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New nano-sized biocompatible MR contrast agents based on lysine-dendri-graft macromolecules

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Abstract

Paramagnetic nano-materials for use as magnetic resonance imaging (MRI) contrast agents have higher relaxivity than conventional low molecular weight MRI agents. However, the biocompatibility and pharmacokinetics of such nano-materials will strongly affect the likelihood of clinical approval. We synthesized MRI contrast agents based on biocompatible lysine-dendri-grafts: Gd-BzDTPA-lysineG2 and Gd-BzDTPA-lysineG3. The relaxivity of Gd-BzDTPA-lysineG2 and Gd-BzDTPA-lysineG3 increased with sample temperature, while the relaxivity of Gd-BzDTPA-PAMAMG4 decreased with increasing sample temperature. The increase in relaxivity with increasing temperature may be attributed to inaccessibility of water to the internal Gd chelates with lysine-dendri-grafts, which does not occur with PAMAM dendrimers. Gd-BzDTPA-lysineG3 had a long intravascular half life but were largely excreted by the kidneys. Therefore, Gd-BzDTPA-lysineG3 enhanced the blood vessels for longer periods than Gd-BzDTPA-PAMAMG4, but was still excreted through the kidney. Because of their biocompatibility, desirable magneto-physical characteristics and favorable pharmacokinetics, which are derived from different interior structures rather than the physical size, lysine-dendri-graft MR contrast agents may be feasible for clinical use.

Keywords

Soft nanomaterial; MRI contrast agent; Relaxivity; Lysine-dendri graft; Polyamidoamine dendrimer

Introduction

There are distinct physical and chemical characteristics of nano-sized macromolecules, such as size, charge, and hydrophobicity that greatly influence their behavior, in vivo (1,2). Nano-sized macromolecules can be finely tuned to have optimal characteristics for biological applications (3–5). Although in vivo behavior of nano-sized macromolecules is not easy to be predicted (6), precise physical and biological characteristics of specific nanoparticles must be verified with animal studies.

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Nano-sized magnetic resonance imaging (MRI) contrast agents are attractive because they have numerous attachment sites for chelated Gadolinium (or other chelated lanthanides) and favorable physical properties that lead to higher T1 relaxivity compared with conventional small molecular weight contrast agents. Lysine-dendri-graft is a new nano-sized soft material that is thought to have favorable physico-chemical features, which should increase relaxivity. However, the physico-chemical characteristics, biocompatibility, and pharmacokinetics of such nanoparticles must be determined before clinical translation (7,8).

In this paper, we synthesized two new MRI contrast agents based on generation-2 and -3 lysine-dendri-grafts, and studied their physical and biological characteristics *in vitro* and *in vivo*.

Materials and Methods

Reagents

The poly-L-lysine dendri-grafts (generation-2; G2 and generation-3; G3) were purchased from COLCOM S.A.S. (Montpellier, France). The G2 and G3 dendri-grafts have 50 and 127 terminal primary amino groups, respectively. 2-(4-Isothiocyanatobenzyl)-diethylenetriamine pentaacetic acid (*p*-SCN-BzDTPA) was purchased from Macrocyclics (Dallas, TX). Carrier-free $^{111}\text{InCl}_3$ was purchased from Perkin-Elmer Health Sciences, Inc. (North Billerica, MA). All other chemicals used were of reagent grade. A generation-4 PAMAM dendrimer, which has 64 terminal primary amino groups, was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Conjugation of chelates to the lysine-dendri-grafts

Synthetic scheme is shown in Fig. 1. The poly-L-lysine dendri-graft G2 (1.2 μmol , 1 eq.) was reacted with *p*-SCN-BzDTPA (55.8 μmol , 48 eq.) in a bicarbonate buffer (pH 9, 2 mL) at 40 °C. Following this, *p*-SCN-BzDTPA (55.8 μmol , 48 eq.) was added 48 h later to the reaction solution and maintained at pH 9. The reaction mixture was kept at 40 °C for 48 hours. The resulting solution was purified by diafiltration using Centricon 10 (Millipore, Billerica, MA) to obtain BzDTPA conjugated poly-L-lysine dendri-graft G2 (BzDTPA-lysineG2), and the buffer was changed to PBS. The number of chelates on a dendri-graft was determined using HPLC (column; TSKgel G2000SW (7.8 $\times$ 300 mm, Tosoh Bioscience LLC.), flow rate=0.5mL/min, eluent; PBS (pH 7.2)) by comparing the UV peak area of BzDTPA-lysineG2 and *p*-SCN-BzDTPA. An average of 20 BzDTPA chelates was conjugated to a dendri-graft.

After the buffer was changed to the 0.5 M ammonium citrate (pH 4.8), the BzDTPA-lysineG2 (23 μmol) was reacted with Gd(III) acetate (46 μmol) overnight at 37 °C. Excess Gd(III) was removed by diafiltration using Centricon 10, and the buffer was changed to PBS. The number of chelated Gd was measured by ICP-OES (Columbia Analytical Services, Tucson, AZ). The number of available chelates for binding Gd atom was 17.

Gd-BzDTPA-lysineG3 was prepared in a similar manner as Gd-BzDTPA-lysineG2, by treating G3 poly-L-lysine dendri-graft (0.45 μmol , 1 eq.) with two separated additions of *p*-SCN-BzDTPA (55.8 μmol , 48 eq.). The resulting BzDTPA-lysineG3 (21 μmol) was treated with Gd(III) acetate (42 μmol) to obtain Gd-BzDTPA-lysineG3. The number of chelates on the dendri-graft was 46, and the number of available chelates for binding Gd atom was 38.

Gd-BzDTPA-PAMAMG4 was also prepared as a control by treating PAMAM G4 dendrimer with *p*-SCN-BzDTPA as previously described¹. The number of chelates per dendrimer was 59, and number of available chelates for binding Gd atom was 57. Molecular weights of synthesized Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3 and Gd-BzDTPA-PAMAMG4 are 25.3, 40.4 and 64.5 kDa, respectively.

Size measurement of Gd-BzDTPA-lysineG2 and Gd-BzDTPA-lysineG3

The sizes of Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3 and Gd-BzDTPA-PAMAMG4 were measured by dynamic light scattering (DLS) using a 1 Malvern Zeta Sizer Nano instrument (Malvern Instruments Ltd., Malvern, UK) and by size exclusion HPLC (column; TSKgel G2000SW (7.8×300 mm, Tosoh Bioscience LLC.), flow rate=0.5mL/min, eluent; PBS (pH 7.2)).

Radiolabeling of lysine-dendri-grafts

The Gd-BzDTPA-lysine dendri-grafts (~200 µg) were reacted with carrier-free ^{111}In in 0.15 M NH_4OAc pH 4.25 for 1 hr at room temperature. To remove any non-incorporated free radiometal, 10 µL of 0.1 M EDTA was added and purified using an Amicon Ultra-4 centrifuge with Ultracel-10 (MWCO 10k) (Millipore, Billerica, MA) with an average radiolabeling yield of ~85%.

All radiolabeled [^{111}In]-Gd-BzDTPA dendri-grafts were analyzed by HPLC using TSK 2000SW (Toso Haas) size-exclusion columns connected to Flow-count radio-HPLC detector (Bioscan, Washington, DC, USA). Samples were eluted using 1 × PBS (10 mM phosphate buffer at pH 7.4 in 0.15 M NaCl) at a flow rate of 0.5 mL/min. Radiochemical purity was also assessed using radio-ITLC and was found to be >98%.

Biodistribution of [^{111}In]BzDTPA-lysineG2 and [^{111}In]BzDTPA-lysineG3

All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the NIH Animal Care and Use Committee. The carrier molecule, Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3 or Gd-BzDTPA-PAMAMG4 was added to the radiolabelled solution, and [^{111}In]Gd-BzDTPA-lysineG2 (185 kBq, 300 µg), [^{111}In]Gd-BzDTPA-lysineG3 (185 kBq, 300 µg) or [^{111}In]Gd-BzDTPA-PAMAMG4 (185 kBq, 300 µg) was injected intravenously into female nude mice (National Cancer Institute Animal Production Facility, Frederick, MD). The mice were sacrificed at 15 min, 1, 4 and 24 hr and the organs were weighed and counted in a gamma counter to calculate %ID/g.

Relaxivity

Relaxivity was measured by a custom designed variable field T1–T2 analyzer (Southwest Research Institute, San Antonio, TX) and a 3T MR system (Signa LX, GE Healthcare, Waukesha, WI, USA).

For the variable field analyzer, the field strength was varied from 0.035 to 1.5 T (1.5–62 MHz) and the sample temperature was set at 23 °C. Solutions of Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3, Gd-BzDTPA-PAMAMG4, and Gd-DTPA (Magnevist, Berlex, Princeton, NJ) (1 mM Gd, PBS, pH 7.4) were prepared, and T1 was measured using a saturation recovery pulse sequence with 32 incremental recovery times. T2 was measured using a Carr-Purcell-Meiboom-Gill pulse sequence of 500 echoes and an interecho time of 2 msec. To investigate the temperature dependency, additional measurements were performed at 0.8 T field strength by changing the temperature to 16, 23 and 37 °C.

We also measured the relaxivity at 22 °C using a 3.0T clinical scanner (Signa Excite, GE Healthcare, Waukesha, WI, USA) equipped with a rectangular single loop receiver coil (84 × 126 × 6 mm). Phantom solutions in 1× PBS consisting of 0, 0.1, 0.25, 0.5, 0.75 and 1 mM in Gd(III) together with Gd-DTPA as reference controls were prepared. A series of single-slice 2D inversion recovery (IR) fast-spin echo images of all solutions were obtained at an echo time (TE) of about 7.54 ms using different inversion recovery times (TI) of 50, 100, 350, 750, 1250, 2500, and 5000 ms. The r_1 values for each dilution were determined by fitting ROI intensity

values from variable IR images using Igor Pro (<http://www.wavemetrics.com>). r_2 values were measured from ROI values from T2 maps, which were calculated from the multiecho images in *ImageJ* (<http://rsb.info.nih.gov/ij>) using the MRI analysis plug-in (<http://rsb.info.nih.gov/ij/plugins/mri-analysis.html>). The molar relaxivities, r_1 and r_2 , were obtained from the slope of $1/T_1$ or $1/T_2$ vs $[Gd(III)]$ plots determined from region of interest measurements.

Contrast-enhanced dynamic 3D-micro MRI of mice

All dynamic micro-MRI images were obtained at 3-T with a 1-in. round receive-only modified Alderman-Grant resonators. Mice were chemically restrained with 2% isoflurane (Abbott Laboratories, NJ) in O₂ delivered using a Summit Anesthesia Solutions vaporizer (Bend, OR) at a flow rate of 0.8 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 with body temperature monitored using FOT-M fiber optic sensors (Fiso Technologies Inc., San Jose, CA) and a UMI-8 multichannel instrument (Fiso Technologies Inc.). A tail vein cannula consisting of a 30-gauge needle attached to Tygon tubing (0.01 in i.d) 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 10.888/3.86 ms; flip angles 8° and 24°; bandwidth 31.25 kHz; matrix size 512×128×40; voxel resolution 156×156×600 μm; slice thickness 0.6 mm; two averages; scan time ~2 min). One hundred microliter (100 μL) of the Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3 or Gd-BzDTPA-PAMAMG4 was injected (0.03 mmolGd/kg) from the tail vein. The region of interest was placed on the liver, the cortex and medulla of the kidney, the muscle at the femoral region and the jugular vein, and the time-intensity curves were made. In addition, the slice data were processed into 3D images with the maximum intensity protection (MIP) method.

Results

Size

The size of Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3 and Gd-BzDTPA-PAMAMG4, measured by dynamic light scattering (DLS) was 8.58, 14.59 and 9.28 nm, respectively. The retention time by size-exclusion HPLC was 15.2, 12.4 and 14.3 min for Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3 and Gd-BzDTPA-PAMAMG4, respectively, and the results were matched with the DLS results.

Relaxivity

The r_1 profiles of the Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3, Gd-DTPA and Gd-BzDTPA-PAMAMG4 at 23°C are plotted in Fig. 2. The profiles are similar for Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3 and Gd-BzDTPA-PAMAMG4 and exhibit an increase in peak relaxivity at about 35 MHz as observed previously for macromolecular MRI contrast agents. The low molecular weight contrast agent Gd-BzDTPA exhibits dispersion at around 10MHz and slightly decreasing over 10MHz in this frequency range. All samples exhibited characteristic T_1 and T_2 profiles. Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3 and Gd-BzDTPA-PAMAMG4 had peak relaxivities around 35 MHz (0.8 T). The Gd-BzDTPA-lysineG2 and Gd-BzDTPA showed lowest r_1 values. The r_2 values of Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3 and Gd-BzDTPA-PAMAMG4 increased with frequency as expected. The magnitude of $1/T_2$ for the G3 lysine was greater than G4 PAMAM. This has been attributed to improved access of water to the interior Gd chelates in the lysine-dendri-graft.

The r_1 values of Gd-BzDTPA-lysineG2 and Gd-BzDTPA-lysineG3 increased with sample temperature; in contrast, r_1 decreased with temperature for Gd-BzDTPA-PAMAMG4 and Gd-DTPA. The similar tendency was observed for r_2 values (Fig. 3). The r_1 and r_2 values of Gd-BzDTPA-lysineG2, Gd-BzDTPA-lysineG3, Gd-BzDTPA-PAMAMG4 and Gd-DTPA measured by 3T clinical scanner were 7.53, 6.86, 11.04 and 4.44 /s/mM for r_1 , and 25.72, 26.38, 30.64 and 5.46 /s/mM for r_2 respectively.

Biodistribution of [^{111}In]Gd-BzDTPA-lysineG2, [^{111}In]Gd-BzDTPA-lysineG3 and [^{111}In]Gd-BzDTPA-PAMAMG4

The results of biodistribution experiments are summarized in Fig. 4. High kidney activity for all agents, but was highest for [^{111}In]Gd-BzDTPA-PAMAMG4 and lowest for [^{111}In]Gd-BzDTPA-lysineG2. The liver accumulation was highest for [^{111}In]Gd-BzDTPA-lysineG3. [^{111}In]Gd-BzDTPA-lysineG3 also showed high splenic uptake. The blood clearance was slowest for [^{111}In]Gd-BzDTPA-lysineG3. The clearance from the body was fastest for [^{111}In]Gd-BzDTPA-lysineG2.

3D-micro-MR

The time-intensity curve and MIP images are shown in Fig. 5 and 6, respectively. The blood retention was greatest for Gd-BzDTPA-lysineG3. The profiles of signal intensity in blood were similar for Gd-BzDTPA-lysineG2 and Gd-BzDTPA-PAMAMG4. Relatively high signal intensity was detected in the cortex of the kidneys for Gd-BzDTPA-lysineG2. The signal intensities in the renal pelvis were high for both the G2 and G3 lysine-dendri-grafts, while it was lower for Gd-BzDTPA-PAMAMG4. Gd-BzDTPA-lysineG3 showed higher signal intensity than Gd-BzDTPA-lysineG2 and PAMAMG4 in the liver. The signal in muscle was low for all compounds.

Discussion

An interesting physical characteristic of lysine-dendri-graft based MRI contrast agents is their relaxivity behavior relative to PAMAM-based dendrimers of similar sizes or small molecular weight chelates. Since the Gd-chelates are located on the surface of low-generation PAMAM-based dendrimer contrast agents they constantly interact with water, the rotational correlation time of the chelated Gd ions increases as the temperature decreases leading to an increase in r_1 for these contrast agents. Similar results were previously obtained by Jaszberenyi et al. (9) Low molecular weight gadolinium chelates behave similarly. Conversely, an increase in temperature results in a decrease in r_1 because of the decrease in the rotational correlation time of the single Gd chelate or the Gd chelates on the surface of the low generation dendrimers. In contrast, lysine-dendri-grafts demonstrate higher r_1 s as the temperature increases, as has been previously observed for high-generation PAMAM dendrimers (10). In addition, overall r_1 of the lysine-dendri-graft based MRI contrast agents was lower than that of PAMAM-based dendrimer contrast agents with similar size especially at the low temperature. The lysine-dendri-grafts potentially have terminal amines in the interior structure. Therefore, some of the chelated Gd ions might not be fully accessible by water. As the temperature increases, however, water can access the internal chelates which lead to an increase in relaxivity. Furthermore, the increase in water exchange between the (interior) bound water and the exterior bulk water with rising temperature would also result in an increased relaxivity. However, it is difficult to know the exact extent of the interplay between water accessibility and the water exchange and additional studies are needed over a larger range of lysine-based dendri-graft generations. However, the exchange time of the water bound to the interior Gd chelates and in bulk solution also increases because now the water has to go inside the dendrimer and then back out. With PAMAM, it only has to go to the exterior and then back to the bulk water. So, this increases the water exchange time for the lysine-dendri-graft compared to the PAMAM dendrimer and

increases even further with rising temperature. The effect on increasing the water exchange time is the same effect as increasing the rotational correlation time---an increase in relaxivity. As was observed for the high generation PAMAM, if the water exchange time is increased too much, it actually will start to decrease the relaxivity. Therefore, this unique relaxivity behavior of the contrast agents based on the lysine-dendri-graft compared with the PAMAM-based contrast agent of the similar size could be derived from the less packed, therefore, better flexible interior of the lysine-dendri-grafts than that of PAMAM dendrimer (10,11). The relaxivity change of the macromolecular contrast agents was mostly focused on the size of the molecule because of the rotational correlation time, however, our findings suggested the flexibility of the interior can also affect to the relaxivity change. This theory can be useful for designing nano-sized contrast agents with high relaxivity or chemical exchange saturation transfer (CEST) contrast using various soft nano-materials.

Accelerated renal excretion is important to minimize the potential toxicity of Gd-chelated agents. It is known that excretion from by glomerular filtration primarily depends on molecular size (12). The lysine-dendri-graft based MRI contrast agents are relatively larger in hydro-dynamic physical size than the PAMAM-based dendrimer contrast agents. Nevertheless lysine-dendri-grafts demonstrate renal excretion, an observation likely related to the flexibility of the molecule. In addition, it is known that lysine administration can block renal tubular reabsorption of small-molecular-weight proteins (13,14), and Kobayashi et al. previously reported that lysine co-injection can accelerate the renal excretion of Gd-chelated PAMAMG4 (15). Therefore, Gd-BzDTPA-lysineG3 can visualize the blood vessels longer than Gd-BzDTPA-PAMAMG4, but still can be excreted through the kidney. Since the G8 PAMAM-based dendrimer contrast agent with similar size to Gd-BzDTPA-lysineG3 is not excreted from the kidney, this pharmacokinetic characteristic of lysine-dendri-graft based MRI contrast agents is potentially advantageous.

In conclusion, we have synthesized nano-sized MRI contrast agents using lysine-dendri-graft platforms and compared their physical and pharmacological characteristics with those of a PAMAM-based dendrimer contrast agent of comparable size. Nano-sized MRI contrast agents using lysine-dendri-graft platforms showed different relaxivity characteristics from the PAMAM-based agents of the similar size. In addition to its biocompatibility, preferable physical characteristics and pharmacokinetics of nano-sized MRI contrast agents with lysine-dendri-graft, which are derived from different interior structures rather than the physical size, might make these agents clinically feasible.

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Figure 1.
Synthetic scheme of Gd-BzDTPA-lysineG3.

Figure 2.
Frequency dependency of r_1 (A) and r_2 (B) profiles for each contrast agent at 23 °C.

Figure 3.
Temperature dependency r_1 (A) and r_2 (B) profiles for each contrast agent at 35 MHz.

Figure 4. Biodistribution of [^{111}In]Gd-BzDTPA-lysineG2 (A), [^{111}In]Gd-BzDTPA-lysineG3 (B) and [^{111}In]Gd-BzDTPA-PAMAMG4 (C) in mice.

Figure 5. Plots of the signal intensity in the jugular vein, the liver, the cortex and medulla of the kidney and the muscle at the femoral region obtained from the contrast enhanced dynamic MRI of mice; Gd-BzDTPA-lysineG2 (A), Gd-BzDTPA-lysineG3 (B) and Gd-BzDTPA-PAMAMG4 (C).

Figure 6.

The whole body 3D MR images of mice, which were constructed with the MIP method with preinjection (left), immediately after (middle) and 50 min after (right) injection of Gd-BzDTPA-lysineG2 (A), Gd-BzDTPA-lysineG3 (B) or Gd-BzDTPA-PAMAMG4 (C).