

ELECTRONIC SUPPLEMENTARY INFORMATION

Endothelin B receptors targeted by iron oxide nanoparticles functionalized with a specific antibody: toward immunoimaging of brain tumors

S. Richard,^a M. Boucher,^b A. Herbet,^c Y. Lalatonne,^a S. Mériaux,^{‡b} D. Boquet,^{‡*c} L. Motte^{‡a}

ESI-1. Monoclonal antibody rendomab-B1-AF488 (AF488-RB1)

Rendomab-B1 antibodies were produced in cell culture (hybridoma) using a bioreactor (Integra Bioscience - CL350, INTEGRA Biosciences AG, Switzerland). Antibodies were purified by affinity chromatography on protein A-Sepharose (Merck Millipore, USA). Antibodies were labeled with Alexa Fluor® 488 dye according the supplier's protocol (Life Technologies, USA).

ESI-2. $\gamma\text{Fe}_2\text{O}_3@CA$ -AF488-RB1 NP preparation and characterization

Chemicals

Reagents for particle synthesis and caffeic acid were from Sigma-Aldrich (Saint Louis, MO, USA). To conjugate antibody to the nanoparticles (NPs), we used EDC from Alfa Aesar (Ward Hill, MA, USA), Pluronic F127 from Sigma-Aldrich, H_3PO_4 and ethanolamine from Acros Organics (Geel, Belgium), Tris from Carl Roth (Karlsruhe, Germany) and 0.2% Tween from Merck Millipore. To release antibody, we used FBS from Dominique Dutscher (Brumath, France) and antiprotease from Roche (Basel, Switzerland). The fluorochrome reagent solution (OPA) was from Thermo-Scientific (Waltham, MA, USA).

NP synthesis and characterization

Noncoated $\gamma\text{Fe}_2\text{O}_3$ particles were synthesized by the reaction of ferrous dodecyl sulfate with dimethylamine. After 2 hours at 28°C, the solution was rinsed under acidic conditions. The magnetic particles were then separated from the supernatant using a permanent magnet and dispersed in water at pH 2. After synthesis, the NP surface was directly functionalized with caffeic acid. 10 mL of an aqueous caffeic acid solution (3 mg.mL⁻¹) at pH 10 was directly mixed with 3 mL of bare $\gamma\text{Fe}_2\text{O}_3$ particle suspension (10 mg.mL⁻¹) at pH 10. After mixing for 2 hours at room temperature, the functionalized magnetic particles were washed 7 times using magnetic separation at pH 2. The magnetic $\gamma\text{Fe}_2\text{O}_3@CA$ particles were then dispersed in distilled water at pH 7.

The average number of molecules of CA per nanocrystal (1100 CA per NP) was measured by thermogravimetric analysis using a LabsSys evo TG-DTA-DSC 16000 device from Setaram Instrumentation (Caluire, France).

The stability of the nanopatform $\gamma\text{Fe}_2\text{O}_3@AC$ ([Fe] = 0.25 mM) as a function of pH was investigated by dynamic laser light scattering using a Nano-ZS (Red Badge) ZEN 3600 device (Malvern Instruments, Malvern, UK). Figure SI-1 shows the change in hydrodynamic diameter and zeta potential of $\gamma\text{Fe}_2\text{O}_3@AC$ versus pH.

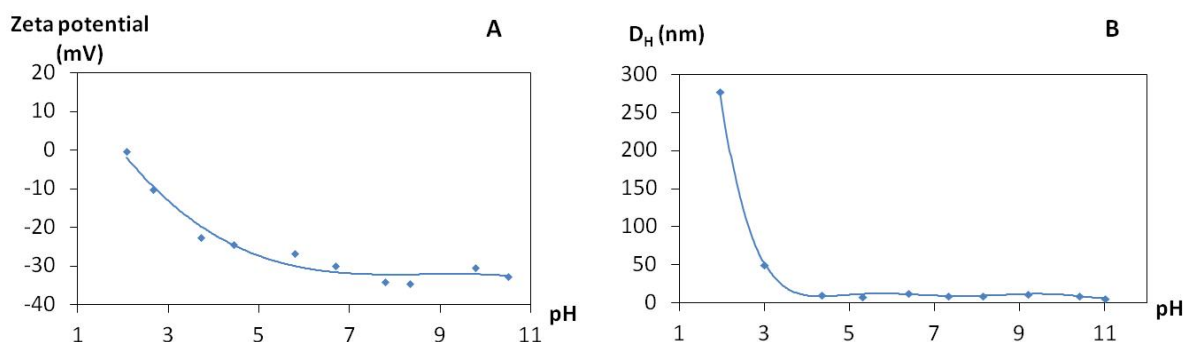


Fig. ESI-1: Zeta potential (A) and hydrodynamic diameter (B) as a function of the pH of $\gamma\text{Fe}_2\text{O}_3@AC$ solution.

Carbodiimide chemistry was used to conjugate random-B1 labeled with AF488 (AF488-RB1) and $\gamma\text{Fe}_2\text{O}_3@CA$ particles. First, the carboxylic acid functions at the outer surface of the nanocrystals were activated using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, $n_{\text{EDC}} = 4 n_{\text{COOH}}$) in grafting buffer (0.3% Pluronic F127, 100 mM H_3PO_4 pH 4) for 10 minutes at 37°C. The second step was the linkage of the amine function of RB1-AF488 with the activated carboxylic acid functions on the nanocrystals. The procedure was carried out with molar ratios $R = n_{\text{AF488-RB1}}/n_{\text{NP}}$ equal to 2 and 4. AF488-RB1 was dissolved in grafting buffer at pH 4 and added to the ferrofluid for 60 minutes at 37°C. The activated carboxylic functions that did not react with the amine function of the antibody were saturated in grafting ethanolamine (EA) buffer ($n_{\text{EA}} = 5 n_{\text{COOH}}$) for 30 minutes at 37°C. The modified particles were isolated with a magnet in blocking buffer (50 mM Tris, 0.2% Tween, pH 8.6) 2 times and washed 2 times with deionized water by centrifugation (14600 rpm, 3 min). The particles were dispersed in water at physiological pH for physicochemical characterization.

TEM images were obtained using a FEI CM10 Microscope (Philips) and samples were prepared by depositing a drop of nanoparticle suspension on carbon coated copper grids placed on a filter paper. The median diameter was deduced from TEM data measurements, simulating the diameter distribution with a lognormal function. The TEM images and the size distributions, Fig. ESI-2, show that the nanoparticles with or without antibody have a diameter around 9.6 nm with a size distribution $\sigma=0.2$.

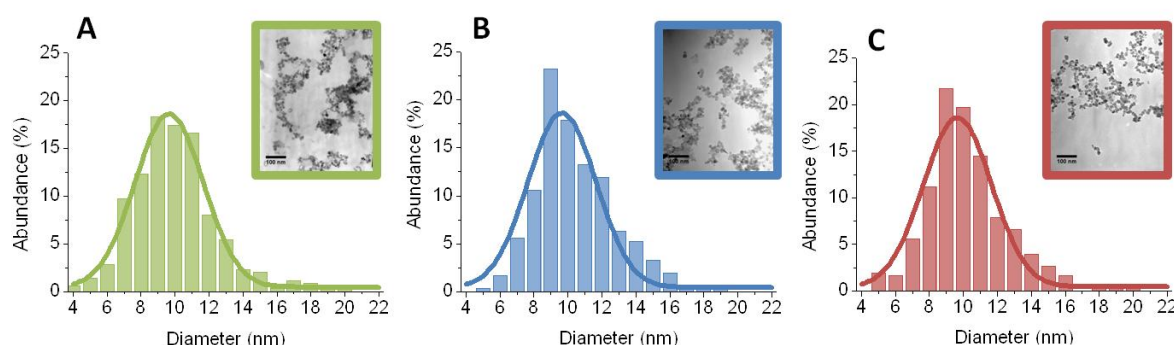


Fig. ESI-2: TEM images and size distributions of $\gamma\text{Fe}_2\text{O}_3@CA$ (A), $\gamma\text{Fe}_2\text{O}_3@CA\text{-AF488-RB1}$ (B) and $\gamma\text{Fe}_2\text{O}_3@CA\text{-(AF488-RB1)}_2$ (C)

The average number of grafted antibodies per nanocrystal was deduced by measuring the fluorescence emission spectrum (excitation wavelength 488 nm) of residual AF488-RB1 in the supernatant or by using the OPA method. For the OPA method, 50 μL of the sample (iron concentration between 2.5 and 4.5 mM) was diluted in 50 μL of 2 M NaOH and left overnight at 60°C. Then, 900 μL of OPA reagent was added to the mixture and fluorescence was recorded. The fluorescence measurements were performed with a Spex FluoroMax spectrofluorometer (HORIBA Jobin-Yvon, France) equipped with a Hamamatsu Photonics K.K. 928 photomultiplier (Hamamatsu, Japan). For each experiment, a calibration curve was plotted (Figure SI-2).

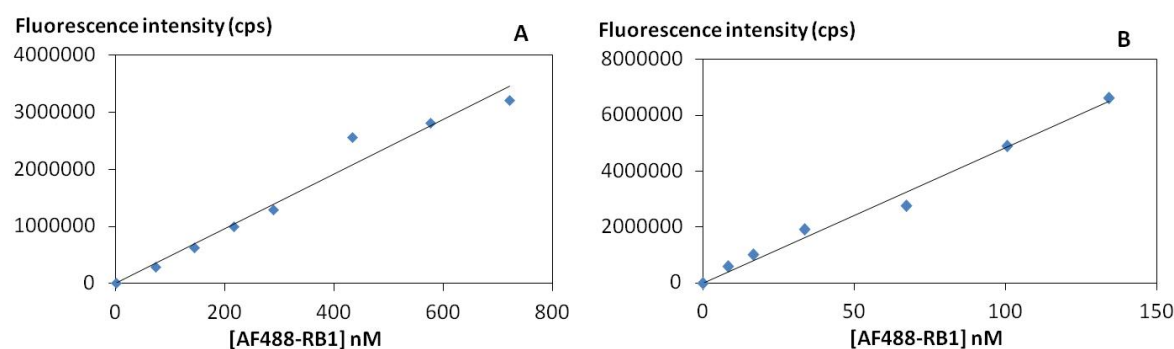


Fig. ESI-3: (A) Calibration curve of AF488-RB1 by fluorescent measurement by the OPA method ($\lambda_{\text{ex}} = 330$ nm) and (B) calibration curve of AF488-RB1 by fluorescence measurement ($\lambda_{\text{ex}} = 488$ nm)

The average number of AF488-RB1 per NP was deduced from these two calibration curves. The quenching factor was deduced considering the fluorescence intensity of the nanocomplexes and the expected intensity considering the number of AF488-RB1 per NP.

The release of $\gamma\text{Fe}_2\text{O}_3@CA\text{-AF488-RB1}$ ($R = n_{\text{AF488-RB1}}/n_{\text{NP}}$: $R=2$ and $R=4$) in 10% FBS with or without antiprotease at 50 nM RB1-AF488 was investigated by fluorescence measurement ($\lambda_{\text{ex}} = 488$ nm).

ESI-3. Cell culture

CHO cells (European Collection of Cell Cultures ECACC Salisbury, UK) were cultured in Ham-F12 medium supplemented with 10% fetal calf serum, 1 mM pyruvate, 1% nonessential amino acids, 2 mM glutamine, 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin, and were maintained at 37°C in a humidified atmosphere of 5% CO_2 . All media and cell culture supplements were from Invitrogen (Life Technologies, Carlsbad, USA).

ESI-4. Flow cytometry

Flow cytometric analysis was performed on CHO-WT (wild-type) and CHO-ETBr cells. Eighty percent (80%) confluent cells in 75-cm² flasks were washed with phosphate buffer saline (D-PBS, Invitrogen) and collected after incubation at 37°C for 15 min with 10 mL of versene buffer (D-PBS/8 mM EDTA). Cells were then seeded (300000 cells/tube) in 15 mL Falcon® tubes, which were centrifuged. Supernatant was discarded and cells were incubated for 2 h at 4°C with 500 μL of D-PBS/0.1% BSA/5% normal goat serum (NGS, Invitrogen) containing 100 nM ligand ($\gamma\text{Fe}_2\text{O}_3@CA$, $\gamma\text{Fe}_2\text{O}_3@CA\text{-AF488-RB1}$ and $\gamma\text{Fe}_2\text{O}_3@CA\text{-(AF488-RB1)}_2$) except for ET-1 (1 μM). Cells were washed twice and resuspended in 500 μL of D-PBS. Fluorescein-labeled ET-1 (ET-1 FAM, PhoenixPharmaceuticals) was used to test for ETBR expression at the cell surface. Fluorescence was assayed using a FACs Calibur (BD Bioscience, Franklin Lakes, USA) flow cytometer and mean fluorescence intensity (MFI) was measured (30000 cells/count). In Figure 2, CHO-WT cells are represented by blue bars and CHO-ETBR by red bars, autofluorescence is indicated by “Cells” and the positive control is represented by ET-1-FAM.

ESI-5. MTT toxicity assays

Cells were seeded at a density of 1.10^4 cells/well in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated in complete culture medium 24 hours. Then medium was removed and replaced by complete culture medium containing increasing concentrations of $\gamma\text{Fe}_2\text{O}_3@CA\text{-AF488-RB1}$ and $\gamma\text{Fe}_2\text{O}_3@CA\text{-(AF488-RB1)}_2$. After 3 hours, medium was removed and PBS with MTT (0.5 mg mL^{-1}) was added for 2 hours at 37°C. Medium was removed again and insoluble formazan crystals were dissolved by addition of DMSO. The optical density was measured at 570 nm using a spectramax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

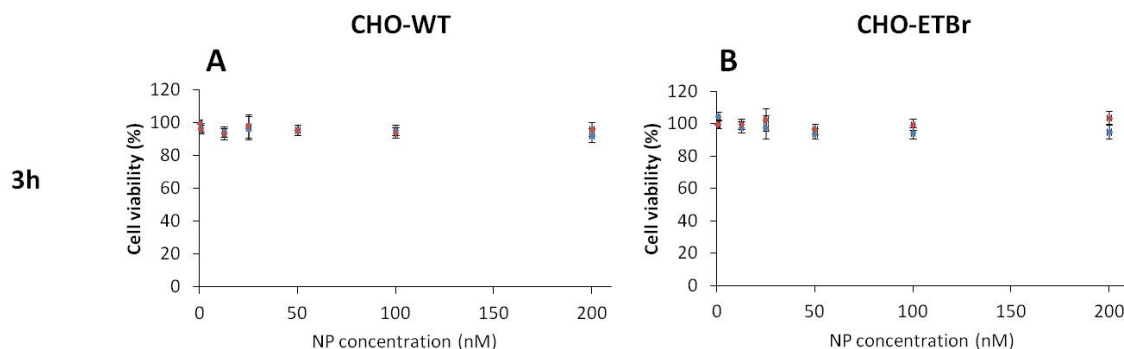


Fig. ESI-4: MTT viability assay following a 3 hours incubation of $\gamma\text{Fe}_2\text{O}_3@CA\text{-AF488-RB1}$ (blue) and $\gamma\text{Fe}_2\text{O}_3@CA\text{-(AF488-RB1)}_2$ (red) with CHO-WT (A) and CHO-ETBR (B) cells.

ESI-6. MRI protocols and data analysis

In vitro measurement of nanocomplex relaxivities.

Longitudinal r_1 and transverse r_2 relaxivities of $\gamma\text{Fe}_2\text{O}_3@CA$, $\gamma\text{Fe}_2\text{O}_3@CA\text{-AF488-RB1}$ and $\gamma\text{Fe}_2\text{O}_3@CA\text{-(AF488-RB1)}_2$ NPs were measured *in vitro* at 37°C and 7 T (Bruker PharmaScan preclinical scanner, Ettlingen, Germany) using dedicated phantoms consisting of 11 tubes filled with different concentrations of contrast agent (from 0.001 to 0.1 mM for functionalized NPs and 0.01 to 0.25 mM for control NPs) suspended in 0.3% w/w agar matrix. T_1 and T_2 maps were acquired respectively with an Inversion Recovery Fast Gradient Echo Sequence (TE = 2.5 ms, 90 inversion times with $\Delta\text{TI} = 100$ ms, 6 segments, TR = 15000 ms, Field Of View = 3×3 cm² in-plane, acquisition matrix = 120 x 120, 6 slices of 1.25 mm thickness, total acquisition time = 54 min) and a Multi-Slice Multi-Echo sequence (TE = 7.7 ms, 64 echoes, TR = 10000 ms, Field Of View = 3×3 cm² in-plane, acquisition matrix = 120 x 120, 6 slices of 1.25 mm thickness, total acquisition time = 1h 20min).

In a first post-processing step, T_1 (resp. T_2) maps were reconstructed by fitting the signal intensity versus inversion time (resp. echo time) using Bloch equations. Then, the mean relaxation rate R_1 (resp. R_2), equal to the inverse of relaxation time T_1 (resp. T_2), was computed for each tube, and a linear fit of R_1 (resp. R_2) values versus iron concentrations was performed to estimate the longitudinal relaxivity r_1 (resp. transverse relaxivity r_2) as the slope of the fitted curve.

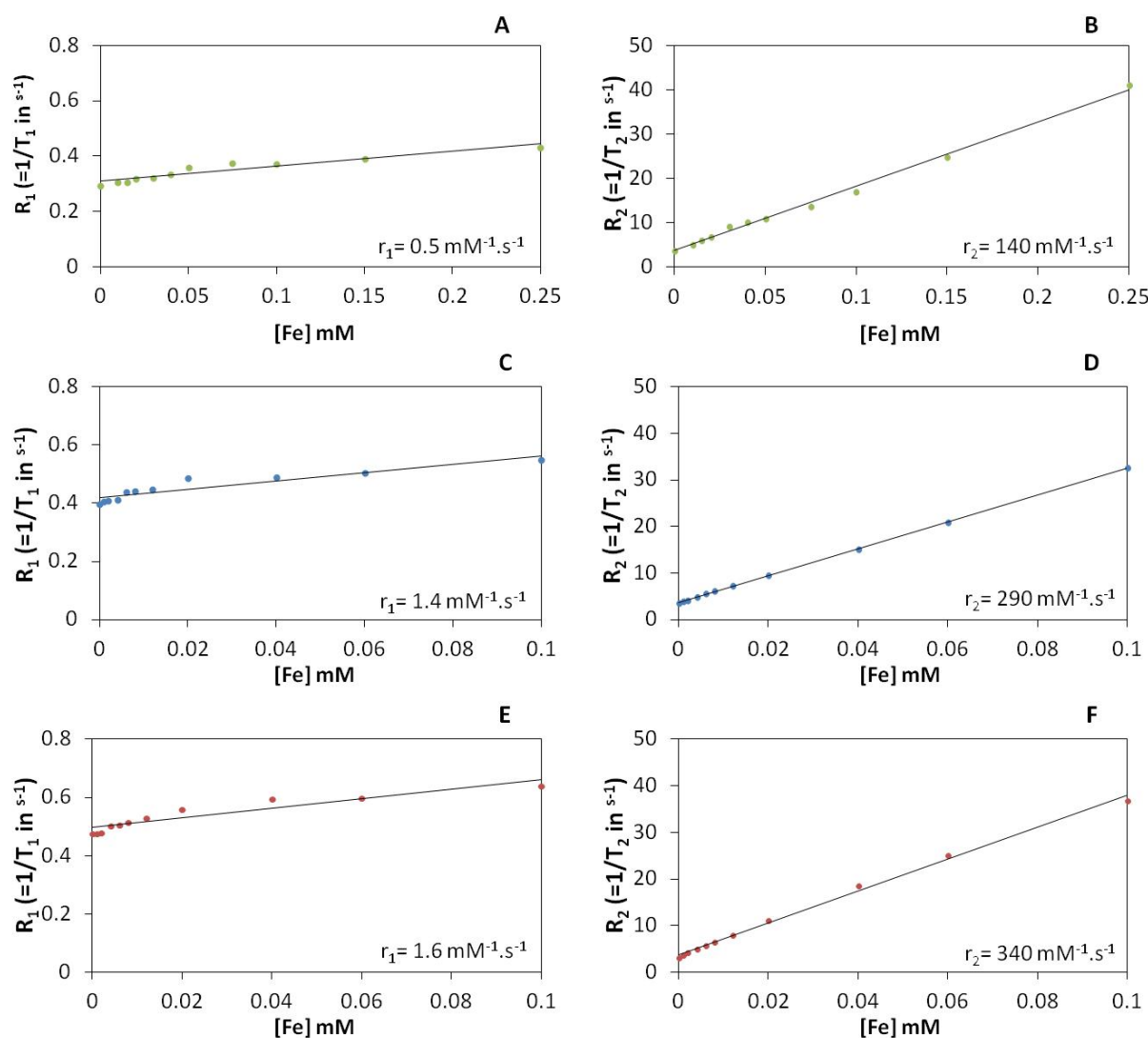


Fig. ESI-5: *In vitro* longitudinal relaxivity measurements (A, C and E) and transverse relaxivity measurements (B, D and F) at 37°C and 7 T of $\gamma\text{Fe}_2\text{O}_3@CA\text{-AF488-RB1}$ ($R = n_{\text{RB1-AF488}}/n_{\text{NP}}$: green $R=0$, blue $R=2$ and red $R=4$)

Animal preparation.

All *in vivo* experiments were performed in accordance with the recommendations of the European Community (86/609/EEC) and French legislation (decree no. 87/848) for use and care of laboratory animals. The protocol for contrast agent injection has been approved by the Comité d’Ethique en Expérimentation Animale du Commissariat à l’Energie Atomique et aux Energies Alternatives – Direction des Sciences du Vivant Ile de France (CETEA / CEA / DSV IdD) under protocol ID 10-025. MRI experiments were carried on male Swiss mice (25 g, Janvier, Le Genest-St-Isle, France) anesthetized with 2% isoflurane in a mixture of air and oxygen and placed in a cradle in prone position. Body temperature and breathing rate were continuously monitored throughout the experiment and a warm air blowing system kept body temperature around 37°C. Between pre- and post-injection scans (Figure SI-4), contrast agent was injected at the caudal vein with an insulin syringe (29G, 300 μL capacity, 150 μL injected volume for an approximate dose of 225 $\mu\text{mol}_{\text{Fe}}/\text{kg}$) by removing the cradle from the MRI scanner tunnel. The post-injection scan was usually launched 15 min after contrast agent injection.

In vivo MRI acquisitions.

Mice were scanned using the same 7 T preclinical scanner to highlight the ability of the NPs to enhance brain vessel contrast as well as to show their access to the brain vasculature. Prior to and after injection, a T_2^* -weighted FLASH (Fast Low Angle SHot) sequence (TE = 16 ms, TR = 485 ms, NEX = 48, Field Of View = 1.92 x 1.68 cm², spatial resolution = 160 x 160 μm^2 in-plane, 22 slices of 0.32 mm thickness, total acquisition time = 40 min) was acquired.

Data post-processing.

FLASH images were reconstructed from raw data using home-made routines written in Matlab (The Mathworks, Natick, USA). To reconstruct brain angiograms, images were zero-filled to 512 x 512 in-plane and then filtered according to the Frangi method¹⁻² which computes the magnitude gradient in 3 dimensions and evaluates the probability for a vessel to cross a given voxel. A mask of the brain was produced manually to compute the filtered image only in the region of interest: the mouse brain. Finally, Anatomist software (CEA NeuroSpin, Gif-sur-Yvette, France, <http://brainvisa.info>) was used to produce a Maximum Intensity Projection of the 3D angiogram for visualization (Figure 3).

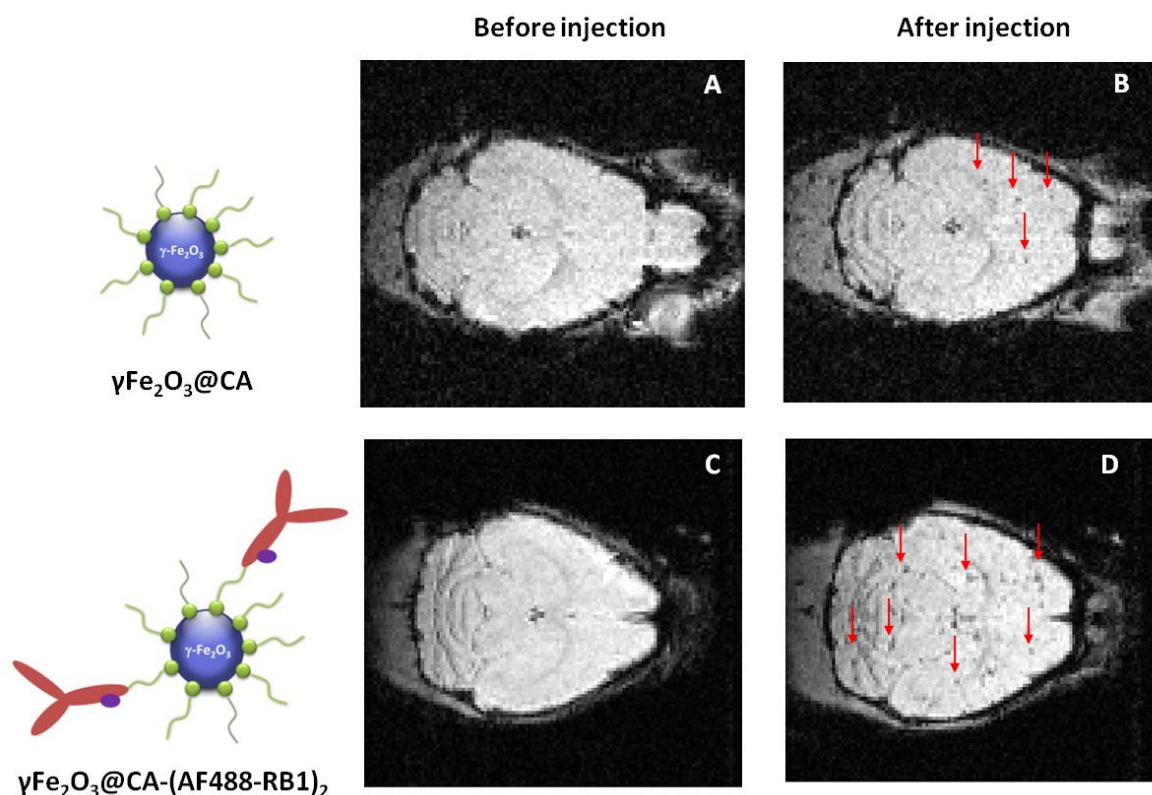


Fig. ESI-6: FLASH images showing the hypo-intense voxels due to the presence of iron circulating into blood stream after NPs injection.

References

1. Frangi AF, Niessen WJ, Vincken KL, and Viergever MA. Multiscale vessel enhancement filtering: In: Medical image computing and computer-assisted intervention MICCAI'98, Wells WM, Colchester A, Delp S editors. *Lecture Notes in Computer Science*. Volume 1496 Berlin: Springer-Verlag; 1998, p. 130-137.
2. Manniesing R, and Niessen W, Multiscale vessel enhancing diffusion in CT angiography noise filtering. *Inf Process Med Imaging*, 2005, **19**, 138.