Electronic Supplementary Information

Zwitterion-coated ultrasmall iron oxide nanoparticles for enhanced T_1-weighted magnetic resonance imaging applications†

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Experimental Section:

Materials

Diethylene glycol and L-cysteine (Cys) were obtained from Sigma-Aldrich (St. Louis, MO). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), and *N*-hydroxysuccinimide (NHS) were from GL Biochem. (Shanghai, China). Polyethylene glycol (PEG) with one amine end group and the other maleimide end group (NH_2-PEG-Mal, Mw = 2,000) and methoxy PEG amine (*m*PEG-NH_2, Mw = 2,000) were from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). HeLa cells (a human cervical carcinoma cell line), Raw264.7 cells (a mouse macrophage cell line), and L929 cells (a mouse fibroblast cell line) were from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and pancreatin were obtained from Gibco (New York, NY). Iron (III) chloride hexahydrate, trisodium citrate dihydrate, sodium acetate anhydrous and other agents were from Sinopharm Chemical Reagent Ltd. (Shanghai, China). Regenerated cellulose dialysis membranes (molecular weight cut-off, MWCO = 8,000) were obtained from Yuanye Biological Technology Ltd. (Shanghai, China). A Mi1li-Q Plus 185 water purification system (Millipore, Bedford, MA) was employed to prepare ultrapure water with resistivity higher than 18.2 MΩ.cm.

Synthesis of ultrasmall citrate-stabilized Fe_3O_4 NPs

Citrate-stabilized ultrasmall Fe_3O_4 NPs were prepared according to the literature.[1, 2] In brief, $FeCl_3.6H_2O$ (1081 mg) was dissolved in 40 mL of diethylene glycol (DEG) under vigorous stirring. Then, $Na_3Cit.2H_2O$ (471 mg) was added to the above solution and the mixture was heated to 80 °C in water bath for 2 h. After that, anhydrous sodium acetate (1312 mg) was added into the above mixture solution under stirring to form a clear solution. The above mixture was transferred to a Teflon-lined stainless-steel reaction kettle with a volume of 100 mL. The reaction kettle was heated to 200 °C for 5 h. After the reaction mixture was cooled down to room temperature, black product was collected by centrifugation (85 00 rpm, 15 min) and the product was washed with anhydrous ethanol for four times.

The sediment was dried in a vacuum oven (50 °C) for further use.

Synthesis of the Fe_3O_4-PEG-Cys NPs

The citric acid carboxyl groups of Fe_3O_4 NPs (58 mg, in 8 mL deionized water) were activated by EDC (215 mg, in 2 mL water) and NHS (87 mg, 1 mL water) for 3 h. Then NH_2-PEG-Mal (12 mg, in 1 mL water) was fed into the aforesaid solution. This mixture was kept stirring at room temperature for 3 days. Afterwards, the product was depurated by dialysis against phosphate buffered saline (PBS, 3 times, 2 L) and water (6 times, 2 L) for 3 days. The dialysis membrane (MWCO = 8,000) was used to exclude the excess reactants and byproducts. Fe_3O_4-PEG-Mal NPs were obtained by lyophilization.

A water solution of the Cys (7 mg, 2 mL) was added into the solution of Fe_3O_4-PEG-Mal (70 mg, 10 mL in water) under vigorous stirring for 72 h at room temperature. Finally, the reaction mixture was dialyzed against PBS (3 times, 2 L) and water (6 times, 2 L) for 3 days using a dialysis membrane (MWCO = 8,000), followed by lyophilization to obtain the Fe_3O_4-PEG-Cys NPs. For comparison, Fe_3O_4 NPs was conjugated with *m*PEG-NH_2 under the same conditions to synthetize the Fe_3O_4-*m*PEG NPs.

Characterization Techniques

Zeta potential and dynamic light scattering (DLS) measurements were conducted using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) equipped with a standard 633 nm laser. Transmission electron microscopy (TEM) was used to observe the morphology of the NPs at an operating voltage of 200 kV. Samples were prepared by depositing a suspension of the particles onto carbon-coated copper grid and air-dried before measurements. X-ray diffraction (XRD) analysis was carried out using a D/max 2550 VB+/PC X-ray diffractometer (Rigaku Cop., Tokyo, Japan) with Cu Kα radiation (λ = 0.154056 nm) at 40 kV and 200 mA and a 2θ scan range of 5-90°. Fourier transform infrared (FTIR) spectra were recorded on a Nicolet 6700 FTIR spectrophotometer (Thermo Electron Corporation, Madison, WI). Samples were mixed with milled KBr crystals and pressed to form 13-mm diameter disks before measurements. A TG 209 F1 (NETZSCH Instruments Co., Ltd., Bavaria, Germany) thermogravimetric analyzer was used for thermogravimetric analysis (TGA) of the samples under nitrogen atmosphere in a temperature range of 10-900 ^oC. Magnetization curves were measured

on an LH-3 vibrating sample magnetometer (VSM, Nanjing, China) at room temperature. UV-vis spectrophotometer was carried out using a Lambda 25 UV-vis spectrophotometer (Perkin Elmer, Boston, MA). The influence of pH and protein on the stability of the NPs were examined by DLS and zeta potential measurements. Fe concentrations of the NPs in solutions were analyzed using a Leeman Prodigy inductively coupled plasma-optical emission spectroscopy (ICP-OES, Hudson, NH). T_1 relaxometry was performed using a 0.5 T NMI20 Analyzing and Imaging system (Shanghai NIUMAG Corporation, Shanghai, China). The parameters were set as follows: TR = 400 ms, TE = 20 ms, resolution = 156 mm × 156 mm, section thickness = 0.5 mm. The r_1 relaxivity was obtained through linear fitting of the inverse T_1 relaxation time ($1/T_1$) as a function of Fe concentration.

Protein Resistance Evaluation

Bovine serum albumin (BSA, 1 mg/mL, in PBS) was incubated with Fe_3O_4-PEG-Cys NPs or Fe_3O_4-*m*PEG NPs ([Fe] = 0-1000 μg/mL) for 2 h, respectively. Then the mixture was centrifuged at 8000 rpm for 5 min. The UV-vis absorbance of BSA solution before incubation of the particles and after incubation of the particles and successive centrifugation was measured. The reduced absorbance at 278 nm (characteristic absorption peak of BSA) representing the protein absorption amount was recorded. To simulate the complex environment *in vivo*, the protein absorption of the Fe_3O_4-*m*PEG or Fe_3O_4-PEG-Cys NPs was also detected in 50% fetal bovine serum (FBS) solutions according to the above-mentioned method.

Cytotoxicity Assay and Cell Morphology Observation

The cytotoxicity of Fe_3O_4-*m*PEG and Fe_3O_4-PEG-Cys NPs was evaluated by CCK-8 assay of L929 cells. The cells were seeded into a 96-well plate (1×10^4 per well) with DMEM supplemented with 10% FBS, 1% penicillin and 1% streptomycin. After incubated overnight, the medium was replaced with fresh medium containing Fe_3O_4-*m*PEG or Fe_3O_4-PEG-Cys NPs with different Fe concentrations (0-100 μg/mL). After 24 h, CCK-8 solution was added (20 μL per well, 5 mg/mL) and the cells were incubated for 4 h at 37 ^oC and 5% CO_2. The absorbance of each well was measured at 450 nm by a Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA).

L929 cells was stained by Calcein-AM (AM) after treated with the Fe_3O_4-*m*PEG or Fe_3O_4-PEG-

Cys NPs for 24 h and the morphology of L929 cells was observed by an Axio Vert. A1 inverted fluorescence microscope (Carl Zeiss, Jena, Germany). The magnification was set at 100 × for each sample.

Hemolysis Assay

Fresh human blood sample stabilized with EDTA was kindly supplied by Shanghai General Hospital (Shanghai, China) and used with the permission by the ethical committee of Shanghai General Hospital. Human red blood cells (HRBCs) were obtained and hemolysis assay was performed according to the literature.[3] In brief, the blood was centrifuged (1000 rpm, 10 min) to remove the supernatant and washed with phosphate buffer saline (PBS) for 5 times to completely remove serum and obtain the HRBCs. Then, the HRBCs were 10 times diluted with PBS. The diluted HRBC suspension (0.1 mL) was transferred into 2-mL Eppendorf tubes prefilled with 0.9 mL water (as positive control), 0.9 mL PBS (as negative control), and 0.9 mL PBS containing Fe_3O_4-*m*PEG or Fe_3O_4-PEG-Cys NPs at different Fe concentration (0-200 μg/mL), respectively. The above mixtures were then incubated at 37 ^oC for 2 h, followed by centrifugation (10 000 rpm, 1 min) and the absorbance of the supernatants related to hemoglobin was recorded with a Lambda 25 UV-vis spectrophotometer (Perkin Elmer, Waltham, MA) at 541 nm. The hemolytic percentage (HP) was calculated using the following equation:

$$HP(\%) = \frac{D_t - D_{nc}}{D_{pc} - D_{nc}} \times 100\%$$

where D_t is the absorbance of the test samples; D_{pc} and D_{nc} are the absorbances of the positive and negative control, respectively.

Macrophage Cellular Uptake Assays

The uptake of the Fe_3O_4-PEG-Cys NPs by Raw 264.7 cells was investigated by ICP-OES analysis. Raw 264.7 cells were seeded in 12-well plates at a density of 2×10^5 cells per well in 1 mL DMEM and incubated at 37 ^oC and 5% CO_2. After overnight incubation, the medium was replaced with 1 mL fresh medium containing PBS (control), and Fe_3O_4-*m*PEG or Fe_3O_4-PEG-Cys NPs at different Fe concentrations (20, 40, 60, 80, and 100 μg/mL, respectively). The cells were incubated for another 4 h,

after counting the cell number in the cell suspensions, the cells were centrifuged, digested by aqua regia solution overnight, and diluted. ICP-OES was performed to determine the Fe content in the cell samples.

To further confirm the cellular uptake of the Fe_3O_4-PEG-Cys NPs, Prussian blue staining was employed.[4] Similar to the ICP-OES analysis, Raw 264.7 cells were treated with the Fe_3O_4-*m*PEG or Fe_3O_4-PEG-Cys NPs for 4 h, then the cells were washed 3 times with PBS, and stained by Prussian blue solution. Afterwards, the stained cells were observed by phase contrast microscopy.

Pharmacokinetics

Animal experiments were carried out according to protocols approved by the ethical committee of Shanghai General Hospital for animal care, and also in accordance with the policy of the National Ministry of Health. Female Sprague Dawley rats (200-240 g) purchased from Shanghai Slac Laboratory Animal Center (Shanghai, China) were intravenously injected with Fe_3O_4-PEG-Cys NPs ([Fe] = 0.1 M, in 500 μL PBS, for each rat) through tail vein, and then the blood samples was collected at different time points (from 0 to 72 h). For comparison, Fe_3O_4-*m*PEG NPs with similar dose were also intravenously injected under the same experimental conditions. The Fe concentration in the blood samples was measured by ICP-OES. The half-decay time ($t_{1/2}$) of both particles was analyzed by DAS Software 2.0 (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

***In Vivo* Blood Pool MR Imaging**

For blood pool MR imaging, female Sprague Dawley rats (200-240 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg). Fe_3O_4-PEG-Cys or Fe_3O_4-*m*PEG NPs ([Fe] = 0.1 M, in 500 μL PBS) was then injected into each rat through the tail vein, respectively. The rats were scanned by a 3.0 T clinical MR imaging system (SOMATON Definition Flash, Siemens, Erlangen, Germany). The parameters of MR imaging *in vivo* were set as follows: TR = 280 ms; TE = 15 ms; FOV = 80×100; matrix = 318×314; thickness = 0.7 mm; gap = 0.14 nm; and NEX = 10.

***In Vivo* MR Imaging of a Xenografted Tumor Model**

Male nude mice (15-20 g) were injected with 5×10^6 HeLa cells/mouse in the left back. When the

tumor nodules reached a volume of about 250 mm^3, the mice were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg), then the Fe_3O_4-*m*PEG or Fe_3O_4-PEG-Cys NPs were injected into the tumor-bearing mice *via* the tail vein ([Fe]= 0.1 M, in 150 μL PBS, for each mouse). The mice were scanned by a 3.0 T clinical MR imaging system (SOMATON Definition Flash, Siemens, Erlangen, Germany). The parameters of MR imaging were set as follows: TR = 300, 600, 900, 1200 ms, TE = 10.7 ms, matrix = 256× 256, section thickness = 2 mm, and FOV = 12 cm. T_1-weighted MR images were obtained before and after intravenous injection of the Fe_3O_4-*m*PEG or Fe_3O_4-PEG-Cys NPs at the time points of 0, 30, 45, 90, and 180 min, respectively.

***In Vivo* Biodistribution**

The tumor-bearing mice after MR scanning were euthanized at 4, 8, and 24 h postinjection and the heart, liver, spleen, lung, kidney, and tumor were extracted and weighed. The organs were then cut into 1-2 mm^2 pieces and digested by aqua regia for 24 h. Then, the Fe content in these organs was measured by ICP-OES.

Statistical Analysis

One-way ANOVA statistical analysis was used to analyze the significance of the experimental data. A p value of 0.05 was selected as the level of significance, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. Zeta potential and hydrodynamic size of the pristine Fe_3O_4, Fe_3O_4-*m*PEG and Fe_3O_4-PEG-Cys NPs, respectively.

Sample	Zeta potential (mV)	Hydrodynamic size (nm)	PDI
Fe_3O_4	-30.2±2.9	26.0±4.2	0.441±0.038
Fe_3O_4-*m*PEG	-16.4±0.6	93.6±0.2	0.144±0.026
Fe_3O_4-PEG-Cys	-15.7±0.3	116.2±0.9	0.221±0.009

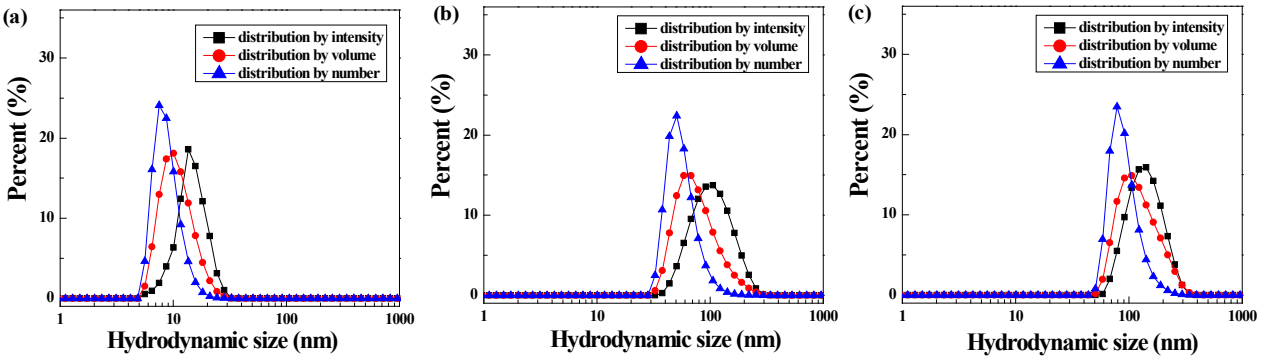

Figure S1. Hydrodynamic size distributions of the Fe_3O_4 NPs (a), Fe_3O_4-*m*PEG NPs (b) and Fe_3O_4-PEG-Cys NPs (c) measured by intensity, volume and number, respectively.

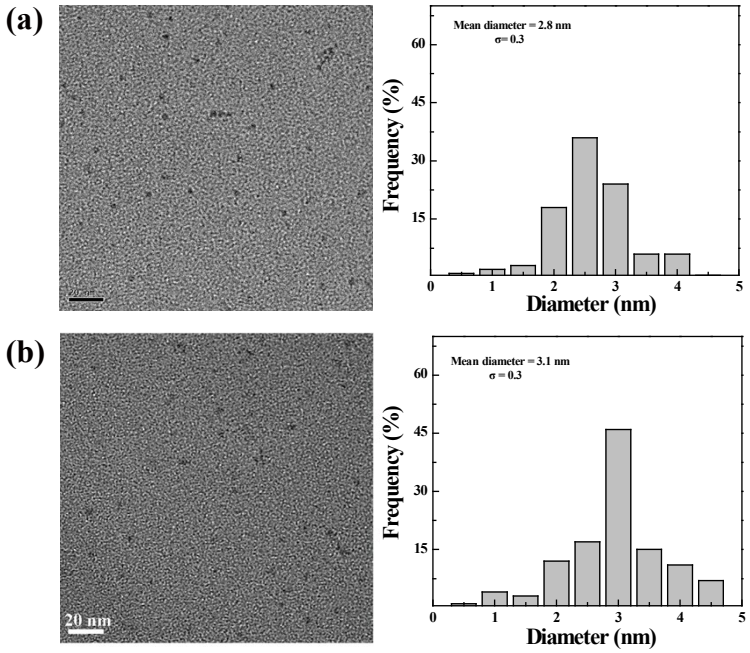

Figure S2. TEM image and size distribution histogram of the ultrasmall Fe_3O_4 NPs (a) and Fe_3O_4-*m*PEG NPs (b). The scale bar in each image represents 20 nm.

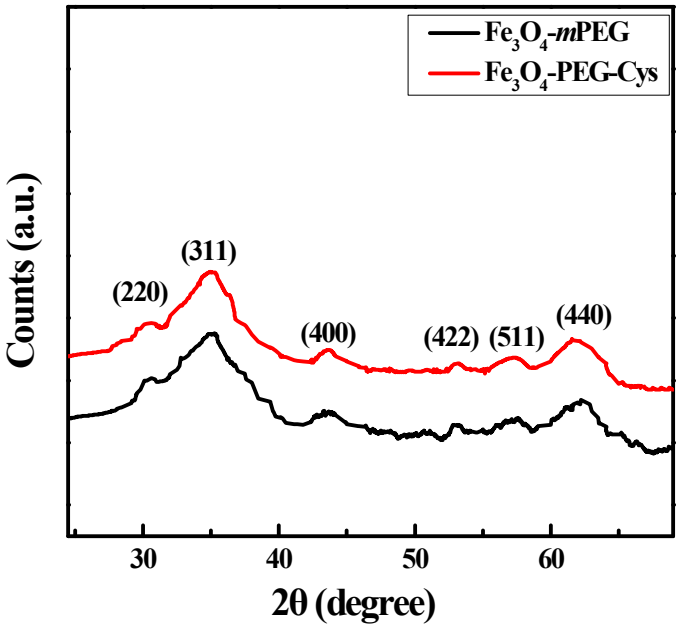

Figure S3. XRD patterns of Fe_3O_4-*m*PEG and Fe_3O_4-PEG-Cys NPs.

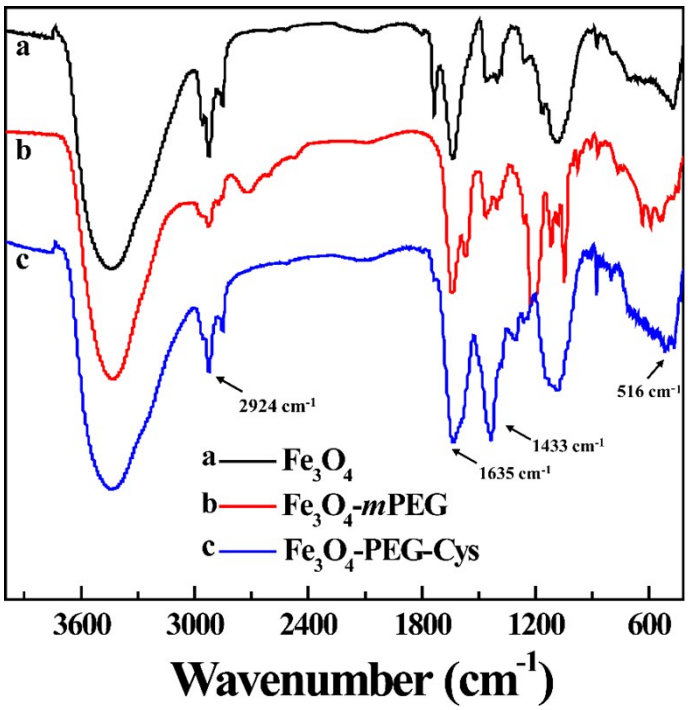

Figure S4. FTIR spectra of Fe_3O_4, Fe_3O_4-*m*PEG and Fe_3O_4-PEG-Cys NPs.

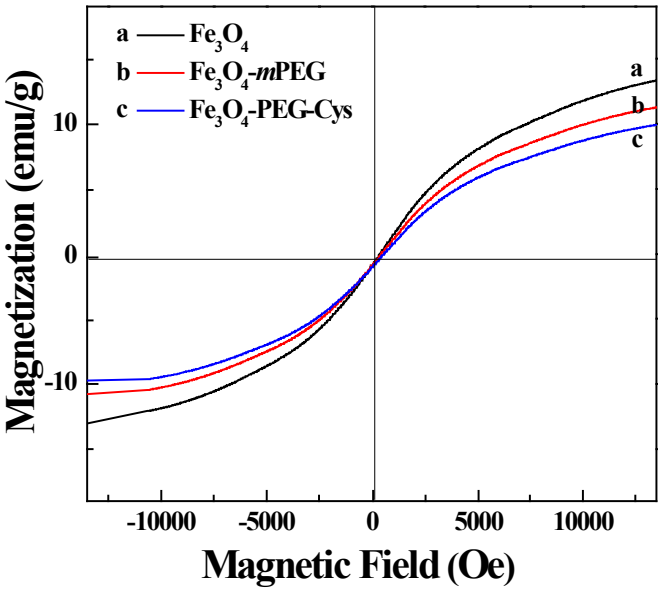

Figure S5. Magnetization curves of Fe_3O_4, Fe_3O_4-*m*PEG and Fe_3O_4-PEG-Cys NPs.

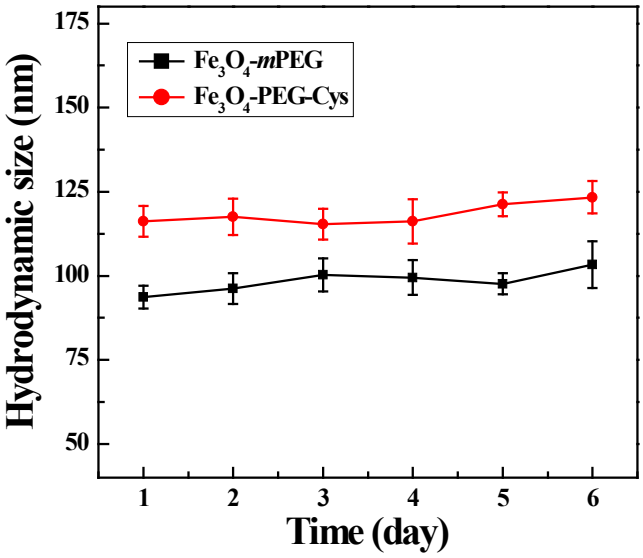

Figure S6. Hydrodynamic size of the Fe_3O_4-*m*PEG and Fe_3O_4-PEG-Cys NPs dispersed in water at different time periods.

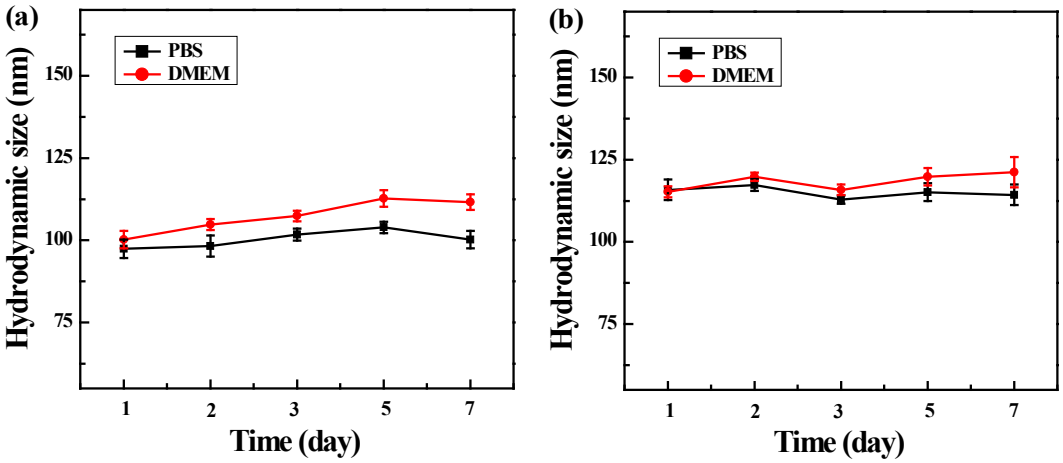

Figure S7. Hydrodynamic size of the Fe_3O_4-*m*PEG (a) and Fe_3O_4-PEG-Cys NPs (b) exposed to PBS and DMEM supplement with 10% FBS at different time periods.

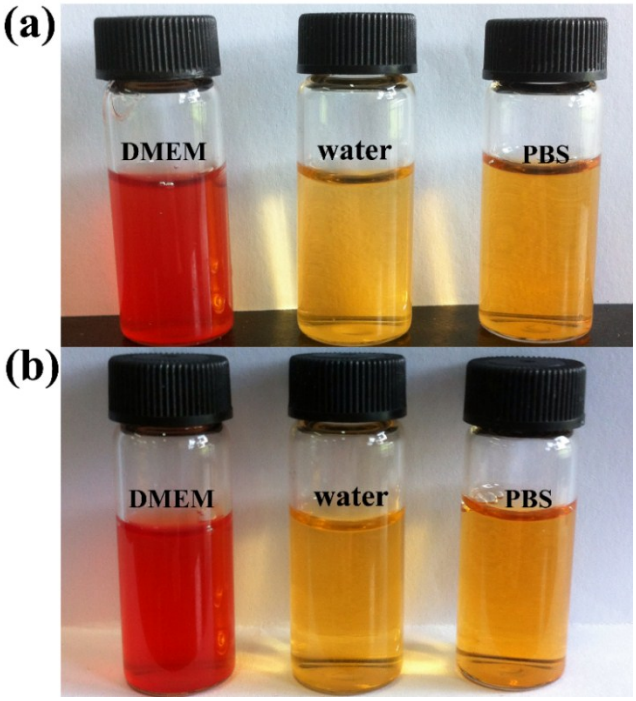

Figure S8. Photographs of the Fe_3O_4-*m*PEG (a) and Fe_3O_4-PEG-Cys NPs (b) dispersed in cell culture medium (DMEM) supplemented with 10% FBS, water and PBS, respectively for 20 days.

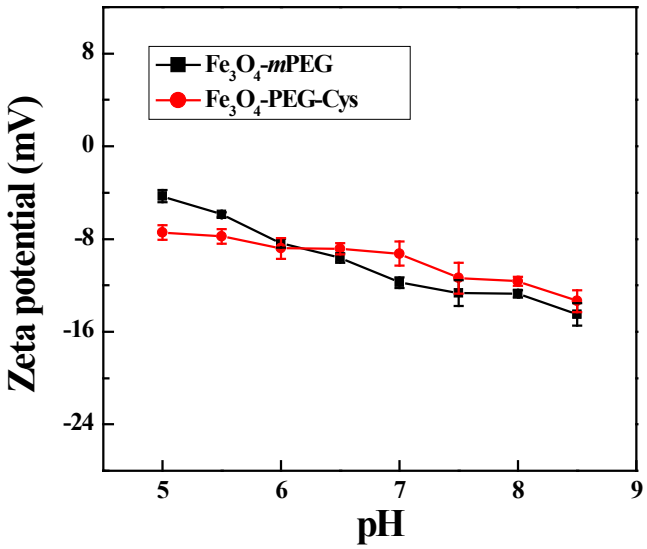

Figure S9. The change of surface potential of Fe_3O_4-*m*PEG and Fe_3O_4-PEG-Cys NPs as a function of pH.

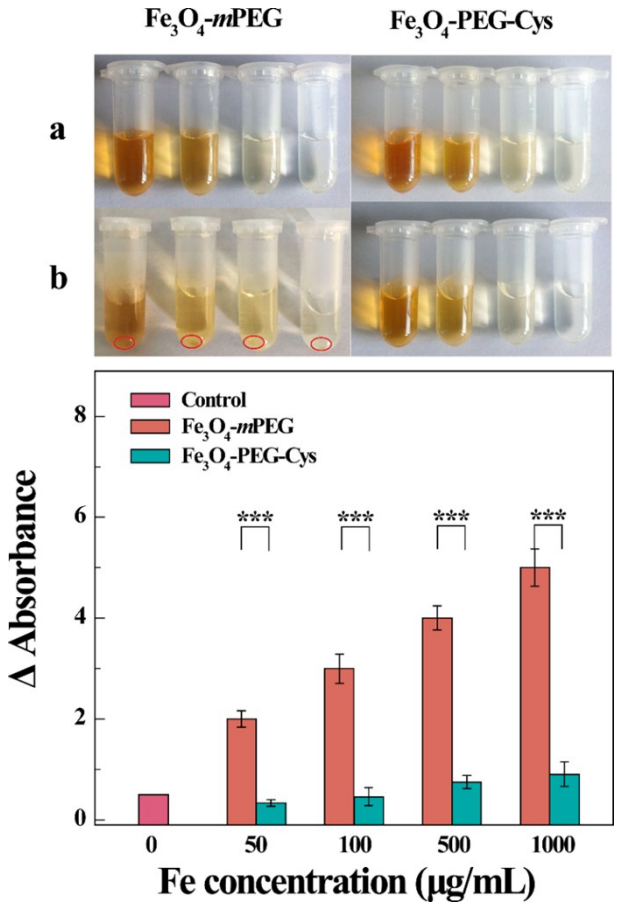

Figure S10. Photographs of Fe_3O_4-*m*PEG or Fe_3O_4-PEG-Cys NPs at different Fe concentrations incubated with FBS solution (50%) for 2 h before (a) and after (b) centrifugation (8000 rpm, 5 min). The absorbance before (a) and after centrifugation (b) were measured. The reduced absorbance was calculated to represent the protein resistance ability (down panel).

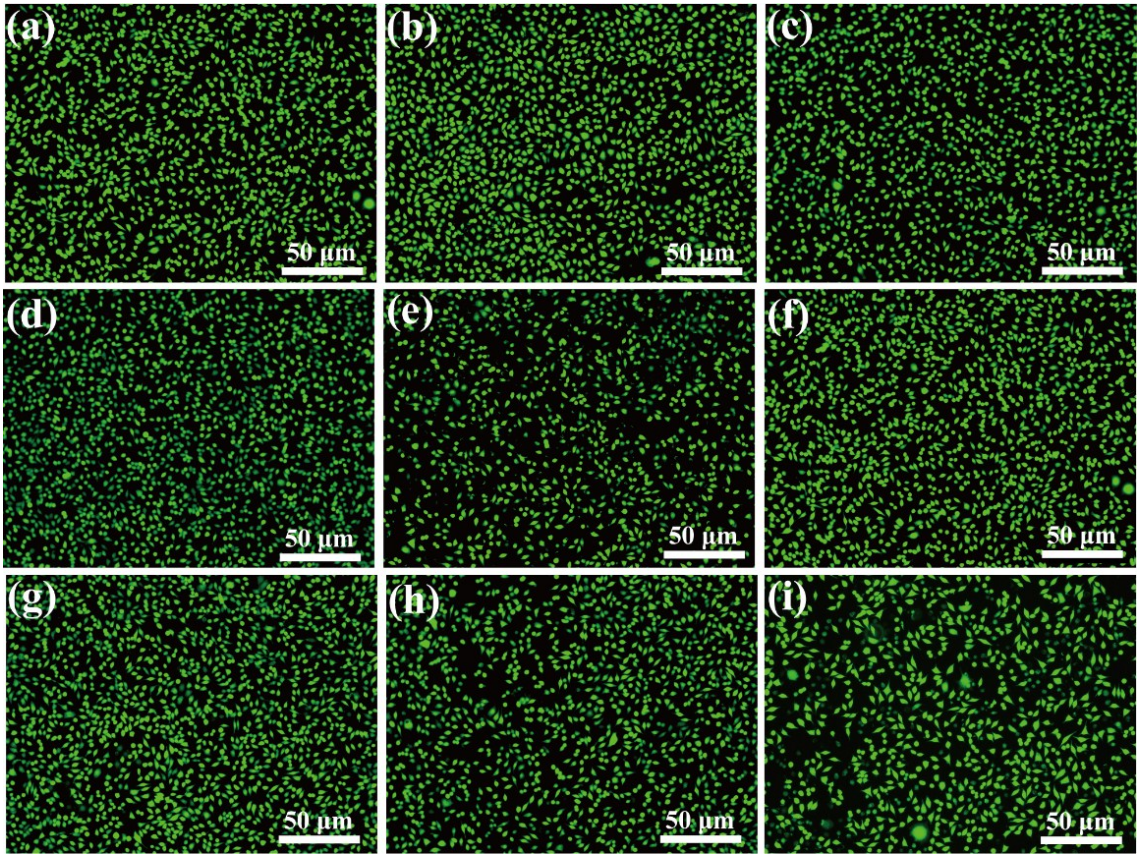

Figure S11. Micrographs of L929 cells treated with PBS (a), Fe_3O_4-*m*PEG NPs at an Fe concentration of 10 (b), 25 (c), 50 (d) and 100 (e) μg/mL, and Fe_3O_4-PEG-Cys NPs at an Fe concentration of 10 (f), 25 (g), 50 (h) and 100(i) μg/mL for 24 h.

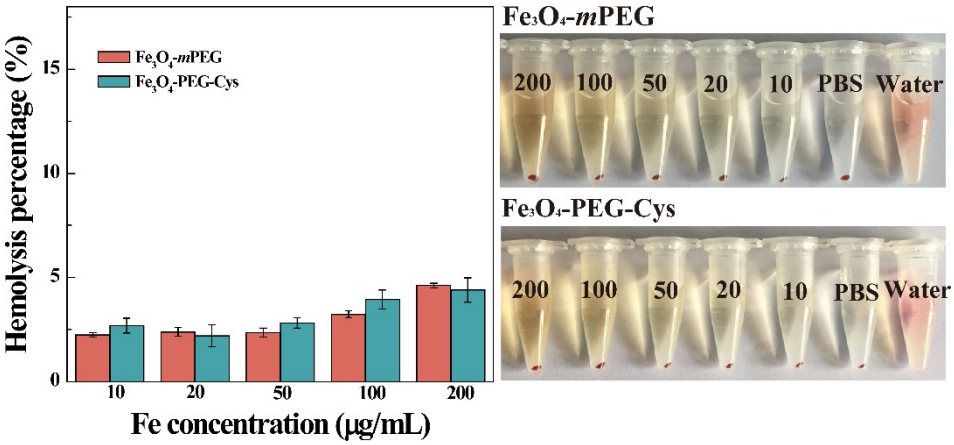

Figure S12. Hemolysis percentage and photographs of the HRBC suspensions treated with the Fe_3O_4-*m*PEG and Fe_3O_4-PEG-Cys NPs at different Fe concentrations. The data are expressed as mean ± SD (n = 3). Water and PBS were used as positive and negative controls, respectively.

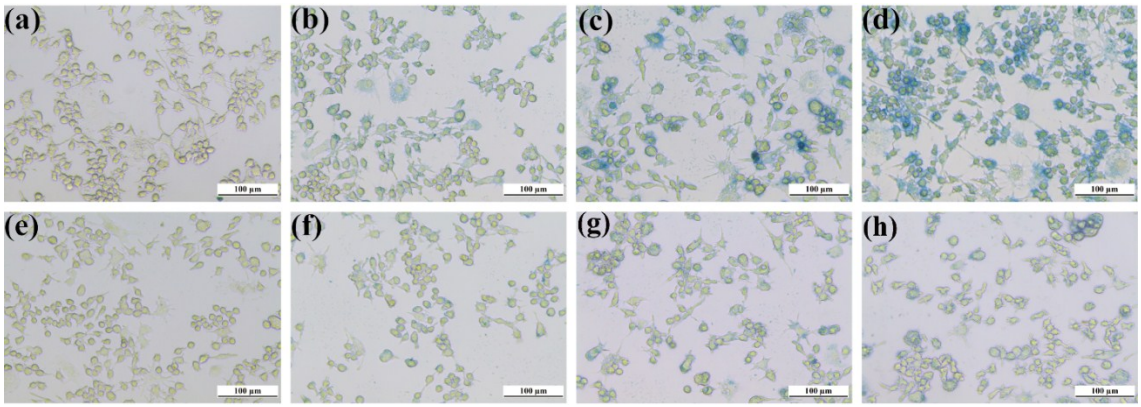

Figure S13. Prussian blue staining of Raw264.7 cells treated with Fe_3O_4-*m*PEG or Fe_3O_4-PEG-Cys NPs. Raw264.7 cells treated with PBS were used as control (a and e). Cells were treated with the Fe_3O_4-*m*PEG NPs at an Fe concentration of 25 (b), 50 (c), and 100 (d) μg/mL, or treated with Fe_3O_4-PEG-Cys NPs at an Fe concentration of 25 (f), 50 (g) and 100 (h) μg/mL. In all case, the incubation time was 4 h. Blue staining indicates the presence of iron element. The scale bar in each panel represents 100 μm.

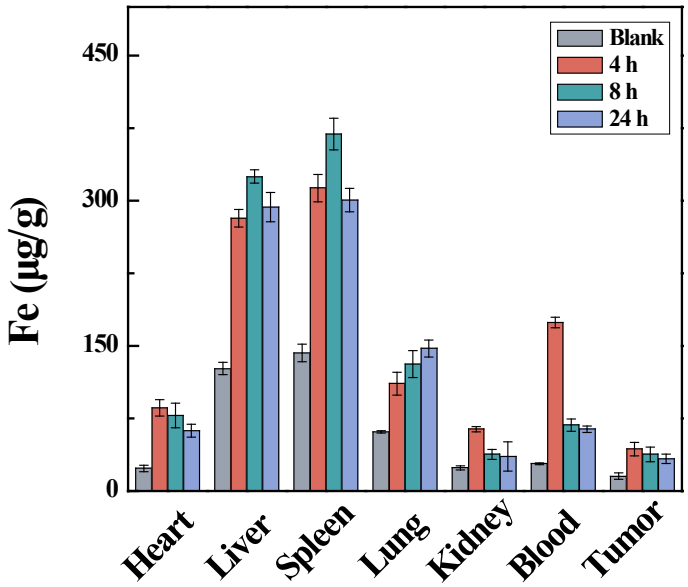

Figure S14. Biodistribution of Fe element in blood, tumor and the major organs of the mice including heart, liver, spleen, lung, and kidney. The data were recorded from the whole organ taken at different time points post intravenous injection of Fe_3O_4-PEG-Cys NPs ([Fe] = 0.1 M, 150 μL in PBS for each mouse).

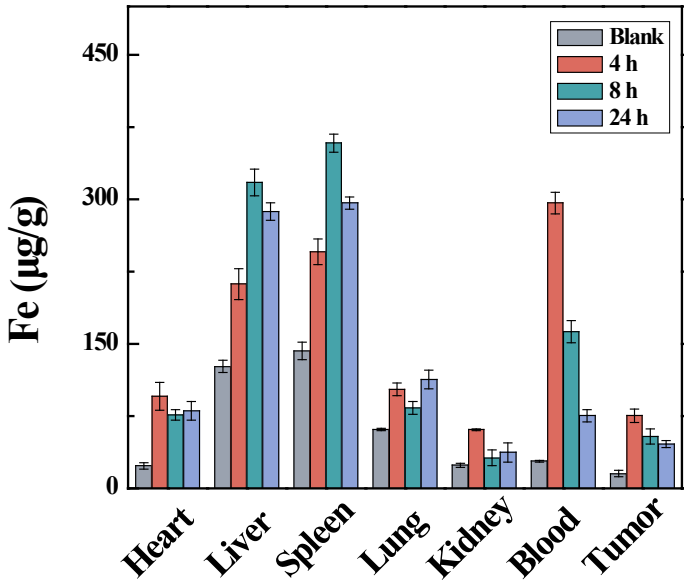

Figure S15. Biodistribution of Fe element in blood, tumor and the major organs of the mice including heart, liver, spleen, lung, and kidney. The data were recorded from the whole organ taken at different time points post intravenous injection of the Fe_3O_4-*m*PEG NPs ([Fe] = 0.1 M, 150 μL in PBS for each mouse).

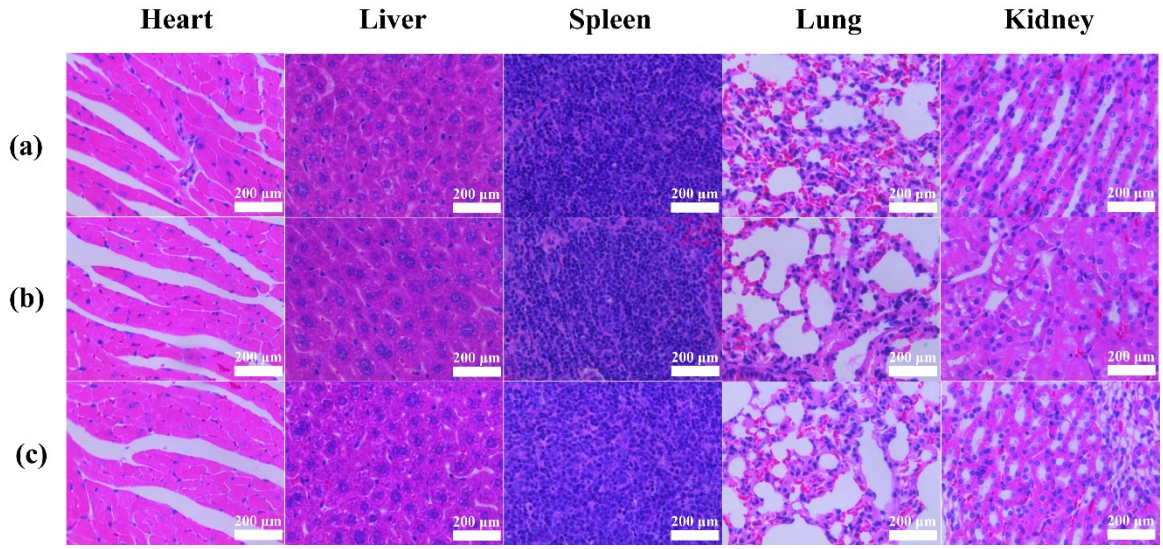

Figure S16. H&E staining of major organs of mice before injection (a) and at 30 days postinjection of Fe_3O_4-*m*PEG NPs (b) or Fe_3O_4-PEG-Cys (c) ([Fe] = 0.1 M, 150 μL in PBS for each mouse).

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