Toward Improved Syntheses of Dendrimer-Based Magnetic Resonance Imaging Contrast Agents: New Bifunctional Diethylenetriaminepentaacetic Acid Ligands and Nonaqueous Conjugation Chemistry

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Two different fourth-generation (G4) polyaminonamido dendrimer-based magnetic resonsance (MR) agents were prepared by a new synthetic approach wherein *tert*-butyl-protected forms of 2-(4-isothiocyanatobenzyl)-6-methyldiethylenetriamine pentaacetic acid (1B4M-DTPA), bearing either an isothiocyanate or a succinimidyl ester moiety, respectively, were conjugated to the primary amines of the dendrimer. Purification was facilitated using a solid phase, *N*-(2-aminoethyl)aminomethyl polystyrene. After Gd(III) incorporation, molar relaxivity measurements of both new dendrimer-based agents as compared to a G4 agent prepared by an aqueous chemistry route indicated no significant changes in relaxivity. Comparative MR imaging revealed equivalent enhancement of the vessels and organs such as the kidney and liver, although slightly different vascular clearance rates were observed. This general synthesis provides a procedure for preparation of dendrimer-based MR agents for clinical applications with higher yields and efficiency while enhancing versatility. The latter aspect is further demonstrated by preparation of a novel maleimide analog of 1B4M-DTPA from a key synthetic intermediate aniline derivative.

Introduction

The development of contrast agents for magnetic resonance imaging in clinical settings continues to receive great attention.[1] Paramagnetic metal chelates, such as Gd(III)-diethylenetriaminepentaacetic acid [Gd(III)-DTPA] (Magnevist) and Gd(III)-*N*,*N*′,*N*″,*N*‴-tetracarboxymethyl-1,4,7,10-tetraazacyclododecane [Gd(III)-DOTA], have proven to increase the relaxation rate of surrounding protons and have been widely used as MR[a] contrast agents.[2,3] However, these low molecular weight agents (LMW) also possess rapid clearance rates from vascular circulation and concomitant rapid renal excretion. These LMW contrast agents also possess relatively low molar relaxivity properties. The combination of these properties may limit time-dependent imaging studies or acquisition of highly resolved images of patients.[4−6]

Macromolecular imaging agents were developed to overcome these limitations and to provide unique properties, e.g., increased molecular size and weight, which would also result in higher molar relaxivities and hence greatly enhanced sensitivity.[7,8] Due to their multivalent surfaces, well-defined architectures, and nanoscale sizes, PAMAM dendrimers are optimal macromolecules as a core platform to carry multiple copies of small molecules such as chelated Gd(III) for MR contrast. In our laboratory, dendrimer-based macromolecular MR agents have been extensively studied in the past decade, an area of interest that continues forward with this report. Concurrently, our research efforts in this area and those in parallel for radioimmunotherapy applications have led to the establishment of a library of bifunctional chelates such as 2-(4-isothiocyanatobenzyl)-6-methyl-diethylenetriamine pentaacetic acid (1B4M-DTPA), *N*-[2-amino-3-(4-isothiocyanatobenzyl)propyl]-*cis*-cyclohexyl-1,2-diamine-*N*,*N*′,*N*′ ,*N*″,*N*″-pentaacetic acid (CHX-A-DTPA), and 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (*p*-SCN-Bz-DOTA), which are all potentially useful for forming Gd(III) complexes and thus MR contrast agents (Figure 1).[9−11] The bifunctional nature of these chelators permits conjugation to antibodies, peptides, dendrimers, and other macromolecular structures.[12] Our research on macromolecular chelate-conjugated dendrimer-based Gd(III) MR contrast agents based on the PAMAM classes of dendrimers has revealed that these agents can be tuned for various applications by virtue of choosing generation size, core elements, and other exterior modification.[13] For example, the use of PAMAM agents permitted imaging tumor vasculature accurately at the 200 μm scale.[14] Recent results applying these agents for imaging the lymph system has revealed that sentinel nodes and the draining connecting lymphatic vessels can in fact be imaged with high resolution and detail superior to current methods.[15]

In previous studies, conjugation of bifunctional chelators to amino-terminated dendrimers was performed in aqueous phase using the isothiocyanate form of 1B4M-DTPA (Figure 1). This conjugation process, dependent on scale and dendrimer generation, can be a lengthy operation. In the course of reaction, a large excess of bifunctional chelator and inorganic base (e.g.,

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[a] Abbreviations: PAMAM, polyaminonamido; G4, generation 4; MR, magnetic resonsance; 1B4M-DTPA, 2-(4-isothiocyanatobenzyl)-6-methyldiethylenetriamine pentaacetic acid; DTPA, diethylenetriaminepentaacetic acid; DOTA, *N*,*N*′,*N*″,*N*‴-tetracarboxymethyl-1,4,7,10-tetraazacyclododecane; LMW, molecular weight agents; CHX-A-DTPA, *N*-[2-amino-3-(4-isothiocyanatobenzyl)propyl]-*cis*-cyclohexyl-1,2-diamine-*N*,*N*′,*N*′,*N*″,*N*″-pentaacetic acid; *p*-SCN-Bz-DOTA, 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; SPECT, single photon emission computed tomography; PET, positron emission computed tomography; NHS, *N*-hydroxysuccinimidyl; EDCI, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; MIP, maximum intensity projection.

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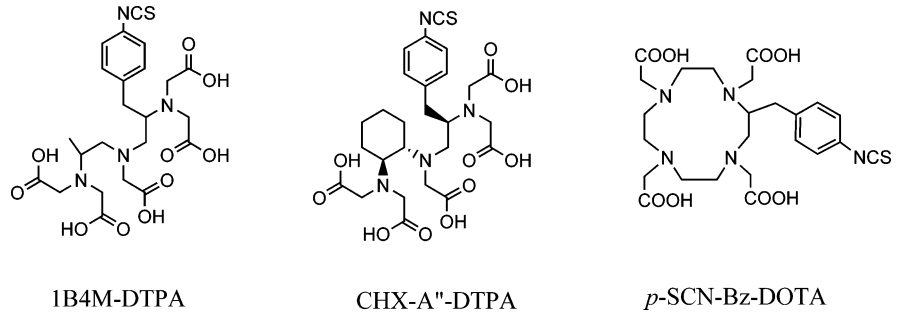

Figure 1. Structures of 1B4M-DTPA, CHX-A″-DTPA, and *p*-SCN-Bz-DOTA.

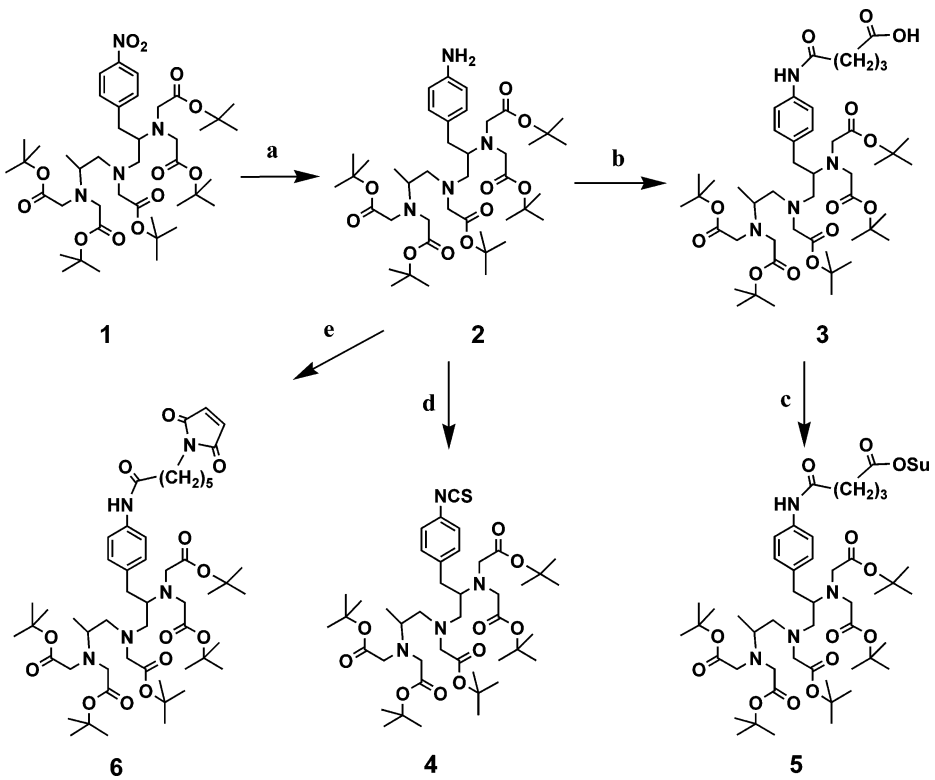

Figure 2. Synthesis of 1B4M-DTPA active ester, maleimide, and *p*-isothiocyanato functionalized *tert*-butyl ester derivative **4**−**6**.

K_2CO_3) were usually added to drive the reaction to maximum saturation of the dendrimer surface.[16] These reaction conditions were derived from the procedures for preparation of monoclonal antibody conjugates with bifunctional chelates.[17] The excessive amount of bifunctional chelator required for preparation of the dendrimer conjugates when using these conditions was necessary to counter the competing decomposition and disproportionation reactions of the reactive isothiocyanate group.[18] Additionally, these organic byproducts and inorganic salts are major components of the crude reaction solution, which complicated monitoring reaction progress. Last, removal of these small molecular weight and size impurities from dendrimer conjugates required lengthy and extensive dialysis or diafiltration.[19]

In this study, we introduce a novel and more efficient synthetic approach to the preparation of dendrimer-based macromolecular imaging agents, specifically for the introduction of bifunctional chelating agents for sequestering paramagnetic ions such as Gd(III), but also radiotracers such as ^{111}In (SPECT) or ^{86}Y (PET). The primary objective was to either minimize or eliminate the decomposition and disproportionation reactions of the reactive isothiocyanate group by moving from a basic aqueous reaction environment to an organic solvent system. Therefore, this modification was hypothesized to facilitate an opportunity to improve the overall efficiency of bifunctional chelator to polyamine surface dendrimers by elimination of loss of the reagent to aqueous basic pH conditions, shorter reaction times, and a potential increase in loading efficiency of chelator onto the dendrimer. Concurrent with that objective, an opportunity to revisit the choice of reactive conjugation functional group was envisioned as a secondary aspect of this study.

Herein, we report on the syntheses and characterization of three novel analogs of the 1B4M-DTPA that are prepared as their respective *tert*-butyl pentaesters (Figure 2). The first 1B4M-DTPA derivative is a direct extension of the well-established parental isothiocyanate reagent, while the other derivative addresses the secondary aspects of improved or alternate linking chemistry, and as such possesses an *N*-hydroxysuccinimidyl (NHS) active ester group tethered at the end of a five-carbon long chain for reaction with terminal primary amines of the dendrimer. Concurrently, we report on the application of a solid-phase reagent, *N*-(2-aminoethyl)aminomethylpolystyrene, which functions as a scavenger at the end of reaction to facilitate removal of the excess reactive chelating agents. Use of this agent significantly reduced the purification efforts in preparing pure functionalized dendrimers. Last, we also report the synthesis of a maleimide derivative of 1B4M-DTPA to demonstrate the possibility of efficiently creating additional analogs of this chelating agent that would have reactive functional groups

potentially suitable for protein or peptide conjugate conjugation to endogenous synthetically introduced thiols.

Results and Discussion

Bifunctional Chelator Syntheses. The synthetic strategies were aimed to provide an entry point to a general synthesis for 1B4M-DTPA derivatives that would possess reactive functional groups suitable for efficient conjugation to amino-terminated PAMAM dendrimers in an organic-phase reaction. The choices of an isothiocyanate and hydroxysuccinimidyl active ester as two initial reactive groups for evaluation were justified by their established general usage, their reaction efficiencies, and the relative stabilities of both thiourea and amide linkages in vivo.[20] A maleimide derivative was also prepared to expand the range of possible conjugation functional group targets. All three agents were also envisioned as being potentially suitable for modification of peptides and other synthetic delivery vectors.

The synthesis of 2-methyl-6-(*p*-nitrobenzyl)diethylene-*N*,*N*,*N*′,*N*″,*N*″-penta-*tert*-butylacetate (**1**) was performed by modification of the previously described procedure (Figure 2).[21] Modifications at this step were to replace the reaction solvent, DMF, with CH_3CN and to also replace the base, Na_2CO_3, with K_2CO_3. A mixture of diastereomers as previously reported[9] was obtained after purification. Although diastereomers could be differentiated by thin layer chromatography (TLC), their separation by either flash chromatography or HPLC remained impractical, even when at the scale of preparation required for dendrimer-based MR contrast agents. Catalytic hydrogenation of the aryl nitro group in **1** cleanly produced aniline **2**, which was the key intermediate for the synthesis of both target products. Chromatographic isolation of this compound was actually found to be more convenient than isolation of the aryl nitro precursor. The aniline was readily converted to isothiocyanate **4** in high yield (thiophosgene, 88%).

To synthesize a 1B4M-DPTA derivative with an NHS ester (Figure 2), aniline **2** was first acylated with glutaric anhydride to produce carboxylic acid derivative **3**, which was in turn transformed to the NHS ester by standard coupling chemistry (EDCI, NHS, 84%). The choice of glutaric anhydride was based on two factors. The use of succinic anhydride to introduce a shorter chain had been noted to result in instability from intramolecular cyclization reaction to form an imide that may pose an unnecessary complication.[22] Second, there were some concerns as to the impact of adding too lengthy a hydrophobic chain. Despite being a relatively rather short chain, very high numbers of this moiety would be introduced into the dendrimer structure, which would be an unknown variable. We had previously noted that the change of the amide carbonyl components to methylene groups within the dendrimer interior significantly altered normal liver uptake of a dendrimer-based MR agent.[23] Therefore, a minimal chain length was chosen for establishing the synthesis of a 1B4M-DPTA derivative with a succinimidyl ester. Last, the choice of the succinimidyl ester was arbitrary from the lengthy list of available active ester chemistry and other options; e.g., tetra- or pentafluorophenyl esters could also be envisioned as possible.

Aniline **2** was recognized as a versatile intermediate that could serve as a nexus for the introduction of numerous reactive functional groups suitable for conjugation chemistry. To explore this option in brief, we chose to convert **2** into maleimide **6**, which can readily and specifically react with sulfhydryl groups of proteins and peptides. Thus, bifunctional agent **6** was formed from aniline **2** by reaction with *N*-ϵ-maleimidocaproic acid in presence of EDCI and then isolated in 84% yield after flash column chromatography.

Purification of **1**, **3**, and **6** was found to be conveniently performed on silica gel by gradient elution. Isothiocyanate **4** and NHS ester **5** were isolated and used directly for conjugation to dendrimers without further purification. The structures of **1**−**6** were confirmed by ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectra.

Conjugation of the Chelating Agents 4 and 5 to Dendrimers. Dendrimers are a well-defined class of highly branched, synthetic polymers with a wide array of possible chemical structures and functional groups.[24−27] Their controlled structure and generational size attributes provide an attractive platform for the development of reproducible chemistry for a host of biomedical applications,[28] in addition to imaging and MR contrast agent development.[29]

Dendrimer-based Gd(III) macromolecular contrast agents have proven to have high relaxivities and to provide MR images with high resolution and details superior to the small molecule DTPA and DOTA Gd(III) complexes.[8,29] In our current studies, PAMAM G4 dendrimers were chosen as the scaffolding to carry multiple copies of the novel chelators primarily due to their appropriate size (e.g., ∼4 nm),[30] established biological behaviors,[31] and their commercial availability to permit comparative studies. Our previous studies demonstrated that PAMAM G4 dendrimers conjugated with chelated Gd(III) were subject to rapid renal excretion and that they were almost exclusively retained in the blood vessels or urinary tracts with minimal extravasation.

For purposes of comparison and validation, conjugations of isothiocyanate **4** and of NHS ester **5** to G4 PAMAM dendrimer were performed in either DMSO or MeOH. A variety of organic solvents, including DMSO, MeOH, $CHCl_3$, CH_2Cl_2, MeCN, DMF, *n*-propanol, *tert*-butyl alcohol, EtOAc, were tested for this conjugation step. Only DMSO and MeOH were found to completely dissolve both compounds **4** and **5** and also the G4 dendrimer. For the synthesis of dendrimer conjugate **9a**, the PAMAM G4 dendrimer was reacted with 2 equiv of ester **5** per each primary amine available on the surface of the dendrimer at pH 7 in DMSO solvent for 1 day while the reaction progress was monitored by TLC. The product, **9a**, had a strong UV absorption due to the large number of aromatic groups originating from the chelator and exhibited a long smeared band by TLC, while the native G4 dendrimer was UV inactive. After the reaction was complete, *N*-(2-aminoethyl)aminomethyl polystyrene resin was added as a reactive scavenger to remove excess **5**. The use of this solid-phase agent with reactive amine groups was found to greatly facilitate this purification step and as such has been adopted as a standard procedure now in our laboratories for this and related conjugation procedures. TLC analysis of this step indicated that excess **5** was completely removed from the reaction mixture. Functionalized dendrimer **9a** with the chelate protected as the per-*tert*-butyl ester was very soluble in common organic solvents, such as diethyl ether, $CHCl_3$, CH_2Cl_2, MeCN, and EtOAc. To minimize any possible complications associated with removal of DMSO, reaction mixtures were first diluted with CH_2Cl_2 and then exhaustively washed with H_2O. No further purification was applied to the resulting dendrimer conjugates at this stage.

Conjugating **5** to G4 dendrimer could also be carried out in MeOH. However, in our experiments, solvent MeOH competed with the amines of the G4 dendrimer and reacted with the NHS ester to form corresponding methyl ester **7**. However, this side reaction did not affect the percent incorporation of 1B4M-DTPA when 2 equiv of **5** was used in the reaction.

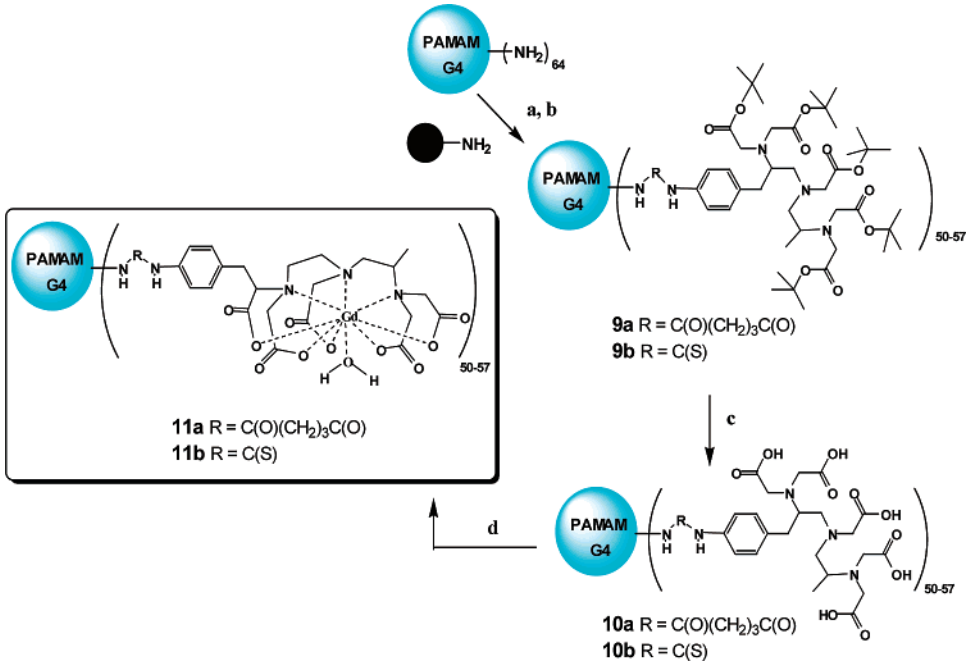

Figure 3. Synthesis of Gd-1B4M-DTPA-functionalized PAMAM G4 dendrimers.

Removal of the *tert*-butyl ester protection from **9a** was quantitatively achieved by treatment with trifluoroacetic acid (TFA). Size-exclusion HPLC was used to assess the purity of the water-soluble dendrimer **10a** following elimination of the TFA. The corresponding chromatogram indicated excellent purity of the crude dendrimer conjugate **10a**, particularly so when considering that **5** was used directly for this conjugation without the benefit of, or need for, any chromatographic purification.

Synthesis of **9b** was accomplished by the reaction of isothiocyanate **4** and the G4 dendrimer again using MeOH as a solvent. The reaction conditions were quite similar with that used with NHS ester **5**. In this case, methylisothiourethane **8**, the MeOH adduct to **4**, was found to be a byproduct in this reaction as identified by high-resolution mass spectrum. And, while addition of the *N*-(2-aminoethyl)aminomethylpolystyrene resin could completely remove the excess isothiocyanate, methylisothiourethane **8** remained with the functionalized dendrimer **9b**. This was not considered to be a complication at this point, since during the subsequent deprotection and purification steps, small molecular weight compounds were eliminated. In purification of dendrimers **10a** and **10b**, small molecules (e.g., unreacted chelating agent) were conveniently removed by diafiltration using a Centriprep Ultracel YM 10. Use of the Centriprep YM10 allowed handling of the G4 dendrimer conjugates on the gram scale.

To prepare macromolecular MR contrast agents **11a** and **11b**, dendrimers **10a** and **10b** were treated with 1.5 equiv of gadolinium acetate $Gd(OAc)_3$ in a citrate buffer solution. The excess Gd(III) was then conveniently removed by diafiltration again using a Centriprep YM 10 (Figure 3).

Previous preparations of PAMAM dendrimer 1B4M-DTPA conjugates for complexing with Gd(III) to be used for MR contrast agents have been made using aqueous media. The conditions employed have usually required a pH range of 9.0−10.0 and have also routinely used 2−3 mol equiv of the 1B4M-DTPA reacted over several days at slightly elevated temperatures. These conditions were derived from those conditions used for conjugation of the 1B4M-DTPA to antibodies, albeit with modifications to time and temperature in an attempt to counter the disproportionation reaction of the isothiocyanate. Considerable quantities of reagent and inconvenience were encountered during previous preparations of PAMAM dendrimer 1B4M-DTPA conjugates, particularly so when moving to larger production scales. Another apparent limitation of the prior preparations is a lack of actual yields and product and at times minimal characterization. While the derivatization and adaptation of reaction conditions from antibody conjugation was simple and direct, we felt that the retention of this aqueous chemistry may have occurred prematurely and without good rationale. Thus, we returned to the penta-*tert*-butyl ester derivative of the 1B4M-DTPA and found that use of a simple 2-fold molar reaction with the reported solvent conditions provided the desired product(s) cleanly and efficiently. Moreover, the products could be purified more conveniently and better characterized than previously possible. A far better grasp of actual yields and thus scalability became possible with this improvement in characterization.

Characterization of macromolecular compounds (e.g., high-generation dendrimers) however still remains a great challenge. ^{1}H NMR, MALDI-TOF/MS, and C, H, N elemental analyses were employed to determine the degree of dendrimer surface functionalization. ^{1}H NMR spectra of dendrimer **10a** produced rather sharp peaks for all protons, while spectra of dendrimer **10b** generated very broad signals. The signals for the aromatic protons from the 1B4M-DTPA residues have relatively sharp and expected signal patterns and are also well-separated from the remaining peaks. However, accurate integration to obtain chelate to dendrimer data remains inconsistent, even when employing lengthy delay times between pulses (see Supporting Information). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry instead proved amenable, gave reliable information on the molecular weights of dendrimers, and was used to determine dendrimer structures and structure defects. Typical MALDI-TOF mass spectra are presented in Figure 4and the mass signal patterns are consistent with the previous MALDI-TOF studies on dendrimers reported by Tomalia and Cloninger.[32,33] Elemental analysis was also used for the determination of the number of chelates on the dendrimer scaffold. Our calculations showed that 50−57 chelates were

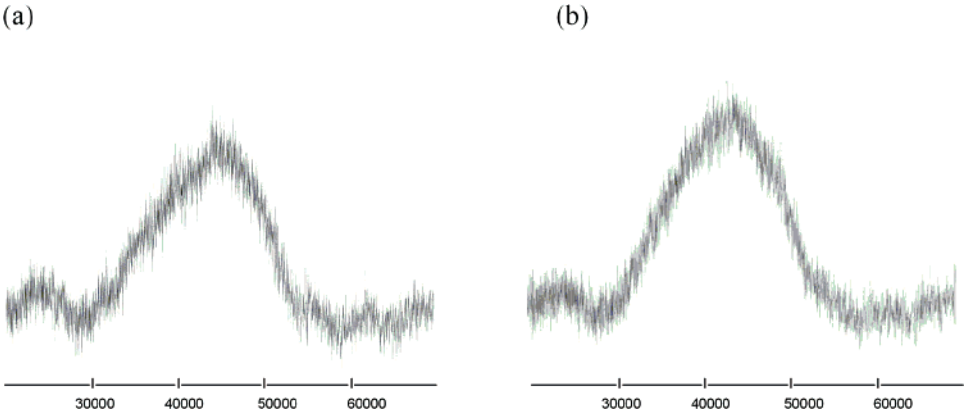

Figure 4. MALDI-TOF/MS of (a) **11a** and (b) **11b**.

Table 1. Comparison of the Number of Chelates on Dendrimers **9**−**11** and the Molar Relaxivities of **11a** and **11b**

compound	no. of chelates[a]	% sat.	$t_{1/2}$, min[b]	relaxivity,[c] mM^{-1} s^{-1} r_1	r_2
9a				ND	ND
9b				ND	ND
10a	53	83		ND	ND
10b	50	78		ND	ND
11a	50	78	87.08 ± 8.15	12.2 ± 0.6	24.9 ± 0.5
11b	57	89	60.28 ± 11.46	14.2 ± 1.4	34.5 ± 2.1
G4-1B4M$_{60}$-Gd$_{42}$	60	94	48.63 ± 14.49	13.9 ± 1.6	33.6 ± 3.1

[a] Reported values are the average values as calculated from the within 0.6% of the elemental analyses (C, H, N, S, and Gd) results, which can be ±10 of the X of the reported number of chelate, and ±5 of the number of Gd. [b] Blood clearance half-life as measured from the R_1 map of the jugular vein in dynamic MR angiographic images. Errors are reported as standard deviations. [c] Molar relaxivity values obtained from phantom measurements. Errors are reported as standard deviations.

covalently attached to the surface of dendrimer, corresponding to 78−89% saturation of a total of 64 amino groups. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) analysis was then employed to determine the Gd(III) content of the final products and SE-HPLC correlated well with the MS results for both **11a** and **11b**.

In order to validate **11a** and **11b** and the use of the two different nonaqueous conjugation routes with these agents, their respective molar relaxivity was determined versus the analogous G4-1B4M$_{60}$-Gd$_{42}$ previously prepared by use of the aqueous conjugation chemistry route.[32] For each concentration, the relaxation rates were determined by linear regression. The relaxivity, r_1, for each agent was then calculated according to the actual molar Gd(III) concentrations as determined analytically and is reported in Table 1. As can be seen, there were no significant differences found among the three agents evaluated, indicating that while no advantages in the preparation methods here were achieved in this property, no deleterious impact was incorporated as well.

Use of the active ester agent, **11a**, was pursued with some anticipation that a more efficient and more rapid conjugation reaction would occur, leading to greater numbers of Gd(III) being complexed. Concerns also existed that the extended linking chemistry associated with the active ester chain might actually result in decreased relaxivity due to an enhancement in free rotation of the Gd(III) complex. A recent study on macromolecular MR agents revealed that the internal flexibility of the dendrimer platform might limit the proton relaxivity of these MR agents.[34] Comparison of the molar relaxivities of these two agents (Table 1), **11a** (12.2 mM^{-1} s^{-1}) and **11b** (14.2 mM^{-1} s^{-1}), showed no significant difference as compared to the molar relaxivity of the G4-1B4M$_{60}$-Gd$_{42}$ (13.9 mM^{-1} s^{-1}) (**11a** vs G4-1B4M$_{60}$-Gd$_{42}$ ($p = 0.312$) and **11b** vs G4-1B4M$_{60}$-Gd$_{42}$ ($p = 0.675$). This result tends to indicate that the five-carbon linker on the agent **11a** most likely does not impact the overall rigidity of the G4 dendrimer platform or the linkage of the chelated Gd(III) as related to the rotational tumbling time, τ_r. This result may be due to the chain length not being long enough to distance the chelated Gd(III) from the dendrimer to permit enhanced independent rotation of the complex. Future studies may be able to address this question by investigating longer conjugation chain lengths in the linking chemistry.

MR imaging studies were also performed to qualitatively assess the image quality obtained in normal mice with the three agents, **11a** and **11b** and G4-1B4M$_{60}$-Gd$_{42}$ previously prepared by aqueous means. Image quality was judged to be equivalent among the three agents (Figure 5).

Meanwhile, MR agent **11a**, with a five-carbon-long hydrophobic spacer chain introduced between the surface of PAMAM G4 dendrimer and 1B4M-DTPA, and **11b** both have a longer clearance rate in mice (**11a** vs G4-1B4M$_{60}$-Gd$_{42}$ $p = 0.0001$) (**11b** vs G4-1B4M$_{60}$-Gd$_{42}$ $p = 0.017$) (Figures 6 and 7). This is unlikely to have clinical significance; persistence of the contrast agent could provide a longer imaging window.

In conclusion, the new synthetic methods for G4 dendrimers (**11a** and **11b**) produce MR contrast agents with very similar relaxivities and clearance properties to the original aqueous-phase synthesis. The current synthesis is more reliable and faster and thus more amenable to translation to clinical production and evaluation. The agent is useful not only by itself as a vascular contrast agent but could also potentially be targeted to image intravascular targets associated with angiogenesis. Dendrimers represent a versatile and reliable carrier molecule for molecular imaging applications with MR, optical, and radionuclides.

Materials and Methods

{[2-(Bis-*tert*-butoxycarbonylmethylamino)-3-(4-nitrophenyl)-propyl]-[2-(bis-*tert*-butoxycarbonylmethylamino)propyl]amino}-acetic acid *tert*-butyl ester (1) was prepared by modification of the previously described procedure.[21] *N*-Hydroxysuccinimide (NHS), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI), thiophosgene, glutaric anhydride, and peptide sequence grade trifluoroacetic acid were purchased from Aldrich/Sigma Chemical Co. and were used as received. Generation 4 (G4) ethylenediamine core PAMAM dendrimer was obtained from Dendritech, Inc. (Midland, MI) as a 15.35% w/w solution in MeOH. The aqueous conjugation chemistry version of the generation 4 1B4M-DTPA Gd(III) (G4-1B4M$_{60}$-Gd$_{42}$) was prepared and characterized as previously reported.[14−16] All experiments with moisture-

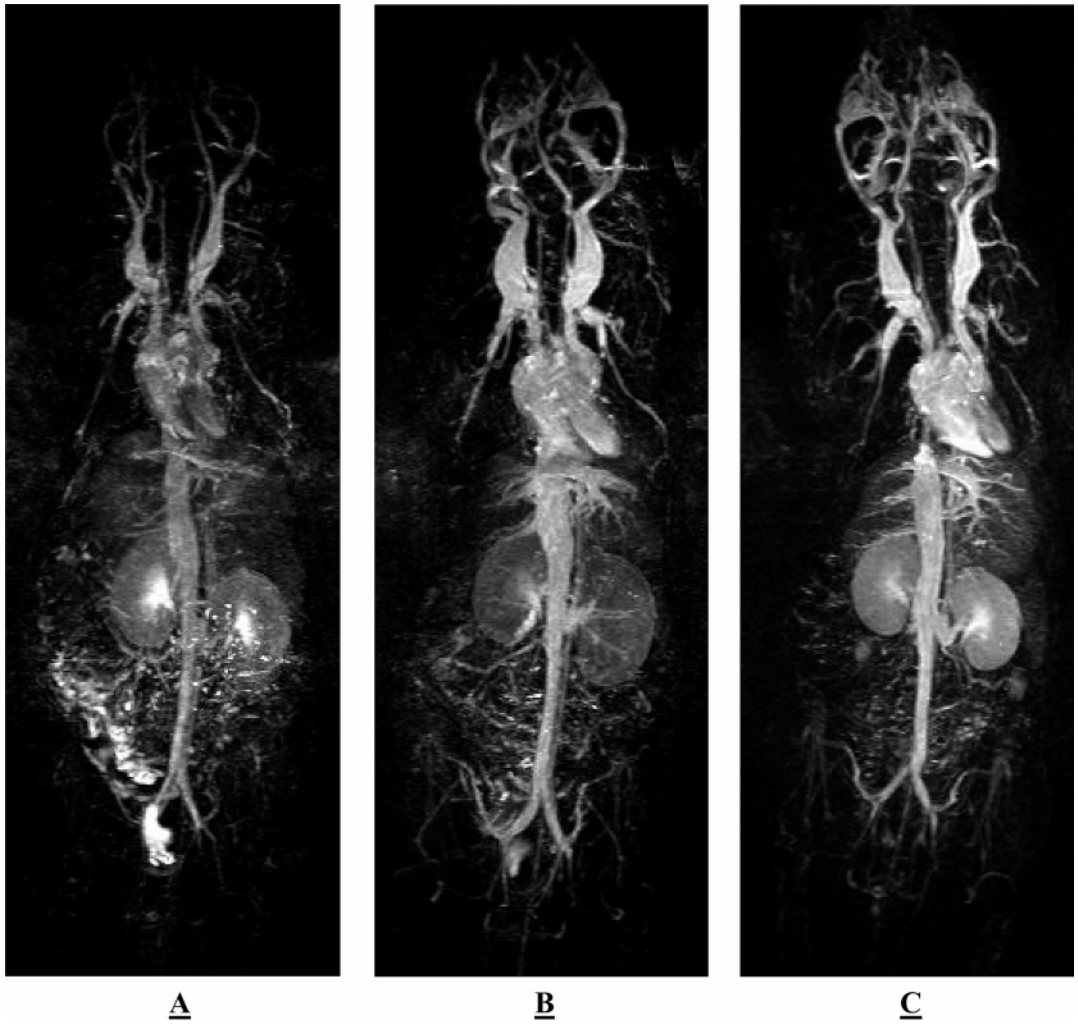

Figure 5. Subtraction maximum intensity projection (MIP) T_1-weighted 3D images of three mice 5−10 min postinjection of G4-1B4M_{60}-Gd_{42} (A), **11b** (B), or **11a** (C) show comparable image quality for each agent. The vascular system is opacified in all cases and there is substantial renal excretion based on the enhancement of the kidneys, ureter, and bladder.

and/or air-sensitive compounds were carried out under a dried N_2 or Ar atmosphere. For column chromatography, Merck 60 silica gel was used (70−230 mesh). Thin-layer chromatography (TLC) was performed on silica gel 60 F-254 plates from EM Reagents. All water used was purified using a Hydro Ultrapure water purification system (Rockville, MD).

Proton and ^{13}C NMR data were obtained using a Varian Gemini 300 MHz instrument, and chemical shifts are reported in ppm on the δ scale relative to TMS, TSP, or residual solvent. Proton chemical shifts are annotated as follows: ppm (multiplicity, coupling constant (Hz), integration). Low- and high-resolution mass spectra (HRMS) were obtained on a Waters' LCT Premier time-of-flight mass spectrometer using electrospray ionization (ESI/TOF/MS) in positive ion mode operated at a resolution of 10 000. The electrospray capillary voltage was 3 kV and the sample cone voltage was 60 V. Desolvation temperature was 225 °C and the desolvation gas was nitrogen at 300 L/h. Accurate masses were obtained using the lock spray mode with Leu-enkephalin as the external reference compound. Elemental analyses were performed by Desert Analytics (Tucson, AZ) using a combustion analysis method for C, H, N, and S and an inductively coupled plasma-atomic emission spectroscopy (ICP-AES) method for determining the percentage of Gd. MALDI-TOF mass spectral data were obtained by the Scripps Center for Mass Spectrometry (La Jolla, CA).

Dendrimer conjugation and purity was assessed by size exclusion HPLC (SE-HPLC) using a Beckman System Gold (Fullerton, CA) equipped with Model 126 solvent delivery module and a Model 168 UV detector (λ 254 and 280 nm) controlled by 32 Karat software. Size exclusion chromatography was performed on a Tosohaas G2000SWxl 10 μm, 7.8 mm × 30 cm column (Montgomeryville, PA) using phosphate-buffered saline (1× PBS) solution as the eluent (0.5 mL/min).

{[3-(4-Aminophenyl)-2-(bis-*tert*-butoxycarbonylmethylamino)-propyl]-[2-(bis-*tert*-butoxycarbonylmethylamino)propyl]amino}acetic Acid *tert*-Butyl Ester (2). A solution of aryl nitro compound **1** (1.98 g, 2.41 mmol) in EtOH (30 mL) was treated with 10% Pd/C (0.2 g) and stirred under an H_2 atmosphere overnight. The mixture was filtered on a glass frit through a pad of Celite 535 (Fluka) washed with EtOH (2 × 20 mL). The filtrate was evaporated at reduced pressure to give a pale, yellow oil. The residue was purified by flash chromatography on silica gel eluted with THF−hexanes 1:2 to 1:1 to afford amine **2** (1.81 g, 95%) as a colorless oil. ^1H NMR (DMSO-d_6): δ 6.91 (d, J = 8.2 Hz, 2 H), 6.53 (d, J = 8.5 Hz, 2 H), 3.40 (m, 10 H), 3.10−2.30 (complicated m, 8 H), 1.46 (m, 45 H), 0.98 (d, J = 6.3 Hz 3 H). HRMS: calcd for $C_{42}H_{73}N_4O_{10}$ [M + H^+] 793.5327, found 793.5349. Anal. Calcd for $C_{42}H_{72}N_4O_{10}$: C, 63.61; H, 9.15; N, 7.06. Found: C, 63.57; H, 9.16; N, 6.94.

4-[4-(2-(Bis-*tert*-butoxycarbonylmethyl-amino)-3-{[2-(bis-*tert*-butoxycarbonylmethylamino)propyl]-*tert*-butoxycarbonylamino}-propyl)phenylcarbamoyl]butyric Acid (3). A solution of **2** (2.10 g, 2.65 mmol) in EtOAc (30 mL) was treated with glutaric anhydride (0.36 g, 3.18 mmol) and stirred at room temperature for 18 h. The solution was evaporated at reduced pressure and the residue was chromatographed on silica gel eluting with EtOH−hexanes 1:4 to 1:1 to yield acid **3** as a colorless solid (1.98 g, 82%).

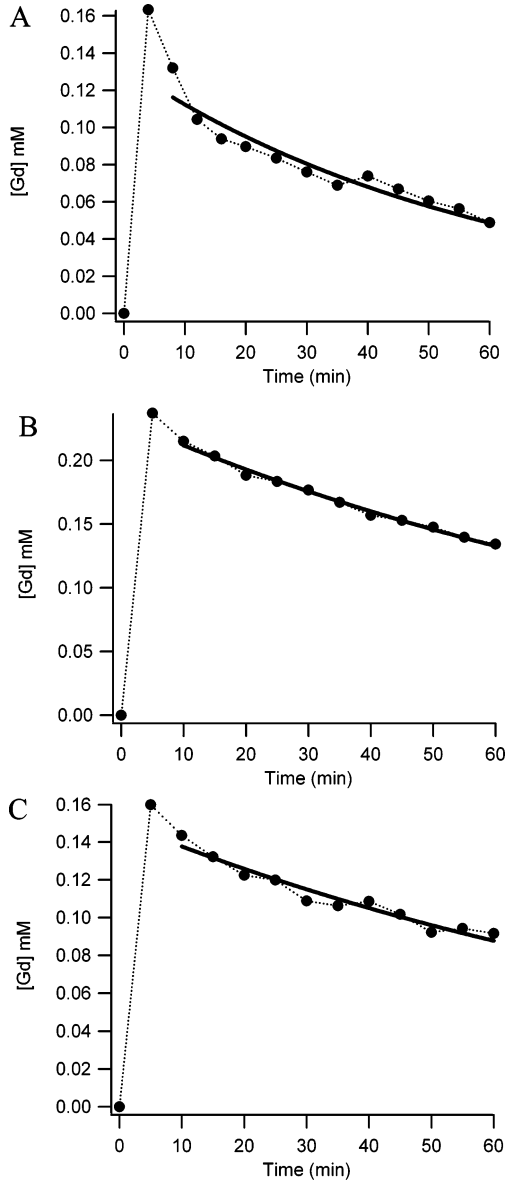

Figure 6. Representative clearance curves derived from the jugular vein demonstrate faster clearance for the aqueous-phase dendrimer G4-1B4M$_{60}$-Gd$_{42}$ (A) as compared with either of the organic-phase dendrimers **11a** (B), and **11b** (C).

^{1}H NMR (DMSO-d_6): 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 (complicated 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). HRMS: calcd for $C_{47}H_{79}N_4O_{13}$ [M + H$^+$] 907.5644, found 907.5645.

{[2-(Bis-*tert*-butoxycarbonylmethylamino)-3-(4-isothiocyanatophenyl)propyl]-[2-(bis-*tert*-butoxycarbonylmethylamino)-propyl]amino}acetic Acid *tert*-Butyl Ester (4). A solution of aniline **2** (8.10 g, 10.20 mmol) in EtOAc (30 mL) was treated with thiophosgene (1.52 g, 13.30 mmol) and stirred at room temperature for 4 h. The solution was evaporated at reduced pressure and dried under vacuum to afford the HCl salt of **4** as a yellow solid (8.20 g, 88%). ^{1}H NMR (DMSO-d_6): δ 7.45 (m, 4 H), 3.80−2.60 (complicated m, 18 H), 1.50 (m, 45 H), 1.01(m, 3 H). HRMS: calcd for $C_{43}H_{71}N_4O_{15}S$ [M + H$^+$] 835.4891, found 835.4880. Anal. Calcd for $C_{43}H_{70}N_4O_{10}S\cdot 2HCl$: C, 56.88; H, 7.99; N, 6.17; S, 3.53. Found: C, 56.84; H, 7.73; N, 6.48; S, 3.63.

4-[4-(2-(Bis-*tert*-butoxycarbonylmethylamino)-3-{[2-bis-*tert*-butoxycarbonylmethylamino)propyl]-*tert*-butoxycarbonylmethylamino}propyl)phenylcarbamoyl]butyric Acid 2,5-Dioxopyrrolidin-1-yl Ester (5). To a solution of acid **3** (2.20 g, 2.40 mmol) in MeCN (50 mL) were added EDCI (0.92 g, 4.80 mmol) and *N*-hydroxysuccinimide (0.41 g, 3.60 mmol). The mixture was stirred at room temperature for 18 h. Afterward, the reaction solution was concentrated at reduced pressure, diluted with CH_2Cl_2 (100 mL), and then washed successively with H_2O (2 × 100 mL), 5% w/v $NaHCO_3$ (2 × 100 mL), and H_2O (2 × 100 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to afford active ester **5** as a yellow solid (2.00 g, 83%). ^{1}H NMR (DMSO-d_6): 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 (complicated 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). HRMS: calcd for $C_{51}H_{82}N_5O_{15}$ [M + H$^+$] 1004.5807, found 1004.5844. Anal. Calcd For $C_{51}H_{82}N_5O_{15}\cdot H_2O$: C, 59.92; H, 8.18; N, 6.85. Found: 59.54; H, 7.95; N, 6.90.

{(2-(Bis-*tert*-butoxycarbonylmethylamino)-3-{4-[7-(2,5-dioxopyrrolidin-1-yl)hexanoylamino]phenyl}propyl)-[2-(bis-*tert*-butoxycarbonylmethylamino)propyl]amino}acetic Acid *tert*-Butyl Ester (6). To solution of aniline **2** (0.22 g, 0.28 mmol) in CH_2Cl_2 (10 mL) were added EDCI (0.12 g, 0.63 mmol) and *N*-ϵ-maleimidocaproic acid (0.06 g, 0.30 mmol). The mixture was stirred at room temperature for 18 h. Afterward, the reaction solution was diluted with CH_2Cl_2 (50 mL) and then washed successively with H_2O (2 × 50 mL), 5% w/v $NaHCO_3$ (2 × 50 mL), and H_2O (2 × 50 mL). The organic layer was dried over anhydrous Na_2SO_4, evaporated, and chromatographed on silica gel eluting with EtOH−hexanes 1:4 to 1:1 to yield **6** as a colorless oil (0.23 g, 84%). ^{1}H NMR (DMSO-d_6): δ 9.82 (s, 1H), 7.55 (d, J = 8.1 Hz, 2H), 7.18 (d, J = 8.4 Hz, 2H), 7.09 (s, 2H), 3.45 (m, 12H), 3.20−2.40 (complicated m, 8 H), 2.32 (t, J = 6.9 Hz, 2H), 1.80−1.20 (m, 51H), 1.00 (d, J = 6.3 Hz 3H). HRMS ES-MS: calcd for $C_{52}H_{84}N_5O_{13}$ [M + H$^+$] 986.6, found 986.6. Anal. Calcd for $C_{52}H_{83}N_5O_{13}\cdot 0.6CH_2Cl_2$: C, 60.91; H, 8.18; N, 6.75. Found: C, 60.75; H, 8.14; N, 6.69.

1B4M-DTPA Functionalized Dendrimer by Active Ester Conjugation (10a). A solution of amine-terminated G4-PAMAM dendrimer (1.34 g of a 15.35% w/w solution in MeOH, 0.014 47 mmol) was evaporated in vacuo and washed with hexane (2 × 10 mL). The residue was dissolved in DMSO (15 mL), and 1B4M-DTPA derivative **5** (1.86 g, 1.85 mmol) was added. The mixture was stirred at room temperature for 24 h, diluted with CH_2Cl_2 (15 mL), and then treated with *N*-(2-aminoethyl)aminomethylpolystyrene (1.0 g, loading ≥ 2.00 mmol/g) (NOVA BioChem). The resulting mixture was again stirred for 24 h and filtered, and the filtrate was concentrated under reduced pressure to provide crude **9a**. TLC: UV-active long band smearing from the origin on a silica gel plate using $CHCl_3$−MeOH (9:1) as the developing solvent. MALDI-TOF/MS: *m*/*z* 49801, calcd for [G4(1B4M-*tert*-butyl pentaester)$_{53}$(H$_2$O)$_{2136}$]$^{2+}$ 49802.

Crude **9a** was then treated with trifluoroacetic acid (20 mL) and stirred for 18 h. The solution was then evaporated, and the resulting residue was washed with CH_2Cl_2 (2 × 30 mL) and then dissolved in H_2O (30 mL). The dendrimer solution was adjusted to pH = 5 with 1 N aqueous NaOH, dialyzed exhaustively with water using a Centriprep Ultracel YM-10 (MWCO 10 000 Da) (Millipore), and lyophilized to give **10a** as a white solid (0.51 g, 89.6% based on dendrimer used). MALDI-TOF/MS: *m*/*z* 39329, calcd for [G4-(1B4M)$_{53}$(Na)$_{25}$(H$_2$O)$_{1751}$]$^{2+}$ 39328. Anal. Calcd for [G4(1B4M)$_{53}$-(TFA)$_6$Na$_{25}$(H$_2$O)$_{215}$]: C, 47.97; H, 6.37; N, 12.53. Found: C, 47.55, 47.75; H, 6.50, 6.56; N, 12.77, 12.73. SE-HPLC: t_R = 12.4 min.

1B4M-DTPA Functionalized Dendrimer by Isothiocyanate Conjugation (10b). MeOH (15 mL) and 1B4M-DTPA derivative **4** (1.00 g, 1.20 mmol) were added to a solution of amine-terminated G4-PAMAM dendrimer (0.87 g of a 15.35% w/w solution in MeOH, 0.00935 mmol). The mixture was stirred at room temperature for 24 h, evaporated, diluted with CH_2Cl_2 (15 mL), and then treated with *N*-(2-aminoethyl)aminomethylpolystyrene (0.6 g, loading ≥ 2.00 mmol/g). The resulting mixture was again stirred for 24 h and filtered, and the filtrate was concentrated under the reduced pressure to afford crude **9b**. TLC: UV-active long band smearing from the origin on a silica gel plate using $CHCl_3$−MeOH (9:1) as

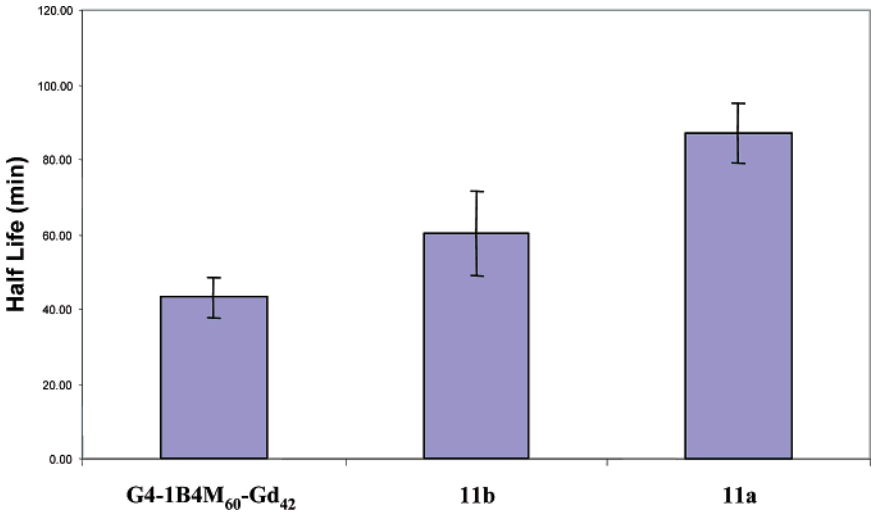

Figure 7. Average blood clearance rate constants measured at the jugular vein demonstrate faster clearance rate (0.016 ± 0.002 min^{-1}) for the aqueous phase dendrimer, G4-1B4M$_{60}$-Gd$_{42}$ vs **11b** (0.012 ± 0.003 min^{-1}) and **11a** (0.0080 ± 0.0008 min^{-1}). This is also reflected in the blood half-life times (graph).

developing solvent. MALDI-TOF/MS: *m*/*z* 42014, calcd for [G4-(1B4M-*tert*-butyl pentaester)$_{50}$(H$_2$O)$_{1548}$]$^{2+}$ 42018.

The residue **9b** was then treated with trifluoroacetic acid (10 mL) and stirred for 18 h. The solution was then evaporated and the resulting residue was washed by CH_2Cl_2 (2×30 mL) and dissolved in H_2O (30 mL). The dendrimer solution was adjusted to pH = 5 with 1 N aqueous NaOH, dialyzed exhaustively with water using Centriprep Ultracel YM-10 (MWCO 10 000Da), and lyophilized to give **10b** as a yellow solid (0.31 g, 66.4% based on dendrimer used). MALDI-TOF/MS: *m*/*z* 28403, calcd for [G4-(1B4M)$_{50}$Na$_{25}$(H$_2$O)$_{796}$]$^{2+}$ 28 408. Anal. Calcd for [G4(1B4M)$_{50}$-(TFA)$_5$Na$_{25}$(H$_2$O)$_{80}$]: C, 48.07; H, 6.60; N, 14.17; S, 3.60. Found: C, 48.93, 48.48; H, 6.65, 6.50; N, 14.51, 14.47; S, 1.06, 1.36. SE-HPLC: t_R = 12.7 min.

Gd Complexation to the Dendrimer−1B4M Conjugates (General Procedure). Gadolinium acetate (Gd(OAc)$_3$•xH$_2$O) was added to a solution of dendrimer−1B4M-DTPA (150 mg) in 0.3 M citrate buffer (10 mL, pH = 4.5). The amount of gadolinium acetate used was calculated to be a 1.5 times molar excess based on the number of 1B4M units conjugated to the dendrimer. The solution was stirred at room temperature for 12 h and then dialyzed exhaustively with water using a Centriprep Ultracel YM-10 (MWCO 10 000 Da) and monitored by SE-HPLC. The retentate was lyophilized and the product was obtained as a yellow solid.

Gd-1B4M-DTPA Functionalized Dendrimer by Active Ester Conjugation (11a). MALDI-TOF/MS: *m*/*z* 44326, calcd for [G4-(1B4M)$_{53}$Gd$_{36}$Na$_{45}$(H$_2$O)$_{1971}$]$^{2+}$ 44326. Anal. Calcd for [G4-(1B4M)$_{53}$Gd$_{36}$(C$_6$H$_7$O$_3$)$_{15}$Na$_{45}$(H$_2$O)$_{110}$]: C, 44.35; H, 5.66; N, 11.16; Gd, 9.76. Found: C, 44.53, 44.18; H, 5.79, 5.76; N, 11.34, 11.29; Gd, 9.57, 10.00. SE-HPLC: t_R = 12.8 min.

Gd-1B4M-DTPA Functionalized Dendrimer by Isothiocyanate Conjugation (11b). MALDI-TOF/MS: *m*/*z* 43312, calcd for [G4(1B4M)$_{57}$Gd$_{41}$Na$_{50}$H$_2$O)$_{1851}$]$^{2+}$ 43311.5. Anal. Calcd for [G4-(1B4M)$_{57}$Gd$_{41}$(C$_6$H$_7$O$_3$)$_8$Na$_{50}$(H$_2$O)$_{170}$]: C, 41.09; H, 5.63; N, 11.57; S, 3.16; Gd, 11.14. Found: C, 41.23, 41.11; H, 5.48, 5.48; N, 11.18, 11.18; S, 2.19, 1.96; Gd, 12.13, 11.28. SE-HPLC: t_R = 13.2 min.

Relaxivity Measurements. Solutions of compounds **11a** and **11b** (0.25−1.0 mM) in 1× PBS (300 μL volume) were prepared along with a corresponding set from the G4-1B4M$_{60}$-Gd$_{42}$ prepared by aqueous chemistry for comparison purposes. Measurements were obtained at ~22 °C using a 3-Tesla clinical scanner (Signa Excite, General Electric Medical System, Waukesha, WI) equipped with a rectangular single loop receiver coil ($84 \times 126 \times 6$ mm). Images of the solutions using an 8-echo 2D-spin echo (2D-SE) sequence were acquired with repetition times of 167, 300, 617, 1250, 2500 and 5000 ms at an echo time of 9.2 ms. T_1 and T_2 maps were calculated using the ImageJ MR Analysis plug-in (http://rsb.info.nih.gov/ij/plugins/mri-analysis.html). T_1 and T_2 relaxivities, r_1 and r_2, were determined from the slopes of the plot of relaxation rates, $R_1 = 1/T_1$ and $R_2 = 1/T_2$, vs [Gd].

Dynamic Contrast-Enhanced MR Angiography Imaging Experiments. All procedures were performed in accordance with the National Institutes of Health guidelines on the use of animals in research and were approved by the Animal Care and Use Committee of the National Cancer Institute.

Normal 6−8 week old female athymic nu/nu mice (Charles Rivers Laboratories) were imaged in pairs to increase throughput on a 3-Tesla clinical scanner (Philips Intera 3.0T, Philips Medical System, Best, The Netherlands) using a parallel receiver coil array comprised of two modified Alderman-Grant resonators (38 mm o.d. × 75 cm) and equipped with a multichannel animal support and monitoring system. Mice ($n = 4-5$ per agent evaluated) were chemically restrained with 2.5% isoflurane (Abbott Laboratories, NJ) in O_2 delivered using a Summit Anesthesia Solutions vaporizer (Bend, OR) at a flow rate of 1.0 L/min. Respiration rate was maintained at 25−30 respirations per min and monitored using a Biopac System MP150 (Biopac Inc., Goleta, CA). The temperature of the mouse was maintained at 32 ± 1 °C using a Polyscience Model 210 heating recirculator with 3M Fluorinert Electronic Liquid FC-77. Body temperature was monitored using FOT-M fiber optic temperature sensors (Fiso Technologies Inc., San Jose, CA) and a UMI-8 Universal Multichannel Instrument (Fiso Technologies Inc.). A tail vein cannula consisting of a 30-gauge needle attached to Tygon tubing (0.010 in i.d. × 10 m length) was then established. Prior to injection of the contrast agent, a T_1 map was obtained by using a 3D-fast spoiled gradient echo image (3D-fSPGR) sequence at two different flip angles (repetition time/echo time 8.8/1.9 ms; flip angles 8° and 24°; bandwidth 31.25 kHz; matrix size 512 × 128 × 40; voxel resolution 156 × 156 × 600 μm; four excitations; scan time 4 min 29 s). Fifty microliters of contrast agent (12 mM Gd; 0.03 mmole Gd/kg mouse) followed by 50 μL of 1× PBS was injected in the tail vein of each mouse at a rate of 150 μL/min using dual 1.0 cm^3 syringes in a Harvard Apparatus PHD2000 (Holliston, MA) syringe pump. Dynamic MR angiography images were obtained immediately after injection by repeating the 3D-fSPGR sequence at the higher flip angle every 5 min for 1 h.

The dynamic 3D images were processed using ImageJ (http://rsb.info.nih.gov/ij/). The baseline precontrast 3D images were subtracted from each of the postcontrast 3D images and a maximum intensity projection (MIP) was calculated of the resulting 3D images. The resulting MIP images were subjectively compared by a board-certified radiologist (P.L.C.) and an MR physicist (M.W.B.) for image clarity and for opacification of vessels and organs.

Clearance Rate Analyses. Time curves for clearance from the blood were measured from a region of interest (ROI) that was drawn over the jugular vein using ImageJ and exported for analysis to

Igor Pro (Wavemetrics, Inc., Lake Oswego, OR). The clearance rates were determined by fitting the decay curves to a single-exponential decay function with the baseline fixed to zero, and clearance pseudo-first-order rate constants were calculated (Table 1). Results were averaged for all animals in each group ($n = 4-5$).

Statistical Analyses. The statistical analysis of the differences between clearance rates and relaxivity values among the three agents was assessed with a student's *t*-test using an Excel spreadsheet (Microsoft, Redmond, WA).

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Supporting Information Available: Characterization of side products, synthesis of the G4-1B4M$_{60}$-Gd$_{42}$, MALDI-TOF/MS, NMR, SE-HPLC chromatograms, and relaxivity curve results for the compounds are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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