

Electronic Supplementary Information

Ultrasmall iron oxide nanoparticles with MRgFUS for enhanced magnetic resonance imaging of orthotopic glioblastoma

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Materials and methods

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Materials

Iron (III) chloride was obtained from Adamas Reagent Co., Ltd. (Shanghai, China). Ethylenediamine (EDA) was purchased from Sinopharm Chemical Reagent Ltd. (Shanghai, China). 1,3-propanesultone (1,3-PS) was obtained from J&K Scientific (Shanghai, China). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from GL Biochem. (Shanghai, China). RPMI-1640 medium, fetal bovine serum (FBS) were obtained from Gibco (Carlsbad, CA). Penicillin and streptomycin, pancreatin, and Cell Counting Kit-8 (CCK-8) were purchased from Gino Biomedical Technology Co., Ltd. (Hangzhou, China). GL261 cells (a Glioblastoma cell line) was from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China).

Synthesis of USIO NPs and USIO NPs-1,3-PS

Citric acid-stabilized USIO NPs were synthesized according to protocols described in our previous work^{1, 2}. Firstly, we dissolved $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.6-0.7 g) in diethylene glycol (DEG, 35-45 mL) under vigorous stirring, and then added $\text{Na}_3\text{Cit} \cdot 2\text{H}_2\text{O}$ (0.35-0.55 g) into the solution with continuous stirring for 2 h under 40 °C. Secondly, we added anhydrous sodium acetate (1.2-1.4 g) into the above mixture solution under stirring, which was transferred to a Teflon-lined stainless-steel reaction kettle (100 mL, 200 °C) for 5 h. USIO NPs was obtained by cooling down to room temperature, washing with anhydrous ethanol and drying in a vacuum oven (50 °C).

Subsequently, we dispersed USIO NPs (56 mg) into water (16 mL) and added EDC (150 mg, 1 mL in water) under stirring for 30 min. We added NHS (95 mg, 1 mL in water) into the above solution under stirring for 3 h to activate the citric acid carboxyl groups, and then added EDA (217 μL , 2 mL in

water) under stirring for 3 days. USIO NPs-NH₂ was obtained by dialyzed (MWCO = 10000 Da) against water for 3 days.

Finally, we dropped 1,3-PS into the USIO NPs-NH₂ (10 mg, 5 mL in water) under stirring for 3 days. Afterward, the mixture solution was dialyzed (MWCO = 500 Da) against water to generate the USIO NPs-1,3-PS.

Nanoparticle characterization

Transmission electron microscopy (TEM) was used to observe the morphology and microstructure of USIO NPs and USIO NPs-1,3-PS at an operating voltage of 80 kV. Samples of USIO NPs and USIO NPs-1,3-PS were prepared by depositing them onto carbon-coated copper grid and air-dried before measurements. Zeta potential and dynamic light scattering (DLS) measurements were explored by a Malvern Zeta sizer Nano ZS system (model ZEN3600, Worcestershire, UK) equipped with a standard 633 nm laser.

Fourier transform infrared (FTIR) spectra were recorded on a Nicolet 6700 FTIR spectrophotometer (Thermo Electron Corporation, Madison, WI). Thermogravimetric analysis (TGA) were assessed by A TG 209 F1 thermogravimetric analyzer (NETZSCH Instruments Co., Ltd., Bavaria, Germany) under nitrogen atmosphere. Fe concentrations were investigated by a Leeman Prodigy inductively coupled plasma-optical emission spectroscopy (ICP-OES, Hudson, NH).

Cytotoxicity assay

We evaluated the in vitro cytotoxicity of USIO NPs and USIO NPs-1,3-PS via CCK-8 cell viability assay. GL261 cells were seeded into 96 well plates at the density of 1×10^4 cells per well with 100 μ L regularly cultured medium under normal cell culture conditions. The medium in each well was replaced with fresh medium containing USIO NPs and USIO NPs-1,3-PS with different Fe

concentrations (0, 20, 40, 60, 80, 100 µg/mL, respectively) solutions. After 24 h, the cells were washed three times with PBS and added CCK-8 solution (10 µL per well) for 2 h. We recorded the absorbance of each well at 450 nm by a Thermo Scientific Multiskan MK3 ELISA reader (Waltham, MA).

The live-dead staining analysis was used to further confirm the cytocompatibility of the USIO NPs and USIO NPs-1,3-PS by Calcein/PI Cell Viability/Cytotoxicity Assay Kit. GL261 cells were treated with USIO NPs and USIO NPs-1,3-PS ([Fe] = 80 µg/mL) for 24 h. Then, the cells were washed three times with PBS and added with 1 mL Calcein AM/PI solution for 30 min, the cells were observed by LEICA DMI8 inverted fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Assessment of cell uptake

GL261 cells were seeded in a 12-well plate at a density of 1×10^6 cells per well for overnight incubation at 5 % CO₂ and 37 °C. The medium was replaced with fresh medium containing USIO NPs or USIO NPs-1,3-PS ([Fe] = 50 µg/mL, [Fe] = 100 µg/mL), and then the cells were collected at different time points (2, 4, or 6 h). After cell counting, the cells were digested by *aqua regia* (nitric acid/hydrochloric acid, v/v = 1:3, 1 mL) for 4 h and diluted with water (5 mL). Then, Fe content in the cells for each sample (n = 3) was analyzed by ICP-OES (Leeman Prodigy, Hudson, NH).

To further confirm the cellular uptake of USIO NPs-1,3-PS, Prussian blue staining was employed. GL261 cells were treated with the USIO NPs or USIO NPs-1,3-PS ([Fe] = 80 µg/mL) for different time points (2 or 4 h). The cells were then washed 3 times with PBS, fixed with Fixation Buffer, stained with Prussian blue solution and Nuclear fast red solution, and observed by inverted fluorescence microscope.

Animal models

A total of 54 Male C57BL/6 mice, including healthy and tumor-implanted animals, were used in this study. Twelve mice were used to detect the feasibility and safety of USIO NPs or USIO NPs-1,3-PS. Fifteen mice were used to optimize the BBBO parameters. Twelve mice were used to improve USIO NPs-1,3-PS deposition in the brain.

To build up the subcutaneously implanted tumor model, each mouse was injected with suspension of GL261 mouse glioma cells (2×10^6 cells/mL, 100 μ L) on the right leg. Tumor sizes were measured using 3.0 T MR after two weeks.

For intracranial injection to build up orthotopic Glioblastoma multiforme, GL261 cells were harvested by trypsinization and washed with phosphate-buffered saline. Male C57BL/6 mice (25-30 g) were anesthetized by intraperitoneal administration of Zoletil (55-60 mg/kg) and immobilized on a stereotaxic apparatus. We made a sagittal incision on the skin overlying the calvarium, and used a small drill to make a hole in the right exposed skull (0.5 mm anterior and 3 mm lateral to the bregma). GL261 cells (2×10^6 cells/mL, 5 μ L) suspension was injected at a depth of 2.5 mm from the exposed skull for 10 minutes, and the needle was withdrawn for another 5 minutes³. One week after implantation, tumor sizes were examined by 3.0 T MR.

Histologic Examination and biodistribution studies *in vivo*

Fifteen tumor-bearing mice were sacrificed after *in vivo* MR analysis for histologic examination. Main organs (heart, liver, spleen, lung, and kidney) were prepared, paraformaldehyde-fixed, paraffin-embedded, sectioned for hematoxylin-eosin (H&E) staining according to standard protocols reported in the literature.

To investigate the distribution and metabolic pathway of USIO NPs-1,3-PS (400 μ g/mouse, in 150 μ L PBS), the mice were sacrificed at different time points after intravenous injection. Vital organs

(heart, liver, spleen, lung, kidney and tumor) were extracted, weighed, cut into small pieces, and processed with *aqua regia* solution (3 mL, hydrochloric acid/nitric acid, v/v = 3:1) for two days. Finally, each sample was diluted with water (7 mL) and quantified by ICP-OES measurements to analyze the Fe content. The data were expressed as mean \pm SD (n = 3).

Table S1. Zeta potential and hydrodynamic size of the pristine USIO NPs, USIO NPs-NH₂ and USIO NPs-1,3-PS. Data are shown as mean \pm SD (n = 3).

Sample	Zeta potential (mV)	hydrodynamic size (nm)	PDI
USIO NPs	-33.0 \pm 0.2	13.26 \pm 2.13	0.235 \pm 0.005
USIO NPs-NH ₂	-16.5 \pm 1.2	50.46 \pm 3.33	0.337 \pm 0.039
USIO NPs-1,3-PS	-14.6 \pm 0.7	68.42 \pm 2.52	0.413 \pm 0.043

Table S2. The T₁ and T₂ relaxation time of USIO NPs and USIO NPs-1,3-PS were measured by this 0.5T NMI20 NMR analyzing and imaging instrument.

Fe concentrations (mM)	relaxation time of USIO NPs (ms)		relaxation time of USIO NPs-1,3-PS (ms)	
	T ₁	T ₂	T ₁	T ₂
0.1	1958.06	2245.50	1847.54	1918.18
0.2	1498.28	1337.36	1445.59	1542.11
0.4	1101.67	791.41	994.99	936.02
0.8	715.84	560.40	678.80	590.15
1.6	401.34	250.99	383.61	279.36

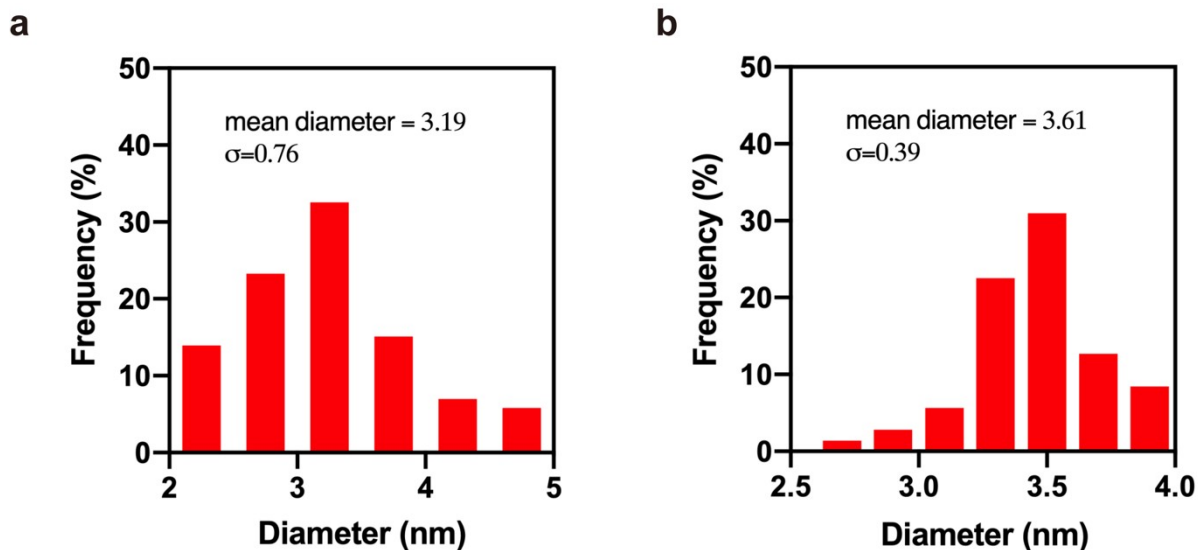


Figure S1. Size distribution histogram of the ultrasmall USIO NPs (a) and USIO NPs-1,3-PS (b).

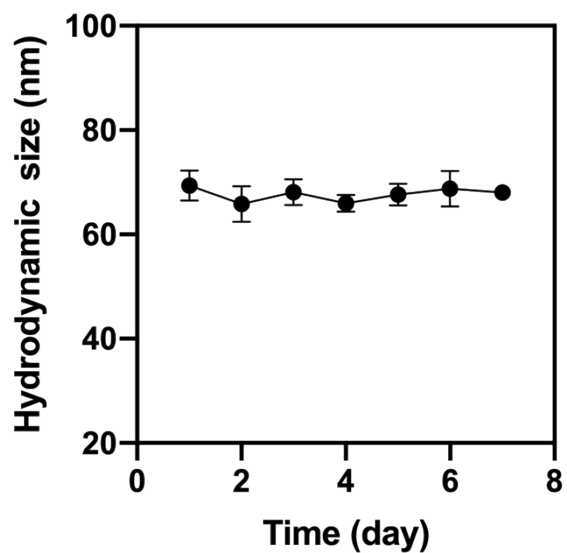


Figure S2. Hydrodynamic size of USIO NPs-1,3-PS in PBS as a function of storage time period at 4°C.

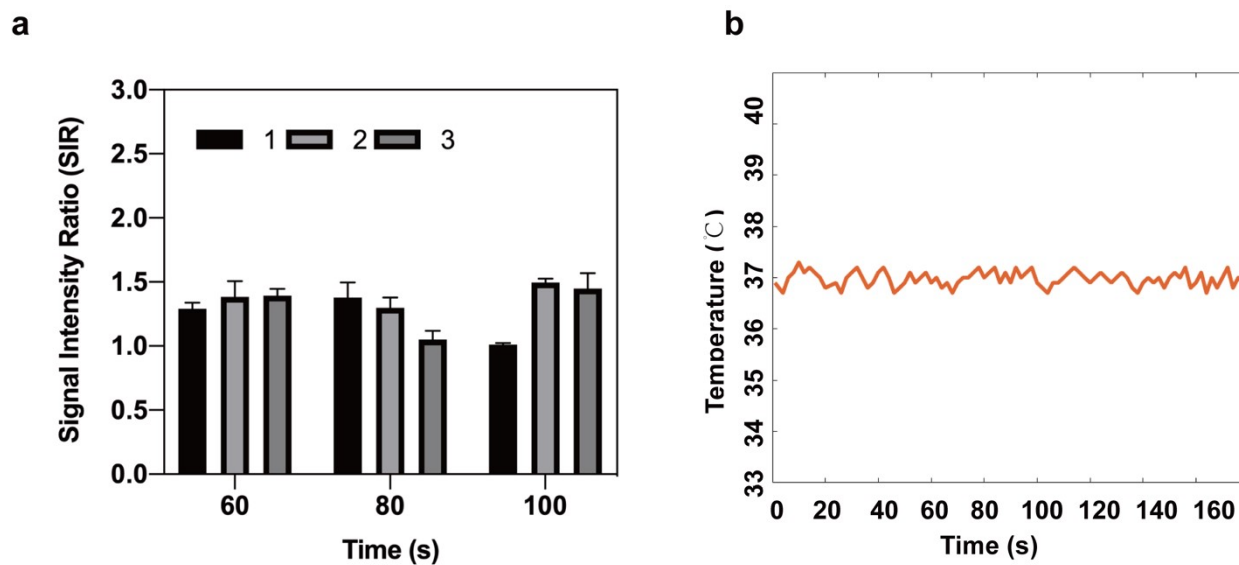


Figure S3. (a) T_1 -weighted MR signal intensity ratio (SIR) of BBB opening area at different time points. (b) The real-time temperature during FUS exposure.

References

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