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Supporting Information

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Fe_5C_2 Nanoparticles with High MRI Contrast Enhancement for Tumor Imaging

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Experimental

Surface modification and conjugation

For surface modification, the as-synthesized Fe_5C_2 nanoparticles in chloroform were added to a DSPE-PEG-COOH chloroform solution. The mixture was agitated for 1 h, and the solvent was slowly evaporated. The nanoparticles were redispersed in water with sonication, and purified using a microcon centrifugal filter unit (YM-100) to remove excess DSPE-PEG-COOH. For conjugation, the solvent was exchanged to borate buffer (PH 8.3, 50 mM) using a microcon centrifugal filter unit. The particles were activated by EDC and NHS (100:1 molar ratio, relative to the amount of nanoparticles) for 30 min, and then collected and purified (3 ×) by a microcon centrifugal filter unit. The nanoparticles were then redispersed in PBS (pH 7.4). Into the solution, c(RGDyK) (100:1, molar ratio) was added, and the mixture was gently

agitated at room temperature for 1 h. The products were again purified using an microcon centrifugal filter to remove the free c(RGDyK). The final products, RGD-Fe_5C_2 nanoparticles, were redispersed in PBS. The conjugation between Fe_3O_4 and RGD followed a similar protocol.

Phantom study

DSPE-PEG-COOH coated Fe_5C_2 nanoparticles and carboxyl groups conjugated Fe_3O_4 nanoparticles (Ocean Nanotech, Inc.) with iron concentrations ranging from 8.93×10^{-4} to 1.79×10^{-2} mM were suspended in 1% agar gel in 300 µl PCR tubes. These tubes were embedded in 1% agar gel, which is held by a home-made tank with appropriate size to fit the MRI coil. MR scans of the phantom were conducted on a 7 T Varian small animal MRI system. In order to demonstrate the T_2 shortening effect of the nanoparticles, T_2-weighted fast spin echo (FSE) images were acquired with the following parameters: repetition time (TR) = 2 s; echo time (TE) = 48 ms; echo train length (ETL) = 8. For quantitative studies, T_2 values of various concentrations of Fe_5C_2 and Fe_3O_4 nanoparticles were derived using the spin echo sequence with 10 different TEs from 10 to 100 ms, with the step size set at 10 ms. Both scans shared the same slice setting: field-of-view (FOV) = 70^2 mm^2; matrix size = 256^2; 4 coronal slices with 1 mm slice thickness.

Cell line and animal models

The U87MG human glioblastoma cell line was obtained from the American Type Culture Collection (ATCC) and cultured with Dulbecco's Modified Eagle Medium (DMEM) in a cell culture flask. Athymic nude mice were purchased from Harlan laboratories. The animal model was established by subcutaneously injecting approximately 5×10^6 U87MG cells into the right hind limb of each mouse. In vivo MR imaging was performed when the tumor size reached ~ 200-500 mm^3. All of these procedures were conducted following a protocol

approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC).

Viability assays

Approximately 1×10^4 U87MG cells were seeded in each well of a 96-well plate. After 24 h incubation, Fe_5C_2 nanoparticles with different iron concentrations (0, 5, 10, 15, and 25 μg Fe/mL) were added to the plate. After incubation for another 24 h, MTT assay was performed to determine the cell viability. The cytotoxicity was also studied by live & dead assays (Invitrogen). Briefly, approximately 1×10^5 U87MG cells were seeded in each well of a 4-well cell culture chamber. After 24-hour incubation, Fe_5C_2 nanoparticles at the same concentration gradient (0, 5, 10, 15, 25 μg Fe/mL) were added, and incubated with the cells for 24 h. The cells were then washed with PBS, and incubated with the working solution (2 μM calcein AM and 4 μM EthD-III) at 37 ˚C for 30 minutes. After removing the solution, the stained cells were observed under a fluorescence microscope.

In vitro Prussian blue staining

The staining followed a previously published protocol (Chen et al. *Biomaterials* **2009**, *30*, 6912-6919). Approximately 1×10^5 U87MG cells were seeded in each well of a 4-well cell culture chamber. After 24 h incubation, the cells were fixed with ice-cold 95% EtOH for 15 min. Next, Fe_5C_2 or RGD-Fe_5C_2 nanoparticles at a concentration of 5 μg Fe/mL in PBS were added and incubated with the cells at room temperature for 1 h with gentle shaking. The particles were then removed and the cells were washed 3 times with PBS buffer. Subsequently, cells were incubated with Prussian blue staining solution (a mixture of equal volume of 20% hydrochloric acid and 10% potassium ferrocyanide solution) for 40 min at room temperature. The cells were then washed twice with PBS buffer and incubated with fast red nuclear

staining solution for 10 min. After consecutive dehydrations with 75%, 90%, and 100% EtOH, the chamber was removed and the slice was mounted.

In vivo MR imaging

The U87MG tumor-bearing mice were anesthetized with isoflurane and intravenously injected with RGD-Fe_5C_2, RGD-Fe_3O_4, and Fe_5C_2 nanoparticles at a dose of 10 mg Fe/kg. T_2-weighted FSE images were acquired on a 7 T Varian small animal MRI system before and 4 and 24 h post particle injections, with the following scan parameters: TR = 2.5 s; TE = 48 ms; ETL = 8; FOV 40^2 mm^2; matrix size = 256^2; 15 axial slices with 1 mm slice thickness. At the end of the 24 h scan, the mice were sacrificed. The tumors as well as the major organs were collected and snap-frozen for histology analyses.

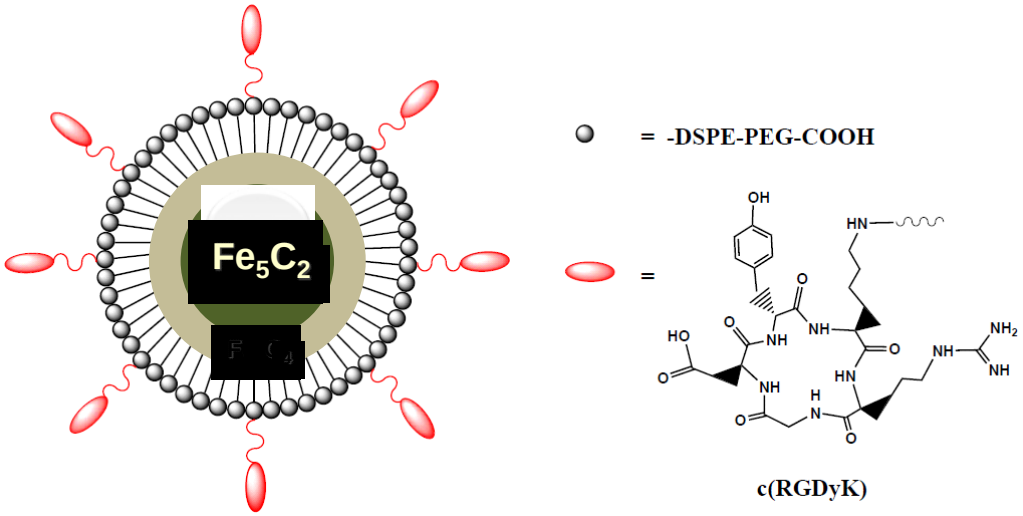
Prussian blue staining

The tumor and major organ were sectioned into 8 μm slices and stored at -80 ˚C. Before staining, the slices were warmed up for 20 min at room temperature and fixed with 10% formalin for 5 min. After the fixation, the samples were dried and immersed in a staining solution (a mixture of equal volume of 20% hydrochloric acid and 10% potassium ferrocyanide solution) for 40 min at room temperature. After being gently washed with PBS and counterstained with fast red nuclear solution for 3 min, the slices were dehydrated consecutively with 75%, 90%, and 100% EtOH. Finally, the samples were cleaned with xylene and mounted.

Prussian blue and integrin β_3 double staining

The frozen U87MG tumor blocks were sectioned into 8 μm slices and were warmed up for 20 min at room temperature. After fixation with ice-cold acetone for 10 min, slides were incubated in 0.3% H_2O_2 PBS solution for 10 min to block endogenous peroxidase activity.

The slides were then rinsed with PBS for 3 times, and then blocked with 100 μL 10% goat serum for 30 min at room temperature. After removing the blocking solution, anti-integrin β_3 primary antibody (ab75872, Abcam) was added and incubated with the slices overnight at 4 ˚C in a humid chamber. The slides were washed with PBS for 3 times (5 min each). Goat polyclonal secondary antibody to rabbit IgG (ab6721, 1:50) was applied and incubated with the slices for 1 h at room temperature. After rinsing 3 times with PBS, the slices were developed with DAB substrate solution until the desired color intensity was reached. The resulting slides were washed with PBS and subjected to Prussian blue staining using the protocol described above.



Scheme S1. Surface modification of Fe_5C_2 nanoparticles and the conjugation of c(RGDyK) to the particle surface.

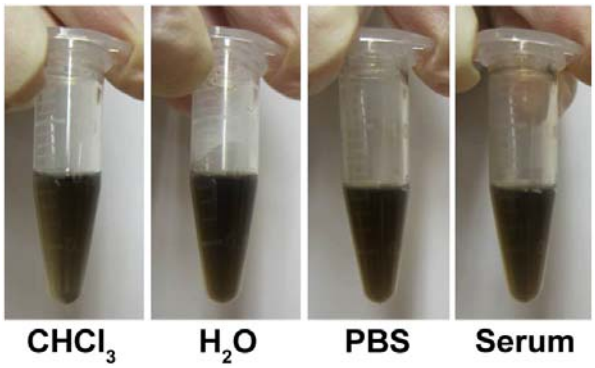


Figure S1. Fe_5C_2 nanoparticles dispersed in $CHCl_3$ (before surface modification), and in H_2O, PBS, and serum (after surface modification). The nanoparticles are stable for more than 24 h in all the solvents.

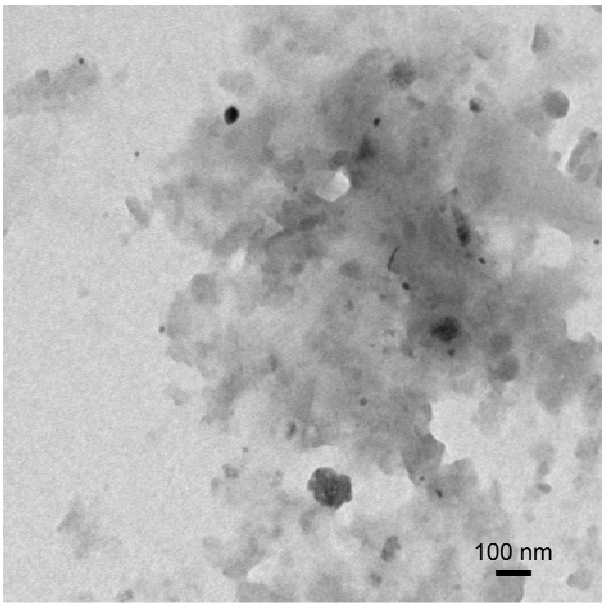


Figure S2. TEM image of Fe_5C_2 nanoparticles after incubation for 72 h in a pH 5.0 solution. Most nanoparticles were found degraded.