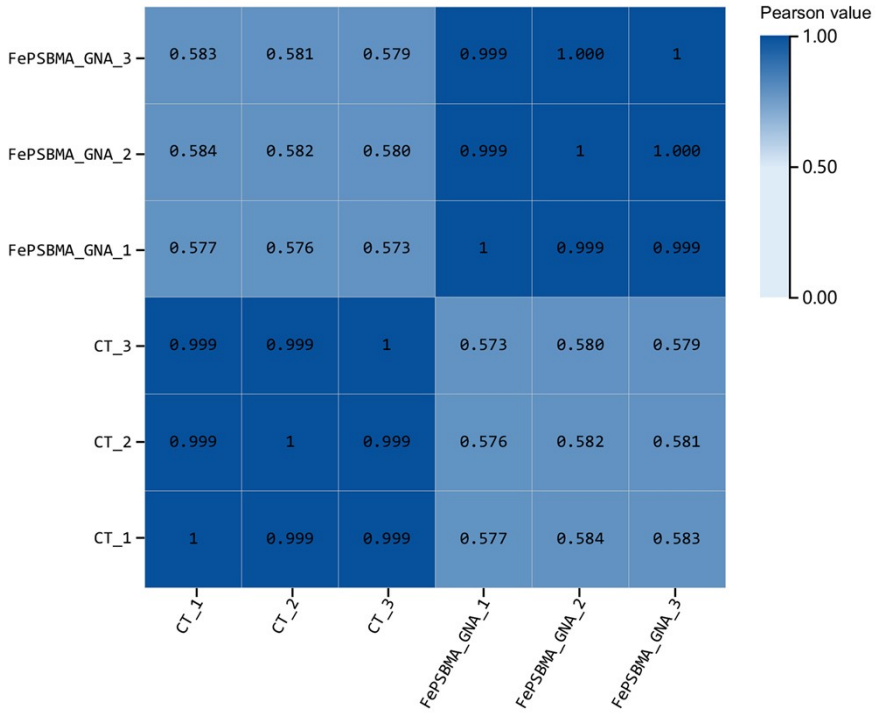

Fig. S9 The correlation between samples in the control and Fe_3O_4@PSBMA-GNA groups. CT: control, FePSBMA_GNA: Fe_3O_4@PSBMA-GNA.

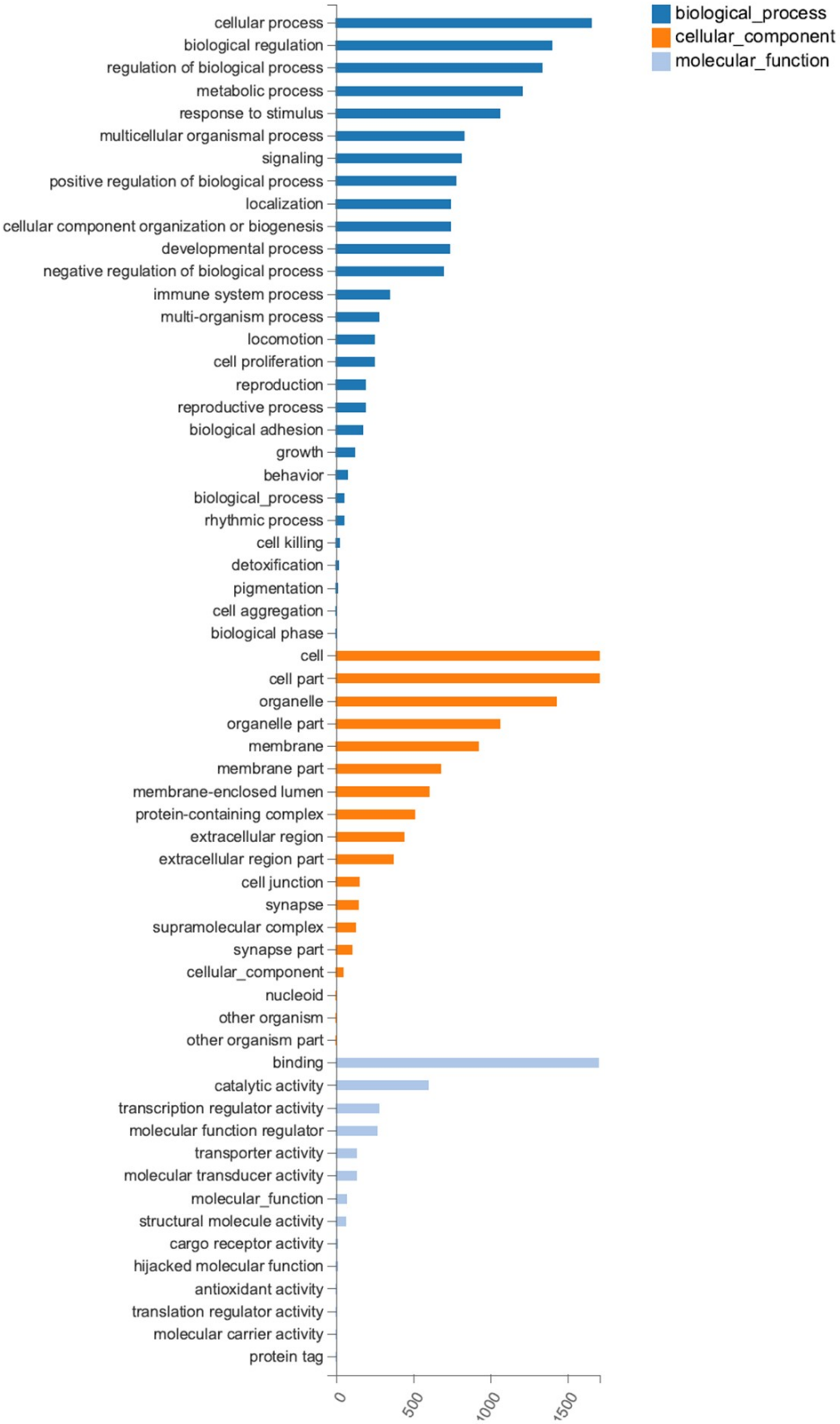

Fig. S10 GO enrichment analysis of DEGs after Fe_3O_4@PSBMA-GNA treatment.

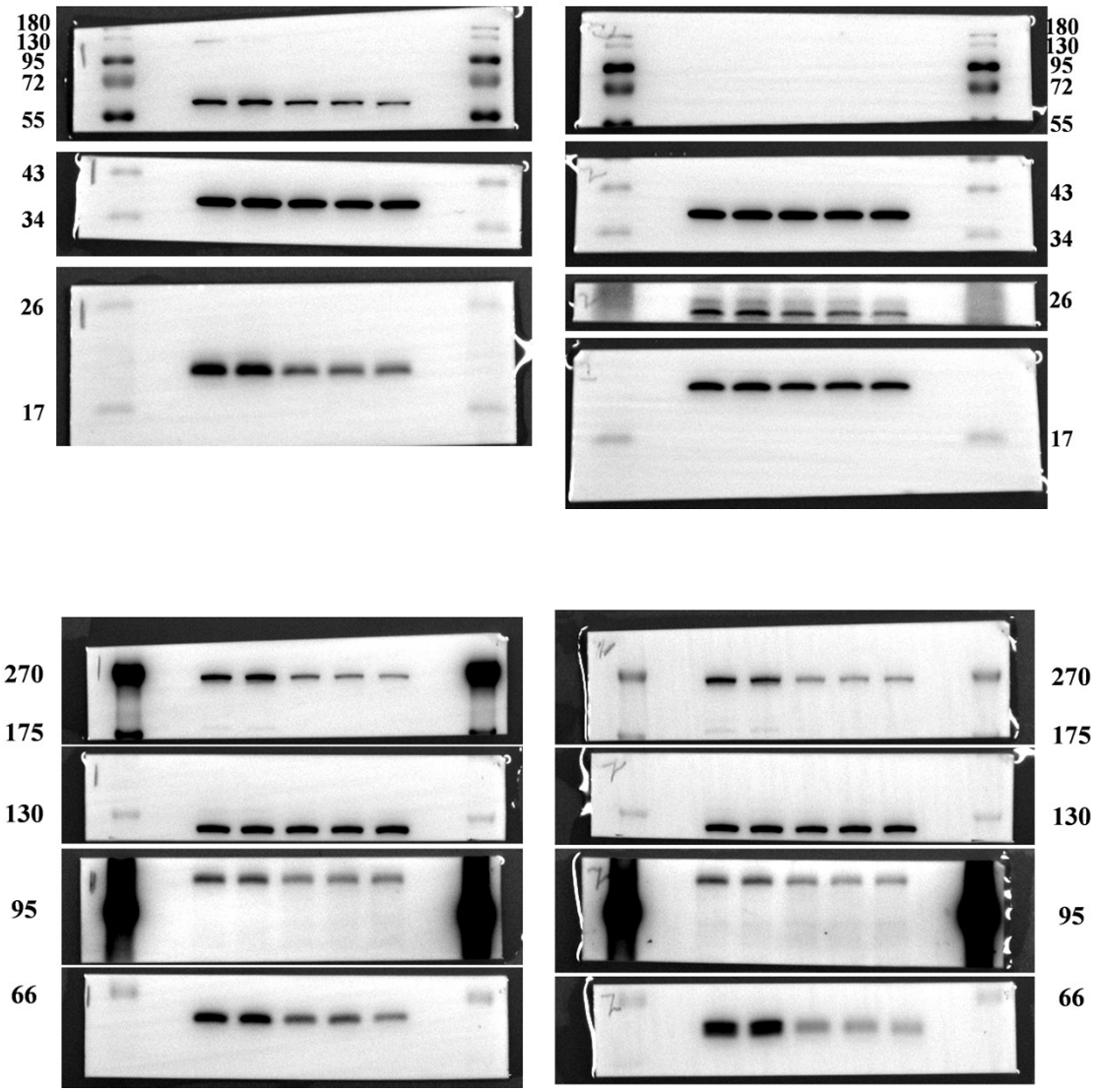

Fig. S11 Intracellular expression of XcT, GPX4, Bcl-2, Bax, p-mTOR, mTOR, PI3K, p-AKT, and AKT proteins of MDA-MB-231 cells after different treatments. Untreated MDA-MB-231 cells were taken as control.

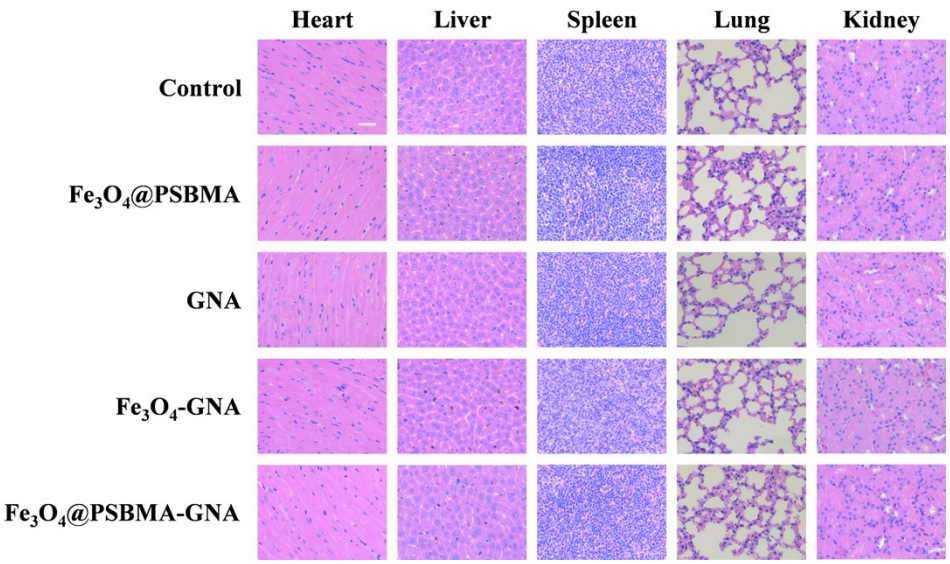

Fig. S12 H&E staining images of major organ (heart, liver, spleen, lung, and kidney) tissues dissected from each group on the 16th day after different treatments. Scale bar: 50 μm.

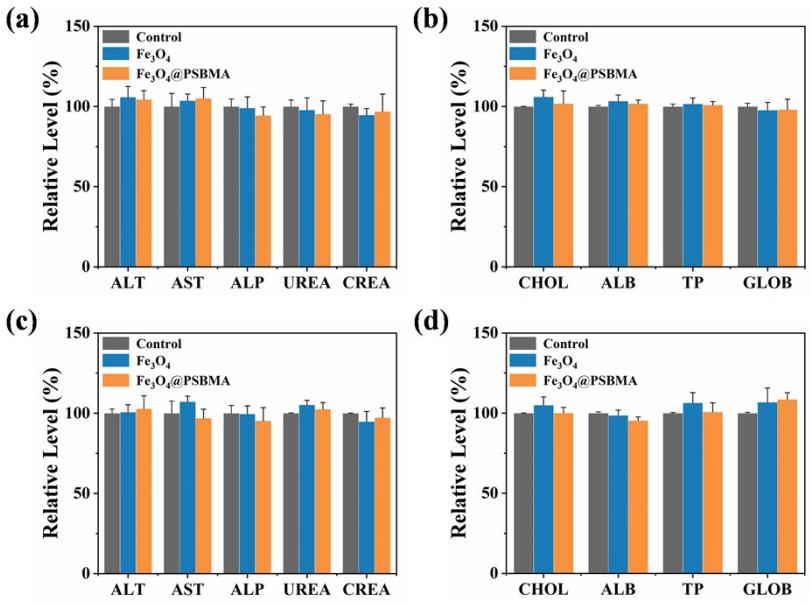

Fig. S13 Blood biochemistry indices after intravenous injection (a, b) 7 days and (c, d) 16 days, including the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), UREA, creatinine (CREA), cholesterol (CHOL), albumin (ALB), total protein (TP), and globulin (GLOB); n = 4.

Table S1 Statistics of RNA-seq for the control and Fe_3O_4@PSBMA-GNA-treatment groups.

Sample		Total Clean Reads (Million)	Total Mapping (%)
Control	CT_1	23.72	92.80
	CT_2	22.80	92.58
	CT_3	23.72	92.66
Fe_3O_4@PSBMA-GNA	FePSBMA_GNA_1	23.72	93.79
	FePSBMA_GNA_2	23.73	94.11
	FePSBMA_GNA_3	23.72	94.00

Table S2 Whole blood panel analysis after intravenous injection of Fe_3O_4 and Fe_3O_4@PSBMA nanoparticles after 7 days. Mice treated with PBS were used as control. Data are the means ± SD; n = 4.

	Unit	Control	Fe_3O_4	Fe_3O_4@PSBMA
WBC	$\times 10^9$/L	6.4±0.7	6.6±0.9	5.7±1.2
RBC	$\times 10^{12}$/L	9.2±0.2	8.9±0.5	9.0±0.7
HGB	g/L	161±5	161±7	157±5
HCT	%	50.9±1.2	48.4±2.7	50.7±3.8
MCV	fL	50.1±0.4	49.7±0.3	49.9±0.5
MCH	pg	15.8±0.4	16.2±0.2	15.7±0.7
MCHC	g/L	316±8	333±4	316±9
RDW	%	13.8±0.5	14.0±0.3	13.5±0.2
PLT	$\times 10^9$/L	1685±82	1713±97	1771±110

Table S3 Whole blood panel analysis after intravenous injection of Fe_3O_4 and Fe_3O_4@PSBMA nanoparticles after 16 days. Mice treated with PBS were used as control. Data are the means ± SD; n = 4.

	Unit	Control	Fe_3O_4	Fe_3O_4@PSBMA
WBC	$\times 10^9$/L	5.6±0.3	6.0±0.6	5.1±0.2
RBC	$\times 10^{12}$/L	9.2±0.3	8.6±0.4	8.5±0.6
HGB	g/L	160±6	152±4	151±4
HCT	%	51.4±1.0	47.3±2.1	47.5±2.7
MCV	fL	50.3±0.3	49.3±0.1	50.4±0.7
MCH	pg	15.6±0.4	15.8±0.3	15.9±0.7
MCHC	g/L	310±8	321±7	317±10
RDW	%	13.1±0.6	13.3±0.4	13.8±0.3
PLT	$\times 10^9$/L	1617±63	1569±78	1631±108