Coating Optimization of Superparamagnetic Iron oxide Nanoparticles for High T_2 Relaxivity

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SUPPORTING INFORMATION

S1. T_2 relaxivity of SPIOs

The T_2 shortening effect of magnetic nanoparticles is determined by the translational diffusion of water molecules and the magnetic field gradient generated by the magnetic nanoparticle. This phenomenon is characterized by two time scales: τ_D, the time required for water to diffuse across the nanoparticle surface, and $1/\Delta\omega_r$, the angular phase shift induced by the magnetic nanoparticle at the equator line on its surface.[1-2]

$$\tau_D = r^2/D \quad \text{(S1)}$$

$$(\Delta\omega_r)^2 = (4/5)\,\gamma^2\,\mu_c^2 / r^6 \quad \text{(S2)}$$

where r is the effective radius of the nanoparticle. D is the diffusion coefficient of water molecules. γ is the proton gyromagnetic ratio. μ_c is the Curie moment of the magnetic nanoparticle. For a SPIO that fulfills $\tau_D < 1/\Delta\omega_r$, the quantum-mechanical outer-sphere theory applies,

$$1/T_2 = (4/9)\,V\,\tau_D\,(\Delta\omega_r)^2 \quad \text{(S3)}$$

$$V = (4\pi r^3/3)\,N_0\,M \times 10^{-3}$$

V and M are the volume fraction and the molarity (moles/L) of the SPIO, respectively. N_0 is Avogadro's number. At high imaging magnetic field, μ_c can be approximated by $(4\pi a^3/3)\,M_s$, where a and M_s are the radius and the saturation magnetization of the iron oxide core, respectively.[3] Accordingly, equation (S3) can be reorganized into the following form,

$$1/T_2 = (256\pi^2\gamma^2/405)\,V^*\,M_s^2\,a^2 / [D\,(1+L/a)] \quad \text{(S4)}$$

$$V^* = (4\pi a^3/3)\,N_0\,M \times 10^{-3}$$

V^* is the volume fraction of the iron oxide core. L is the thickness of the coating layer. It should be noted that the SPIO is assumed to be coated by an impermeable polymer matrix and water diffuses freely beyond the coating in this theory. Analytical analysis for a SPIO coated with a permeable polymer matrix is not available, but we can examine the effects of coating in two extreme cases. When the coating is not permeable,

$$1/T_2 \propto 1 / (1+L/a) \quad \text{(S5)}$$

Therefore, T_2 relaxivity decreases as the coating becomes thicker. On the other hand, if the SPIO is coated with a diluted polymer matrix that spreads to the whole solution and allows water diffuses in it,

$$1/T_2 \propto 1 / D^* \quad \text{(S6)}$$

where D^* are the effective diffusion coefficient of water molecules. In this case, T_2 relaxivity increases with the coating density if the decrease in water proton density is neglected.

S2. Synthesis of iron oxide cores of different sizes.

The iron oxide cores were synthesis by thermo-decomposition of iron tri(acetyl acetonate) in the presence of oleic acid and oleylamine using a protocol modified according to a previous publication (see methods).[4] The core size was controlled by varying the ratio between oleic acid/ oleylamine and the reaction temperature, which included three temperature ramping processes (Table S1).

Table S1. Reaction Conditions for Iron Oxide Core Synthesis

Size (nm)	Oleic acid / Oleylamine	r.t – 120 °C (°C/min)	Holding (hour)	120-220 °C (°C/min)	Holding (Hour)	220-300 °C (°C/min)	Holding (Hour)
5	2/3	10	1	5	0.5	2	0.5
14	1/1	5	2	5	0.5	2	0.5

The synthesized iron cores were photographed with TEM (Figure S1). The size distribution of the iron oxide cores were measured using an image processing software, ImagePro Plus. The two types of iron oxide cores used in this study were measured to be 4.8±0.9 nm and 13.8±2.2 nm in diameter, which are referred to as 5 nm and 14 nm, respectively. Given that the density of Fe_3O_4 is 5.17 g/cm^3, the weight of a 4.8 nm core is 2.99×10^{-19} g and contains 2329 iron atoms. Likewise, a 13.8 nm core contains 55352 iron atoms. The molar concentration of the SPIOs was estimated by the molar concentration of iron divided by the number of iron atoms per SPIO.

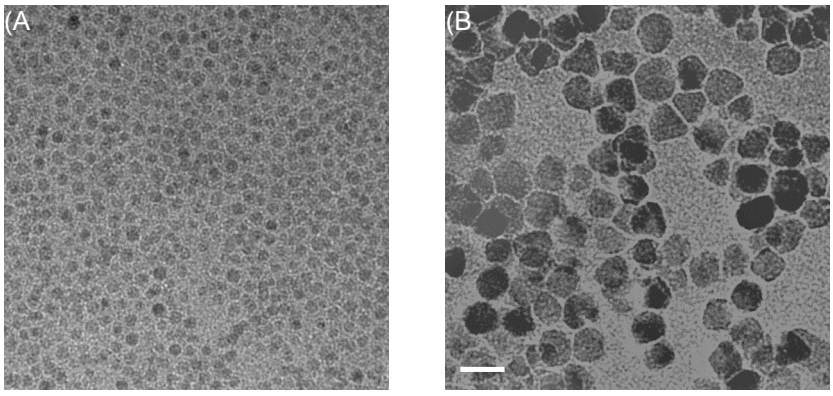

Figure S1. TEM images of iron oxide cores. (A) and (B) are representative TEM images of 5 nm and 14 nm cores, respectively. Bar equals 20 nm.

S3. Comparison of film hydration and solvent exchange methods

To compare the film hydration and solvent exchange methods for coating SPIOs, we performed gel electrophoresis of the SPIOs synthesized with the film hydration method and the solvent exchange method respectively. As shown in Figure S2, in each group, the solvent exchange method yielded better SPIOs with respect to the size and charge distribution. Therefore, the solvent exchange method is superior in achieving uniform distribution of functional groups among the SPIOs, which is advantageous for subsequent conjugation of targeting molecules. Furthermore, we used the maleimide group on the SPIO to conjugate antibodies through thiol-maleimide reaction, and found that the solvent exchange method preserves sensitive functional groups, e.g. maleimide, during the coating process, because it does not involve heating and sonication in aqueous buffers as required by the film hydration.

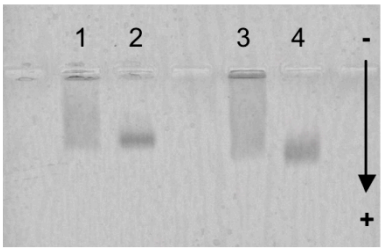

Figure S2. Electrophoresis of SPIOs synthesized with film hydration and solvent exchange methods. SPIOs with 5 nm iron oxide core and DSPE-PEG2000 coating were synthesized with film hydration method (1 & 3) or solvent exchange method (2 & 4). The SPIOs 1 & 2 were functionalized with 2% DSPE-PEG-COOH, and the SPIOs 3 & 4 with 4% DSPE-PEG-COOH. In each group, the SPIO was synthesized with identical starting materials. Electrophoresis was performed with 200 μg Fe/ml of SPIOs in each group (electrophoresis conditions: 0.5% agarose gel, Voltage 135V, running buffer tris-acetate-EDTA pH 8.0 and running time 75 minutes). The SPIOs were used without purification after coating procedures.

S4. Thickness of PEG coating layer of SPIOs

The thickness of the PEG layer of a SPIO in aqueous solutions can be estimated with a scaling model, which was originally developed by Johnsson et al. for the PEG layer of DSPE-mPEG formed micelles.[5]

$$L = \left[N l^{1/\nu} \frac{8 f^{(1-\nu)/2\nu}}{3\nu 4^{1/\nu}} + R_c^{1/\nu} \right]^{\nu} - R_c \qquad \text{(S7)}$$

where L is the thickness of the PEG layer, N is the number of monomeric units per PEG chain, l is the statistical length of a monomer (= 0.39 nm), f is the number of PEG chains on the surface, ν is the Flory exponent (= 0.583), and R_c is the radius of the core. R_c of the SPIOs includes the contribution from both iron oxide cores and the lipid bilayer formed by DSPE and oleic acid / oleylamine. The thickness of the lipid bilayer is assumed to be the same as that of the cell membrane (~ 3 nm). f can be estimated as the number of amino groups on the SPIOs coated with DSPE-PEG2000-NH_2 alone, which is 632 PEG chains for a 13.8 nm SPIO (see section S6). The density of DSPE-PEG2000-NH_2 on a 13.8 nm SPIO (0.51 PEG / nm^2) agrees well with the density of DSPE-mPEG2000 on a micelle obtained by Johnsson et al. (0.59 PEG / nm^2).[5] Since L is not very sensitive to the change in f, we assumed that the PEG density remains unchanged for all core sizes and PEG chain lengths. The calculated PEG layer thicknesses are listed in Table S2.

Table S2. Thickness of the PEG coating on SPIOs.

Size (nm)	PEG550 (N = 12)	PEG750 (N = 16)	PEG1000 (N = 22)	PEG2000 (N = 45)	PEG5000 (N = 113)
13.8	1.11 nm	1.46 nm	1.97 nm	3.80 nm	8.43 nm
4.8	1.07 nm	1.40 nm	1.88 nm	3.53 nm	7.47 nm

Based on the values in Table S2, the SPIOs with 4.8 nm and 13.8 nm cores and DSPE-PEG1000 coating have a hydrodynamic diameter of 14.56 nm and 23.74 nm, respectively.

S5. Stability of SPIOs in biological solutions

The DSPE-PEG coating of the SPIOs is formed by the hydrophobic interaction between DSPE and oleic acid / oleylamine on the iron oxide surface. Similar to DSPE-PEG formed micelles, the SPIOs may dissemble in aqueous solutions at low concentration or due to competition of lipid binding proteins. In this study, we found that the DSPE-PEG coated SPIOs were stable in deionized water at room temperature for up to several months. Further, we tested the stability of the SPIOs in two common biological buffers, phosphate buffered saline (PBS) and serum. The stability of the SPIOs in buffers was examined based on SPIO sedimentation because the SPIOs tend to aggregate and precipitate if the coating is broken. The SPIOs were dispersed in PBS and serum at either 10 μg/mL or 100 μg/mL, and incubated at 37°C for 24 hours. After incubation, the solutions were centrifuged at 500 g and the SPIOs remained in the supernatant were quantified using Ferrozine assay (see methods). To compensate particle sedimentation due to centrifugation, the SPIOs in water were used as a control to normalize the values obtained for PBS and serum. As shown in Figure S3, the SPIOs are stable at physiologically relevant concentrations in both PBS and serum.

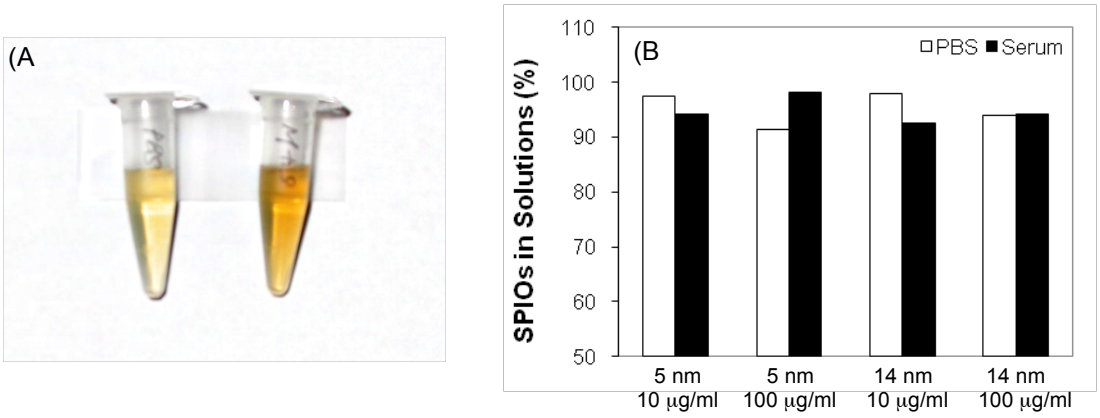

Figure S3. Stability of SPIOs in PBS and Serum. (A) Image of 5 nm SPIO (100 μg Fe/ml) after incubated with PBS (Left) or mouse serum (Right) at 37°C for 24 hours. (B) The amount of SPIOs remained in the solutions after incubation.

S6. Effects of functional groups and buffers on T_2 relaxivity

Charged functional groups, such as NH_2 and COOH, on the SPIO surface may change T_2 relaxivity by affecting hydrogen bond formation. To determine the effects of functional groups and buffers on T_2

relaxivity, we synthesized 14 nm SPIOs with three different combinations of functionalized DSPE-PEGs (Molar loading ratio: NH_2, 20%, COOH, 20% or NH_2+COOH, 10% each). The SPIOs were dispersed in three buffers: (1) 200 mM ammonium acetate (pH = 4.0), (2) deionized water or (3) PBS. T_2 relaxivity of SPIOs was measured at 40°C using Hahn Spin Echo pulse sequence with a 0.47T Bruker Minispec Analyzer (see methods). The T_2 relaxivity of the SPIOs with NH_2 functional groups alone is slightly lower than that of other SPIOs (Figure S4). The buffer conditions did not induce significant changes in T_2 relaxivity.

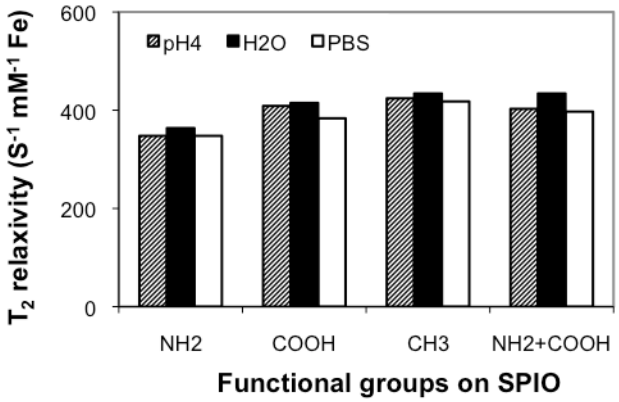

Figure S4. Effects of functional groups on T_2 relaxivity of SPIOs.

S7. Functionalization of SPIOs.

Functionalization of SPIOs can be achieved by mixing DSPE-mPEG and DSPE-PEG ended with functional groups at desired ratio before coating. Here the 14 nm SPIOs with amino groups were prepared by coating iron oxide cores with a mixture of DSPE-mPEG and DSPE-PEG-NH_2. The percentage of DSPE-PEG-NH_2 in the mixture was 10%, 20%, 50% or 100%. To increase the accessibility of functional groups for chemical conjugation, each functionalized DSPE-PEG2000 has twice more PEG units than that of a DSPE-mPEG1000 in the mixture. After coating and purification, the concentration of NH_2 group in the final solution was measured by fluorescamine method. In brief, fluorescamine was dissolved in acetone at 0.3% (w/v). The SPIOs was dispersed in deionized water at 100 μg Fe/ml. The fluorescamine was mixed with the SPIO solutions in a 96-well quartz plate. The mixture was incubated at room temperature for 20 minutes. The standard solutions contained DSPE-PEG-amine and 100 μg Fe/ml of the SPIOs coated with DSPE-mPEG1000 alone. The fluorescence intensity was quantified with a microplate reader (ex: 395 nm, em: 470 nm). The number of amino groups per SPIO was estimated by dividing the molar concentration of amino groups with that of the SPIOs. As shown in Figure S5, our measurements indicated that the number of functionalized DSPE-PEG per nanoparticle was proportional to the initial DSPE-PEG loading ratio and the maximum number of NH_2 groups on each 14 nm SPIO is approximately 600.

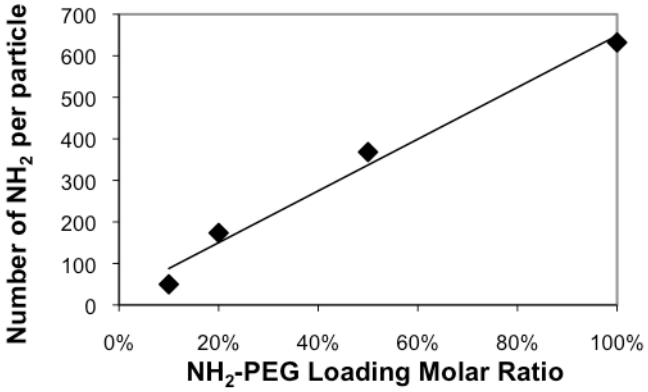

Figure S5. Density of amino groups on 14 nm SPIOs.

S8. Targeting tumor with SPIOs conjugated with antibody

5 nm and 14 nm SPIOs were conjugated with an antibody targeting VEGF receptor-1 (VEGFR-1). An in vitro ELISA assay was used to test the specificity of the SPIOs. In brief, plates were coated with purified mouse VEGFR-1 and saturated with BSA. 5 and 14 nm SPIOs were loaded to the ELISA plate at 9.7 nM particle concentration. After incubated 1 hour at 37°C, the plate was washed with PBS containing 0.05% TWEEN-20. Iron content of bound SPIOs in each well was quantified using Ferrozine method. Both SPIOs showed excellent specificity to mouse VEGFR-1 (Figure S6).

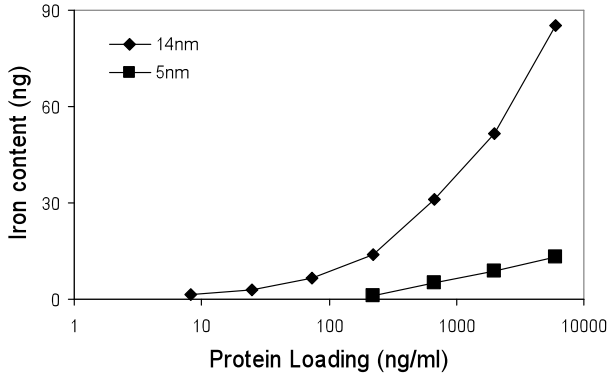

Figure S6. Targeting specificity of SPIOs in vitro.

5 nm and 14 nm SPIOs conjugated with antibody were administrated to nude mice carrying U87-MG tumor by tail vein injection. The tumors were imaged before and one hour after injection with a Bruker 7T small animal MRI instrument using spin-echo pulse sequence. T_2 values were calculated for individual pixels in the tumor cross sections. The histograms of $1/T_2$ were plotted for animals treated with 5 nm or 14 nm SPIOs, respectively (Figure S7). Only 14 nm SPIO induced a detectable increase in T_2 relaxivity of the tumor tissue.

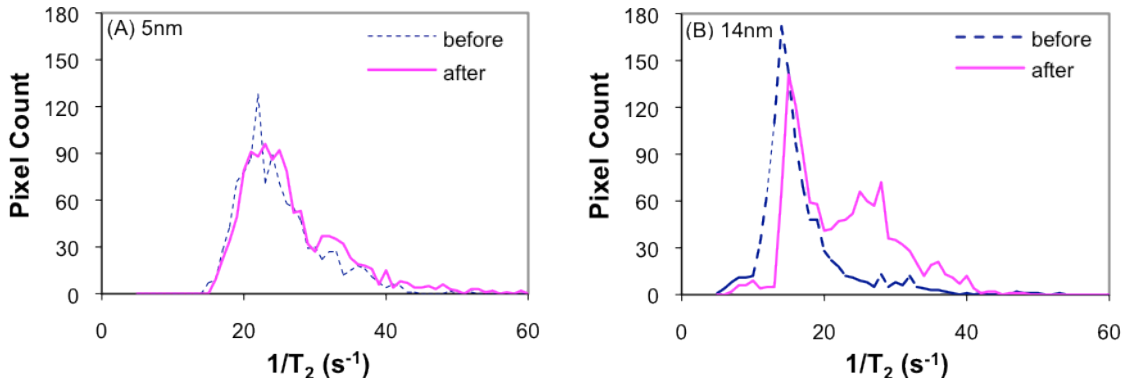

Figure S7. Histograms of $1/T_2$ map of tumors. The $1/T_2$ histograms were plotted for tumors before and after injection of either 5 nm (A) or 14 nm (B) SPIOs conjugated with the antibody targeting VEGFR1.

S9. Materials and methods:

Materials. Iron tri(acetyl acetonate), oleic acid, oleylamine, hexane, toluene, hydrochloric acid, hydroxylamine HCl, sodium hydroxide, ammonium acetate, fluorescamine, phosphotungstic acid and ferrozine were purchased from Sigma-Aldrich. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-X] (ammonium salt), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (ammonium salt) were purchased from Avanti Polar Lipids. X is the molecular weight of methoxy (polyethylene glycol) ranging from 550 Dalton to 5000 Dalton. For simplicity, the three molecules are referred to as DSPE-mPEG, DSPE-PEG-amine and DSPE-PEG-maleimide, respectively. All chemicals were used without further modification.

Mouse IgG and goat anti mouse IgG were purchased from Vector Laboratories. Goat anti mouse IgG conjugated with horseradish peroxidase was purchased from Santa Cruz Biotechnology. Goat anti-mouse VEGFR-1 antibody was purchased from R&D systems. ABTS ELISA peroxidase substrate was purchased from Rockland Immunochemicals. 2-Mercaptoethylamine•HCl was purchased from Pierce Biotechnology. Human U87-MG glioblastoma cell line was purchased from ATCC.

Synthesis of iron oxide cores. Iron oxide cores were synthesized by thermodecomposition of iron tri(acetyl acetonate) in a mixture of oleic acid and oleylamine.[4] The core size was controlled by modulating the ratio between oleic acid and oleylamine and the temperature ramping (see section S2). For 14 nm cores, iron tri(acetyl acetonate) (1.40 g), oleic acid (8.90 g), oleylamine (8.13 g) were mixed in an 100 mL two-neck flask. Cycles of vacuum-Ar purging were carried out for three times. Temperature was ramped from room temperature until 120°C during 30 minutes. The reaction was kept at 120°C for two hours. Then the temperature was ramped again to 220°C during 30 minutes and kept at 220°C for 30 minutes. Finally the temperature was ramped to 300°C during one hour and kept at 300°C for 30 minutes. During the whole reaction, the reaction mixture was kept under argon. After cooled down to room temperature, the reaction mixture was washed with ethanol and hexane for four times. In each wash, the black liquid was dripped into ethanol. After centrifugation at 3000 g overnight, the

precipitate was collected, dried in air and dissolved in hexanes. The solution was centrifuged at 3000 g for 30 minutes and the supernatant was collected. After the last wash, the deposit was dissolved in toluene and the solution was stored at 4°C under Argon.

Surface coating with DSPE-mPEG. To make water soluble SPIO, iron oxide cores were coated with DSPE-mPEG using a dual solvent exchange method. DSPE-mPEG with PEG size of 1000 to 5000 Da was dissolved in chloroform. Iron oxide cores (dispersed in toluene) and DSPE-mPEG were mixed at 1:1 weight ratio (iron : DSPE-mPEG). Then, 4 volume of DMSO was added gradually to the mixture. After chloroform and toluene were completely vaporized under vacuum, DMSO was substituted by water using an ultrafiltration centrifugal device with polyethersulfone membrane (100K Da cutoff size). The empty micelles were removed by ultracentrifugation at 100,000 g. Finally, SPIO solutions were centrifuged at 3000 g and the precipitates were discarded to remove large aggregates.

When the molecular weight is smaller than 1000 Da, DSPE-mPEG has a strong tendency to form lamellar structures. We found that iron oxide cores would precipitate after DMSO was added to chloroform solution. To overcome this problem, the solutions were heated to break down the lamellar structures.[5] DSPE-mPEG was dissolved in toluene instead of chloroform. The mixture of iron oxide cores and DSPE-mPEG was heated to 70°C. After DMSO was added, the solution was maintained at 70°C for 3 hours. The rest of the procedure is the same as described above.

Functionalization of SPIOs. Amino, carboxylic and maleimidyl groups were added to the SPIOs by coating the iron oxide cores with mixtures of DSPE-mPEG and functionalized DSPE-PEG. In order to increase the accessibility of functional groups, functionalized DSPE-PEG2000 (45 PEG units) were mixed with DSPE-mPEG1000 (22 PEG units) in chloroform at desired ratios and this mixed solution was used to coat iron oxide core as described previously.

To conjugate antibodies on the SPIOs, the disulfide bond in the antibody was reduced with 2-Mercaptoethylamine•HCl using the standard protocol. The reduced antibody was added to the SPIOs with maleimide groups in 0.1 M PBS solution at 4°C for overnight. Free antibody fragments were removed by dialysis.

Transmitted electron microscopy. 5 μl of the SPIO solution in toluene was dropped onto a carbon coated copper grid. After dried for 15 minutes, the copper grid was ready for TEM observation. High resolution TEM was performed with Hitachi 3600 transmitted electron microscope connected to a CCD camera. TEM images of DSPE-mPEG coated SPIOs were obtained by negatively staining the SPIOs with phosphotungstic acid.

Size determination with dynamic light scattering. The hydrodynamic diameter of SPIOs was determined by dynamic light scattering using a particle sizer - NICOMP 380ZLS (NICOMP Particle Sizing Systems, Santa Barbara, CA). The SPIO solution in deionized water was centrifuged at 10000 g for 5 minutes and the supernatant was collected for size determination. Number-weighted NICOMP analysis was used to calculate the size distribution of SPIOs. The size distribution data were collected after one hour of measurement according to the manufacturer's manual.

Iron measurement. To measure the iron content of SPIOs, the SPIOs was dissolved in concentrated hydrochloric acid and the iron concentration was measured using ferrozine method modified according

to previous literature [6]. In brief, 50 μl sample was mixed with equal volume of 12 N HCl and incubated at room temperature for 60 minutes. Thereafter, the solution was mixed with 240 μl 2 M NaOH, 50μl 4M ammonium acetate and 110 μl 5% hydroxylamine HCl. After 30 minutes incubation, the solution was mixed with 0.02% ferrozine solution. Light absorption was read at 562 nm with 810 nm as reference.

T_2 relaxivity. T_2 spin echo relaxation time of SPIOs was measured with a 0.47 T Bruker Minispec Analyzer MQ20 (Bruker, Fremont, CA). SPIOs were dissolved in 1ml deionized water and loaded to the sample tube. T_2 was measured at 40 °C using Hahn Spin Echo method. T_2 relaxivity was calculated as $1/T_2$ divided by molar concentration of iron.

In vitro targeting using SPIOs conjugated with antibody. Mouse IgG was purified with Melon Gel IgG spin purification kit (Pierce Biotechnology, Rockford, IL). The final concentration of IgG was quantified with coomassie blue method. IgG was diluted at various concentration in PBS containing 2 μg/ml BSA. IgG solutions were load to a 96 well ELISA plate and incubated at 4°C for 24 hours. After washing, the plates were blocked with 1% BSA solution at 4°C for 24 hours. The plates were incubated with either goat anti mouse IgG conjugated with horseradish peroxidase (2 μg/ml) or SPIOs conjugated with goat anti mouse IgG. The concentrations of 5 nm and 14 nm SPIOs were fixed at 9.7 nM. After one hour incubation at 37°C, all wells were washed with PBS containing 0.05% Tween-20. ABTS substrate was added to the wells containing peroxidase conjugated antibody. Light absorption was read at 405 nm using a microplate reader. The wells treated with SPIOs were incubated with 50 μl of 6N HCl for 30 minutes at room temperature. Then, 120 μl of 2 N NaOH, 25 μl of ammonium acetate buffer, 25 μl of 5% hydroxylamine HCl and 30 μl of ferrozine were added sequentially. Iron content of bound SPIOs was determined by light absorption at 562 nm with 810 nm as reference. To estimate the T_2 effect of bound SPIOs in MRI, the same amount of SPIOs were suspended in 50 μl water and loaded to untreated wells. MRI images were collected using spin-echo sequence in a Bruker 7 T small animal MRI instrument equipped with a 38 mm surface coil. The map of T_2 effect was calculated using a program developed in Matlab.

In vivo MR imaging. All animal procedures were conducted with the approval of the Institutional Animal Care and Use Committee at the Georgia Institute of Technology. Tumors were induced in nude mice by injecting human U87-MG glioblastoma cells subcutaneously. In vivo MR imaging were performed using a Bruker 7 T small animal MRI instrument with a 38 mm surface coil (Pharmascan, Bruker). Tumor-bearing mice were imaged using a spin-echo sequence (TR = 1000 ms. TE increased from 12 ms to 144 ms with an increment of 12 ms. Matrix size = 256×256, FOV = 30 mm). Images were taken before and one hour after tail vein injection of SPIOs conjugated with antibody against mouse VEGFR-1 (Dose = 0.15 ml of 200 nM SPIOs). The map of T_2 effect was calculated using a program developed in Matlab.

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