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Gd-Conjugated Dendrimer Nanoclusters as a Tumor-Targeted T1 Magnetic Resonance Imaging Contrast Agent**

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Contrast agents are increasingly being used in diagnostic magnetic resonance (MR) imaging to help detect and characterize pathological abnormalities. In fact, it has been estimated that nearly 50% of all MR examinations already involve the use of MR contrast agents, with chelated gadolinium compounds being by far the most widely used.[1,2] Most clinically relevant Gd-based agents are small, non-targeted compounds that passively distribute into the intravascular and interstitial space.[3] However, there has recently been emerging interest in the development of paramagnetic contrast agents that are capable of probing the molecular profile of tissues via ligand targeting, enzymatic activity and multiplexing.[4,5] It is envisioned that these agents could be used to acquire a more specific clinical diagnosis and thus improve patient management.

To compensate for the low signal enhancement generated by individual Gd ions, most targeted Gd compounds have relied on the development of nanoplateforms that can (1) carry a high payload of Gd and (2) enhance the longitudinal relaxivities (R_1), per Gd. A wide range of macromolecules and other nanoparticulate systems have already been tested as platforms for Gd labeling, including dendrimers,[6–12] polymers,[13] emulsions,[14] silica nanoparticles,[15–17] and vesicles.[18–21] Some of these agents have exhibited relaxivities on the order of 10^5 to 10^6 $\text{mM}^{-1} \text{s}^{-1}$ per nanoparticle.[14,17,18] Since the R_1 for chelated Gd is typically only between 5 and 30 $\text{mM}^{-1} \text{s}^{-1}$ when attached to these nanoparticulate carriers, these contrast agents clearly benefited most from their ability to carry a high Gd payload. Further, since the theoretical maximum R_1 for Gd is estimated to be only ~ 80 $\text{mM}^{-1} \text{s}^{-1}$ (1.5T),[22] it can be argued that any major future improvements in the R_1 per particle will be achieved through the development of nanoplateforms that support higher Gd payloads. Considering that most current nanoplateforms are only labeled with Gd chelates on their outer surface, to ensure high water accessibility, we hypothesized that higher Gd payloads could be achieved through the development of highly porous nanoparticles that contained a high Gd content throughout the intraparticulate volume. Here, we show that this could be accomplished by creating “dendrimer nanoclusters” (DNCs) composed of individual Gd-labeled PAMAM dendrimers that have been cross-linked to form larger nanoparticulate carriers. We also demonstrate that these Gd-labeled DNCs can readily be functionalized with targeting ligands (e.g. folic acid) and used for in vivo molecular

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imaging. A synthetic scheme of a folate-receptor targeted Gd-labeled DNC is shown in Figure 1.

Paramagnetic DNCs were prepared by first crosslinking PAMAM dendrimers (Generation 5) with the homobifunctional amine-reactive crosslinking agent, NHS-PEG-NHS. The presence of the polyethylene glycol (PEG) spacer arm helped maintain the high water solubility of the formed dendrimer clusters. To control nanocluster size, the molar ratio between NH_2 -containing PAMAM dendrimer and NHS-containing BS(PEG)₅ cross-linker was varied. It was found that at a molar ratio of 50:1 [NH_2]:[NHS] it was possible to obtain DNCs with an average hydrodynamic diameter of 150 nm and a relatively narrow size distribution, as determined by dynamic light scattering (DLS) (see Supporting Information, Figure SI-1A). It should be mentioned that non-crosslinked individual dendrimers, with an average diameter of 5.8 nm, were removed through repeated washes on a 100 nm centrifugal filter device. The purified DNCs were labeled with Gd by reacting the functional groups (amines) with the chelating agent diethylenetriaminopentaacetic acid (DTPA)-dianhydride. The resulting paramagnetic DNCs were further functionalized with the optical imaging dye fluorescein isothiocyanate (FITC) and the tumor-targeting ligand folic acid.

Transmission electron microscopy (TEM) confirmed the labeling of the DNCs with Gd (see Supporting Information, Figure SI-1B). Due to the presence of the electron-dense gadolinium ions, DNCs were directly placed on a carbon-coated copper grid and observed without using any additional staining agents, which is often required to enhance the contrast of unmodified dendrimers.[23] The DNCs observed by TEM were approximately spherical in shape and 75–150 nm in diameter. This was slightly smaller than the size measurements acquired by DLS and may reflect the difference between the hydrodynamic diameter, measured by DLS, and the physical diameter, measured by TEM. The smaller average DNC size, based on TEM measurements, could also be a consequence of the limited number of DNCs analyzed in TEM micrographs ($n = 20$).

To assess the paramagnetic properties of the Gd-conjugated DNCs, the amount of Gd within the sample was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The relaxivity was then calculated as the slope of the curves $1/T_1$ vs. Gd concentration, as shown in Figure 2. T_1 -relaxation times were determined using a Bruker mq60 MR relaxometer operating at 1.41 T (60 MHz) and at 40 °C. It was found that Gd-conjugated DNCs had an R_1 relaxivity value of $12.3 \text{ mM}^{-1} \text{ s}^{-1}$ per Gd. This was only slightly higher than Gd-labeled individual PAMAM (G5) dendrimers, which had an R_1 relaxivity of $10.1 \text{ mM}^{-1} \text{ s}^{-1}$ per Gd. It is likely that only a marginal increase in R_1 was observed, despite the larger size of DNCs, due to large amount of internal motion within the DNCs caused by the PEG linkers. “Saturation” of ion relaxivity, which has previously been reported for high generation dendrimers, could also be a contributing factor.[24] As a point of comparison, Gd-DTPA was determined to have an R_1 of $3.9 \text{ mM}^{-1} \text{ s}^{-1}$ per Gd.

In order to determine the R_1 per DNC particle, it was first necessary to determine the number of particles within a given aqueous sample. This was accomplished by using Einstein’s viscosity equation to determine the volume fraction of the DNCs and DLS measurements to determine the average volume of individual DNCs.[25] Subsequent measurements of Gd content in the same samples by ICP-AES revealed that there were approximately 300,000 Gd per DNC. For comparison, individual G5 dendrimers possess a maximum of 128 functional groups for attachment of single Gd chelates. Higher generation dendrimers can, of course, be used to carry higher payloads, but these dendrimers are difficult to synthesize and costly. Further, even a generation 10 dendrimer can only accommodate a maximum of 4096 single Gd chelates.[24] Accordingly, Gd payloads are typically reported to be in the range of 4 to 1860 Gd per dendrimer.[8,24] The dramatic

difference in Gd payload between DNCs and individual dendrimers arises from both the larger size of the DNCs and the ability of DNCs to carry chelated Gd throughout the intraparticular volume, not just on its outer shell. Based on the average Gd content of each DNC and the relaxivity per Gd, it is estimated that the relaxivity per DNC is approximately $3.6 \times 10^6 \text{ mM}^{-1} \text{ s}^{-1}$.

The paramagnetic properties of the DNCs reported here compare very favorably with Gd-based agents that have previously been reported in the literature. For example, Gd-labeled shell-crosslinked nanoparticles (40 nm diameter) exhibit an R1 of $39 \text{ mM}^{-1} \text{ s}^{-1}$ per Gd (0.47 T) but possess only 510 Gd per particle, which results in an R1 of $2 \times 10^4 \text{ mM}^{-1} \text{ s}^{-1}$ per nanoparticle.[26] Paramagnetic silica nanoparticles (~100 nm) have been found to exhibit an R1 of $9.0 \text{ mM}^{-1} \text{ s}^{-1}$ per Gd (4.7 T) and contain 16,000 Gd per nanoparticle, which results in an R1 of $1.4 \times 10^5 \text{ mM}^{-1} \text{ s}^{-1}$ per nanoparticle.[17] Gd-encapsulated porous polymersomes (~125 nm) possess nearly 44,000 Gd per particle and exhibit an R1 of $3.2 \times 10^5 \text{ mM}^{-1} \text{ s}^{-1}$ per nanoparticle.[18] Consequently, all three particles exhibit relaxivities that are significantly lower than the paramagnetic DNCs presented here.

Perfluorocarbon nanoparticles have a reported R1 of $25.3 \text{ mM}^{-1} \text{ s}^{-1}$ per Gd (1.5 T) and 94,200 Gd per particle, which results in an R1 of $2.38 \times 10^6 \text{ mM}^{-1} \text{ s}^{-1}$ per nanoparticle;[14] however, while this relaxivity is similar to that of the 150 nm DNCs, it should be noted that the perfluorocarbon particles are much larger with a diameter of 273 nm. Scaling with volume, a paramagnetic DNC of this size would possess 1,800,000 Gd and exhibit an R1 of $2.2 \times 10^7 \text{ mM}^{-1} \text{ s}^{-1}$.

To confirm the folate receptor-targeting capabilities of the DNCs, KB cells were incubated with DNCs for two hours and subsequently analyzed by fluorescence microscopy. KB cells are known to express the folate receptor and are often used as a model cell line to evaluate the targeting capabilities of folic acid-labeled nanoparticles. Note that all of the DNCs were labeled with FITC, in addition to the folic acid and Gd-DTPA. Indicative of cell labeling, all of the KB cells exhibited a bright fluorescent signal (Figure 3). To verify that uptake of the DNCs was mediated through folate-receptor-dependent targeting; competitive inhibition studies were conducted by adding DNCs to cell cultures in the presence of excess free folic acid. Under these conditions, fluorescence was significantly reduced. Moreover, when folate-targeted DNCs were incubated with NIH 3T3 cells, which are negative for the folate receptor, very little cellular fluorescence was observed (see Supporting Information, Figure SI-2). These findings confirm that cellular binding of DNCs was specifically mediated by the folate receptor.

Cell labeling with DNCs was further assessed by acquiring T1-weighted magnetic resonance (MR) images of KB cells that were pelleted in PCR tubes, following incubation with DNCs in the presence and absence of free folic acid (see Supporting Information, Figure SI-3). It was found that KB cells that were incubated with DNCs alone exhibited a significant enhancement in MR signal intensity, compared with unlabeled cells. Conversely, cells that had been incubated with DNCs in the presence of excess free folic acid only exhibited a slight increase in signal intensity, indicating that the free folic acid was able to specifically block the binding of the DNCs. These results further confirm that DNCs can efficiently bind KB cells via the folate receptor.

Prior to evaluating DNCs in living subjects, their cytotoxic effects were examined in a MTT cell proliferation assay (where MTT is 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide). Specifically, various concentrations of the folate receptor-targeted DNCs were incubated with KB cells, which are known to express the folate receptor, and NIH 3T3 cells which do not, for 24 hours. The data indicate the cell viabilities for each cell type

normalized to a control cell sample that was not incubated with any DNCs (see Supporting Information, Figure SI-4). In general, DNCs did not have much of an effect on the viability of NIH 3T3 cells up to a Gd concentration of 5 mM (i.e. ~90% viability). However, Gd concentrations of 0.1 mM and above did seem to have an effect on the viability of KB cells, with viability falling to ~66% at Gd concentrations of 5 mM. It is suspected that the observed level of KB cell death is attributable to the high driving force for cell internalization, imparted by the folic acid on the DNCs, and the long incubation time (24 hours).

To examine whether DNCs could be used to effectively identify folate-positive tumors in living subjects, axial MR images of mice with subcutaneous KB cell xenografts were acquired precontrast and at various times after intravenous (i.v.) injection of DNCs (0.3 mmol Gd/kg) (Figure 4). In the precontrast images, there was little intrinsic contrast between the implanted KB tumors and surrounding muscle. At 1 hour following administration of the DNCs, a slight contrast enhancement was observed within the KB tumor. The signal enhancement increased significantly by 4 hours and by 24 hours the signal within the tumor was extremely bright and the boundary of the tumor was clearly demarcated.

Control experiments to assess specificity were performed by i.v. injection of DNCs in the presence of free folic acid (50 mM), into mice with subcutaneous KB cell xenografts. In these animals, a slight enhancement in signal within the tumor was observed; however, the signal was clearly lower than when DNCs were administered alone for each of the time points studied. Similarly, when DNCs were injected into mice with folate receptor-negative tumors (i.e. subcutaneous T6-17 cell xenografts), very little enhancement in contrast was observed between pre- and postcontrast images. As an additional control, we also compared the signal enhancement of DNCs to Gd-labeled G5 dendrimers, which were also functionalized with folic acid and administered at the same Gd dose. In agreement with previous reports, the targeted dendrimers did exhibit a slight enhancement in signal within the KB tumor, compared the precontrast images; however, the signal was noticeably lower than that observed with DNCs. Quantitative analysis of the MR images is presented in Figure 5. These results confirm that the presence of free folic acid led a statistically significant reduction in DNC binding to KB tumor cells ($p < 0.05$), confirming the specificity of the folate-receptor targeting. The residual signal that was observed even in the presence of folic acid (i.e. relative signal enhancement > 1) is suspected to be due to incomplete blocking of the folate receptor (especially considering the rapid clearance of free folic acid) and because of the enhanced permeability and retention effect within the KB tumor. Analysis of the MR images also revealed that DNCs exhibited a statistically significant improvement in image contrast compared with targeted dendrimers ($p < 0.05$).

In conclusion, we have provided a facile method for the synthesis of nanometer-sized dendrimer nanoclusters. Due to the many chemically functional amine groups present within DNCs, a high capacity for Gd labelling was achieved via simple and efficient DTPA chelation. Further, we have demonstrated the utility of these DNCs as optical and MR imaging contrast agents for the in vitro and in vivo detection of tumor cells overexpressing the folate receptor. By conjugating appropriate cancer-targeting ligands, the ultrasensitive MR detection of various types of cancers may be possible. Therefore, we believe that the DNCs described here provide a powerful new platform for the early detection of disease. Of course, before any new Gd-based contrast can be extended to the clinic their toxicological profile must be carefully evaluated, especially with the increasing concerns over Gd-mediated toxicity (i.e. Nephrogenic Systemic Fibrosis). This is particularly relevant for Gd-labeled nanoparticles, which generally exhibit longer circulation and residence times compared with chelated Gd. As a consequence, future work will be aimed at developing biodegradable dendrimers and/or crosslinkers that can be eliminated from the body in a

reasonable period of time after carrying out their diagnostic function. Efforts will also be made to reduce the size distribution of the DNCs. It is likely that DNCs within the reported population exhibited size-dependent variability in their retention, biodistribution and mechanism of nanoparticle uptake. In general, it is preferable to have a more monodisperse sample with uniform pharmacokinetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Bellin MF. *Eur J Radiol.* 2006; 60:314–323. [PubMed: 17005349]
2. Shellock FG, Kanal E. *JMRI-J Magn Reson Imaging.* 1999; 10:477–484.
3. Caravan P, Ellison JJ, McMurry TJ, Lauffer RB. *Chem Rev.* 1999; 99:2293–2352. [PubMed: 11749483]
4. Major JL, Meade TJ. *Accounts Chem Res.* 2009; 42:893–903.
5. Song Y, Kohlmeir EK, Meade TJ. *J Am Chem Soc.* 2008; 130:6662–6663. [PubMed: 18452288]
6. Boswell CA, Eck PK, Regino CAS, Bernardo M, Wong KJ, Milenic DE, Choyke PL, Brechbiel MW. *Mol Pharm.* 2008; 5:527–539. [PubMed: 18537262]
7. Kobayashi H, Brechbiel MW. *Adv Drug Deliv Rev.* 2005; 57:2271–2286. [PubMed: 16290152]
8. Langereis S, de Lussanet QG, van Genderen MHP, Meijer EW, Beets-Tan RGH, Griffioen AW, van Engelshoven JMA, Backes WH. *NMR Biomed.* 2006; 19:133–141. [PubMed: 16450331]
9. Langereis S, Dirksen A, Hackeng TM, van Genderen MHP, Meijer EW. *New J Chem.* 2007; 31:1152–1160.
10. Rudovsky J, Botta M, Hermann P, Hardcastle KI, Lukes I, Aime S. *Bioconjugate Chem.* 2006; 17:975–987.
11. Swanson SD, Kukowska-Latallo JF, Patri AK, Chen CY, Ge S, Cao ZY, Kotlyar A, East AT, Baker JR. *Int J Nanomed.* 2008; 3:201–210.
12. Zhu WL, Mollie B, Bhujwalla ZM, Artemov D. *Magn Reson Med.* 2008; 59:679–685. [PubMed: 18302223]
13. Duarte MG, Gil MH, Peters JA, Colet JM, Vander Elst L, Muller RN, Geraldes C. *Bioconjugate Chem.* 2001; 12:170–177.
14. Morawski AM, Winter PM, Crowder KC, Caruthers SD, Fuhrhop RW, Scott MJ, Robertson JD, Abendschein DR, Lanza GM, Wickline SA. *Magn Reson Med.* 2004; 51:480–486. [PubMed: 15004788]
15. Lin YS, Hung Y, Su JK, Lee R, Chang C, Lin ML, Mou CY. *J Phys Chem B.* 2004; 108:15608–15611.
16. Rieter WJ, Kim JS, Taylor KML, An HY, Lin WL, Tarrant T, Lin WB. *Angew Chem-Int Edit.* 2007; 46:3680–3682.
17. Santra S, Bagwe RP, Dutta D, Stanley JT, Walter GA, Tan W, Moudgil BM, Mericle RA. *Adv Mater.* 2005; 17:2165–2169.
18. Cheng ZL, Tsourkas A. *Langmuir.* 2008; 24:8169–8173. [PubMed: 18570445]
19. Hak S, Sanders H, Agrawal P, Langereis S, Grull H, Keizer HM, Arena F, Terreno E, Strijkers GJ, Nicolay K. *Eur J Pharm Biopharm.* 2009; 72:397–404. [PubMed: 18940253]
20. Terreno E, Castelli DD, Cabella C, Dastru W, Sanino A, Stancanella J, Tei L, Aime S. *Chem Biodivers.* 2008; 5:1901–1912. [PubMed: 18972531]
21. Unger EC, Macdougall P, Cullis P, Tilcock C. *Magn Reson Imaging.* 1989; 7:417–423. [PubMed: 2811620]
22. Caravan P, Farrar CT, Frullano L, Uppal R. *Contrast Media Mol Imaging.* 2009; 4:89–100. [PubMed: 19177472]
23. Jackson CL, Chanzy HD, Booy FP, Drake BJ, Tomalia DA, Bauer BJ, Amis EJ. *Macromolecules.* 1998; 31:6259–6265.

24. Bryant LH, Brechbiel MW, Wu CC, Bulte JWM, Herynek V, Frank JA. *JMRI-J Magn Reson Imaging*. 1999; 9:348–352.
25. Reynolds F, O'Loughlin T, Weissleder R, Josephson L. *Anal Chem*. 2005; 77:814–817. [PubMed: 15679348]
26. Turner JL, Pan DPJ, Plummer R, Chen ZY, Whittaker AK, Wooley KL. *Adv Funct Mater*. 2005; 15:1248–1254.

Figure 1.

Schematic diagram illustrating paramagnetic targeted dendrimer nanoclusters (DNCs). Nanoclusters were fabricated by crosslinking polyamidoamine (PAMAM) dendrimers (G5) using a bifunctional amine-reactive crosslinker. Following DNC formation, paramagnetic Gd^{3+} ions were conjugated to DNCs via DTPA. The resulting paramagnetic DNCs were further functionalized with the tumor-targeting ligand folic acid and the fluorescence dye FITC.

Figure 2. Relaxivity (R_1) measurements for Gd-labeled DNCs and Gd-DTPA. T_1 measurements were acquired with a Bruker mq60 MR relaxometer operating at 1.41 T (60 MHz) at 40 °C.

Figure 3.

Cellular uptake of fluorescently labeled DNCs. Phase contrast and fluorescent images of (A&B) untreated KB cells; (C&D) KB cells following 2 hours incubation with FITC-labeled DNCs. The DNCs are also labeled with folic acid and Gd-DTPA; (E&F) KB cells following 2 hours incubation with FITC-labeled DNCs in the presence of 5 mM free folic acid.

Figure 4.

Magnetic resonance images of nude mice with subcutaneous tumor xenografts at various time points following the intravenous injection of Gd-contained nanoparticles. The local hyperintensity generated by the Gd-contained nanoparticles was visualized using a 4.7 T small animal MR. The top row shows mice with KB cell tumor xenografts before and after i.v. injection with folate receptor-targeted DNCs. The middle row shows mice with KB cell tumor xenografts before and after i.v. injection with folate receptor-targeted DNCs in the presence 50 mM free folic acid (FA). The third row shows mice with KB cell tumor xenografts before and after i.v. injection with folate receptor-targeted, Gd-labeled dendrimers (individual dendrimers, G5). The bottom row shows mice with T6-17 cell tumor xenografts (i.e. folate receptor-negative) before and after i.v. injection with folate receptor-targeted DNCs. Images were acquired pre-injection and 1, 4 and 24 hr post-injection. White arrow shows location of tumor.

Figure 5.

Quantitative analysis of MR images. The average MR signal intensity (SI) was measured for each tumor and the relative signal enhancement, rSE, was then calculated as the quotient of the SI in the 24 hr post-contrast image and the pre-contrast image. A t-test (two-tailed, unequal variance) was used to compare the rSE for each group of animals. A $p < 0.05$ was considered statistically significant and is indicated by an asterisk.