Video Article

Preparation and *In Vitro* Characterization of Dendrimer-based Contrast Agents for Magnetic Resonance Imaging

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Abstract

Paramagnetic complexes of gadolinium(III) with acyclic or macrocyclic chelates are the most commonly used contrast agents (CAs) for magnetic resonance imaging (MRI). Their purpose is to enhance the relaxation rate of water protons in tissue, thus increasing the MR image contrast and the specificity of the MRI measurements. Current clinically approved contrast agents are low molecular weight molecules that are rapidly cleared from the body. The use of dendrimers as carriers of paramagnetic chelators can play an important role in the future development of more efficient MRI contrast agents. Specifically, the increase in local concentration of the paramagnetic species results in a higher signal contrast. Furthermore, this CA provides a longer tissue retention time due to its high molecular weight and size. Here, we demonstrate a convenient procedure for the preparation of macromolecular MRI contrast agents based on poly(amidoamine) (PAMAM) dendrimers with monomacrocyclic DOTA-type chelators (DOTA – 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate). The chelating unit was appended by exploiting the reactivity of the isothiocyanate (NCS) group towards the amine surface groups of the PAMAM dendrimer to form thiourea bridges. Dendrimeric products were purified and analyzed by means of nuclear magnetic resonance spectroscopy, mass spectrometry, and elemental analysis. Finally, high resolution MR images were recorded and the signal contrasts obtained from the prepared dendrimeric and the commercially available monomeric agents were compared.

Video Link

The video component of this article can be found at https://www.jove.com/video/54776/

Introduction

Magnetic resonance imaging (MRI) is a powerful and non-ionizing imaging technique widely used in biomedical research and clinical diagnostics due to its noninvasive nature and excellent intrinsic soft-tissue contrast. The most commonly used MRI methods utilize the signal obtained from water protons, providing high-resolution images and detailed information within the tissues based on differences in the density of the water signals. The signal intensity and the specificity of the MRI experiments can be further improved using contrast agents (CAs). These are paramagnetic or superparamagnetic species that affect the longitudinal (T_1) and transverse (T_2) relaxation times, respectively[1,2].

Complexes of the lanthanide ion gadolinium with polyamino polycarboxylic acid ligands are the most commonly used T_1 CAs. Gadolinium(III) shortens the T_1 relaxation time of water protons, thus increasing the signal contrast in MRI experiments[3]. However, ionic gadolinium is toxic; its size approximates that of calcium(II), and it seriously affects calcium-assisted signaling in cells. Therefore, acyclic and macrocyclic chelates are employed to neutralize this toxicity. Various multidentate ligands have been developed so far, resulting in gadolinium(III) complexes with high thermodynamic stability and kinetic inertness[1]. Those based on the 12-membered azamacrocycle cyclen, in particular its tetracarboxylic derivative DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate) are the most investigated and applied complexes of this CA class.

Nevertheless, GdDOTA-type CAs are low molecular weight systems, displaying certain disadvantages such as low contrast efficiency and fast renal excretion. Macromolecular and multivalent CAs may be a good solution to these problems[4]. Since CA biodistribution is mainly determined by their size, macromolecular CAs display much longer retention times within tissues. Equally important, the multivalency of these agents results in an increased local concentration of the monomeric MR probe (*e.g.,* GdDOTA complex), substantially improving the acquired MR signal and the measurement quality.

Dendrimers are amongst the most preferred scaffolds for the preparation of multivalent CAs for MRI[4,5]. These highly branched macromolecules with well-defined sizes are prone to various coupling reactions on their surface. In this work, we report the preparation, purification, and characterization of a dendrimeric CA for MRI consisting of a generation 4 (G4) poly(amidoamine) (PAMAM) dendrimer coupled to GdDOTA-like chelates (DCA). We describe the synthesis of the reactive DOTA derivative and its coupling to the PAMAM dendrimer. Upon complexation with Gd(III), the standard physicochemical characterization procedure of DCA was performed. Finally, MRI experiments were performed to demonstrate the ability of DCA to produce MR images with a stronger contrast than those obtained from low molecular weight CAs.

Protocol

1. Preparation of DCA

1. **Synthesis of the monomeric unit 4[6].**
    1. Synthesis of 4-(4-nitrophenyl)-2-(4,7,10-tris-*tert*-butoxycarbonylmethyl-1,4,7,10-tetraazacyclododec-1-yl)butyric acid *tert*-butyl ester (**2**).
        1. Dissolve (4,7-bis-*tert*-butoxycarbonylmethyl-1,4,7,10-tetraaza-cyclododec-1-yl)-acetic acid *tert*-butyl ester **1** (1.00 g, 1.94 mmol) in *N*,*N*-dimethylformamide (DMF, 5 ml), add potassium carbonate (0.67 g, 4.86 mmol, 2.5 equiv.) and stir the mixture at room temperature for 45 min.
        NOTE: Macrocycle **1** was prepared from cyclen and *tert*-butyl bromoacetate according to the previously published procedure[7].
        2. Add *tert*-butyl-2-bromo-4-(4-nitrophenyl)butanoate (0.87 g, 2.53 mmol, 1.3 equiv.) portionwise over 1 hr. Continue stirring the mixture under the same reaction conditions for the following 18 hr.
        Note: *Tert*-butyl-2-bromo-4-(4-nitrophenyl)butanoate was prepared from 4-(4-nitrophenyl)-butyric acid, thionyl chloride, and bromine according to the previously published procedure[8].
        3. Remove DMF by means of bulb-to-bulb vacuum distillation at 40-60 °C[9].
        4. Purify the residue by column chromatography (silica gel, 7% methanol/dichloromethane) to obtain product **2** as a brown amorphous solid (1.09 g, 72%)[10].

    2. Synthesis of 4-(4-aminophenyl)-2-(4,7,10-tris-*tert*-butoxycarbonylmethyl-1,4,7,10-tetraazacyclododec-1-yl)butyric acid *tert*-butyl ester (**3**).
        1. Dissolve the nitrobenzene derivative **2** (1.00 g, 1.28 mmol) in ethanol (10 ml) and 7 N ammonia solution in methanol (150 µl). Add palladium on activated carbon as a catalyst (Pd/C, 150 mg, 15 wt %) to the solution.
        2. Shake the heterogeneous mixture for 16 hr under a hydrogen atmosphere (2.5 bar) in the Parr hydrogenator apparatus.
        3. Prepare a cake of diatomaceous earth by suspending it in ethanol and filtering the suspension through a sintered glass funnel. Pour the suspension from 1.1.2.2 over the prepared cake to remove the Pd/C catalyst by filtration.
        4. Remove the solvent by gentle distillation on a rotary evaporator (water bath temperature ~40 °C) to obtain compound **3** as a brown amorphous solid (0.91 g, 95%).

    3. Synthesis of 4-(4-isothiocyanatophenyl)-2-(4,7,10-tris-*tert*-butoxycarbonylmethyl-1,4,7,10- tetraazacyclododec-1-yl)butyric acid *tert*-butyl ester (**4**).
        1. Add thiophosgene (0.124 ml, 1.58 mmol, 1.3 equiv.) to a mixture of 3 (0.91 g, 1.22 mmol) and triethylamine (0.685 ml, 4.87 mmol, 4 equiv.) in dichloromethane (15 ml).
        2. Vigorously stir the reaction mixture with a magnetic stirrer at room temperature for 16 hr.
        3. Remove the solvent by gentle distillation on a rotary evaporator (water bath temperature ~40 °C), and then purify the crude product by column chromatography (silica gel, 5% methanol/dichloromethane) to obtain the product **4** as a light brown amorphous solid (0.51 g, 53%).

2. **Synthesis of the dendrimer DCA.**
    1. Synthesis of the dendrimer **5**.
        1. Take G4-PAMAM dendrimer (667 mg, 10% dendrimer solution in methanol, 4.67 µmol), evaporate the methanol by gentle distillation on a rotary evaporator (water bath temperature ~40 °C), and dissolve the residue in DMF (4 ml).
        2. Add triethylamine (0.105 ml, 0.75 mmol, 160 equiv.), stir for 45 min at 60 °C, and add isothiocyanate **4** (354 mg, 0.45 mmol, 1.5 equiv. relative to the amino surface groups of the dendrimer) portionwise over 1 hr.
        3. Stir the reaction mixture with a magnetic stirrer at 45 °C for 48 hr.
        4. Remove the solvent by means of bulb-to-bulb vacuum distillation at 40-60 °C.
        5. Purify the residue by size-exclusion chromatography using a lipophilic gel filtration medium and methanol as the eluent. To pack the column, swell the filtration media in methanol for at least 3 hr at room temperature (>4 ml of methanol per 1 g of powder) without applying pressure. Perform gravity separation by collecting 1 ml fractions.
        6. Analyze the collected fractions with thin-layer chromatography (TLC). Develop the TLC plate in 15% methanol/dichloromethane (only the most polar spot located on the base line is derived from dendrimeric product). Evaporate the collected fractions by gentle distillation on a rotary evaporator (water bath temperature ~40 °C) to obtain product **5** (270 mg, 91%).

    2. Synthesis of the dendrimer **6**.
        1. Dissolve the protected dendrimeric chelator **5** (270 mg, 4.23 µmol) in formic acid (5 ml) and stir the mixture at 60 °C for 24 hr.
        2. Evaporate the formic acid by distillation on a rotary evaporator (~15 mbar pressure, water bath temperature ~40 °C) and freeze-dry the product to give **6** (pressure ~0.2 mbar)[9].

    3. Synthesis of the dendrimeric contrast agent (**DCA**)
        1. Dissolve the dendrimeric chelator **6** (4.35 µmol) in water and adjust the pH to 7.0 with 0.1 M sodium hydroxide.
        2. Dissolve $GdCl_3 \cdot 6H_2O$ (113 mg, 304 µmol) in water (1 ml) and add it dropwise to the solution of chelator **6** over a period of 4 hr; maintain the pH at 7.0 with aqueous sodium hydroxide solution (0.05 M) by measuring pH with a pH meter.
        3. Stir the mixture with a magnetic stirrer at room temperature for 24 hr.
        4. Add ethylenediaminetetraacetic acid (EDTA, 158 mg, 426 µmol) to the solution portionwise over 4 hr to remove the excess of Gd(III) while maintaining the pH at 7.0 with aqueous sodium hydroxide solution (0.05 M). Stir the mixture at room temperature for 24 hr.

5. Perform size-exclusion chromatography to remove the majority of GdEDTA and the excess of EDTA. Use a hydrophilic gel filtration medium swollen in water to pack the column. Reduce the mixture to a suitable volume and load the column. Elute the column with deionized water without applying pressure.
6. Centrifuge the sample using a 3 kDa centrifugal filter unit for 30 min at centrifugal force 1,800 x g to remove the residues of GdEDTA and EDTA. Repeat this step (around five times) until the filtrate shows the absence of EDTA and GdEDTA. Transfer the sample into a flask, evaporate it, and then freeze-dry the solvent to obtain an off-white product as the final DCA (186 mg, 71%).
NOTE: Check absence of EDTA and GdEDTA by means of ESI-MS.
7. Confirm the absence of Gd(III) as a free ion using the xylenol orange test. Dissolve the filtrate (0.5 ml) in an acetate buffer solution (pH 5.8). Add a few drops of a xylenol orange solution and track the color change (yellow or violet color indicates the absence or presence of free Gd(III) ions in solution, respectively)[11].

2. *In Vitro* Characterization of Dendrimeric Products

1. **Estimation of the number of macrocyclic DOTA-units coupled to the PAMAM dendrimer (loading of the dendrimer with DOTA-like macrocycles)**
   1. Estimation with ^{1}H NMR (NMR — nuclear magnetic resonance spectroscopy).
   NOTE: This procedure is possible on dendrimers **5** and **6**, but not on DCA.
      1. Record the ^{1}H NMR spectrum[12].
      2. Integrate the aromatic region and the two separate aliphatic regions (1. signals of the aliphatic dendrimer and macrocyclic protons; 2. signals of the *t*-Bu groups) or just an aliphatic region for dendrimers **5** and **6**, respectively.
      Note: There is no separate signal in the aliphatic region originated from the *t*-Bu groups in dendrimer **6** since they have been hydrolyzed.
      3. Use Eq. 1 or Eq. 2 to estimate the number of macrocyclic units (n), where R = the ratio of integrals (aliphatic/aromatic in Eq. 1 or aliphatic-dendrimer/aliphatic-*t*-Bu in Eq. 2), H_{dend} = the number of protons in dendrimer, H_{Ar} = the number of aromatic protons, H_{tBu}= the number of protons in *t*-Bu groups, and H_{mac} = the number of protons in one macrocycle.
      Note: Either Eq. 1 or Eq. 2 can be used for dendrimer **5**, while only Eq. 1 can be used for dendrimer **6**. Since exchangeable protons (on amines, amides, thioureas, or carboxylates) are typically being replaced with deuterium, they were not assumed in the calculations. Here, H_{dend} = 1,128 (for **5**) or 1,000 (for **6**), H_{Ar} = 4, and H_{mac} = 27 were used.
      $$n = \frac{H_{dend}}{R \times H_{Ar} - H_{mac}} \quad (1)$$
      $$n = \frac{H_{dend}}{R \times H_{tBu} - H_{mac}} \quad (2)$$
   2. Estimation from elemental analysis by using the ratio of nitrogen to sulphur.
      1. Perform the elemental analysis on the solid dendrimeric sample (DCA in this work).
      2. Use Eq. 3 to estimate the number of macrocyclic units (n), where R = the ratio of determined %N and %S, N_{dend} or S_{dend} = the number of nitrogen or sulphur atoms in the dendrimer, and N_{mac} or S_{mac} = the number of nitrogen or sulphur atoms in one macrocyclic unit.
      Note: The factor 2.29 is obtained from the ratio in atomic masses of sulphur and nitrogen. In this work, N_{dend} = 250, S_{dend} = 2, N_{mac} = 5, and S_{mac} = 1 were used.
      $$n = \frac{2.29 \times N_{dend} - R \times S_{dend}}{R \times S_{mac} - 2.29 \times N_{mac}} \quad (3)$$
   3. Estimation with matrix-assisted laser desorption/ionization time of flight (MALDI-TOF).
      1. Perform the MALDI-TOF MS analysis[13].
      2. Calculate the number of macrocyclic units (n) according to Eq. 4, where M_z = the observed mass (m/z), z = the charge of the species, M_{dend} = the mass of the dendrimeric part, and M_{mac} = the mass of one macrocyclic unit.
      NOTE: M_{dend} = 14,306 and M_{mac} = 719 were used in this work.
      $$n = \frac{M_z \times z - M_{dend}}{M_{mac}} \quad (4)$$

2. **Determination of DCA concentration ([DCA]): Bulk magnetic susceptibility measurement (BMS)**
   1. Dissolve DCA (5-10 mg) in water (360 µl) in a plastic vial tube ([DCA] ~5-10 mM).
   NOTE: [DCA] should be in the range of 5-10 mM to avoid possible overlap of *t*-BuOH resonances at sample concentrations >15 mM, with the resonance of water at δ = 4.7 ppm.
   2. Add 60 µl of D_2O:*t*-BuOH mixture (2:1 v/v) to the aqueous solution of DCA and mix the resulting solution (420 µl) using a Vortex mixer.
   3. Transfer 400 µl of the sample into an outer NMR tube and place a coaxial NMR insert tube with a *t*-BuOH:H_2O mixture (10:90 v/v) into the sample tube.
   4. Record the ^{1}H NMR spectrum and measure the frequency shift between resonance signals deriving from *t*-BuOH in the inner and outer NMR tubes (reference)[12].
   5. Use Eq. 5 to determine the [DCA], where T = the absolute temperature, Δχ = the recorded shift, μ_{eff} = the effective magnetic moment for a lanthanide ion (μ_{eff} = 7.94 for Gd(III)[14], and s = a constant dependent upon the shape of the sample and its position in the magnetic field (0, 1/3, and 1/6 in the case of a sphere, cylinder parallel to, and cylinder perpendicular to the magnetic field, respectively).
   NOTE: The calculated value obtained for the [DCA] should be corrected to the original concentration due to the addition of the D_2O:*t*-BuOH solution (60 µl).

$$c = \frac{T\,\Delta\chi\,(\frac{2.84}{\mu_{eff}})^2}{4\,\pi\,s} \quad (5)$$

3. **Dynamic light scattering (DLS) measurements.**
    1. Prepare a filtered DCA solution (0.2 µm polytetrafluorethylene/PTFE filter, 0.75 mM per Gd(III)) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (25 mM, pH 7.4) and transfer it into a cuvette for the DLS measurement.
    2. Place the cuvette into the DLS apparatus and set the following parameters: 5 repetitions of 15 scans (1 scan = 12 sec, refractive index = 1.345, absorption = 1%) without delays in between the scans and with temperature equilibration 30 sec prior to recording.
    3. Export the acquired data and obtain the size distribution histogram by plotting population (%) as a function of size (hydrodynamic diameter).

4. **Measurement of the longitudinal and transverse relaxivities.**
NOTE: A similar procedure was already described using the relaxation time analyzer[15]; this procedure was performed using a 300 MHz NMR spectrometer with Topspin software.
    1. Prepare a set of DCA solutions in H_2O:D_2O (500 µl, 10% D_2O in H_2O, [DCA] = 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mM, [HEPES] = 25 mM) from the DCA stock sample (see section 2.2).
    2. Transfer 450 µl of solution into an NMR tube and place it into the instrument.
    3. Optimize the acquisition parameters (90° excitation pulse duration (p1), and irradiation frequency offset (O_1)) and then perform the T_1 and T_2 experiments using the inversion recovery (IR) and Car-Purcell-Meiboom-Gill (CPMG) pulse sequences, respectively.
    4. Determination of T_1 and T_2 relaxation times.
        1. Select the recorded measurement, process the 2D spectrum in the F2 dimension, and perform the interactive phase correction.
        2. Select the appropriate slice (peak with maximum intensity) in the *Analysis/ T_1/T_2 relaxation* window, integrate it, and export the region to the relaxation module.
        3. Select the appropriate fitting function (*invrec* or *uxnmrt2* for IR and CPMG experiments, respectively) to obtain the T_1 or T_2 relaxation times.
    5. Repeat steps 2.4.4.2-2.4.4.4 for all the remaining [DCA] solutions.
    6. Calculate the relaxation rates (R_1 and R_2) from the obtained T_1 values ($R_{1,2}=1/T_{1,2}$).
    7. Plot R_1 and R_2 (sec^{-1}) as a function of Gd(III) concentration in mM.
    8. Determine the longitudinal and transverse relaxivities, r_1 and r_2 ($mM^{-1}\,sec^{-1}$), from the slope of the fitted line, as defined by Eq. 6, where $R_{i,obs}$ = the longitudinal (i=1) or transverse (i=2) diamagnetic relaxation rate of water in the absence of paramagnetic species and [Gd] = the concentration of Gd(III) used in the experiment.
    $$R_i = R_{i,obs} + [Gd] \times r_i \quad i = 1,2 \quad (6)$$

3. *In Vitro* MRI; Comparison Between DCA and GdDOTA

1. **Preparation of tube phantoms**
    1. Prepare aqueous solutions of DCA (4 x 350 µl) and GdDOTA (4 x 350 µl) as well as water samples (4 x 350 µl) for two sets of experiments where the concentration of the contrast agents is calculated: (3.1.1.1) per Gd(III) or (3.1.1.2) per molecule.
        1. Prepare two DCA samples and two GdDOTA samples with concentrations of 0.5 and 1.0 mM per Gd(III), respectively. Additionally, prepare two water samples (as control tubes).
        2. Prepare two DCA samples (2.5 and 5.0 mM per Gd(III) or 0.05 and 0.1 mM per dendrimeric molecule), two GdDOTA samples (0.25, 0.5 mM) and two water samples (control tubes).
        NOTE: The appropriate DCA and GdDOTA concentrations should be prepared by diluting the respective stock samples with concentrations determined via the BMS method (see section 2.2) with HEPES buffer (pH 7.4). In order to simplify the calculations, n = 50 was assumed for the average number of macrocyclic units per dendrimer molecule. Therefore, the ratio of DCA:GdDOTA was 1:5, calculated on a per molecule basis.
    2. Place the samples in 300 µl plastic vial tubes, avoiding the presence of air bubbles in the solution.
    NOTE: The size of the plastic vial tubes depends on the type and size of the radiofrequency coil used (here, an example with the volume coil is given).
    3. Insert the samples inside a syringe (60 ml volume), fill it with 1 mM GdDOTA solution, and place it in the scanner.
    NOTE: Samples were placed in the aqueous solution of GdDOTA to avoid susceptibility effects (variations in the magnetic field strength that occur near interfaces between substances of different magnetic susceptibility).

2. **Parameter optimization and imaging.**
    1. Use the anatomical scan (Localizer/Tripilot) to position the syringe with the samples in the isocenter of the magnet.
    2. Press the traffic light (adjustment scan) to perform adjustments for shimming (adjustment of magnetic field homogeneity) of the whole volume, the central frequency (O_1), the receiver gain (RG), and the transmit gain (TX0 and TX1).
    3. For T_1-weighted (T_{1w}) imaging, select the fast low angle shot (FLASH) method.
    4. Choose coronal slice for the samples placed vertically (syringe horizontally) in the scanner using the Localizer scan.
    5. Use Eq. 7 for optimization of the contrast-to-noise (CNR) acquisition parameters[16], where α = the flip angle, *TE* = the echo time, *TR* = the repetition time, and $T_{1,A}$, $T_{1,B}$ = the T_1 times of sample A ($T_{1,A}$) and sample B ($T_{1,B}$) for which the CNR should be maximized (the same is valid for T_2 times: $T_{2,A}$ and $T_{2,B}$).
    NOTE: T_1 and T_2 relaxation times should be set to values obtained from the measurements of longitudinal and transverse relaxivities (section 2.4), while *TE*, *TR*, and α should be obtained from the CNR optimization calculation.

$$CNR_{FLASH} = \frac{\left| \sin(\alpha) \times \frac{1-e^{-\frac{TR}{T_{1,B}}}}{1-e^{-\frac{TR}{T_{1,B}}} \times \cos(\alpha)} - \sin(\alpha) \times \frac{1-e^{-\frac{TR}{T_{1,A}}}}{1-e^{-\frac{TR}{T_{1,A}}} \times \cos(\alpha)} \right|}{\sqrt{TR}} \quad (7)$$

6. Acquire the image using the parameters obtained in the previous step (3.2.5).
7. Calculate the signal-to-noise ratio (SNR).
    1. Load the acquired T_{1w} image (scan) into the *Image display & processing window*, and click on *Define region of interest* (ROI).
    2. Choose a circular ROI and draw it at the sample position and background. Subsequently, click on *display* to obtain the average signal amplitude (S_{signal}) and standard deviation of the background (S_{noise}).
    3. Repeat step 3.2.7.2 for the DCA, GdDOTA, and water samples.
    4. Calculate the SNR using the formula: SNR = S_{signal} / S_{noise}.
8. Following a slightly modified procedure, perform T_2-weighted (T_{2w}) imaging using the rapid acquisition with relaxation enhancement (RARE) method. For optimization of the CNR acquisition parameters, use Eq. 8.

$$CNR_{RARE} = \frac{\left| \left(1-e^{-\frac{TR}{T_{1,B}}}\right)\left(e^{-\frac{TE}{T_{2,B}}}\right) - \left(1-e^{-\frac{TR}{T_{1,A}}}\right)\left(e^{-\frac{TE}{T_{2,A}}}\right) \right|}{\sqrt{TR}} \quad (8)$$

Representative Results

The preparation of **DCA** consisted of two stages: 1) synthesis of the monomeric DOTA-type chelator (**Figure 1**) and 2) coupling of the chelator with the G4 PAMAM dendrimer and subsequent preparation of the dendrimeric Gd(III) complex (**Figure 2**). In the first stage, a cyclen-based DOTA-type chelator containing four carboxylic acids and an orthogonal group suitable for further synthetic modifications was prepared. The preparation commenced from **1** (DO3A-*tert*-butyl ester)[7], which was alkylated with *tert*-butyl 2-bromo-4-(4-nitrophenyl) butanoate[8] to provide DOTA-derivative **2**. The palladium-catalyzed hydrogenation reduced the aromatic nitro group in **2** to yield the aniline **3**. The conversion of **3** with thiophosgene resulted in the isothiocyanate **4**, which was previously used as an amine-reactive agent for the preparation of dendrimeric CAs[17].

In the following stage, the macrocycle **4** was used as the basic monomeric unit in a coupling reaction to the commercially available G4 PAMAM dendrimer. The amine surface groups of the dendrimer react with the isothiocyanate groups of the monomer **4** in the presence of a base. The excess of **4** was removed by size-exclusion chromatography using a lipophilic gel filtration medium with methanol as the eluent. The *tert*-butyl esters on the obtained dendrimer-macrocyclic conjugate **5** were hydrolyzed with formic acid to yield **6**, which was then lyophilized and used in the next step without purification. The formation of Gd(III) complexes of DOTA-type macrocycles was performed by adding $GdCl_3 \cdot 6H_2O$ to an aqueous solution of **6** while maintaining the pH at about 7. The excess of Gd(III) was complexed with a common chelator ethylenediaminetetraacetic acid (EDTA). The GdEDTA complex and excess EDTA were removed from the system by size-exclusion chromatography using a hydrophilic gel filtration medium with water as the eluent. The remaining small-size impurities were removed from the solution by centrifugation using 3 kDa centrifugal filtration units.

Following the synthesis of the dendrimer-macrocycle conjugates, a combined analytical approach has been employed to characterize the products. To determine the surface-amine occupancy of **5** and **6**, ^{1}H NMR spectra have been analyzed. The results were compared and confirmed with the final product (DCA), where the loading of the dendrimer with macrocycles has been estimated using elemental analysis and MALDI-TOF mass spectrometry (**Figure 3**). A combination of these three methods resulted in an average of 49 macrocyclic units being conjugated to the G4 dendrimer, which corresponds to ~75% amine surface group occupancy.

Further characterization of the dendrimeric complex included determination of the relaxivity values, resulting in 6.2 ± 0.1 $mM^{-1}sec^{-1}$ per Gd(III) (or roughly around 300 $mM^{-1}sec^{-1}$ per dendrimer) for the longitudinal relaxivity and 30.5 ± 0.6 $mM^{-1}sec^{-1}$ per Gd(III) (almost 1,500 $mM^{-1}sec^{-1}$ per dendrimer) for the transverse relaxivity. DLS measurements indicated a hydrodynamic diameter of 7.2 ± 0.2 nm for DCA (**Figure 4**).

Finally, to demonstrate the effect of the dendrimeric MRI contrast agent, MR imaging was performed on two sets of phantoms with DCA and the clinically available GdDOTA for comparison (**Figure 5**). The first set of phantoms were prepared for the purpose of comparing these two contrast agents at identical Gd(III) concentrations, while the second set was designed to demonstrate the effect at comparable molecule concentrations of the dendrimeric and monomeric contrast agents, respectively.

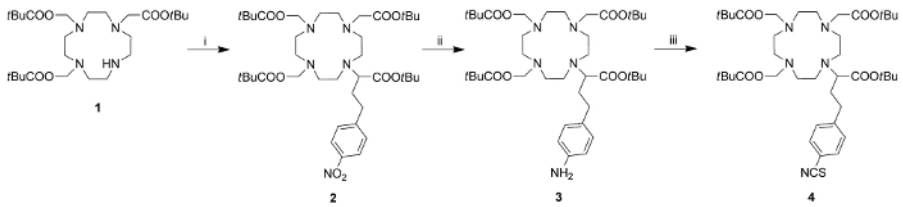

Figure 1: Synthesis of the macrocyclic DOTA-type chelator 4. Reagents, conditions, and isolated yields: (i) *tert*-butyl 2-bromo-4-(4-nitrophenyl)butanoate, K_2CO_3, DMF, 45 °C, 16 hr, 72%; (ii) H_2, Pd/C, EtOH, RT, 16 hr, 95%; (iii) $CSCl_2$, Et_3N, RT, 2 hr, 53%. Please click here to view a larger version of this figure.

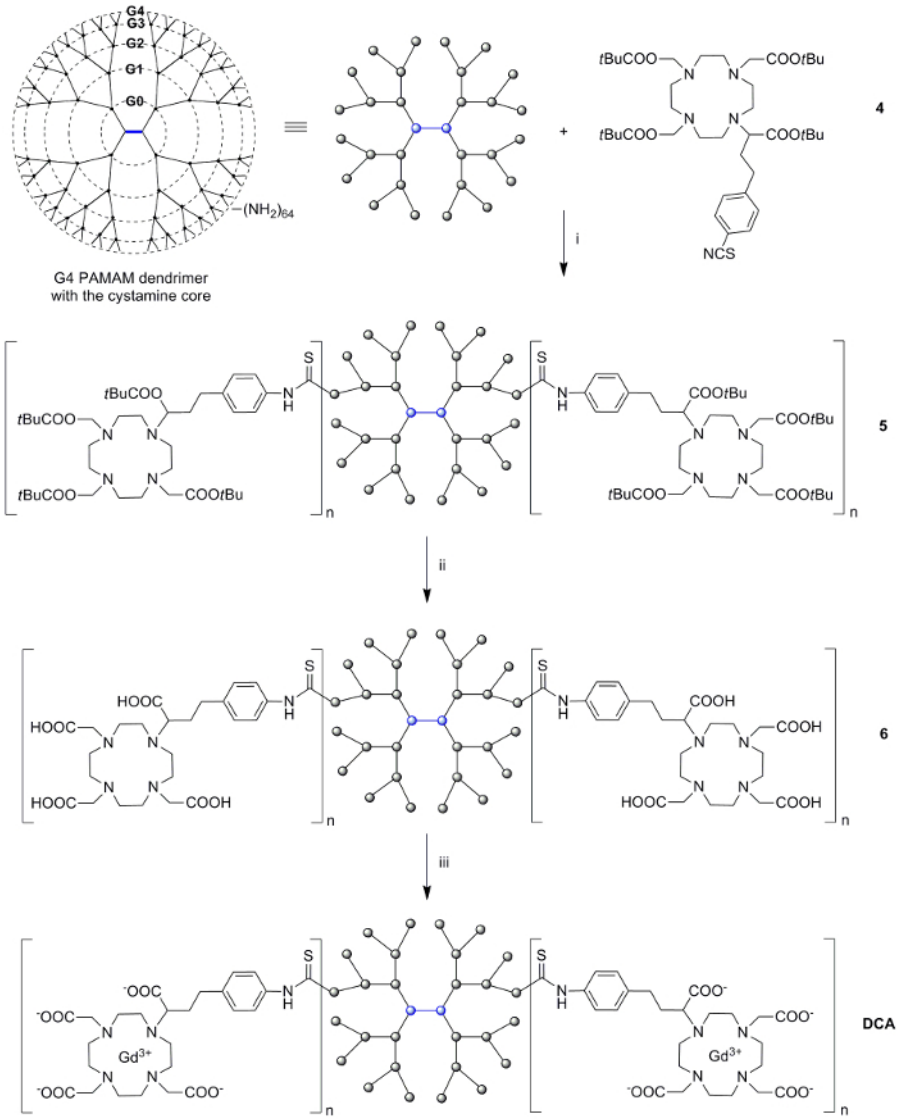

Figure 2: Synthesis of the dendrimeric MRI contrast agent DCA. Reagents and conditions: (i) **4**, Et_3N, DMF, 45 °C, 48 hr, 91%; (ii) formic acid, 60 °C, 24 hr, quant; (iii) $GdCl_3 \cdot 6H_2O$, pH 7.0, RT, 24 hr, 71%. Please click here to view a larger version of this figure.

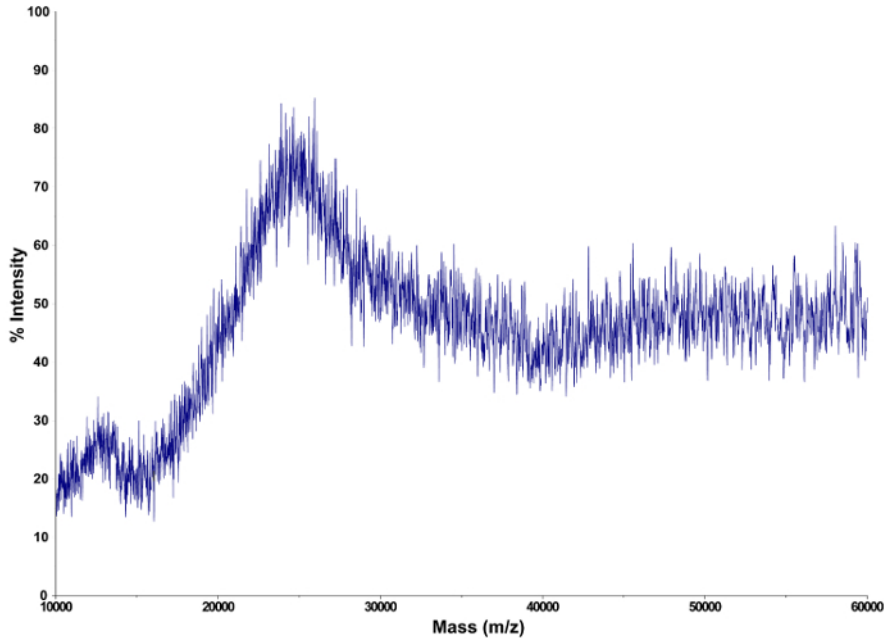

Figure 3: Characterization of the dendrimeric product by means of MALDI-TOF mass spectrometry. A typical MALDI-TOF mass spectrum obtained for DCA. Please click here to view a larger version of this figure.

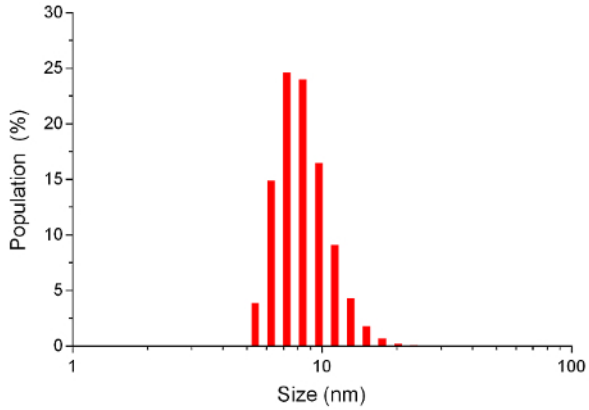

Figure 4: Characterization of the dendrimeric product by means of dynamic light scattering (DLS). DLS measurement of DCA (HEPES, pH 7.4). Please click here to view a larger version of this figure.

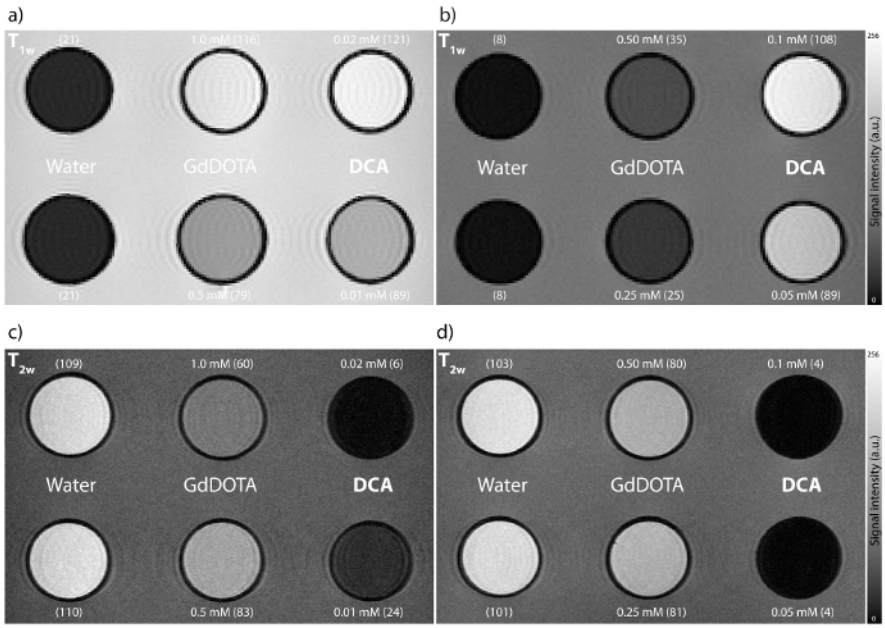

Figure 5: *In vitro* MRI experiments on tube phantoms at 7 T magnetic field. (**a**,**b**) T_1-weighted and (**c**,**d**) T_2-weighted MRI of **DCA** and GdDOTA. Each MRI experiment was performed with two different concentrations of the contrast agent: (**a**,**c**) with comparable Gd(III) concentrations (HEPES, pH 7.4); (**b**,**d**) with a DCA:GdDOTA concentration ratio of 1:5 (HEPES, pH 7.4). The concentrations are expressed per molecule and the SNR values are displayed in the parentheses. The parameters used in these experiments were: field-of-view (FOV) = 40 x 40 mm^2, slice thickness = 0.5 mm, number of excitations (NEX) = 30; (**a**) matrix size (MTX) = 256 x 256, repetition time (TR) = 100 msec, echo time (TE) = 2.95 msec, flip angle (FA) = 90°, acquisition time (TA) = 12 min 48 sec; (**b**) MTX = 256 x 256, TR/TE = 20/2.95 msec, FA = 90°, TA = 2 min 34 sec; (**c**) MTX = 512 x 512, TR/TE = 10,000/130 msec, Rare factor (RF) = 16, TA = 26 min 40 sec; (**d**) MTX = 512 x 512, TR/TE = 10,000/100 msec, RF = 16, TA = 26 min 40 sec. Please click here to view a larger version of this figure.

Discussion

Preparation of the dendrimeric MRI contrast agent requires appropriate selection of the monomeric unit (*i.e.,* the chelator for Gd(III)). They reduce the toxicity of this paramagnetic ion and, to date, a wide variety of acyclic and macrocyclic chelators serve this purpose[1-3]. Among these, macrocyclic DOTA-type chelators possess the highest thermodynamic stability and kinetic inertness and, hence, are the most preferred choice for the preparation of inert MRI contrast agents[1,18]. Furthermore, they are prone to various synthetic transformations, which result in bifunctional chelators, capable of linking to various functional molecules (*e.g.,* targeting vectors or nano-carriers) while still forming stable Gd(III) complexes[19]. To this end, the DOTA-type monomeric unit described in this procedure was prepared from DO3A-*tert*-butyl ester, the common and readily available precursor, and the bromide derivative of the 4-(4-nitrophenyl) butanoic acid. This molecule is derived from DOTA and possesses a similar structure to coordinate Gd(III). The synthetic modification aims to make this chelator prone to coupling reactions to various functional molecules and carriers. Namely, the preparation of the DOTA-modified molecule results in a chelator still with four carboxylic groups available for coordination to Gd(III) to form an inert complex and an orthogonal nitrophenyl group, which upon conversion attaches this chelator to the dendrimer surface. This procedure also allows for flexibility in the choice of the orthogonal reactive group (*e.g.,* NH_2 or COOH), which can serve to couple the Gd(III) chelator to a desired carrier in a preferred manner.

The obtained bifunctional chelator can be coupled to other molecules in two different ways (*i.e.,* synthetic procedures). When the nitro group is reduced to an amino group, the resulting aniline can undergo a condensation reaction with the carboxylic acid group of the other molecule[8]. Moreover, an aromatic primary amine functional group in the presence of thiophosgene can be easily converted into an isothiocyanate, a group which readily reacts with amines in polar organic solvents as well as water, offering more reaction possibilities for the coupling of monomeric units to dendrimers[17,20,21].

For coupling the bifunctional chelator to the dendrimeric carrier, an appropriate dendrimeric scaffold should be selected. Several factors related to the final dendrimer conjugate structure and the desired application should be accounted for in this step. Due to wide commercial availability of dendrimeric carriers, products with different core structures, surface-reactive groups, or generations can be chosen. Consequently, the conjugation reaction will depend on the surface group of the dendrimer and the orthogonal group of the chelator, while the final conjugate may be neutral, charged, or have different sizes (up to 15-20 nm, depending on dendrimer generation)[22]. All these aspects should be taken into account prior to preparing the dendrimeric CA, since they may affect the solubility, relaxivity (MRI signal enhancement), diffusion, and other pharmacokinetic properties of the contrast agent, which can potentially endanger its application in MRI. For instance, cationic dendrimers may exhibit toxicity in biological systems. However, this effect can be reduced by conjugation of negatively charged groups on the dendrimer surface, thereby reducing their overall positive charge[23].

In this protocol, we have prepared the dendrimeric contrast agent DCA using the procedure in which the isothiocyanate group of the monomeric macrocycle **4** was coupled to a commercial cystamine-core G4-PAMAM equipped with 64 primary amine surface groups. The initial purification of the hydrophobic dendrimeric product **5** was performed by gel chromatography using a column with a lipophilic gel filtration medium and methanol as the eluent in order to remove most of the unreacted monomeric units. The hydrolysis of *t*-butyl esters with formic acid is straightforward,

resulting in a water-soluble dendrimeric product that can be purified with size-exclusion chromatography using a hydrophilic gel filtration medium. The complexation of the multimeric and dendrimeric chelators with Gd(III) was performed whilst maintaining the solution at a neutral pH in order to facilitate the complex formation. Otherwise, the complexation of Gd(III) (added as the chloride salt) reduces the pH, slowing down the reaction. Finally, it is worth noting that amine groups in the dendrimer core also tend to coordinate with Gd(III), but only with the excess that could not be chelated with the DOTA units. Avoiding the presence of Gd(III) outside the DOTA chelator is essential, since leakage of Gd(III) from the CA may have undesired effects; namely, it can induce toxicity *in vivo*[18]. The excess Gd(III) can be effectively removed by complexation with EDTA followed by ultrafiltration of GdEDTA and free EDTA using 3 kDa molecular weight cut-off (MWCO) filters. Lower MWCO filters might be used when the dendrimeric conjugates have lower molecular weights.

There are two major troubleshooting issues related to the preparation of DCA. Due to the large broadening effect of Gd(III) on NMR signals, the analysis of DCA by means of NMR spectroscopy is not informative. Instead, this analysis should be performed in earlier steps (compounds **5** and **6**). Next, the conjugation of monomacrocyclic units to the dendrimer surface is never accomplished with 100% conversion, but it is likely to be between 50-90% (see below). Typically, the reaction yields can be increased by adding a second portion of the monomeric reactive unit after the first conjugation of dendrimer and monomeric unit is completed[24]. However, every preparation batch results in somewhat different average numbers of chelators conjugated on the dendrimer surface, even when identical dendrimer and DOTA units are used as materials for coupling. Although the final amount of Gd(III) present in DCA can be determined independently via the BMS method (see section 2.2), for better characterization of dendrimeric conjugates, it is necessary to perform the estimation of bound monomeric units each time a new batch of DCA is prepared (see 2.1 and discussion below).

The analytical characterization of the isolated dendrimeric products can be performed by means of ^{1}H NMR spectroscopy (only on products **5** and **6**), elemental analysis, and MALDI-TOF MS. Typical yields for the conversion of amino surface groups lie between 50-90%, depending on the dendrimer generation, the type of chelator, and the reaction conditions used (solvent and temperature)[6,20,24,25]. In this particular case, the calculated masses obtained from the combined analyses correspond to an average of 49 monomeric chelates being coupled to the dendrimer (*i.e.,* ~75% occupancy of the dendrimer surface amines). Although a slight mismatch in the final number of reacted amino groups could be expected between these methodologies[25], their direct comparison provides reasonable evidence for the formation of the desired **DCA** with a particular average number of attached chelating units.

The *in vitro* characterization aiming to assess the potential of DCA to enhance the contrast in MRI experiments consisted of DLS, relaxometric, and MRI experiments. The hydrodynamic diameter of DCA was determined to be 7.2 ± 0.2 nm by DLS measurements, which is in agreement with previously reported conjugates of this kind with G4 generation 4 PAMAM dendrimers[26]. Determination of the longitudinal relaxivity of DCA followed the previously described procedure[15] and revealed the value of 6.2 ± 0.1 $mM^{-1}sec^{-1}$ per Gd(III). About 50% of the enhancement in the r_1 of paramagnetic Gd(III) in DCA relative to small-size molecules of a similar type (*e.g.,* GdDOTA) can be explained with the intermediate size of the dendrimeric contrast agent. Namely, the reduced motion of the Gd-chelates attached to the dendrimer surface increases the rotational correlation time and, hence, r_1; this effect can still be observed at high magnetic fields for smaller nano-sized agents. Otherwise, the increase in rotational correlation time dominantly contributes to r_1 enhancement at low magnetic fields[27]. On the other hand, the size of the dendrimeric contrast agent had a pronounced effect on the transverse relaxivity[28], resulting in the value of 30.5 ± 0.6 $mM^{-1}sec^{-1}$ per Gd(III). In summary, the methods for *in vitro* assessment of DCA are straightforward and require only careful sample preparation, so no difficulties are expected when acquiring data and analyzing the results.

To demonstrate the performance of the dendrimeric contrast agent and its power to affect the image contrast, we performed MRI experiments on tube phantoms with the newly prepared contrast agent DCA. We also used a solution of a commercially available and clinically approved MRI contrast agent, GdDOTA, as a comparison and tubes with water as a control. In the first T_1-weighted MRI experiment, when equal Gd(III) concentrations were used (0.5 or 1 mM of Gd(III) in DCA or GdDOTA), the SNR in the tubes with DCA was already up to 12% higher due to an increase of about 50% in longitudinal relaxivity of DCA compared to GdDOTA (**Figure 5a**). The second T_1-weighted MRI experiment was designed to demonstrate the effect of DCA when the concentrations were calculated per molecule. Although 5 times less DCA was applied compared to GdDOTA (50 vs. 250 µM or 100 vs. 500 µM DCA vs. GdDOTA, respectively), a high loading of DCA with Gd(III) resulted in a significant increment in the image contrast, which in turn resulted in the observed SNR values being at least three times higher in the phantom tubes filled with DCA. Expectedly, both T_2-weighted MRI experiments exhibited large (3-20 times) differences in the SNR between the phantom tubes filled with DCA and GdDOTA.

In conclusion, this protocol describes a convenient preparation of a dendrimeric CA for MRI using common synthetic procedures to provide DCA with improved properties compared to small-size CAs. DCA exhibits preferred thermodynamic stability and kinetic inertness when compared to its monomeric CA analogues. Nevertheless, the multivalency of DCA and, hence, the high local concentration of the paramagnetic species in the target region induces high contrast in the MR images. Considering the often preferable pharmacokinetic properties (*e.g.,* longer tissue retention time) compared to their monomeric CA analogues, or the ability to carry further functionalities (*e.g.,* targeted vectors), these dendrimeric-macrocycle conjugates represent a promising and valuable class of contrast agents for various future MRI and molecular imaging applications.

Disclosures

The authors have nothing to disclose.

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