Supporting Information for

Multifunctional Dendrimer-Entrapped Gold Nanoparticles Modified with RGD Peptide for Targeted CT/MR Dual Modal Imaging of Tumors

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Supporting Information Available: Additional experimental details, materials characterization (^{1}H NMR, DLS, UV-vis spectrometry, and stability evaluation data), and *in vitro* (cell morphology observation and hemolysis assay data) and *in vivo* (quantitative MR and CT imaging of the tumors at different time points postinjection, and biodistribution of Au element in different organs and tumor) experimental data.

Part of Experimental Details

Materials. Ethylenediamine core amine-terminated G5.NH_2 PAMAM dendrimers with a polydispersity index less than 1.08 were purchased from Dendritech (Midland, MI). 2,2',2''-(10-(2-(2,5-Dioxopyrrolidin-1-yloxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetic acid (DOTA-NHS) was purchased from CheMatech (Dijon, France). PEG monomethyl ether with the other end of carboxyl group (*m*PEG-COOH, Mw = 2 000) and a dual functional PEG with one end of amine group and the other end of carboxyl group (NH_2-PEG-COOH, Mw = 2 000) were from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). The thiolated cyclo(Arg-Gly-Asp-Phe-Lys(mpa)) (RGD), 6-maleimidohexanoic acid N-hydroxysuccinimide ester (6-MAL), acetic anhydride, triethylamine, sodium hydroxide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), hydrochloric acid, and all the other chemicals and solvents were purchased from Aldrich (St. Louis, MO) and used as received. U87MG cells (an $\alpha_v\beta_3$ integrin-overexpressing human glioblastoma cell line) and L1210 cells (a mouse lymphocytic leukemia cell line) were obtained from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Minimum Essential Medium (MEM) and Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with a resistivity higher than 18 MΩ·cm. Regenerated cellulose dialysis

membranes with molecular weight cut-off (MWCO) of 14,000 or 1,000 were acquired from Fisher Scientific (Pittsburgh, PA).

Synthesis of RGD-PEG-COOH. One molar equivalent of 6-MAL (8.48 mg, 27.50 μmol) was added to a dimethylsulfoxide (DMSO) solution of NH_2-PEG-COOH (55.00 mg, 27.50 μmol, 5 mL) under vigorous magnetic stirring for 8 h to form the raw product of MAL-PEG-COOH. Then, one molar equivalent of RGD (19.00 mg, 27.50 μmol) dissolved in DMSO (10 mL) was dropwise added to the above solution of the raw MAL-PEG-COOH product under stirring at room temperature. The reaction was stopped after 12 h. Then, the reaction mixture was extensively dialyzed against phosphate buffered saline (PBS, 3 times, 2 L) and water (3 times, 2 L) for 3 days using a dialysis membrane with MWCO of 1 000 to remove the excess reactants and by-products. This was followed by lyophilization to obtain the product RGD-PEG-COOH.

Synthesis of Gd-Au DENPs-RGD. Briefly, DOTA-NHS (13.25 mg) dissolved in DMSO (5 mL) was dropwise added to a DMSO solution of G5.NH_2 dendrimers (45.26 mg, 1.74 μmol, 15 mL) under vigorous magnetic stirring for 24 h. This led to the formation of the raw product of G5.NH_2-DOTA dendrimers. Then, RGD-PEG-COOH (40.16 mg) pre-activated by EDC (26.69 mg, 139.2 μmol, in 5 mL DMSO) for 3 h was dropwise added to the above solution of the G5.NH_2-DOTA dendrimers under vigorous magnetic stirring for 3 days. The formed raw product of G5.NH_2-DOTA-(PEG-RGD) was further modified with *m*PEG-COOH (52.2 mg, 26.1 μmol) that was pre-activated by EDC for 3 h. The reaction was performed for 3 days under magnetic stirring to get the raw product of the G5.NH_2-DOTA-(PEG-RGD)-*m*PEG dendrimers.

The G5.NH_2-DOTA-(PEG-RGD)-*m*PEG dendrimers (the above raw product) were then used as templates to synthesize Au NPs using protocols described in our previous reports with slight

modifications.[1, 2] Briefly, an aqueous solution of $HAuCl_4 \cdot 4H_2O$ (200 molar equivalents of the G5 dendrimer, 30 mg/mL, 3.18 mL) was added into the solution of the above raw product of G5.NH_2-DOTA-(PEG-RGD)-*m*PEG dendrimers under vigorous stirring. After 30 min, an icy cold aqueous $NaBH_4$ solution (43.88 mg, 1.56 mmol, 5 mL) with 5 times molar excess to the Au salt was added to the above mixture solution under stirring. The reaction mixture changed to be wine red within a few seconds after addition of the $NaBH_4$ solution, indicating the formation of Au NPs. The reaction mixture was stirred for 2 h to complete the reaction. After that, an aqueous $Gd(NO_3)_3$ solution (18.22 mg, 1 mL) with 35 molar equivalents to the G5 dendrimer was dropwise added to the above mixture solution under stirring for 24 h to form the raw product of the $\{(Au^0)_{200}$-G5.NH_2-DOTA(Gd)-(PEG-RGD)-*m*PEG} DENPs.

A final acetylation step was used to convert the remaining dendrimer terminal amines to acetamide groups according to a procedure described in the literature.[1] Briefly, triethylamine (88.9 μL) was added to the above solution of the raw product of the $\{(Au^0)_{200}$-G5.NH_2-DOTA(Gd)-(PEG-RGD)-*m*PEG} DENPs under magnetic stirring for 30 min. Then, acetic anhydride (72.4 μL) with 5 molar excess of the total G5 dendrimer primary amines was added to the above mixture solution under vigorous stirring. The reaction was stopped after 24 h. The reaction mixture was then extensively dialyzed against PBS (3 times, 2L) and water (3 times, 2L) for 3 days to remove the excess reactants and by-products, followed by lyophilization to obtain the target Gd-Au DENPs-RGD product. The intermediate products of G5.NH_2-DOTA-(PEG-RGD) and G5.NH_2-DOTA-(PEG-RGD)-*m*PEG were also collected and purified in order to quantify the numbers of RGD and *m*PEG moieties attached onto each G5 dendrimer.

Characterization Techniques. ^{1}H NMR spectra were recorded on a Bruker DRX 400 nuclear

magnetic resonance spectrometer. Samples were dissolved in D_2O before measurements. UV-vis spectra were collected using a Lambda 25 UV-vis spectrophotometer (PerkinElmer, Boston, MA). Samples were dissolved in water before the experiments. TEM was performed using a JEOL 2010F analytical electron microscope (JEOL, Tokyo, Japan) operating at 200 kV. A TEM sample was prepared by depositing a diluted particle suspension (1 mg/mL, 5 mL) onto a carbon-coated copper grid and air dried before measurements. Dynamic light scattering (DLS) and zeta potential measurements were performed using a Malvern Zetasizer NanoZS model ZEN3600 (Worcestershire, UK) equipped with a standard 633 nm laser. A Leeman Prodigy inductively coupled plasma-atomic emission spectroscopy (ICP-AES) system (Hudson, NH) was used to analyze the composition of Au and Gd in the multifunctional NPs. CT scans were performed using a GE LightSpeed VCT imaging system (GE Medical Systems, Milwaukee, WI) with 100 kV, 80 mA, and a slice thickness of 0.625 mm. Solutions of Gd-Au DENPs-RGD (0.2 mL) with different Au concentrations were prepared in 2.0-mL Eppendorf tubes and placed in a self-designed scanning holder. Contrast enhancement was determined in Hounsfield units (HU) for each sample. T_1 relaxometry of Gd-Au DENPs-RGD was performed using a 3.0 T Signa HDxt superconducting MR system (GE Medical Systems, Milwaukee, WI) with a wrist receiver coil. The Gd-Au DENPs-RGD were dissolved in PBS (1 mL) at different concentrations in 2-mL Eppendorf tubes. T_1 relaxation times were measured using an SE/2D sequence. A total of four echoes were used with the following parameters: TR = 300, 600, 900, 1200 ms, TE= 10.7 ms, matrix = 256 × 256, section thickness = 2 mm, and FOV= 12 cm. The T_1 relaxivity (r_1) was determined *via* a linear fit of the inverse relaxation time as a function of the Gd(III) concentration.

***In Vitro* Cytotoxicity Assay.** The cytotoxicity of the Gd-Au DENPs-RGD probe was first

evaluated *via* 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, 1×10^4 U87MG cells were seeded in each well of 96-well cell culture plates with regular MEM containing 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin the day before the experiments. The next day, the medium was replaced with fresh MEM containing Gd-Au DENPs-RGD with a final Au concentration ranging from 0 to 200 μM. After 24 h incubation at 37 ^oC and 5% CO_2, MTT (10 mL, 5 mg/mL) in PBS was added to each well and the cells were incubated for another 4 h. The medium was then carefully removed, and DMSO (200 mL) was added to dissolve the formazan crystals. The absorbance at 570 nm for each well was measured using a Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA). Mean and standard deviation for the triplicate wells were reported. After treatment with the Gd-AuDENPs-RGD for 24 h, the cell morphology was also observed using a Leica DM IL LED inverted phase contrast microscope (Wetzlar, Germany) with a magnification of 200 × for each sample.

Hemolysis Assay. Hemolysis assay was performed using human blood from healthy adult volunteers provided by Shanghai First People's Hospital (Shanghai, China) and approved by the Ethical Committee of Shanghai First People's Hospital. The blood stabilized with heparin was centrifuged and washed with PBS for 5 times according to the procedure reported in literature[3] in order to completely remove the serum and obtain human red blood cells (HRBCs). Thereafter, the HRBCs were diluted 10 times with PBS. The diluted HRBC suspension (0.2 mL) was transferred into 2-mL Eppendorf tubes prefilled with 0.8 mL water (as positive control), 0.8 mL PBS (as negative control), and 0.8 mL PBS containing the Gd-Au DENPs-RGD probe with different Au concentrations (66.5-540 μM), respectively. The mixtures were incubated at 37° for 2 h, followed by

centrifugation (10 000 rpm, 2 min). Then, the photos of the samples were taken and the absorbance of the supernatants (hemoglobin) was recorded by a Perkin Elmer Lambda 25 UV-vis spectrophotometer. The hemolysis percentages (HPs) of different samples were calculated by dividing the difference in absorbances at 541 nm between the sample and the negative control by the difference in absorbances at 541 nm between the positive and negative controls.[4]

***In Vitro* Cellular Uptake Assay**. The cellular uptake of the Gd-Au DENPs-RGD probe was evaluated by ICP-AES. In brief, U87MG and L1210 cells were separately seeded into a 24-well plate at a density of 5×10^5 cells/well. After culturing overnight to bring the cells to confluence, the medium was replaced with fresh medium containing Gd-Au DENPs-RGD ([Au] = 10 mM and 40 mM, respectively) and the cells were then incubated at 37 °C and 5% CO_2 for 3 h. The cells were then washed 3 times with PBS, lifted with trypsinization, and resuspended in 1 mL PBS. The cell suspensions (100 mL) were counted and the remaining cells were centrifuged to form pellets and lysed using an aqua regia solution (0.5 mL) to digest both the cells and the Au NPs. The digested samples were diluted with 1.3 mL PBS before quantification of Au content by a Leeman Prodigy ICP-AES system. For comparison, U87MG cells pretreated with free RGD (2.5 μM) for 1 h were also treated under similar experimental conditions in order to test the specific cellular uptake of Au.

References

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(2) Peng, C.; Zheng, L.; Chen, Q.; Shen, M.; Guo, R.; Wang, H.; Cao, X.; Zhang, G.; Shi, X. *Biomaterials* **2012**, *33*, 1107-1119.

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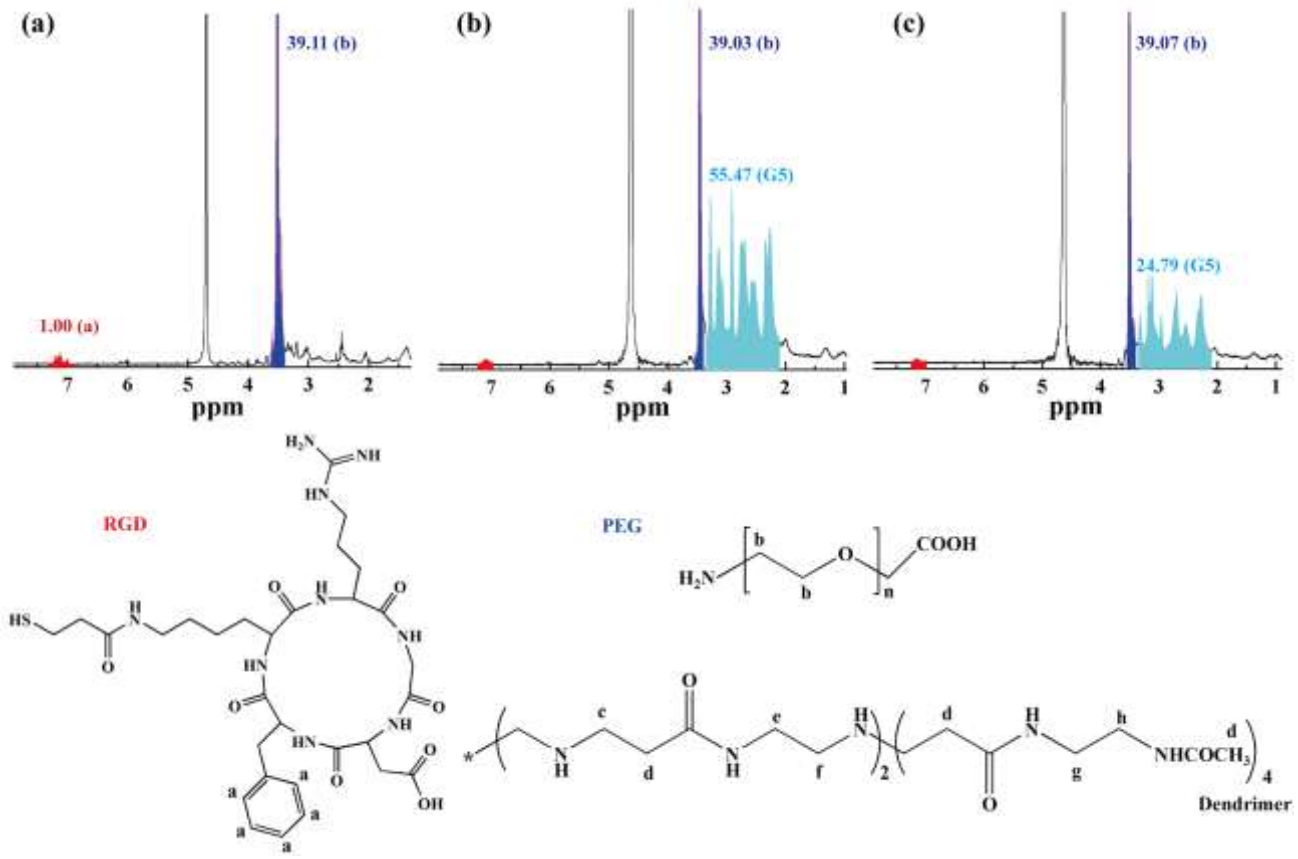

Figure S1. ^{1}H NMR spectrum of RGD-PEG-COOH segment (a), G5.NH_2-DOTA-(PEG-RGD) conjugate (b), and G5.NH_2-DOTA-(PEG-RGD)-*m*PEG conjugate (c).

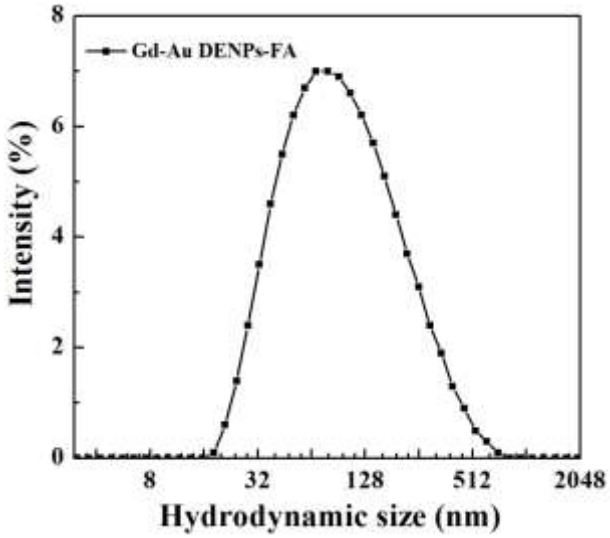

Figure S2. Hydrodynamic size distribution of the Gd-Au DENPs-RGD probe dispersed in water.

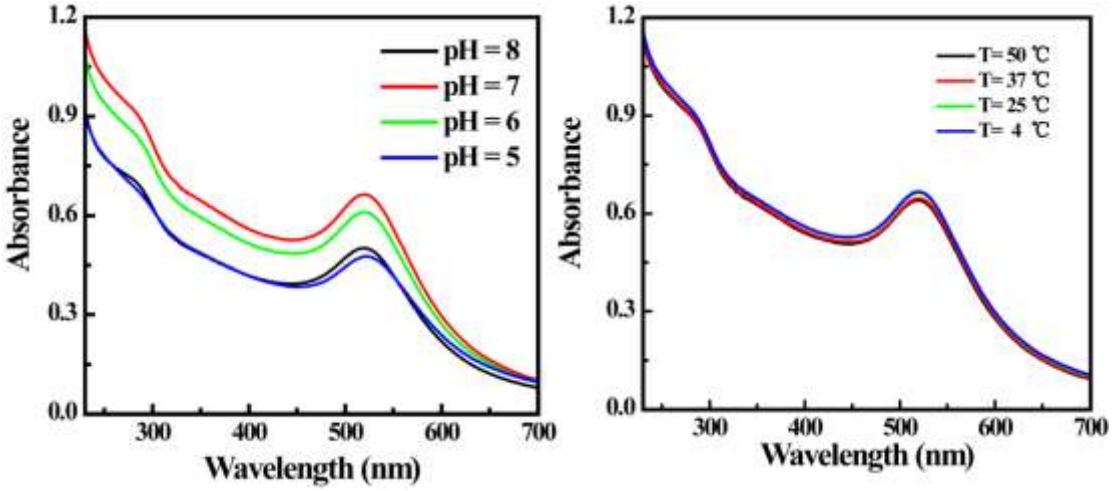

Figure S3. UV-vis spectra of Gd-Au DENPs-RGD dispersed in water under different pHs (a) and temperatures (b).

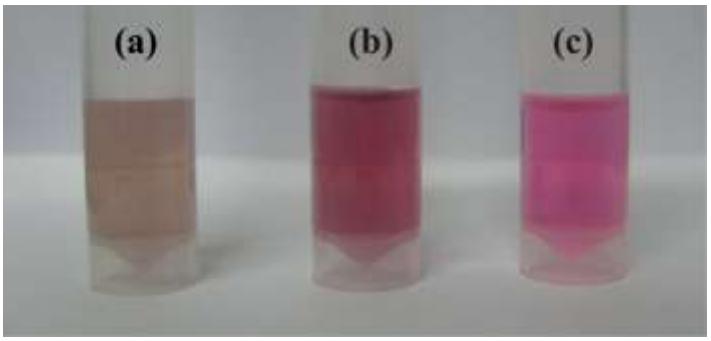

Figure S4. Photograph of the Gd-Au DENPs-RGD probe dispersed in PBS (a), cell culture medium (b). (c) shows the blank cell culture medium.

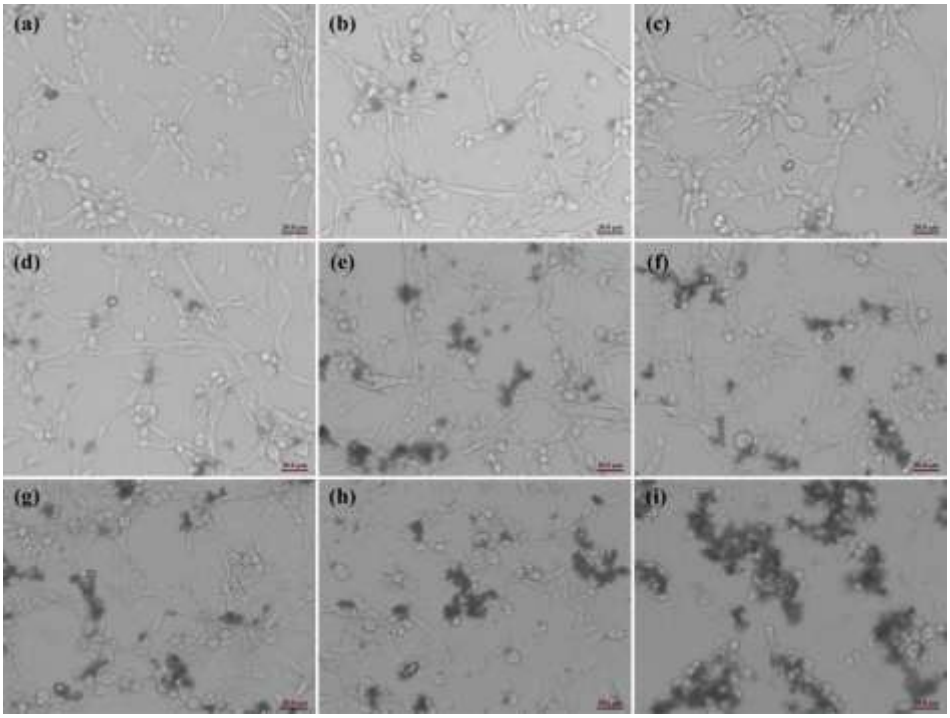

Figure S5. The morphology of U87MG cells treated with PBS (a), and Gd-Au DENPs-RGD at an Au concentration of 5 μM (b), 10 μM (c), 20 μM (d), 30 μM (e), 40 μM (f), 50 μM (g), 100 μM (h), and 200 μM (i), respectively for 24 h.

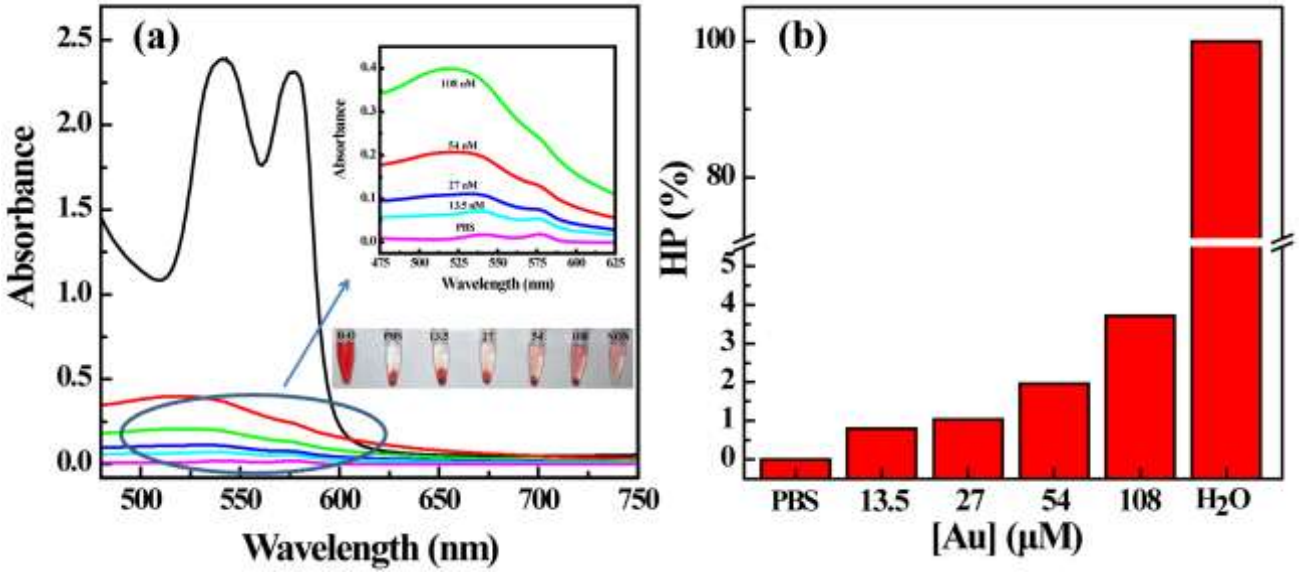

Figure S6. UV-vis spectra (a) and photographs (inset of (a)) of the HRBC suspensions treated with Gd-Au DENPs-RGD at different Au concentrations. (b) shows the hemolysis percentage as a function of Au concentration. H_2O (1) and PBS (2) were used as positive and negative control, respectively. Inset of (a) shows the enlarged UV-vis spectra at the indicated region.

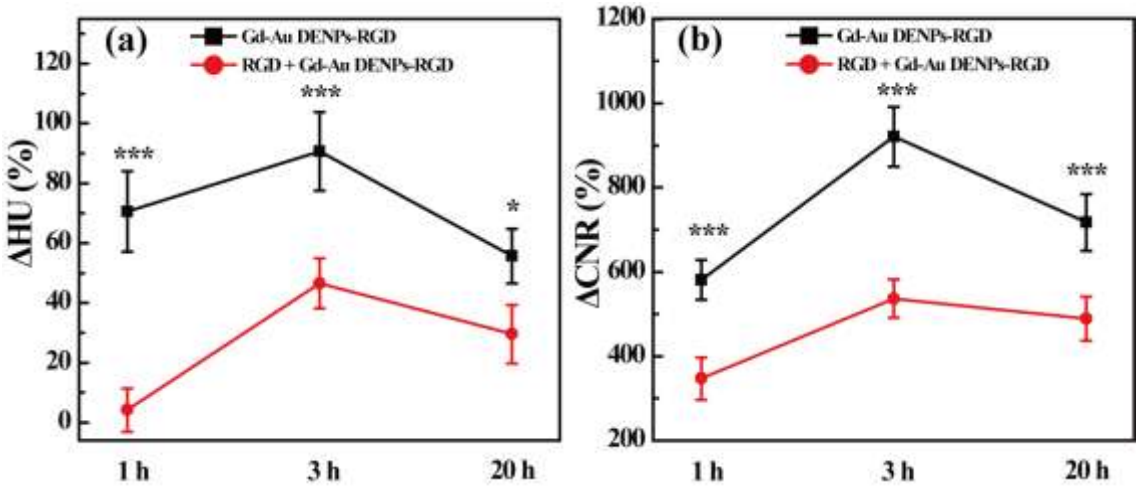

Figure S7. The ΔHU (a) and ΔCNR (b) of the tumor region at different time points. The mice were intravenously injected with the Gd-Au DENPs-RGD probe ([Au] = 0.1 M, 200 μL in PBS) *via* the tail vein.

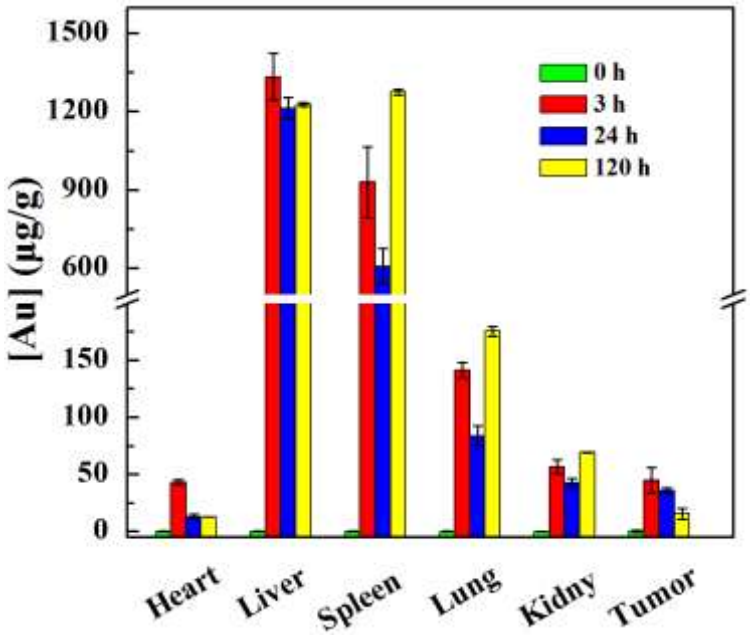

Figure S8. Biodistribution of Au in the major organs of the mice including heart, liver, spleen, lung, kidney, and the tumor. The data was recorded from the whole organ or the whole tumor at different time points post intravenous injection of the Gd-Au DENPs-RGD probe ([Au] = 0.1 M, 200 μL in PBS).