Supporting Information

Ultra-Small c(RGDyK)-coated Fe_3O_4 Nanoparticles and Their Specific Targeting to Integrin $\alpha_v\beta_3$-rich Tumor Cells

Jin Xie,[†] Kai Chen,[‡] Ha-Young Lee,[‡] Chenjie Xu,[†] Andrew R. Hsu,[‡] Sheng Peng,[†] Xiaoyuan Chen,*[,‡] Shouheng Sun*[,†]

Department of Chemistry, Brown University, Providence, Rhode Island 02912; and Molecular Imaging Program at Stanford (MIPS) and Bio-X Department of Radiology, Stanford University, 1201 Welch Rd P095, Stanford, California 94305-5484

ssun@brown.edu; shawchen@stanford.edu

METHODS

Synthesis of 4.5 nm 4-methylcatechol (MC) coated Fe_3O_4 nanoparticles (NPs). 20 mg of 4-methylcatechol was dissolved in 20 ml of benzyl ether, and the solution was heated at 120˚C under N_2 protection and kept at this temperature for 30 minutes. The solution was further heated to 220°C when 0.5 ml of $Fe(CO)_5$ was injected. After 10 minutes, the mixture solution was heated to reflux for 1 hour. Afterwards, it was cooled down to room temperature, and exposed to air for oxidation. The particles were precipitated by adding ethanol to the solution and collected by centrifugation. The product was washed twice with 1:1 mixture of ethanol and hexanes. The final product was re-dispersed in DMF. Other sizes of NPs were synthesized by tuning the ratios between the MC and $Fe(CO)_5$. More MC gives smaller iron oxide NPs. Some representative TEM images of the Fe_3O_4 NPs are shown in Figure S1.

Conjugation of c(RGDyD) peptide to MC-Fe_3O_4 NPs via Mannich reaction. 2 ml dispersion of MC-Fe_3O_4 NPs at ~3 mg/ml in DMF were mixed with 1 ml of the peptide solution (c(RGDyK), M.W. = 619.7, 2 mg/ml), 200 μl of formaldehyde (37% in water) as well as 100 μl of 1 M HCl and stirred for 3 hours at r.t. The reaction mixture was centrifuged at 9000 rpm for 30 minutes to recollect the particles at the bottom (saturated NaCl solution was added to facilitate the precipitation of the particles). The particles were re-dispersed in 2 ml of water with the help of sonication. The free peptide and formaldehyde were removed by PD-10 desalting column or dialysis against water.

Cell uptake experiment. U87MG and MCF-7 cell lines were cultured in DMEM (containing 10%FBS and 1% antibiotics) in T25 flasks. Before the incubation, the growth medium was removed, and the cells were washed twice with PBS, and blocked with 0.1% BSA for 5 minutes at r.t. c(RGDyK)-MC-Fe_3O_4 NPs at concentration of 5 μg Fe/ml in growth medium was added, and incubated with the cells (U87MG and MCF-7) at 37 °C, in 5% CO_2 for 30 minutes. For control, 2 μM c(RGDyK) was incubated with the particles. After 30 minutes incubation, the cells were washed twice with PBS, detached by trypsine, collected by centrifuge, and redispersed in medium. Cell concentration was determined

[†] Brown University.
[‡] Stanford University.

by hemacytometer counting. After this, the cells were lysed by nitric acid and the Fe concentration was determined by ICP-AES. Each test was run in parallel of three.

NP characterization. TEM images were taken on Philips EM 420 (120kV). Hydrodynamic sizes of the NPs were measured by Malvern Zeta Sizer S90 dynamic light scattering instrument. Mass analysis was performed on Voyager-DE PRO MALDI-MS.

Determination of c(RGDyK):NP ratio. It is technically challenging to measure the number of RGD peptides per iron oxide nanoparticle. We chose to conjugate cysteine onto the particle surface under the same Mannich condition and used ICP to quantify the sulphur content. Considering the fact that the cysteine might be partially oxidized to form disulfide bond, and larger steric hindrance of peptide that cysteine, we estimated that there were about 100-200 peptides per particle.

Cell Binding Assay. In vitro integrin $\alpha_v\beta_3$-binding affinities and specificities of c(RGDyK)-MC-Fe_3O_4 NPs were assessed via displacement cell-binding assays using ^{125}I-echistatin as the integrin $\alpha_v\beta_3$-specific radioligand. Experiments were performed on U87MG human glioblastoma cells using a previously described method (Li, Z. B.; Cai, W. B.; Cao, Q. Z.; Chen, K.; Wu, Z. H.; He, L. N.; Chen, X. Y. *J. Nucl. Med.* **2007***, 48,* 1162-1171.). Experiments were performed with triplicate samples and the best fit 50% inhibitory concentration (IC_{50}) values for the U87 MG cells were calculated by fitting the data with nonlinear regression using Graph-Pad Prism (GraphPad Software, Inc.).

In Vivo MRI Studies. Mice were anesthetized by inhalation of 1-2 % inhaled isoflurane anesthesia (IsoFlo, Abbott Laboratories, North Chicago, IL) in 1:2 O_2/N_2 and c(RGDyK)-MC-Fe_3O_4 NPs (15 mg/kg), and c(RGDyK)-MC-Fe_3O_4 NPs plus blocking dose of cold c(RGDyK) (10 mg/kg) were then injected intravenously through a tail vein. MR imaging was performed using a 3.0-Tesla whole body clinical MR scanner (GE Medical System, Systems Revision 12.0 M5, Milwaukee, WI, USA). The MR frame consisted of a nonmagnetic stereotactic wrist coil with a cylindrical surface coil (5 cm internal diameter) positioned directly over the mouse neck. T_2-weighted fast spin-echo imaging was performed under the following conditions: receiver bandwith = ± 16 kHz, repetition time (T_R) = 5000 ms, echo time = 86 ms, flip angle = 90 °C, echo train length = 8, field of view (FOV) = 4 × 4 cm^2, section thickness = 1 mm, 16 slices, matrix = 256 × 256, scan time = 5min 25sec. MR images were acquired either perpendicular to the anterior-posterior (long) axis of the animal (coronal) or parallel to the anterior-posterior direction (axially). Signal intensities (SI) were measured in defined regions of interest (ROIs) which were in similar locations within the tumor center using Image J software (US NIH, Bethesda, MD).

Histological Examination. Tumor mice were sacrificed 4 h after injection of c(RGDyK)-MC-Fe_3O_4 NPs with or without the presence of blocking dose of c(RGDyK) peptide for the confirmation of integrin $\alpha_v\beta_3$ specific binding of these NPs. Liver, spleen, kidneys,

tumor, and muscle were collected and placed into OCT using a plastic mold and the samples were immediately frozen using dry ice and placed into liquid nitrogen at –80 °C. Tissue sections were cut into 10 μm-thick slices and then stained with Prussian Blue. Briefly, the tissue slices were fixed with acetone and incubated with 10% potassium ferrocyanide in 20% hydrochloric acid for 20 min and washed in distilled water 3 times. The cells were incubated with 1 % eosin solution for 10 min and washed in distilled water for counterstaining with nuclear fast red. For dehydration, the cells were incubated with absolute ethanol for 3-5 min. Mounting medium was then placed on the slides along with a coverslip and the iron particles in cells were observed as blue dots using an optical microscope with phase contrast.

Histopathological staining. Tumor vasculature was stained with CD31. For CD31 staining, frozen section were dried at r.t. for 20 min and then put in ice-cold acetone for 10 min. After fixation, sections were dried at r.t. for 30 min, rinsed with PBS twice and incubated with primary rat anti-mouse CD31 antibody (BD Biosciences) at r.t. for 2 h. After rinse with PBS twice, sections were incubated with biotinylated anti-rat IgG secondary antibody (BD Biosciences) for 30 min at r.t. Then streptravidin-HRP (BD Biosciences) was applied to sections for 30 min at r.t. CD31 staining with brown color can be reached using glucose oxidase-diaminobenzidine (DAB) method. Slides were then put in Prussian blue staining solution and incubated at r.t. for 30 min. After rinse with PBS, slides were counterstained with nuclear fast red for 10 min at r.t. After rinse, slides were dehydrated by 70%, 80% and 100% EtOH and transferred into xylene for 5 min and were then mounted. Slides can be used for taking picture under light microscope after mounting media is completely dry. Tumor macrophages were stained with F4/80 antigen. The procedure for the overlay stain of F4/80 and Prussian blue is virtually the same as that of CD31/Prussian blue, except that the primary antibody for F4/80 is rat anti-mouse F4/80 antibody (Abcam).

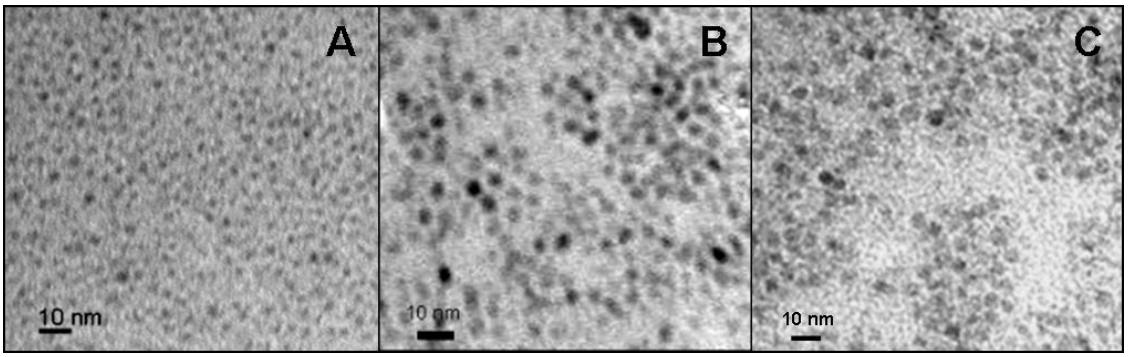


Figure S1. TEM images of the MC-Fe_3O_4 NPs at the sizes of (A) ~2.5 nm, (B) ~3.5 nm, (C) ~5nm.

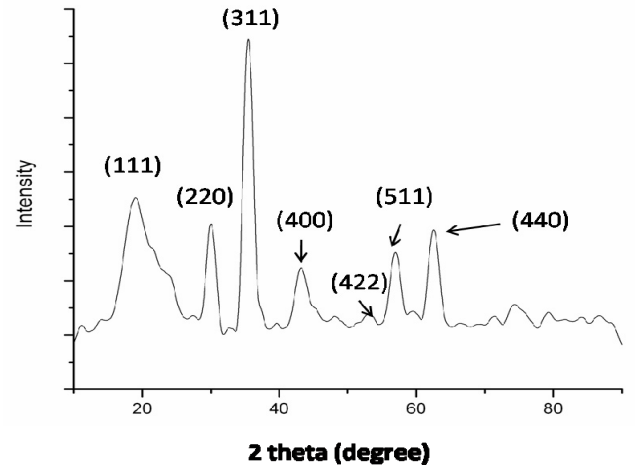
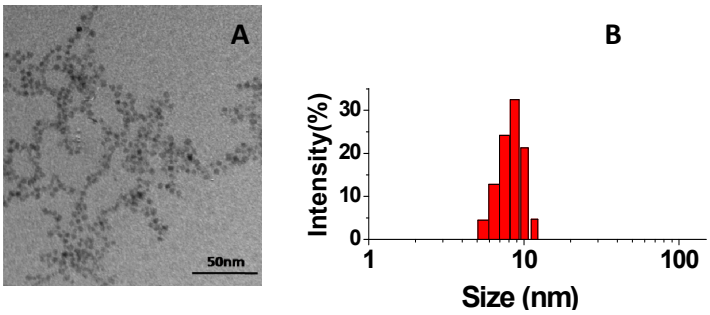


Figure S2. XRD analysis of the 5 nm MC-Fe_3O_4 NPs.



Figured S3. A) TEM of c(RGDyK)-MC-Fe_3O_4 NPs dispersed in water. B) Hydrodynamic size distribution of the c(RGDyK)-MC-Fe_3O_4 NPs dispersed in water.

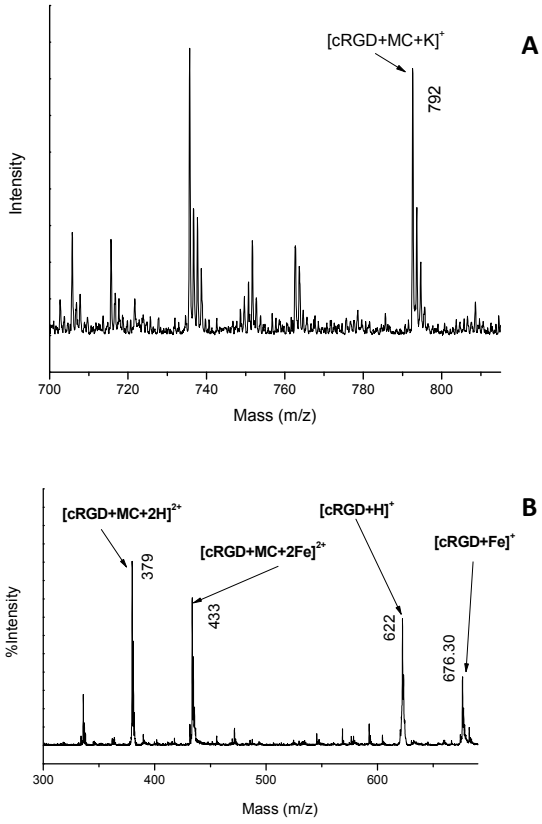


Figure S4. MALDI-MS of (A) c(RGDyK)-MC obtained from Mannich reaction between c(RGDyK) and 4-MC, and (B) c(RGDyK)-MC-Fe_3O_4 NPs. In (B), the NPs were subject to HCl incubation for 30 minutes first to destroy the Fe_3O_4 cores. HCl was then evaporated and the residues were redispersed in water. Note that K cannot be seen in (B) due to the high concentration of iron in the mixture.

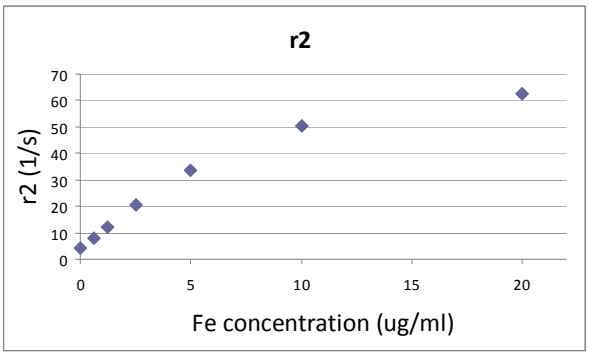


Figure S5. r_2 relaxivity of c(RGDyK)-MC-Fe_3O_4 NPs.

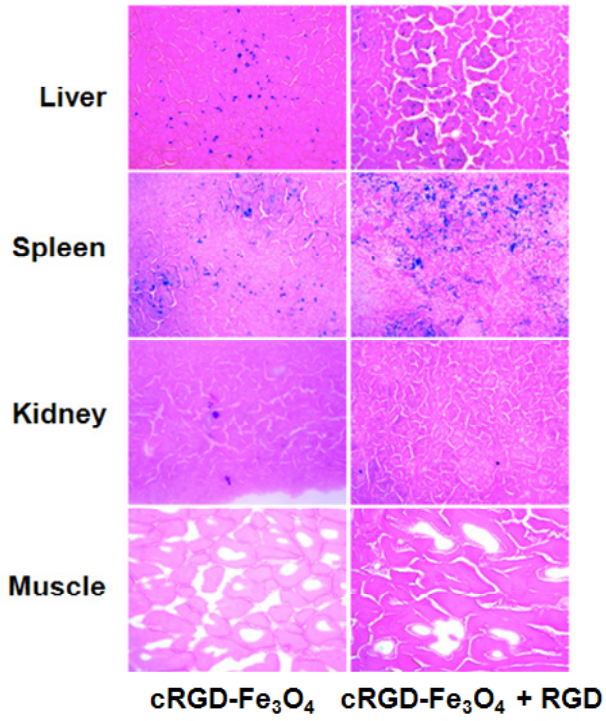


Figure S6. Prussian staining of liver, spleen, kidney and muscle.

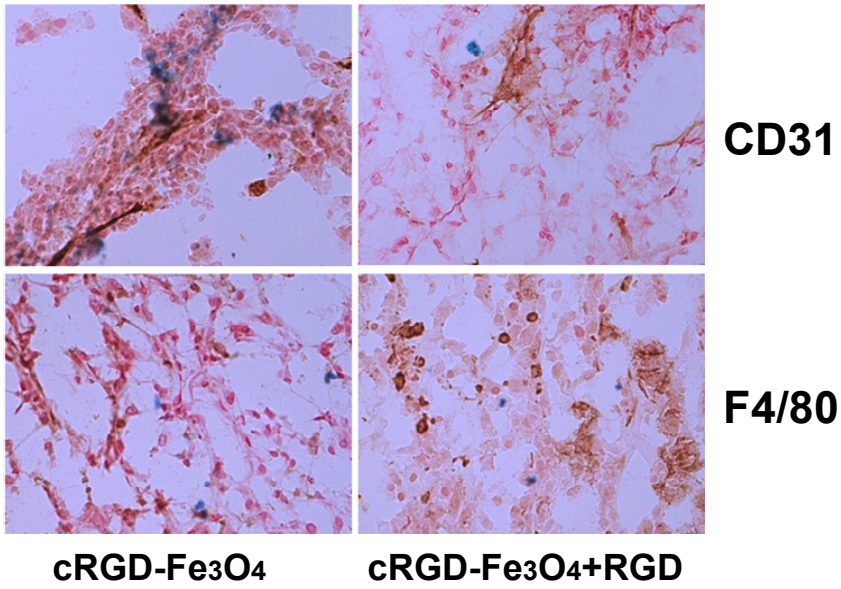


Figure S7. Overlay of Prussian blue/CD31 and Prussian blue/F4/80 of tumor slices. Note that most of the iron oxide nanoparticles are localized on the tumor vasculature (CD31 positive cells) with little or no uptake by the macrophages (F4/80 positive cells).