Electronic Supplementary Information†

Design of multifunctional dendrimer-based nanoplatform for targeted dual mode SPECT/MR imaging of tumors

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Experimental section

Materials: Ethylenediamine core G5.NH_2 PAMAM dendrimers with a polydispersity index less than 1.08 were purchased from Dendritech (Midland, MI). DOTA-NHS was purchased from CheMatech (Dijon, France). FA, FI, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were supplied by J&K Chemical Ltd. (Shanghai, China). $MnSO_4.H_2O$, acetic anhydride, triethylamine and all other chemicals and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). ^{99m}Tc-pertechnetate was supplied by Shanghai GMS Pharmaceutical Co., Ltd. (Shanghai, China). Cellulose dialysis membranes having molecular weight cut-off (MWCO) of 14 000 were acquired from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). HeLa cells (a human cervical carcinoma cell line) were from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were supplied from Hangzhou Jinuo Biomedical Technology (Hangzhou, China) and Shanghai ExCell Biology, Inc. (Shanghai, China), respectively. Water used in all experiments was processed using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) to have a resistivity higher than 18.2 MΩ cm.

Synthesis of the G5.NHAc-FI-DOTA(Mn)-FA dendrimers: G5.NH_2 dendrimers were first conjugated with DOTA according to our previous report.[1] The formed G5.NH_2-DOTA dendrimers were then modified with FA *via* 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) chemistry. In brief, FA (2.25 mg), EDC (4.8 mg), and N-

hydroxysuccinimide (NHS, 2.9 mg) were separately dissolved in 2 mL DMSO, respectively. These three solutions were mixed together and stirred for 3 h to activate the carboxyl group of FA. After that, the activated FA was dropwise added into a DMSO solution containing G5.NH_2-DOTA dendrimers (42.75 mg) under stirring for 3 days to get the raw product of the G5.NH_2-DOTA-FA dendrimers. The G5.NH_2-DOTA-FA dendrimers were then reacted with FI (0.79 mg, in 1 mL DMSO) under magnetic stirring in the dark at room temperature for 1 day, leading to the formation of the raw product of G5.NH_2-DOTA-FI-FA.

The above G5.NH_2-DOTA-FI-FA dendrimers were used to complex Mn(II) ions ($MnSO_4.H_2O$, 3.6 mg, in 2 mL water) under stirring in dark at room temperature for 1 day. After that, the remaining amine groups of the formed G5.NH_2-DOTA(Mn)-FI-FA complexes were acetylated by reaction with acetic anhydride according to a method described in our previous report.[2] The product of G5.NHAc-FI-DOTA(Mn)-FA complexes were purified and lyophilized according to our previous report.[1] For comparison, G5.NHAc-FI-DOTA(Mn) complexes without FA were also prepared in a similar manner.

^{99m}Tc labeling of the G5.NHAc-FI-DOTA(Mn)-FA dendrimers: Briefly, 100 μL (10 mCi) technetium-99m sodium pertechnetate solution and the reducing agent $SnCl_2$ (100 μg) were mixed with the G5.NHAc-FI-DOTA(Mn)-FA dendrimers (200 μg, in 200 μL phosphate buffered saline (PBS)) with continuous stirring at room temperature for 30 min. Then, the excess of reactants and byproducts were removed from the reaction mixture by PD-10 desalting columns and the ^{99m}Tc-labeled dendrimers were collected in 20 tubes. The radioactivity of each aliquot tube was measured with a CRC-15R radioisotope dose calibrator (Capintec, Inc., Ramsey, NJ). The collected product of the ^{99m}Tc-labeled G5.NHAc-FI-DOTA(Mn)-FA dendrimers (G5.NHAc-FI-DOTA (Mn/^{99m}Tc)-FA) were obtained by

lyophilization. For comparison, nontargeted G5.NHAc-FI-DOTA (Mn/^{99m}Tc) dendrimers without FA were also prepared in a similar manner.

Characterization techniques: ^{1}H NMR spectra were collected using a Bruker AV400 nuclear magnetic resonance spectrometer. Samples were dissolved in D_2O before measurements. Zeta potential and dynamic light scattering (DLS) measurements were carried out using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) equipped with a standard 633 nm laser. UV-vis spectroscopy was performed using a Lambda 25 UV-vis spectrophotometer (PerkinElmer, Boston, MA). The Mn concentration and the T_1 relaxometry of the particle solution were measured according to our previous work.[3, 4]

Radiochemical purity analysis: The radiochemical purity of the G5.NHAc-FI-DOTA(Mn/^{99m}Tc)-FA and G5.NHAc-FI -DOTA(Mn/^{99m}Tc) was analyzed by instant thin-layer chromatography (ITLC) using silica gel-coated fiber glass sheets (Macherey-Nagel, GmbH & Co. KG, Düren, Germany). Saline was utilized as the mobile phase, and the sheets were analyzed with a Thin-Layer Chromatogram Scanner (Bioscan Inc., Tucson, AZ).

In vitro radio stability study: The stability of the G5.NHAc-FI-DOTA(Mn/^{99m}Tc)-FA and G5.NHAc-FI-DOTA(Mn/^{99m}Tc) in vitro was studied by measuring the radiochemical purity of the dendrimers *via* ITLC at different time intervals. Briefly, the G5.NHAc-FI-DOTA(Mn/^{99m}Tc)-FA (100 μL, 18.5 MBq) or G5.NHAc- FI-DOTA(Mn/^{99m}Tc) (100 μL, 18.5 MBq) was mixed with 1 mL PBS at 37 °C. ITLC was then utilized to analyze the radiochemical purity of the ^{99m}Tc-labeled dendrimers after incubation at 37 °C for 1, 3, and 6 h, respectively. A similar procedure was applied to evaluate the radiochemical stability of the G5.NHAc-FI-DOTA(Mn/^{99m}Tc)-FA and G5.NHAc-FI-DOTA (Mn/^{99m}Tc) dendrimers in fetal bovine serum (FBS) at 37 °C.

Hemolysis, cytotoxicity and cellular uptake assays: Fresh human blood stabilized with EDTA was kindly supplied by Shanghai General Hospital (Shanghai, China) and used with the permission of the ethical committee of Shanghai General Hospital. Human red blood cells (HRBCs) were obtained and the hemolysis assay was performed according to the literature.[5, 6] In brief, HRBCs were 10 times diluted with PBS. Then, 0.2 mL of the diluted HRBC suspension was transferred to a 1.5-mL Eppendorf tube, which was filled with 0.8 mL of water (as positive control), 0.8 mL of PBS (as negative control), 0.8 mL of PBS containing the G5.NHAc-FI-DOTA(Mn) or G5.NHAc-FI-DOTA(Mn)-FA at different Mn concentrations (5-100 μg/mL), respectively. The above mixtures were then incubated at 37 ^{o}C for 1 h, followed by centrifugation (10 000 rpm, 2 min). Then, the supernatant was determined by Perkin Elmer Lambda 25 UV-vis spectrophotometer to record the absorbance at 541 nm. The hemolysis percentage (HP) was calculated using Equation 1, where D_t is the absorbance of the test samples; D_{pc} and D_{nc} are the absorbances of the positive and negative controls, respectively.

$$HP = \frac{Dt - Dnc}{Dpc - Dnc} \times 100\% \qquad (1)$$

The cytotoxicity of both the G5.NHAc-FI-DOTA(Mn)-FA and G5.NHAc-FI-DOTA(Mn) dendrimers was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay of HeLa cells after treated with the dendrimers at an Mn concentration range of 0-100 μg mL^{-1} according to the literature.[1] Briefly, HeLa cells were seeded into a 96-well plate with a density of 1 × 10^4 per well and incubated in regular DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin for 24 h. Afterwards, the medium was replaced with fresh medium containing the G5.NHAc-FI-

DOTA(Mn) or G5.NHAc-FI-DOTA(Mn)-FA dendrimers at different Mn concentrations. After 24 h incubation at 37 ^{o}C and 5% CO_2, MTT (20 mL, 5 mg/mL) in PBS was added into each well and the cells were incubated for another 4 h under regular culture conditions. The medium was then carefully removed, and DMSO (200 μL) was added to dissolve the formed formazan crystals. The absorbance at 570 nm in each well was measured using a Thermo Scientific Multiskan MK3 ELISA reader (Thermo, Waltham, MA). Mean and standard deviation for the triplicate wells were reported.

The cellular uptake of the G5.NHAc-FI-DOTA(Mn) or G5.NHAc-FI-DOTA(Mn)-FA dendrimers was investigated *via* flow cytometry according to the literature.[4, 7] HeLa cells were seeded in 12-well plates at a density of 2×10^5 cells per well in 1 mL of DMEM and incubated at 37 ^{o}C and 5% CO_2. After overnight incubation to bring the cells to confluence, the medium was replaced with 1 mL fresh medium containing PBS (control), G5.NHAc-FI-DOTA(Mn) or G5.NHAc-FI-DOTA(Mn)-FA dendrimers at different Mn concentrations (0, 10, 25, 50, 75, and 100 μg/mL, respectively). In comparison with FAR-overexpressing HeLa cells (H-FAR, unless otherwise stated, HeLa cells always represent the H-FAR HeLa cells), FAR-blocked HeLa cells (L-FAR, HeLa cells were pretreated with free FA (2.5 μM) for 12 h) were also incubated with the G5.NHAc-FI-DOTA(Mn)-FA dendrimers at different Mn concentrations (0, 10, 25, 50, 75, and 100 μg/mL, respectively). After 4 h incubation, the cells were washed 3 times with PBS, trypsinized, centrifugated, and resuspended in 1 mL PBS before flow cytometry analysis using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The FL1-fluorescence of 10 000 cells was measured and the measurement was repeated for 3 times.

In vivo SPECT/MR imaging of a xenografted tumor model: All animal experiments were carried out according to protocols approved by the institutional committee for animal care, and also in accordance with the policy of the National Ministry of Health. Male 4 to 6-week-old BALB/c nude mice with a bodyweight of 15-20 g (Shanghai Slac Laboratory Animal Center, Shanghai, China) were subcutaneously injected with 5×10^6 cells/mouse in the left back. When the tumor nodules reached a volume of about 200 mm^3 at approximately 3 weeks postinjection, the mice were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg), and then the G5.NHAc-FI-DOTA(Mn/^{99m}Tc)-FA or G5.NHAc-FI-DOTA(Mn /^{99m}Tc) dendrimers were intravenously delivered into the tumor-bearing mice via the tail vein (3 mCi per mouse, in 0.2 mL PBS, n = 3 for each group). SPECT images were obtained before and after administration of the particles at 5, 30, 60, 120, and 240 min postinjection, respectively.

For MR imaging, after the G5.NHAc-FI-DOTA(Mn)-FA or G5.NHAc-FI-DOTA(Mn) dendrimers were intravenously delivered into each tumor-bearing mouse via the tail vein ([Mn] = 1 mg/mL, in 0.3 mL PBS, n = 3 for each group). A 1.5 T GE Discovery MR750 scanner with an animal coil (Magtron Inc., Shanghai, China) was used to image each mouse with an FSE sequence (slice thickness = 1.8 mm, TR = 600 ms, TE = 21.2 ms, FOV = 3 × 3 cm, and point resolution = 320 × 256). For each mouse, two dimensional (2D) spin-echo T_1-weighted MR images were obtained before and at 30, 60, 90, 120, 150, 180, 210, or 240 min postinjection of the particles.

In vivo biodistribution: Each tumor-bearing mouse was intravenously injected with the G5.NHAc-FI-DOTA(Mn)-FA dendrimers (300 μg Mn, in 0.3 mL PBS). The mice were sacrificed at 24 h postinjection and major organs such as heart, liver, spleen, lung, kidney,

and tumor were extracted and weighted. The organs or tumor were then cut into 1-2 mm^2 pieces and digested by aqua regia solution (nitric acid/hydrochloric acid, v/v = 1:3) for 24 h. Then, the Mn content in different organ pieces was quantified by Leeman Prodigy inductively coupled plasma-optical emission spectroscopy (ICP-OES, Hudson, NH). For comparison, the mice injected with PBS (0.3 mL) and the mice injected with the nontargeted G5.NHAc-FI-DOTA(Mn) dendrimers (300 μg Mn, in 0.3 mL PBS) were used as controls (n = 3 for each group).

Statistical analysis: One-way ANOVA statistical analysis was performed to evaluate the significance of the experimental data. A p value of 0.05 was selected as the level of significance, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. Zeta potential and hydrodynamic size of the G5.NHAc-FI-DOTA(Mn) and G5.NHAc-FI-DOTA(Mn)-FA dendrimers.

Sample	Zeta potential (mV)	Hydrodynamic size (nm)	PDI
$G5.NH_2$	+36.9	217.4	0.372
G5.NHAc-FI-DOTA(Mn)	+ 0.7	549.5	0.593
G5.NHAc-FI-DOTA(Mn)-FA	-0.5	863.6	0.931

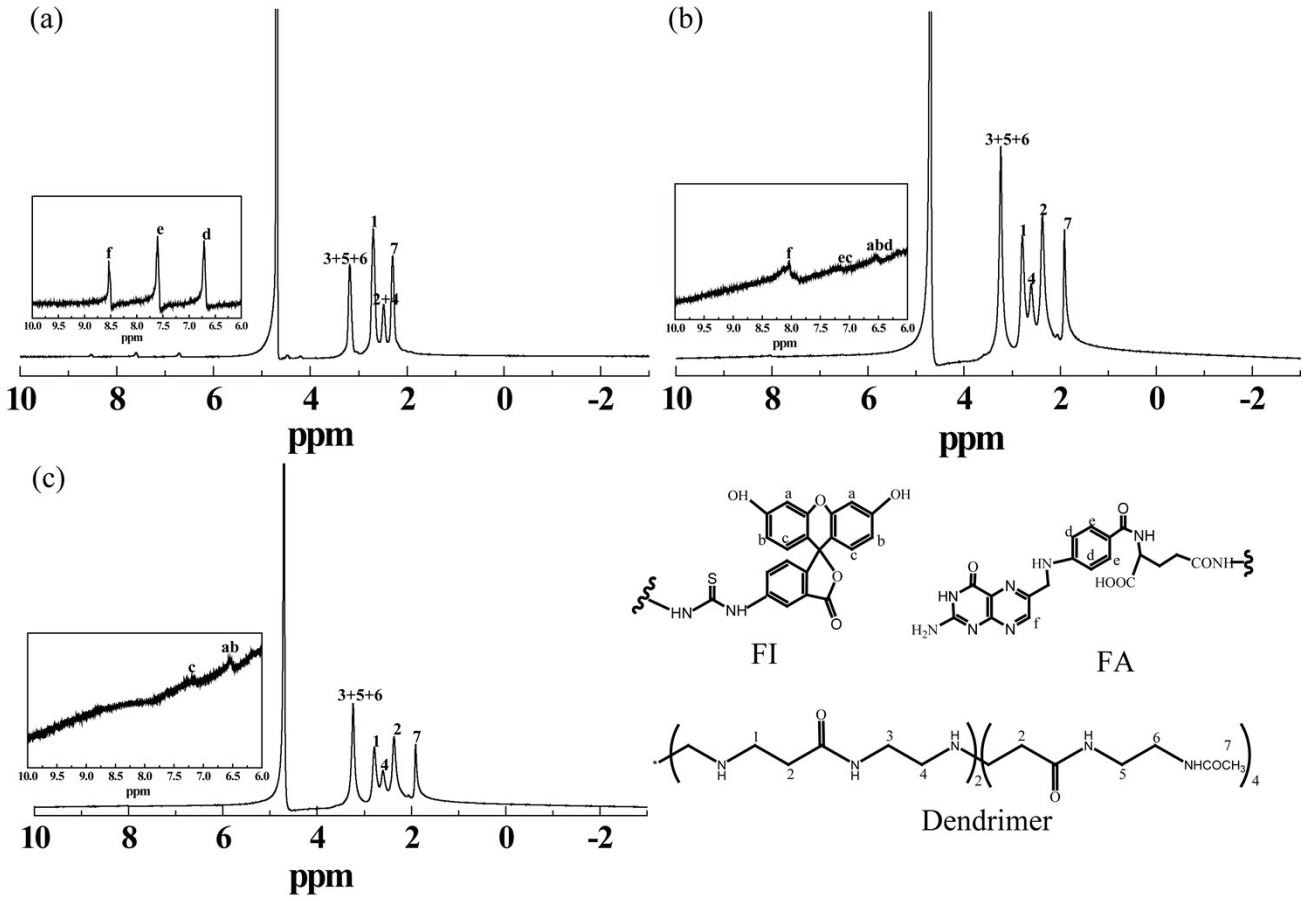

Fig. S1. ^{1}H NMR spectra of the G5.NH_2-DOTA-FA (a), G5.NH_2-DOTA-FI-FA (b), and G5.NH_2-DOTA-FI (c) dendrimers.

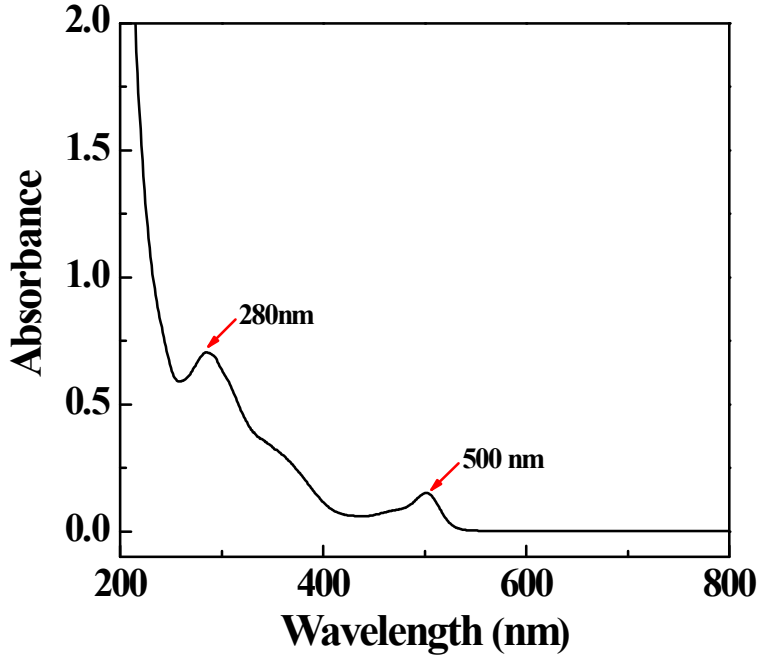

Fig. S2. UV-vis spectrum of the G5.NHAc-FI-DOTA(Mn)-FA dendrimers dissolved in water.

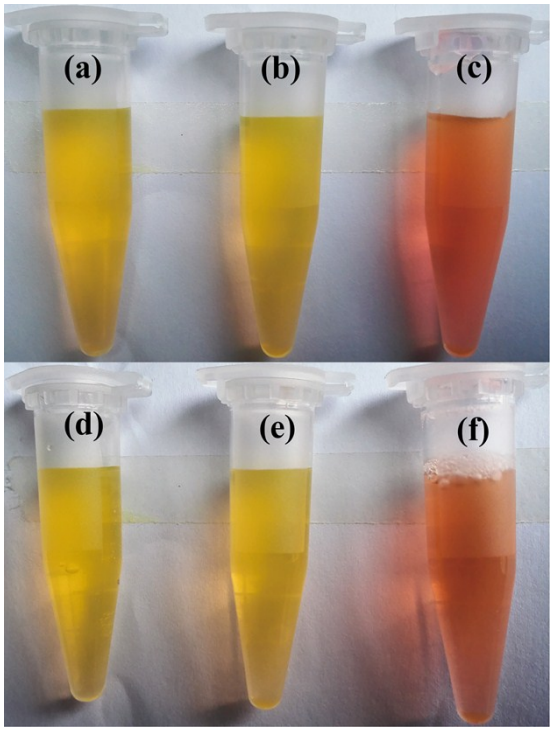

Fig. S3. Photographs of the G5.NHAc-FI-DOTA(Mn) (a-c) and G5.NHAc-FI-DOTA(Mn)-FA (d-f) dendrimers dissolved in water (a, d), PBS (b, e), and cell culture medium (c, f) at 4 ^{o}C for one month.

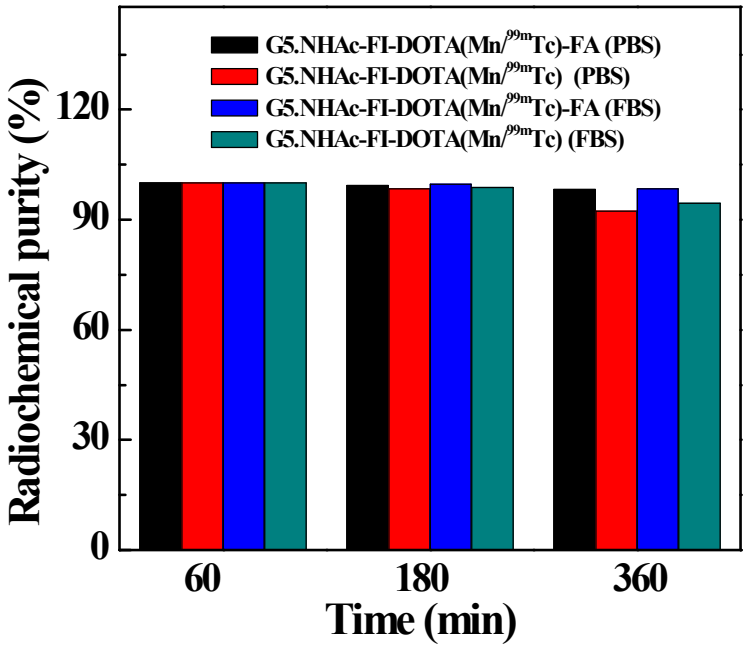

Fig. S4. The radiochemical purity of the G5.NHAc-FI-DOTA(Mn/^{99m}Tc)-FA or G5.NHAc-FI-DOTA(Mn/^{99m}Tc) dendrimers after exposure to PBS or FBS at 37 °C for 1, 3, and 6 h, respectively.

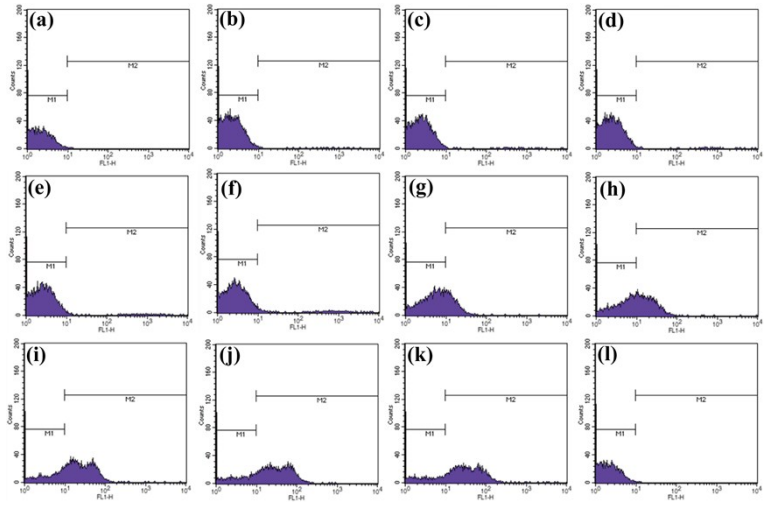

Fig. S5. Flow cytometric analysis of HeLa cells with high-level FAR (H-FAR) after treated with PBS (a, l), G5.NHAc-FI-DOTA(Mn) dendrimers at the Mn concentration of 20 (b), 40 (c), 60 (d), 80 (e), and 100 (f) μg/mL, and G5.NHAc-FI-DOTA(Mn)-FA dendrimers at the Mn concentration of 20 (g), 40 (h), 60 (i), 80 (j), and 100 (k) μg/mL for 4 h, respectively.

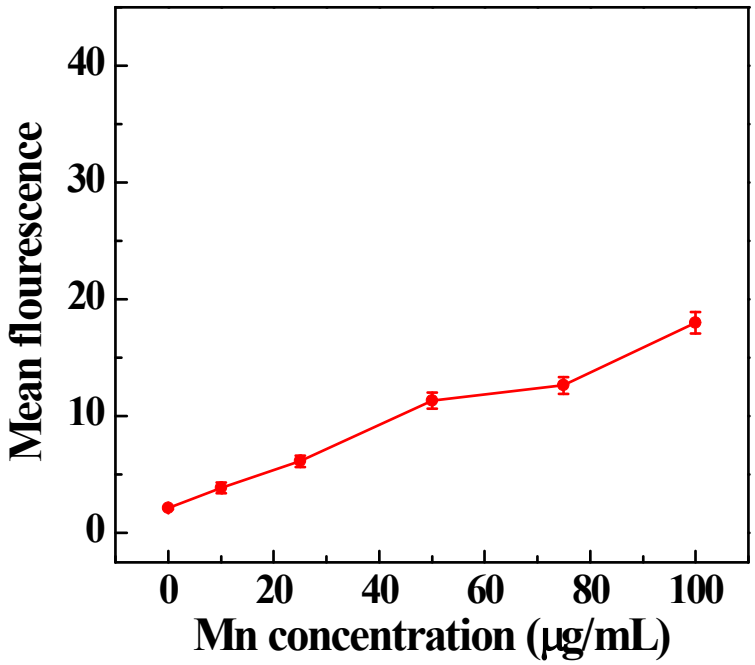

Fig. S6. Flow cytometric analysis of L-FAR HeLa cells after they were treated with G5.NHAc-FI-DOTA(Mn)-FA dendrimers at the Mn concentration of 0, 10, 25, 50 75 and 100 μg/mL for 4 h, respectively.

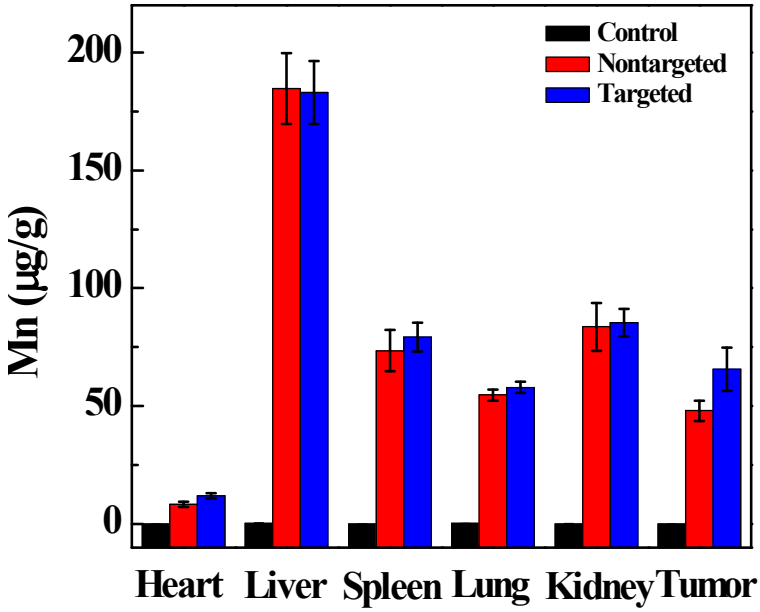

Fig. S7. *In vivo* biodistribution of Mn in the major organs of the mice including the heart, liver, spleen, lung, kidney, and tumor at 24 h post intravenous injection of the nontargeted G5.NHAc-FI-DOTA(Mn) or targeted G5.NHAc-FI-DOTA(Mn)-FA dendrimers (300 μg Mn, in 0.3 mL PBS) into each mouse. Mice treated with PBS were used as control.

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