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Supplementary Information

Size-Dependent Lymphatic Delivery of Gd-Based MRI Contrast Agents: Insights from In Vivo Studies

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Experimental Details

Materials

Gd-DTPA (Magnevist) was purchased from Beijing Beilu Pharmaceutical Co., Ltd. Sodium polyacrylate (PAANa, Mw: 5100), diethylenetriamine (DET), DTPA, citric acid and sodium citrate were purchased from Aladdin Co. Ltd. Gadolinium chloride hexahydrate ($GdCl_3 \cdot 6H_2O$, 99.9%) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMTMM) were provided by Shanghai Macklin Biochemical Co., Ltd. Sodium hydroxide (NaOH, 96%) and Sodium bicarbonate ($NaHCO_3$) were obtained from Beijing Chemical Reagents Co. Ltd. Oleic acid (OA), 1-octadecene (ODE), and NH_4F were purchased from Sigma-Aldrich. Anhydrous ethanol, carbinol, cyclohexane and tetrahydrofuran (THF) were purchased from Damao Chemical Reagent Co., Ltd. The dp-PEG-mal molecules with the molecular weight 2000 were ordered from Beijing Oneder Hightech Co. Ltd. The mouse embryonic fibroblast cell line NIH/3T3 and the murine breast cancer 4T1 cells were purchased from ATCC.

Synthesis of PAA-Gd

(1) *Synthesis of PAA-DET Polymer.* To synthesize the PAA-DET polymer, 120 mg of PAANa was initially dissolved in 12 mL of Milli-Q water by using magnetic stirring in a round-bottom flask. Subsequently, 1.1 mL of DET and 1.15 g of DMTMM (previously dissolved in 21 mL of 0.01 M $NaHCO_3$) were added to the solution. The resulting mixture was stirred at room temperature overnight, followed by purification by using dialysis for 48 h to eliminate unreacted DET molecules. After freeze-drying, the PAA-DET polymer was obtained.

(2) *Synthesis of PAA-DTPA Polymer.* 8 g of DTPA was dissolved in 11.4 mL of Milli-Q water, with the pH adjusted to 8.5 by adding 5 M NaOH aqueous solution. 1.5 g of DMTMM was then incorporated swiftly into the solution, and the mixture was stirred at room temperature for 10 minutes to activate the carboxyl groups in DTPA. Following this, the previously synthesized PAA-DET was added to this activated DTPA solution

to react for 4 hours. The PAA-DTPA product was purified through the dialysis for 48 h and subsequently freeze-dried.

(3) *Synthesis of PAA-Gd.* 30 mg of PAA-DTPA was mixed with 60.2 mg of $GdCl_3 \cdot 6H_2O$ dissolved in 2 mL and 3.24 mL citric acid and sodium citrate buffer solution. The Gd^{3+}-containing solution was then added slowly to the PAA-DTPA solution while stirring at low speed for 2 h. Finally, free Gd^{3+} ions were removed using the dialysis, and the resultant PAA-Gd solution was adjusted to 1× PBS before being stored at 4 °C for future applications.

Synthesis of $NaGdF_4$@PEG

(1) *Synthesis of Oleate-Capped $NaGdF_4$ Nanoparticles.* The synthesis of oleate-capped $NaGdF_4$ nanoparticles was achieved through a high-temperature coprecipitation method. Initially, 0.8 mmol of $GdCl_3 \cdot 6H_2O$, 6 ml of OA, and 14 ml of ODE were combined in a 100-ml flask and the mixture was heated to 150 °C for 30 minutes under a vacuum, resulting in a homogeneous solution. Then the temperature drops to 50 °C. In the second step, 2.5 mmol of NaOH and 4 mmol of NH_4F were dissolved in 10 ml of methanol and subsequently added dropwise to the cooled solution from the previous step. The reaction system was stirred at 50 °C overnight. Following this, the methanol was evaporated at temperatures below 100 °C under vacuum. The reaction temperature was then rapidly increased to 265 °C and reacted for 35 min under a nitrogen atmosphere. After cooling down, the resultant nanoparticles were precipitated out by using ethanol, collected by centrifugation multiple times, and redispersed in cyclohexane for subsequent applications.

(2) *Synthesis of PEGylated $NaGdF_4$ ($NaGdF_4$@PEG) Nanoparticles.* In the process of PEGylation, 10 mg of oleate-capped $NaGdF_4$ nanoparticles were combined with 100 mg of asymmetric polyethylene glycol (PEG) in 14 ml of tetrahydrofuran (THF) within a flask. This mixture was stirred magnetically at 40 °C for 12 h. The resultant PEGylated nanoparticles were then precipitated by using cyclohexane, followed by collection through several centrifugation cycles. The product underwent vacuum drying

at room temperature. Further purification was conducted by using ultrafiltration with 30 kDa MWCO centrifugal filter (Millipore, YM- 50) to remove the free ligand, and then dispersed in Milli- Q water for further use.

Characterizations

PAANa, PAA-DET and PAA-DTPA were characterized by ^{1}H NMR to analyze their structures. The analysis of particle size, morphology, and uniformity of $NaGdF_4$ nanoparticles was conducted by using a transmission electron microscope (TEM, JEM-2100) at an operating voltage of 200 kV. The diameter of nanoparticles was quantified through ImageJ software by averaging measurements from a minimum of 150 particles. DLS measurements were carried out at 298.0 K with Nano ZS (Malvern) equipped with a solid state He-Ne laser (λ = 632.8 nm). Additionally, the gadolinium (Gd) content in different systems was determined using inductively coupled plasma-mass spectrometry (ICP-MS, Thermo, ICAP-Qc).

Relaxivity measurements

The relaxivity values of all contrast agent solutions were assessed by using a 7.0 T animal MRI system (Bruker BioSpec). Aqueous solutions with varying concentrations of Gd were prepared in small Eppendorf tubes and analyzed via MR studies. The detailed parameters for T_1-weighted imaging and T_1map sequence were set as follows: T_1-weighted imaging: TE = 5.01 ms, TR = 300 ms, NEX = 6, and FoV = 35 × 35 mm; T_1map: TE = 5.90 ms, TR = 3000, 1500, 1000, 500, and 298 ms, and FoV = 35 × 35 mm.

Cell culture

Both 3T3 cells and 4T1 cells were cultured in a Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 1% penicillin-streptomycin solution (100 ×) at 37 °C under a 5% CO_2 atmosphere in the cell incubator.

Cell viability assays

The cytotoxicity of the three nanomaterials was evaluated *via* Cell Counting Kit-8 (CCK-8) assay. In detail, 3T3 cells were seeded into a 96-well cell culture plate with a density of ~5 × 10^3 cells/well and cultured at 37°C in CO_2 incubator overnight for adhesion. Thereafter, nanomaterials with different concentrations were introduced into the wells and incubated with the cells for 24 h. Following the replacement of clean culture medium, 10 μL of CCK-8 solution was added to each well and incubated for 1 h in incubator. Thereafter, the optical density of each well at 450 nm was recorded on a microplate reader (Thermo, Varioskan Flash).

Hemolysis test

The hemolysis rate of the three nanomaterials was determined following standard procedures. Briefly, 2 mL blood samples of mice were mixed with excess normal saline (NS) and purified by centrifugation at 250 g for 10 minutes. The resulting clean red blood cells were then diluted in 4 mL of normal saline and the suspension was then diluted 1: 4 into NS (negative control); water (positive control); nanomaterials solutions (in NS) at different concentrations. These red blood cell mixtures were incubated in the dark at 37 °C for 4 h and subsequently centrifuged at 400 g for 5 minutes. Finally, the absorbance of the supernatant from each tube was measured at 541 nm.

The hemolysis rate was calculated by the following equation:

$$\text{Hemolysis rate} = \frac{D_t - D_{nc}}{D_{pc} - D_{nc}} \times 100\%$$

where D_t, D_{nc} and D_{pc} are the absorbance of the tested sample, the negative control and the positive control, respectively.

Animal studies

The female BALB/c mice (7 weeks old) mice were provided from SPF (Beijing) Biotechnology Co., Ltd.. All mice were housed on a 12-hour light-dark cycle with free access to food and autoclaved water in an air-conditioned SPF level animal room (22 ± 1 °C, 50-60% humidity, 6 mice/cage). Every mouse has acclimatized for 3 days prior

to any experimental manipulation. Mice were randomized into groups for all studies, with a sample size of n $\geqslant$ 3 per group for all experiments.

Tumor model establishment

4T1 cells were used for tumor model construction. A total of 6 BALB/c mice were selected randomly and inoculated subcutaneously with~5 × 10^6 4T1 cells (suspended in 100 μL of PBS) into the hind leg. The tumor imaging studies were carried out at 7 days after the inoculation of tumor cells. Three mice with tumors of similar size were included in the study, and those with excessively small were excluded prior to imaging.

MRI studies *in vivo*

The MRI experiments were performed on a 7.0 T animal MRI system (Bruker BioSpec 70/20). The mice were anesthetized with 1-2% isoflurane delivered in oxygen via a nose cone during the imaging period, with respiratory rate continuously monitored to maintain stable anesthesia. The MRI scan was acquired at pre- and post-injection time points, with only a single injection performed per mouse. The 3D DCE-MRI and 2D T_1-weighted imaging sequences were acquired at designed time points. The detailed parameters for 3D DCE-MRI were set as follows: TE = 1.61 ms, and TR = 14.1 ms, FOV = 40 mm × 27 mm ×18 mm, MTX = 285 × 193 × 80, slice thickness = 0.20 mm. The detailed parameters for 2D T_1-weighted imaging were set as follows: TE = 5.2 ms, and TR = 354.63 ms, FOV = 35 mm × 35 mm, MTX = 256 × 256, slice thickness = 1 mm.

(1) *MRI in healthy mice.* A total of 12 BALB/c mice were prepared randomly, and divided into three groups (n = 4), and received footpad injection of one of the following contrast agents: Gd-DTPA, PAA-Gd, or $NaGdF_4$@PEG. For each group, three mice were used for MRI of the lymphatics and lymph nodes in the right lower limb, and one mouse was used for imaging of the lymphatics and lymph nodes in the right upper limb. The injection dose was set at 0.06 mmol Gd per kilogram of mouse body weight, with a maximum volume of 50 μL per injection.

(2) *MRI in tumor-bearing mice.* Tumor-bearing mice (n = 3, screened as described above) were injected at footpad with PAA-Gd at 0.06 mmol Gd per kilogram, with a maximum volume of 50 µL per injection.

Biosafety evaluation

A total of 18 BALB/c mice were randomized into three groups (n = 6). Mice in each group received a single tail intravenous injection of one of the following formulations: PAA-Gd (0.1 mmol Gd/kg), $NaGdF_4$@PEG (0.1 mmol Gd/kg), or PBS aqueous solution. The body weight of each mouse was monitored and recorded daily for 14 days. Finally, the statistical analysis of body weight changes was performed using GraphPad Prism software and the data were presented as mean ± standard deviation (SD). At the end of the 14-day period, the mice were sacrificed. Major organs, including the heart, liver, spleen, lung, and kidney, were carefully excised. These organs were then fixed in paraformaldehyde, sectioned, and stained with hematoxylin and eosin (H&E) for histological analysis.

Statistical analysis

Data are shown as the mean ± standard deviation, as indicated in the figure captions. Statistical differences between two groups were determined *via* Student's t-test. The p value less than 0.05 ($p < 0.05$) was regarded as statistically significant. All the statistical tests were carried out using OriginPro (9.0 and 2019b) and GraphPad Prism software (v8.0).

Live Subject Statement

All animal experiments reported herein were performed according to the ARRIVE guidelines 2.0 and the protocol approved by the Peking University Institutional Animal Care and Use Committee (LA2019083).

Additional Figures

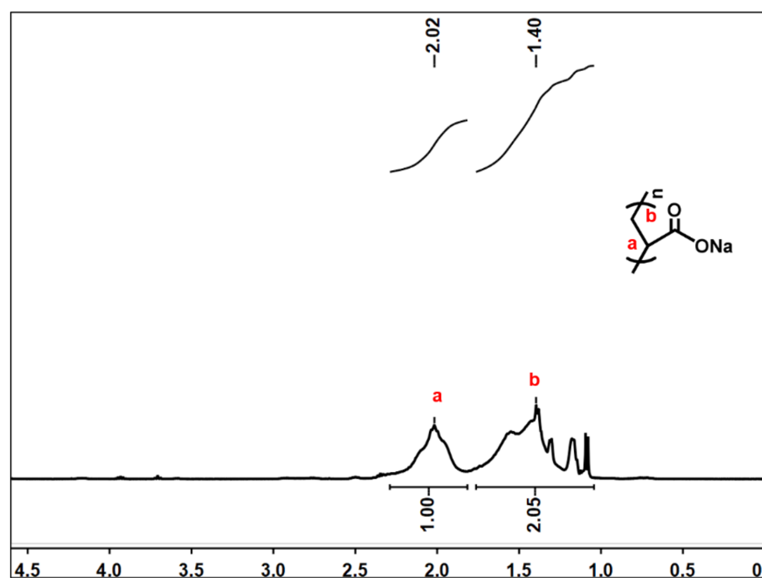

Figure S1. ^{1}H NMR Spectra (D_2O) of PAANa with the numbers of hydrogen atoms determined by integration of the peak areas.

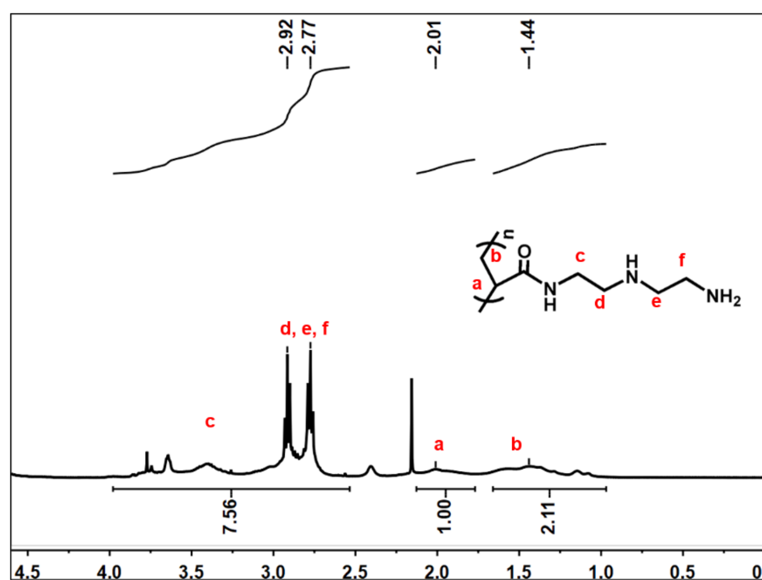

Figure S2. ^{1}H NMR Spectra (D_2O) of PAA-DET with the numbers of hydrogen atoms determined by integration of the peak areas.

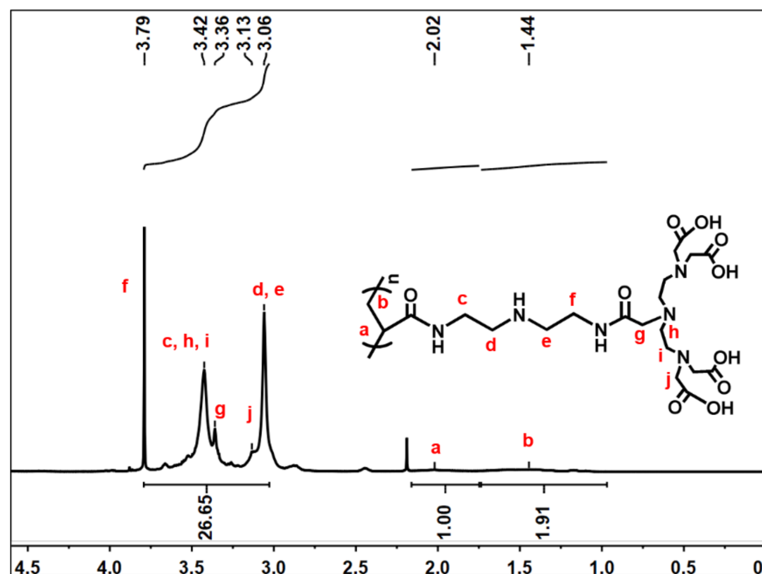

Figure S3. ^{1}H NMR Spectra (D_2O) of PAA-DTPA with the numbers of hydrogen atoms determined by integration of the peak areas.

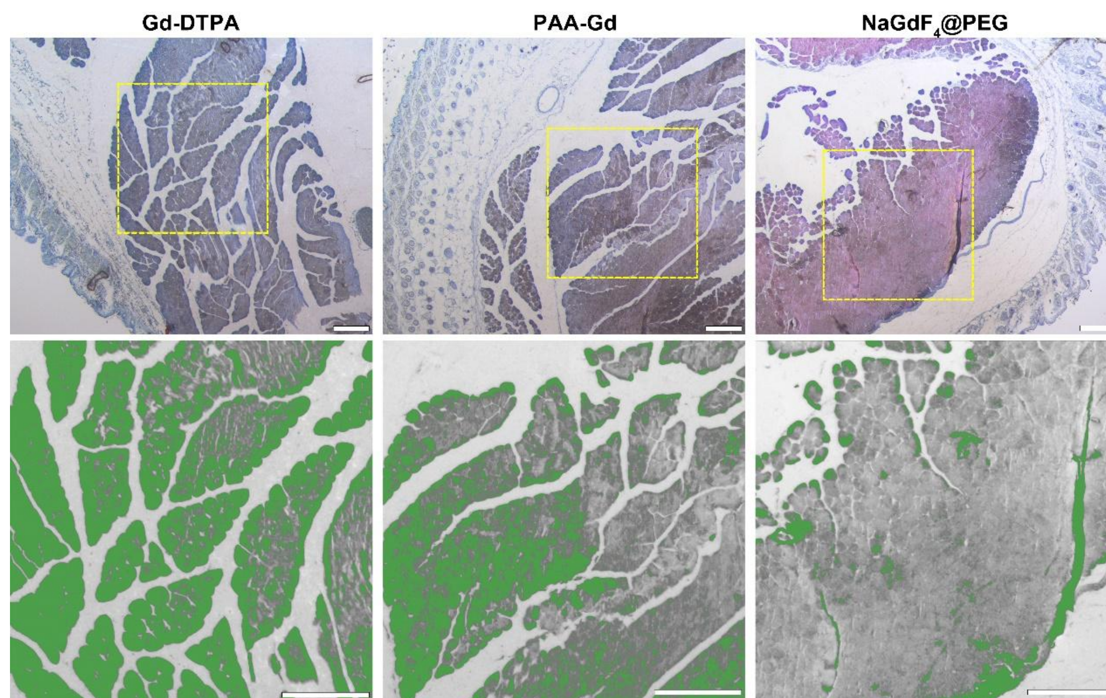

Figure S4. CPN III staining slice and the distribution of Gd^{3+} in the magnified images managed by Image J. The embedded scale bar corresponds to 200 μm.

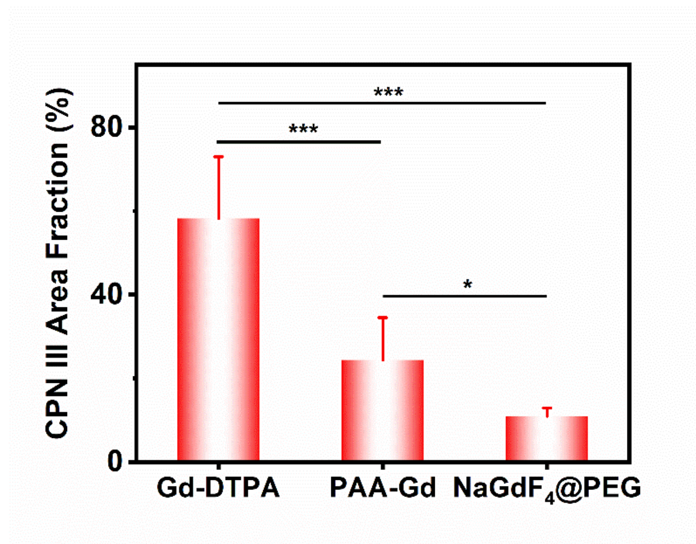

Figure S5. The quantitative analysis of CPN III staining results with Gd-DTPA, PAA-Gd and $NaGdF_4$@PEG.

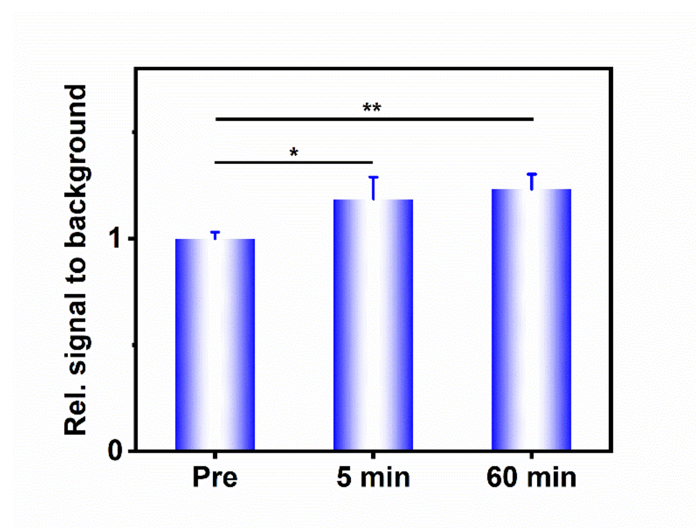

Figure S6. The signal intensity ratio between the tumor and the surrounding normal tissue.

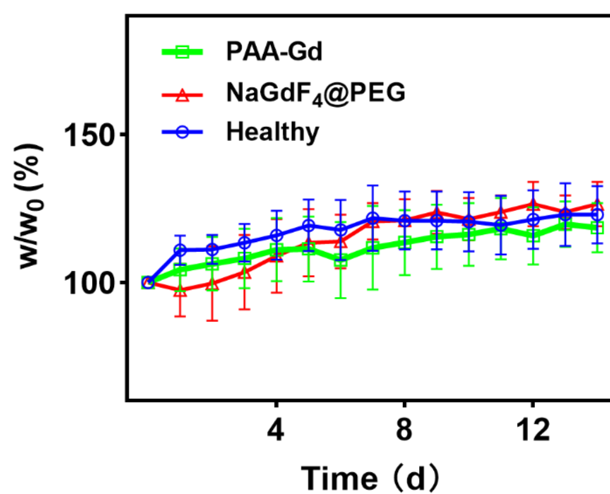

Figure S7. The body weight after PAA-Gd and $NaGdF_4$@PEG administration, respectively (n = 6).

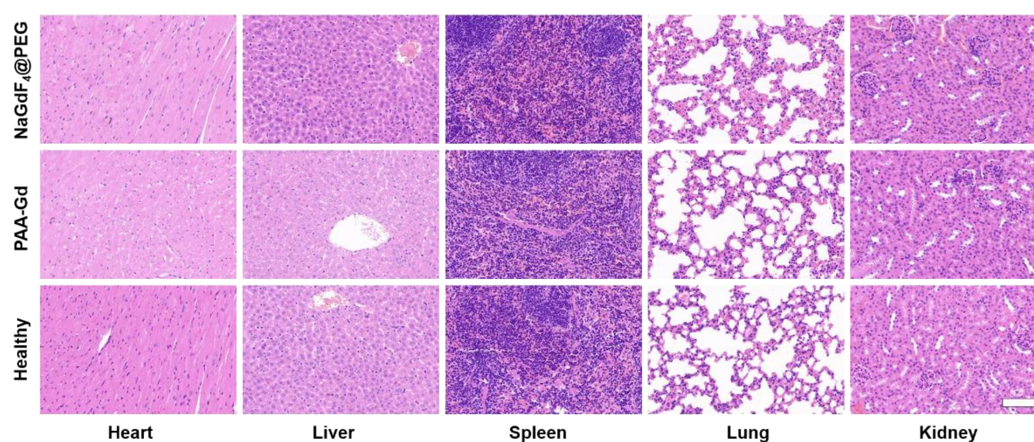

Figure S8. Biosafety of section staining of PAA-Gd and $NaGdF_4$@PEG. The embedded scale bar corresponds to 100 μm.