

Syntheses of Gd-(1B4M)DTPA Functionalized PAMAM Dendrimer MRI Agents

Haitao Wu

Imaging Probe Development Center, National Heart, Lung, and Blood Institute, National Institutes of Health, 9800 Medical Center Drive, Rockville, MD 20850, ipdc@nhlbi.nih.gov

Background

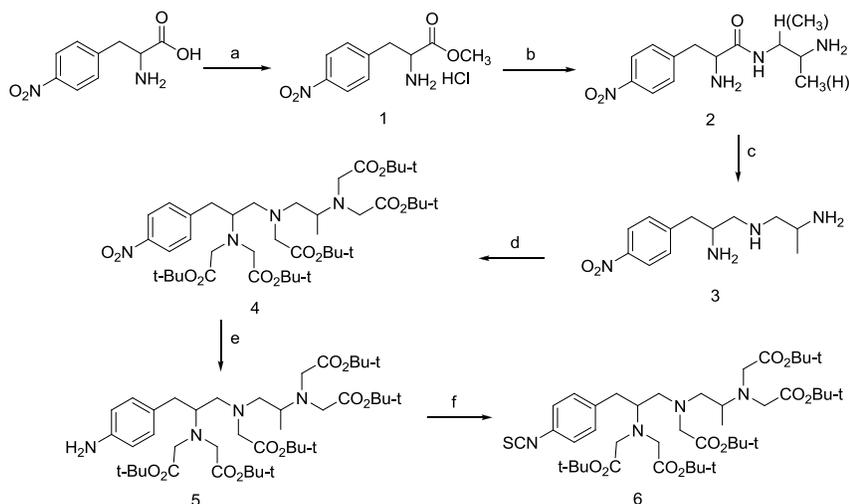
Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) has been successfully used to assess vascular permeability in the microvasculature of tumors undergoing treatment with angiogenic inhibitors.¹ Unfortunately, the currently FDA-approved low molecular weight gadolinium chelates leak readily from both neoplastic vessels and non-neoplastic vessels owing to their small sizes, which makes the leakage of contrast agents a non-specific phenomenon.^{2,3} Dendrimers are monodisperse nanoparticles that allow bioconjugation of multiple gadolinium chelates for MRI. Dendrimeric MRI agents differ in size, but not in their chemical properties.⁴ Therefore, the effects of the size of the contrast agent on permeability and vascular volume estimates can be isolated from other chemical properties. These agents can be used to monitor angiogenic therapies in tumor models, as markers of angiogenesis and as surrogates for drug delivery.⁵

Chemistry

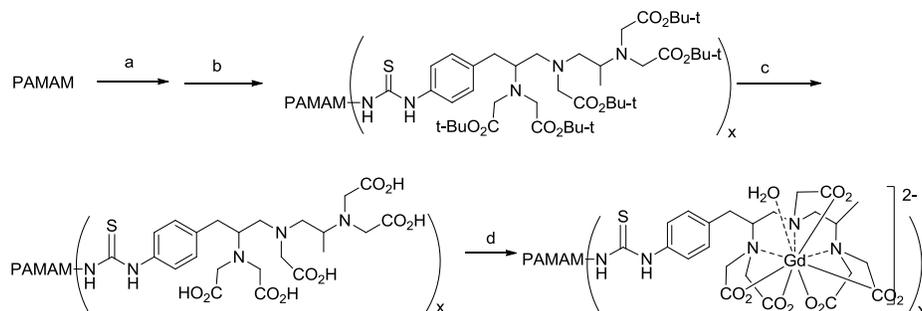
The dendrimeric MRI contrast agents were synthesized according to published methods with modifications (**Scheme 1**).^{6,7} The triamine compound **3** was extracted as free amine from an aqueous basic reaction mixture, instead by precipitation as a hydrochloride salt from hydrogen chloride-saturated ethanolic solution in order to avoid contamination by boric acid. For the synthesis of compound **4**, a higher yield was obtained by switching from potassium carbonate as base to diisopropylethylamine (DIPEA)⁸, thereby eliminating a reflux step (how?) and prevented further undesirable reaction of product **4** with excess *t*-butyl bromoacetate. Free isothiocyanate compound **6** was isolated from its hydrochloride salt form whereas it has been used previously without such purification.⁷ The chelation of diethylenetriaminepentaacetic acid (DTPA) moiety with Gd was carried out in citrate buffer (**Scheme 2**). The *N*-hydroxysuccinimide ester-activated version of DTPA including a spacer (**Scheme 3**) was also synthesized as an alternative

composition in order to explore whether such a spacer could decrease steric hindrance seen during the conjugation of non-spacer-containing DTPA moiety and dendrimers. However, no significant difference was observed in the DTPA substitution level between the two types of DTPA-dendrimer conjugates based on the MALDI-TOF MS and elemental analysis results.

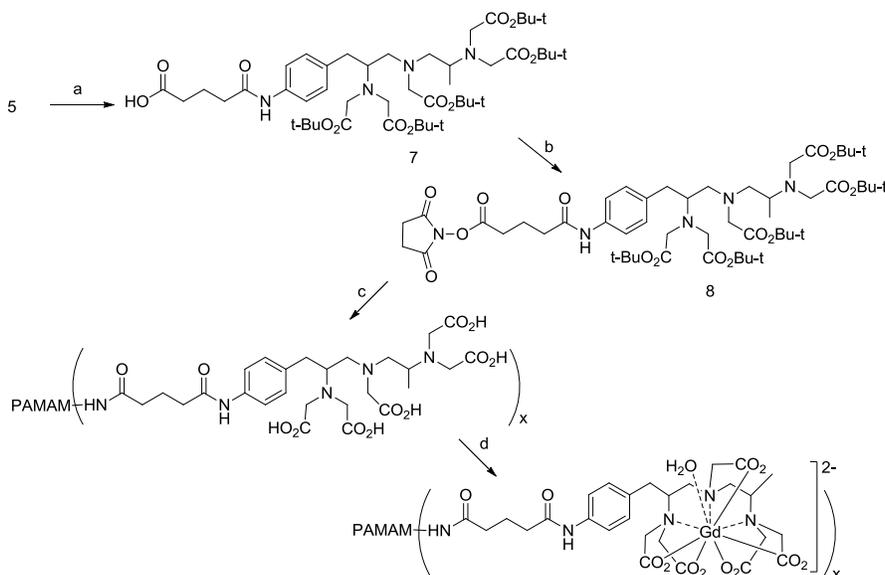
Scheme 1. Synthesis of ITC-Bn-(1B4M)DTPA-t-Bu ester reagent. Reagents and conditions: a. anhydrous MeOH/HCl(g), 12h; b. i). TEA/ether, filtration, ii). 1, 2-diaminopropane, r.t., 24h; c. BH₃·THF, reflux, 6h; d. t-butyl bromoacetate, DIPEA, acetonitrile, r.t., 12h; e. H₂/Pd-C, EtOH, 1atm, r.t., 4h; f. CSCI₂, ethyl acetate, r.t., 4h.



Scheme 2. Synthesis of Gd-Bn-(1B4M)DTPA-functionalized PAMAM dendrimer conjugates. Reagents and conditions: a. **6**/MeOH, r.t., 48h; b. *N*-(2-aminoethyl)-aminomethyl polystyrene, DCM, r.t., 12h; c. TFA, r.t., 6h; d. Gd(OAc)₃·xH₂O, 0.3M citric buffer, pH 4.5.



Scheme 3. Synthesis of spacer-linked Gd-Bn-(1B4M)DTPA-functionalized PAMAM dendrimer conjugates. Reagents and conditions: a. Glutaric anhydride, ethyl acetate, r.t. 12h; b. NHS, EDC, acetonitrile, r.t. 12h; c. i). PAMAM, MeOH, r.t. 48h, ii). *N*-(2-aminoethyl)-aminomethyl polystyrene, DCM, r.t. 12h, iii). TFA, r.t. 6h; d. Gd(OAc)₃·xH₂O, 0.3M citric buffer, pH 4.5.



Experimental

General. All reagents and the hydrogen chloride gas delivery system were purchased from Sigma-Aldrich (Milwaukee, WI). Millipore Centriprep centrifugal filter devices were purchased

from Millipore (Billerica, MA). The ^1H NMR and the ^{13}C NMR spectra were recorded on a Varian 400 MHz spectrometer operating at 400 MHz and 100 MHz respectively. Chemical shifts are reported in part per million (δ) and referenced internally to deuterated solvents. MS data were measured on an Agilent 1200 series LC equipped with an Agilent G2440A LC/MSD-Trap-XCT ion trap mass spectrometer. Matrix-Assisted Laser Desorption Ionization (MALDI-TOF) mass spectrometry data were determined by The Scripps Research Institute (La Jolla, CA). HPLC was performed on a Beckman Coulter Gold system analytical instrument. Size exclusion HPLC analysis was carried out using a TSK G2000SWxl column (7.8 mm x 300 mm, 5 μm) eluted with PBS 1X buffer at 0.5 ml/min flow rate. Elemental analysis was provided by Desert Analytics Laboratory (Tucson, AZ).

Synthesis of 2-(*p*-isothiocyanatobenzyl)-6-methyl-diethylenetriamine-*N, N, N', N'', N'''*-penta-*tert*-butyl acetate (ITC-Bn-(1B4M)DTPA-*t*-Bu ester) (Scheme 1)

***p*-Nitrophenylalanine methyl ester (1)** *p*-nitrophenylalanine (10 g, 47.6 mmol) was suspended in dry methanol (100 mL) in a 300 mL round bottomed flask and the mixture was saturated with dry gaseous hydrogen chloride by bubbling a gentle flow of hydrogen chloride for about 2 h until absorption stopped. The flask was sealed with a rubber septum and allowed to stand at room temperature overnight. The white solid was filtered and washed with the minimum amount of methanol (5 mL). The filtrate was concentrated and cooled in ice to allow any product still remaining in solution to precipitate. The combined white solids were dried *in vacuo* at 50 $^\circ\text{C}$ to remove all volatile components and stored under anhydrous conditions. Yield: 12.2 g (98 %). TLC (CHCl_3 : MeOH v/v, 4: 1) R_f = 0.86. ^1H NMR (400 MHz, D_2O) δ : 8.29 (d-d, J = 8.4, 2.0 Hz, 2H), 7.57 (d-d, J = 8.4, 2.0 Hz, 2H), 4.56 (t, J = 5.0 Hz, 1H), 3.87 (s, 3H), 3.47 (m, 2H). MS (ES)(positive): calcd. for $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_4$ 224.2, found 225.1 (MH^+).

***N*-(2-Aminopropyl)-*p*-nitrophenylalanine amide (2).**⁶ Into a 500 mL Erlenmeyer flask containing a solution of *p*-nitrophenylalanine methyl ester hydrogen chloride salt **1** (9.8 g, 37.6 mmol) in methanol (25 mL), was added triethylamine (7 mL), forming a yellow-brown solution. Diethyl ether (300 mL) was added resulting in formation of a white precipitate, which was filtered off. The filtrate was then concentrated to afford a yellow oil. The yellow oil was

dissolved in methanol (5 mL) and then added to a 200 mL round bottomed flask containing 1, 2-diaminopropane (50 mL). The mixture was stirred at room temperature for 24 h and then concentrated to dryness to yield a green oil (10.7 g). The oil was dissolved in THF (30 mL), filtered through Celite, evaporated, and dried *in vacuo* to give a yellow-brown oily product (9.9 g, yield 99 %). The product was used in the next step without further purification. TLC (ethanol/ ammonium hydroxide, v/v, 4 : 1) $R_f = 0.65$. $^1\text{H NMR}$ (400MHz, D_2O) δ 8.17 (d-d, $J = 8.4$, 2 Hz, 2H), 7.41 (d-d, $J = 8.4$, 2 Hz, 2H), 3.68 (m, 1H), 3.32 (m, 2H), 3.02 (m, 2H), 2.92 (m, 2H), 1.45 (s, 8H), 1.10 (m, 4H). MS(ES)(positive): calculated for $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_4$ 266.3, found 267.3 (MH^+).

2-(*p*-Nitrobenzyl)-6-methyl-diethylenetriamine (3).^{6,9} Amide **2** (9 g, 0.034 mol) was dried *in vacuo* overnight and then dissolved in anhydrous tetrahydrofuran (100 mL) in a dry 500 mL three necked round bottomed flask. A dry distillation condenser with a drying tube on top was mounted on the flask. The other two mouths were sealed with a rubber septa and the flask was cooled in an ice-bath. Nitrogen was bubbled in via a long needle through the septum for 5 minutes. $\text{BH}_3 \cdot \text{THF}$ solution (170 mL, 1M, 0.17 mol) was slowly transferred *via* a cannula into the reaction flask. After the addition was complete, the pale yellow solution was heated to reflux for 6 h. Half of the solvent was evaporated by rotary evaporation. The remaining solution was cooled and ethanol (100 mL) was added slowly to destroy the excess borane. The solution was concentrated *in vacuo* to dryness. The residue was dissolved in absolute ethanol (200 mL) and then saturated with gaseous hydrogen chloride. The reaction mixture was heated to reflux for 6 h and allowed to cool to room temperature. The solvents were removed *in vacuo*. The residue was dissolved in water (30 mL) and the mixture was adjusted to pH 10 using 10 N NaOH. Chloroform (200 mL) was added and the mixture was stirred at room temperature for 8 h. The organic layer was separated and the aqueous layer was again stirred with chloroform (100 mL) for 8 h. The organic layers were combined, dried over anhydrous potassium carbonate and filtered. The solvents were evaporated and the residue was dried *in vacuo* to afford an orange-brown liquid (6.3 g, 74 %). The product was used in the next step without further purification. TLC (ethanol/ ammonium hydroxide, v/v, 4 : 1) $R_f = 0.41$. MS (ES) (positive): calculated for $\text{C}_{11}\text{H}_{16}\text{N}_4\text{O}_3$ 252.3, found 253.1 (MH^+).

2-(*p*-Nitrobenzyl)-6-methyl-diethylenetriamine-*N, N, N', N'', N''*-penta-*tert*-butyl acetate (4).⁸ Triamine **3** (6 g, 0.024 mol) and diisopropylethylamine (DIPEA) (20 g, 0.155 mol) were dissolved in acetonitrile (100 mL) in a dry 300 mL two-necked flask equipped with an addition funnel and a condenser with a drying tube on top. *t*-Butyl bromoacetate (27 g, 0.14 mol) was added slowly with stirring *via* the addition funnel. The reaction mixture was stirred at room temperature for 12 h. Completion of the reaction was confirmed using TLC (hexanes/THF/TEA, v/v/v, 4:1:0.1, product R_f = 0.43). The white solid (DIPEA·HBr salt) was filtered off and the filtrate was concentrated to dryness *in vacuo*. The residue was dissolved in ethyl acetate (100 mL), washed with brine (60 mL) and dried over anhydrous potassium carbonate. After filtration to remove the potassium carbonate, the yellow filtrate was taken to dryness. Flash chromatography was performed using hexanes /THF, v/v, 6:1 as solvents. The yellow product fraction was concentrated to afford a yellow oily product **4** (14.7 g, yield 77 %). TLC showed the two isomers (hexane/THF,v/v, 4:1; R_f = 0.31 and 0.16) or a single spot (hexane/THF/TEA, v/v/v 4:1:0.1, R_f =0.43). ¹H NMR (400 MHz, CDCl₃) δ : 8.09 (dd, J = 8.8, 1.2 Hz, 2H), 7.46 (dd, J = 8.8, 1.2 Hz, 2H), 3.43-3.30 (m, 10 H), 3.03-2.74 (m, 8H), 2.5-2.3(m, 2H), 1.46 (m, 45 H), 1.04 (d, J = 6.7 Hz, 3H). MS (ES) (positive): calculated for C₄₂H₇₀N₄O₁₂ 823.0, found 824.0 (MH⁺).

2-(*p*-Aminobenzyl)-6-methyl-diethylenetriamine-*N, N, N', N'', N''*-penta-*tert*-butyl acetate (5).⁷ A 500 mL Parr hydrogenation flask was purged with nitrogen for 2 minutes. Compound **4** (14.4 g, 17.5 mmol) and 10 % Pd/C (1 g) were added to the flask followed by addition of absolute ethanol (120 mL) (CAUTION: Fire hazard). The flask was sealed and mounted on a Parr hydrogenation apparatus. The hydrogenation was carried out at room temperature at 1-2 atmospheres for 4h. Completion of the hydrogenation reaction was confirmed by TLC (hexanes/THF/TEA, v/v/v, 2 : 1 : 0.1; product R_f = 0.46). The mixture was filtered through Celite and washed with ethanol (200 mL). The filtrate was taken to dryness to give a yellow oil (11.6 g, yield 84 %). The product was used in the next step without further purification. ¹H NMR (400MHz, CDCl₃) δ 7.00 (d-d, J = 8, 3.0 Hz, 2 H), 6.59 (d-d, J = 8, 3.0 Hz, 2 H), 3.43-3.30 (m, 10 H), 3.03-2.74 (m, 8H), 2.5-2.3(m, 2H), 1.46 (m, 45 H), 1.04 (d, J = 6.7 Hz, 3H). MS (ES) (positive): calculated for C₄₂H₇₂N₄O₁₀ 793.0, found 794.0 (MH⁺).

2-(*p*-Isothiocyanatobenzyl)-6-methyl-diethylenetriamine-*N, N, N', N'', N''*-penta-*tert*-butyl acetate (6) (ITC-Bn-(1B4M)DTPA-*t*-Bu ester).⁷ A dry 50 mL round bottomed flask containing a solution of compound **5** (8.5 g, 10.7 mmol) in ethyl acetate (30 mL) was sealed with a rubber septum. A nitrogen balloon was connected to the flask *via* a syringe needle. Thiophosgene (1.6 g, 14 mmol) was added *via* syringe and the mixture was stirred at room temperature for 4 h. The mixture was concentrated to dryness and further dried *in vacuo* to remove volatile components to afford the product as a yellow solid (10 g, yield 99 % based on 3HCl salt form).⁷ Hydrogen chloride-free product was obtained as follows: the solid was dissolved in diethyl ether (20 mL) and 4 molar equivalents of triethylamine (4.3g, 43 mmol) were added. The suspension was filtered and the filtrate was quickly washed with cold, aqueous, saturated sodium bicarbonate (10 mL) and brine (10 mL) and dried over magnesium sulfate. Concentration of the solution *in vacuo* to remove any remaining volatile components afforded yellow-brown oily product **6** (8.8 g, yield 98 %). TLC (hexanes/THF/TEA, v/v/v, 4:1:0.2): R_f = 0.39 and 0.47 for the two isomer. ¹H NMR (400MHz, DMSO-*d*₆) δ : 6.91 (d, 2H, J = 8.2 Hz), 6.53 (d, 2H, J = 8.5 Hz), 3.40 (m, 10 H), 3.10-2.30 (m, 8 H), 1.46 (m, 45 H), 0.98 (d, 3H, J = 6.3 Hz); MS (ES) (positive): calculated for C₄₃H₇₁N₄O₁₅S 835.5, found 836.3 (MH⁺).

Synthesis of Gd-(1B4M)DTPA-functionalized PAMAM dendrimer conjugates.

Bn-(1B4M)DTPA functionalized PAMAM G2 (G2-(1B4M)DTPA).¹⁰ To a solution of second generation PAMAM dendrimer (3.4 g, 20 % in methanol, 0.21 mmol) was added a solution of compound **6** (3.3 g, 3.95 mmol) in MeOH (20 mL). The mixture was stirred at room temperature for 2 days and then concentrated to dryness. The residue was dissolved in CH₂Cl₂ (5 mL). *N*-(2-aminoethyl)aminomethyl polystyrene (1 g, loading: \geq 2.0 mmol N/g) was added at room temperature and stirred for 12 h. The resin was filtered off and the filtrate was concentrated to dryness. Trifluoroacetic acid (3mL) was added to the residue and the mixture was stirred at room temperature for 4 h. TFA was removed and the residue was dissolved in H₂O (10 mL). The pH of the mixture was adjusted to 6-7 using 1N NaOH and the solution was filtered through a Millipore (0.45 μ m) syringe filter unit. The filtrate was dialyzed against water using a Centriprep centrifugal filter device with MW cutoff of 3 kDa membrane to remove salts and small molecules until size exclusion HPLC analysis showed a sole peak. Size exclusion HPLC: t_R =

14.6 min. The solution was lyophilized to afford an off-white solid G2-(1B4M)DTPA. Yield: 2.2 g (88%). MS calculated for $C_{510}H_{768}N_{122}O_{188}S_{16}$ 12128.2; MALDI-TOF MS found 9,245 Da.

Bn-(1B4M)DTPA functionalized PAMAM G4 (G4-(1B4M)DTPA). The general protocol described for the G2 generation dendrimer was also used for the fourth generation PAMAM dendrimer (2.84 g, 10 % w/w solution in MeOH, 0.020 mmol) and compound **6** (1.35 g, 1.6 mmol). Dialysis was carried with 10kDa MW cutoff membrane affording 0.74 g (75%) of conjugate. Size exclusion HPLC: $t_R = 14.4$ min. MS calculated for $C_{2094}H_{3168}N_{506}O_{764}S_{64}$ 49702.2; MALDI-TOF MS found: 40.9 kDa.

Bn-(1B4M)DTPA functionalized PAMAM G6 (G6-(1B4M)DTPA). The general protocol described for the G2 generation was used for the reaction of sixth generation PAMAM dendrimer (4.53 g, 5 % in methanol, 0.0039 mmol) and compound **6** (0.93 g, 1 mmol, 24 h). Dialysis was carried out with a 10 kDa cutoff membrane affording 0.66 g (86%) of conjugate. Size exclusion HPLC: $t_R = 11.1$ min. MS calculated for $C_{8430}H_{12768}N_{2042}O_{3068}S_{256}$ 199998, MALDI-TOF MS found: 110 kDa.

Gd-Bn-(1B4M)DTPA-functionalized PAMAM G2 {(G2-Gd-(1B4M)DTPA)}.¹⁰ To a solution of G2-(1B4M)DTPA (0.90 g, ~ 0.074 mmol) in 0.3 M citric buffer (3 mL, pH 4.5) was added $Gd(OAc)_3 \cdot xH_2O$ (0.60 g, 1.8 mmol). The mixture was stirred at room temperature for 12 h and then dialyzed against water using a Centriprep centrifugal filter device with 10 kDa cut-off membrane until low molecular weight contaminants were removed. The solution was lyophilized to afford 1.1 g (100%) off-white solid. Size exclusion HPLC: $t_R = 13.6$ min. MS calculated for $C_{510}H_{720}Gd_{16}N_{122}O_{204}S_{16}$ 14852.7; MALDI-TOF MS found: 9,859 Da. Elemental analysis found: S, 2.85 %; Gd, 12.8 %.

Gd-Bn-(1B4M)DTPA-functionalized PAMAM G4 {(G4-Gd-(1B4M)DTPA)}. The protocol described above for generation G2 dendrimer was used to carry out the complexation reaction with G4 dendrimer. G4-(1B4M)DTPA (0.40 g, ~ 0.0081) and $Gd(OAc)_3 \cdot xH_2O$ (0.17 g, 0.5 mmol) afforded 0.32 g (66%) of product. Elemental analysis found: S, 2.6 %; Gd, 11.7 %. MS calculated for $C_{2094}H_{2976}Gd_{64}N_{506}O_{828}S_{64}$ 60600; MALDI-TOF MS found: 46.5 kDa.

Bn-(1B4M)DTPA-Gd functionalized PAMAM G6 (G6-(1B4M)DTPA-Gd). The protocol described above for the G2 generation was used to carry out the complexation reaction with G6 dendrimer. G6-(1B4M)DTPA (0.30 g, ~ 0.0015 mmol) and Gd(OAc)₃·xH₂O (0.13 g, 0.39 mmol) afforded 0.34 g (93%) of an off-white solid product. Size exclusion HPLC: t_R = 10.9 min. Elemental analysis found: S, 2.46 %; Gd, 12.6 %. MS calculated for C₈₄₃₀H₁₂₀₀₀Gd₂₅₆N₂₀₄₂O₃₃₂₄S₂₅₆ 243590; MALDI-TOF MS found: 135 kDa.

Syntheses of spacer linked Gd-Bn-(1B4M)DTPA functionalized PAMAM dendrimer conjugates.

2-[*p*-(*N*-(5-oxopentanoic acid))aminobenzyl]-6-methyl-diethylenetriamine-*N*, *N*, *N'*, *N''*, *N''*-penta-*tert*-butyl acetate (7).⁷ A solution of compound **5** (11.5 g, 14.5 mmol) and glutaric anhydride (2.0 g, 17 mmol) in ethyl acetate (60 mL) in a 200 mL dry round bottomed flask was stirred at room temperature overnight. The solution was taken to dryness. The residue was purified by flash chromatography on a silica column eluted with hexane/ethanol (v/v) using a gradient from 4:1 to 2:1. The product fraction was collected and concentrated to afford a colorless oily product which solidified upon standing. Yield: 10.8 g (82 %). ¹H NMR (400MHz, DMSO-d₆) δ 10.32 (s, 1H), 7.59 (d, *J* = 8.5 Hz, 2 H), 7.15 (d, *J* = 8.5 Hz, 2 H), 3.40 (m, 10 H), 3.20-2.20 (m, 8 H), 2.35 (t, *J* = 7.1 Hz, 2 H), 2.12 (t, *J* = 6.9 Hz, 2 H), 1.89 (m, 2 H), 1.46 (m, 45 H), 0.98 d, *J* = 6.3 Hz 3 H). MS (ES) (negative): calculated for C₄₇H₇₉N₄O₁₃ 907.1; found 905.3 [M-H⁺].

2-[*p*-(*N*-(succinimidyl 5-oxopentanoate))aminobenzyl]-6-methyl-diethylenetriamine-*N*, *N*, *N'*, *N''*, *N''*-penta-*tert*-butyl acetate (8) (NHS-Bn-(1B4M)DTPA-*t*-Bu ester).⁷ To a solution of compound **7** (8.5 g, 9.37 mmol) in acetonitrile (150 mL) in a 300 mL dry round bottomed flask was added 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (3.6 g, 18.7 mmol) and *N*-hydroxysuccinimide (1.6 g, 14 mmol). The flask was sealed with a rubber septum and the mixture was stirred at room temperature overnight. The reaction mixture was concentrated *in vacuo* to dryness to afford a yellow solid. The crude product was dissolved in dichloromethane (100 mL) and quickly washed with water (50 mL), 5 % sodium bicarbonate (50

mL), brine (30 mL), dried over sodium sulfate and then filtered. The filtrate was concentrated *in vacuo* to afford a yellow solid product which was stored at -20 °C under anhydrous conditions. Yield: 8.5 g (90 %). ¹H NMR (400MHz, DMSO-d₆) δ 9.90 (s, 1H), 7.57 (d, *J* = 8.5 Hz, 2 H), 7.10 (d, *J* = 8.5 Hz, 2 H), 3.40 (m, 10 H), 3.20-2.20 (m, 8 H), 2.90 (s, 4 H), 2.82 (t, *J* = 7.1 Hz, 2 H), 2.50 (t, *J* = 6.9 Hz, 2 H), 2.08 (m, 2 H), 1.45 (m, 45 H), 0.98 (d, *J* = 6.9 Hz, 3 H). MS (ES) (positive): calculated for C₅₁H₈₂N₅O₁₅ 1004.6; found 1006.0 [MH⁺].

Spacer linked Bn-(1B4M)DTPA functionalized PAMAM G4 {sG4-(1B4M)DTPA}.^{7, 10} A solution of fourth generation PAMAM dendrimer (2.07 g, 10 % w/w solution in MeOH, 0.015 mmol) was evaporated to dryness *in vacuo* and washed with hexane (2 x 10 mL). The residue was dissolved in DMSO (15 mL) and compound **8** (1.63 g, 1.63 mmol) was added. The mixture was stirred at room temperature for 24 h and diluted with CH₂Cl₂ (15 mL). N-(2-aminoethyl)-aminomethyl polystyrene (0.65 g, loading: ≥ 2.00 mmol N/g) was added to this mixture and stirred at room temperature for 12 h. The resin was filtered off and the filtrate was concentrated to afford an off-white solid. Trifluoroacetic acid (10 mL) was added to the solid and the resulting solution was stirred at room temperature for 12 h and then taken to dryness. The residue was washed with CH₂Cl₂ (2 x 20 mL) and again taken to dryness. The residue was dissolved in H₂O (10 mL) and the pH of the solution was adjusted to 6-7 with 2N aqueous NaOH. The solution was filtered through a 0.45µm Millipore syringe filter unit and then dialyzed against water using a Centriprep centrifugal filter device (with MW cut-off of 10 kDa membrane) until small molecules were removed as indicated by size exclusion HPLC analysis (conjugate t_R = 11.9 min). The solution was lyophilized to afford off-white powder. Yield: 0.60 g (75 %). MS calculated for C₂₄₁₄H₃₆₈₀N₅₀₆O₈₉₂ 54062, MALDI-TOF MS found: 34 kDa.

Spacer linked Bn-(1B4M)DTPA functionalized PAMAM G2 (sG2-(1B4M)DTPA). The protocol described above was used for the reaction of second generation PAMAM dendrimer (0.086 mmol) and compound **8** (2 × 0.7g, 1.4 mmol, added in two portions, each followed by stirring for 12 h). The reaction gave 0.63 g (yield 56 %) of product. Size exclusion HPLC: t_R = 13.9 min. MS calculated for C₅₉₀H₈₉₆N₁₂₂O₂₂₀ 13218.1, MALDI-TOF MS found: 9.8 kDa.

Spacer linked Bn-(1B4M)DTPA functionalized PAMAM G6 (sG6-(1B4M)DTPA). The protocol described for the G4 generation was used for the reaction of sixth generation PAMAM dendrimer (0.0026 mmol) and compound **8** (2×0.7 g, 1.4 mmol, added in two portions, every 12 h, with stirring). The reaction gave 0.30 g (yield 54 %) of product. Size exclusion HPLC: $t_R = 11.1$ min. MS calculated for $C_{9710}H_{14816}N_{2042}O_{3580}$ 217437, MALDI-TOF MS found: 102 kDa.

Spacer linked Gd-Bn-(1B4M)DTPA-functionalized PAMAM G4 {Gd-sG4-(1B4M)DTPA}.^{7,}

¹⁰ A solution of sG4-(1B4M)DTPA (0.54 g, ~ 0.01 mmol) in 0.3 M citric buffer (5 mL, pH 5) and $Gd(OAc)_3 \cdot xH_2O$ (0.32 g, 0.96 mmol) dissolved in 5 mL of 0.3 M citric buffer were combined and stirred at room temperature for 12 h. The solution was filtered through a 0.45 μm Millipore syringe filter unit and then dialyzed against water using a Centriprep centrifugal filter device (with MW cut-off of 10 kDa membrane) until small molecules were removed as determined by analytical HPLC. Size exclusion HPLC: $t_R = 12.8$ min. The solution was lyophilized to afford 0.60 g (92%) an off-white product. Elemental analysis found: Gd, 13.0 %. MS calculated for $C_{2414}H_{3488}Gd_{64}N_{506}O_{956}$ 64957, MALDI-TOF MS found: 44.4 kDa.

Spacer linked Gd-Bn-(1B4M)DTPA functionalized PAMAM G2 {Gd-sG2-(1B4M)DTPA-Gd}. The protocol described above for the G4 reaction was used in the reaction of sG2-

(1B4M)DTPA (0.40 g, 0.030 mmol) and $Gd(OAc)_3 \cdot xH_2O$ (0.23 g) to afford 0.42 g (88%) of product. Size exclusion HPLC: $t_R = 14.7$ min. Elemental analysis found: Gd, 12.4 %. MS calculated for $C_{590}H_{848}Gd_{16}N_{122}O_{236}$ 15942, MALDI-TOF found: 11 kDa.

Spacer linked Gd-Bn-(1B4M)DTPA-functionalized PAMAM G6 (Gd-sG6-(1B4M)DTPA).

The protocol described above for the G4 reaction was used in the reaction of sG6-(1B4M)DTPA (0.20 g, ~ 0.00092 mmol) and $Gd(OAc)_3 \cdot xH_2O$ (0.12 g) to afford 0.22 g (92%) of product. Size exclusion HPLC: $t_R = 10.9$ min. Elemental analysis found: Gd, 10.9 %. MS calculated for $C_{9710}H_{14048}GdN_{2042}O_{3836}$ 261016, MALDI-TOF MS found: 146 kDa.

References

1. Goh, V.; Padhani, A. R., Imaging tumor angiogenesis: functional assessment using MDCT or MRI? *Abdom Imaging* 2006, 31 (2), 194-9.

2. Bellin, M. F., MR contrast agents, the old and the new. *Eur J Radiol* 2006, 60 (3), 314-23.
3. Lorusso, V.; Pascolo, L.; Ferneti, C.; Anelli, P. L.; Uggeri, F.; Tiribelli, C., Magnetic resonance contrast agents: from the bench to the patient. *Curr Pharm Des* 2005, 11 (31), 4079-98.
4. Tomalia, D. A.; Adel, M. N.; William, A. G., III, Starburst Dendrimers: Molecular-Level Control of Size, Shape, Surface Chemistry, Topology, and Flexibility from Atoms to Macroscopic Matter. *Angewandte Chemie International Edition in English* 1990, 29 (2), 138-175.
5. Kobayashi, H.; Kawamoto, S.; Saga, T.; Sato, N.; Hiraga, A.; Konishi, J.; Togashi, K.; Brechbiel, M. W., Micro-MR angiography of normal and intratumoral vessels in mice using dedicated intravascular MR contrast agents with high generation of polyamidoamine dendrimer core: reference to pharmacokinetic properties of dendrimer-based MR contrast agents. *J Magn Reson Imaging* 2001, 14 (6), 705-13.
6. Brechbiel, M. W.; Gansow, O. A.; Atcher, R. W.; Schlom, J.; Esteban, J.; Simpson, D. E.; Colcher, D., Synthesis of 1-(para-isothiocyanatobenzyl) derivatives of DTPA and ADTA - antibody labeling and tumor imaging studies. *Inorganic Chemistry* 1986, 25 (16), 2772-2781.
7. Xu, H.; Regino, C. A.; Bernardo, M.; Koyama, Y.; Kobayashi, H.; Choyke, P. L.; Brechbiel, M. W., Toward improved syntheses of dendrimer-based magnetic resonance imaging contrast agents: new bifunctional diethylenetriaminepentaacetic acid ligands and nonaqueous conjugation chemistry. *J Med Chem* 2007, 50 (14), 3185-93.
8. Moore, J. L.; Taylor, S. M.; Soloshonok, V. A., An efficient and operationally convenient general synthesis of tertiary amines by direct alkylation of secondary amines with alkyl halides in the presence of Huenig's base. *Arkivoc* 2005, 287-292.
9. Renn, O.; Meares, C. F., Large-scale synthesis of the bifunctional chelating agent 2-(p-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid, and the determination of its enantiomeric purity by chiral chromatography. *Bioconj Chem* 1992, 3 (6), 563-9.
10. Kobayashi, H.; Kawamoto, S.; Choyke, P. L.; Sato, N.; Knopp, M. V.; Star, R. A.; Waldmann, T. A.; Tagaya, Y.; Brechbiel, M. W., Comparison of dendrimer-based

macromolecular contrast agents for dynamic micro-magnetic resonance lymphangiography.
Magn Reson Med 2003, 50 (4), 758-66.