Electronic Supporting Information for

Gd_2O_3 and GH Combined with Red Blood Cells to Improve Sensitivity of Contrast Agents for Cancer Targeting MR Imaging

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Experimental Section

Reagents and materials

$GdCl_3 \cdot 6H_2O$, D-glucuronic acid, urethane, sodium pyruvate and FITC were obtained from Sigma-Aldrich. cRGDyK was synthesized by GL Biochem Ltd. (Shanghai, China). Roswell Park Memorial Institute Medium (RPMI-1640) and fetal bovine serum (FBS) were acquired from HyClone (Logan, Utah, USA). Gd-DTPA was obtained from Consun Pharmaceutical Co., Ltd. (Guangzhou, China). All the other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Preparation of RBC-cRGD.

Synthesis of Gd_2O_3: The synthesis procedure of Gd_2O_3 was carried out according to the published method[5]. 1 mmol of $GdCl_3 \cdot 6H_2O$ and 3 mmol of D-glucuronic acid were dissolved in 30 mL of triethylene glycol. The mixture solution was stirred at 110^{o}C under atmospheric condition until it was completely dissolved. Then NaOH solution (3 mmol NaOH in 5 mL triethylene glycol) was added to the mixture solution. Followed by stirring for another 6 h, the obtained solution was diluted with 500 mL DI water. Then, the sample was purified by collecting precipitate after placement for three times. The aqueous sample solution was freeze-dried. The dried Gd_2O_3 were dissolved in PBS (5 mM, pH7.4) for use.

Synthesis of gadolinium-based hybrid nanoparticles: GH was synthesized in a simple method according to the reported procedure[6]. Briefly, aqueous gadolinium chloride solution (56.0 mg in 15.0 mL DI water) was added to BSA solution (15 mL, 50 mg mL^{-1}) , followed by addition of NaOH (1.5 mL, 1.0 M). After stirring for 1h, the solution was dialyzed against DI water to

remove excess Gd^{3+}. The prepared GH were freeze-dried and dissolved in PBS (5 mM, pH 7.4) for use.

Preparation of RBCs: RBCs were separated from the other components in the whole blood from the orbital sinus of Balb/C mice by centrifuging at 500 × g, 4°C, for 10 min and washed thrice with precooling PBS (300 mOsm, pH 7.4). The loading of GH has been achieved by hypotonic swelling procedure. RBCs were stirred gently at 4°C for 1 h in a hypotonic solution (150 mOsm) contained Gd-DTPA/GH/Gd_2O_3 which was resealed by adding a small amount of hypertonic solution for 1 h at 37°C.[1] The resealed RBCs were washed thrice with precooling PBS (300 mOsm, pH 7.4) followed by centrifuging at 500 × g for 10 min. Native RBCs were obtained by the addition of PBS in the loading procedure and served as control groups. The vast majority of Gd-DTPA@RBCs, GH@RBCs and Gd_2O_3@RBCs prepared under these conditions exhibited intact structure without significant hemolysis.

Surface Modification of RBCs: The RBCs were mixed with 0.8 mM glutaraldehyde under agitation for 1 h at 4°C. After reaction, glutaraldehyde was removed completely from RBCs by washing thrice. cRGD (0.5 mg / 8×10^8 RBCs) was added into RBCs and stirred for 1 h at 4°C and the unreacted cRGD was multiple washing. The as-prepared Gd-DTPA@RBCs-cRGD, GH@RBCs-cRGD and Gd_2O_3@RBCs-cRGD exhibited intact structure without significant hemolysis.

Characterization of RBC-cRGD.

Transmission electron microscope (TEM, 200 kV) was taken to characterize the morphology of Gd_2O_3 and GH. To investigate the morphological feature of RBCs during the loading

process, the native RBCs, Gd-DTPA@RBCs and Gd-DTPA@RBCs-cRGD were analyzed using scanning electron microscope (SEM). The concentration of gadolinium loaded RBCs was determined by ICP-AES (Perkin-Elmer Optima 8000).

The relaxivity measurements.

The longitudinal relaxation time (T_1) of Gd-DTPA, Gd-DTPA@RBCs-cRGD, GH, GH@RBCs-cRGD, Gd_2O_3 and Gd_2O_3@RBCs-cRGD were performed on a 0.5 T NMR-analyzer (GY-PNMR-10). The relaxivity values (r_1) were calculated by the curve fitting of $1/T_1$ relaxation time (ms^{-1}) and the concentration of Gd^{3+} (mM). T_1 relaxation was carried out by inversion recovery method.

Cellular MR images.

The U87 human brain glioma cells line was originally obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in low-glucose DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37^{o}C under 5% CO_2. For cellular MR images, U87 cells were cultured in 10 mm culture dishes. After addition of Gd-DTPA, GH, Gd_2O_3, Gd-DTPA@RBCs, Gd-DTPA@RBCs-cRGD, GH@RBCs, GH@RBCs-cRGD, Gd_2O_3@RBCs, Gd_2O_3@RBCs-cRGD with the Gd^{3+} concentration of 0.01 mM, the cells were incubated for another 1 h. Then, the medium was removed and the cells were washed with PBS (300 mOsm, pH 7.4) thrice. After treating with trypsin, cells were harvested and washed (1000 × g, 5 min) with PBS. After removing supernatant completely, MR images of cells were measured under

the following parameters: TR=100 ms, TE=8.6 ms and NS=3.

Flow cytometry analysis.

U87 cells were seeded into 24-well plate at a density of 3×10^4 cells/per well and incubated for 24 h. Afterwards, FITC-labeled RBC-cRGD were added at a RBCs number of 7.5×10^7 per well. The FITC-labeled RBCs with the same number were added in culture medium and blank cells were applied as control groups. For flow cytometry analysis, the cells were washed with 1.0 mL PBS thrice after incubation under 4°C for 30 min and resuspended in 0.3 mL PBS. The fluorescence was determined with a BD Accuri C6 cytometer (USA) by counting 10 000 events.

Inverted fluorescence microscope images.

For inverted fluorescence microscope images, 1×10^4 cells/per well U87 cells were seeded in 24-well plate. After incubated for 24 h, cells were washed with PBS and FITC-labeled RBC-cRGD with the number of 7.5×10^7 per well were added in 24-well plate and incubated under 4°C for 30 min. The FITC-labeled RBCs with the same number were applied under the same conditions as control groups.

Half-life and the interaction of RBCs with proteins.

To study the *in vivo* stability property of RBCs, we measured the half-life of Gd_2O_3@RBCs-cRGD in blood circulation. Gd_2O_3@RBCs-cRGD was intravenous injected under the concentration of 0.0625 mmol/kg. Blood was collected in 0 min, 15 min, 1 h, 2 h, 6 h and 12

h from venous plexus of orbital cavity. The concentrations of Gd^{3+} in blood were measured by ICP-MS. The half-life was calculated by the following equations:

$$lgC_t = lgC_0 + (-K_e/2.303)t$$

$$t_{1/2} = 0.693/K_e$$

We also evaluated the interaction of RBCs with proteins to study the stability of RBCs *in vivo*, using bovine serum albumin (BSA) as a model of plasma proteins. The RBCs with reduced nonspecific protein adsorption would be expected to exhibit prolonged blood circulation time. BSA was used as a model protein to determine the protein adsorption of Gd_2O_3@RBCs-cRGD. The Gd_2O_3@RBCs-cRGD was incubated with BSA in PBS (300 mOsm, pH 7.4) with the concentration at 2.5 mg/mL. After incubating at 37 ^oC for 2 h and 6 h, each sample was centrifuged at 8000 g for 10 min. The absorption of supernatant was determined using UV-Vis spectroscopy by measuring at 280 nm wavelength.[2]

Hematoxylin-eosin (HE) staining for toxicity assay.

Hematoxylin-eosin (HE) staining was carried out to investigate the toxicity of Gd_2O_3@RBCs-cRGD. Femal athymic nude mice (4 weeks old, ~20g) were purchased from Nanjing Sikerui Biological Technology Co. Ltd and acclimated for 1 week. Mice were raised in an animal facility under filtered air and fed with standard pellet diet and pure water. The mice were tail intravenously injected 400 μL of Gd_2O_3@RBCs-cRGD. Meanwhile, 400 μL of native RBCs was injected as control group. After injection for 48 h, mice were sacrificed to collect organs (heart, liver, spleen, lung and kidney) for histology analysis.[3]

The biodistribution of Gd in organs.

The experimental procedure for the injection of Gd_2O_3@RBCs-cRGD to mice was same as above. After injecting for 24 h, mice were sacrificed to collect organs (heart, liver, spleen, lung and kidney) for measuring the concentration of Gd^{3+} in the organs. The samples were completely digested in 5 mL of nitric acid through heating, and centrifuged at 13000 rpm for 5 min, the Gd^{3+} concentration was then measured by ICP-AES. Afterwards, the averaged Gd content in each organ or tissue was calculated and converted to percentage of injected dose (ID) per organ.[4]

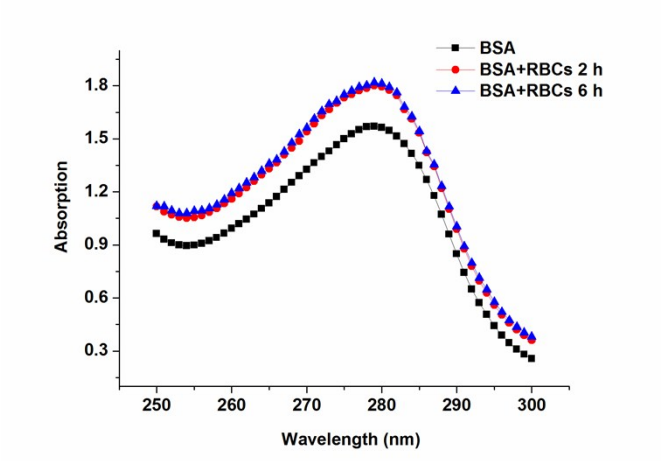

Figure S1. The absorption of BSA before and after incubation with Gd_2O_3@RBCs-cRGD for 2 h and 6 h.

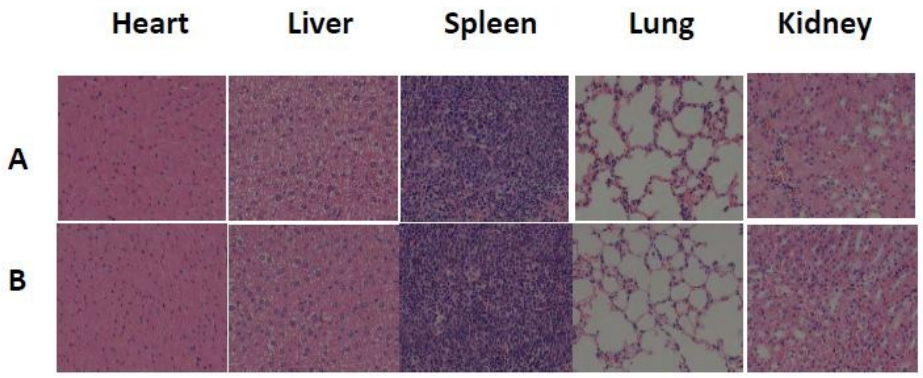

Figure S2. HE staining for major organs (including heart, liver, spleen, lung and kidney). (A) native RBCs.(B) Gd_2O_3@RBCs-cRGD with the injection of Gd^{3+} concentration of 0.0625 mmol/kg.

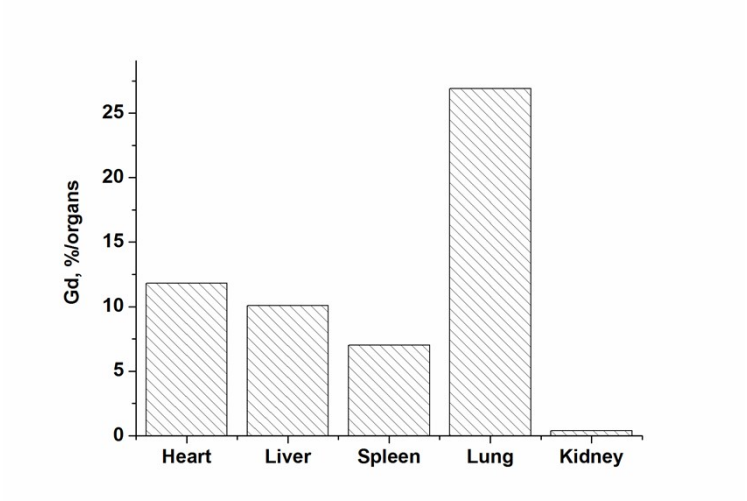

Figure S3. %ID (injected dose) of Gd^{3+} in organs after injecting Gd_2O_3@RBCs-cRGD at the dose of 0.0625 mmol Gd/kg for 24 h.

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