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Manganese G8 Dendrimers Targeted to Oxidation-Specific Epitopes: *In vivo* MR imaging of Atherosclerosis

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Abstract

Objective—To determine if manganese (Mn) G8 dendrimers targeted to oxidation-specific epitopes (OSE) allow for *in vivo* detection of atherosclerotic lesions.

Background—OSE have been identified as key factors in atherosclerotic plaque progression and destabilization. Mn offers a potentially clinically translatable alternative to gadolinium-based agents when bioretention and potential toxicity of gadolinium is anticipated. However, to be effective, high payloads of Mn must accumulate intracellularly in macrophages. It was hypothesized that G8 dendrimers targeted to OSE may allow delivery of high Mn payloads, thereby enabling *in vivo* detection of macrophage rich plaques.

Methods—G8 dendrimers were modified to allow conjugation with MnDTPA (758 Mn ion) and the antibody MDA2 that is targeted to malondialdehyde (MDA)-lysine epitopes. Both the untargeted and targeted G8 dendrimers were characterized and their *in vivo* efficacy evaluated in apoE^{-/-} mice over a 96-hour time period after bolus administration of a 0.05 mmol Mn/kg dose using a clinical MR system (3 Tesla).

Results—Significant enhancement (normalized enhancement >60%, p=0.0013) of atherosclerotic lesions was observed within a 72-hour time period following administration of the targeted dendrimers. The presence of Mn within atherosclerotic lesions was confirmed using spectroscopic methods (>8 µg Mn/g). Limited signal attenuation (<18%) and Mn deposition (< 1 µg Mn/g) was observed in the arterial wall following injection of the untargeted material.

Conclusions—This study demonstrates that manganese labeled dendrimers, allowing a high Mn payload, targeted to OSE may allow *in vivo* image of atherosclerotic lesions.

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Keywords

G8 Dendrimers; manganese; Oxidation-Specific Epitopes

Introduction

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality in developed nations (1). In the United States, the majority of annual CVD-related deaths are attributed to coronary heart disease and stroke, complications of atherosclerotic lesions. New therapies and interventions are emerging that may promote lesion regression and/or prevent the destabilization of high-risk atherosclerotic lesions (2,3). In order to evaluate the efficacy of these emerging therapies, non-invasive diagnostic imaging techniques that surpass morphologic and anatomical evaluation are required. It is widely accepted that the progression and destabilization of a lesion is more closely associated with cellular composition and molecular inflammatory mediators than the degree of luminal narrowing (4). High-resolution magnetic resonance imaging (MRI) is a promising diagnostic imaging modality for the characterization of plaque morphology (5-7). In order to detect molecular mediators of atherosclerosis, MR molecular imaging probes which target cellular and molecular events in atherosclerotic lesions are required (8).

Oxidation of lipids in the vessel wall is a key factor in the pathogenesis and de-stabilization of atherosclerotic plaques (9,10). The immune response to the generation of oxidation-specific epitopes (OSE) creates chronic inflammatory responses. In atherosclerotic plaque progression, oxidized phospholipids promote expression of chemoattractants and adhesion molecules on endothelial cells resulting in recruitment of monocytes (11). Since OSE are immunogenic, unique monoclonal antibodies have been identified and isolated from both mice and humans. For example, the murine monoclonal antibodies MDA2 that specifically binds to malondialdehyde (MDA)-lysine epitopes and E06 that binds to the phosphocholine head group of oxidized phospholipids, and human antibody IK17 that binds to an advanced MDA epitope, have been validated to image atherosclerotic lesions (5,12-15). The clinical relevance of OSE as targets for imaging high-risk plaques has been demonstrated by the strong correlation between the presence of OSE and advancing and ruptured plaques (10,16).

The current work builds upon studies that used oxidation-specific antibody (MDA2, E06, IK17) labeled gadolinium (Gd) micelles to allow in vivo diagnostic imaging of OSE and macrophage enriched atherosclerotic lesions (12-14). However, bioretention and biotransformation of Gd was observed in the liver, suggesting potential toxicity. Recent work with targeted (i.e. containing oxidation-specific antibodies) Mn-micelles suggests that intraplaque macrophages are able to effectively degrade and release the paramagnetic ions from the particle (12). Since cells are able to safely excrete intracellular Mn, toxicity issues associated with bioretention and transmetallation are expected to be nominal as long as the Mn remains chelated in the bolus/vascular phase. Maximizing the payload of Mn to macrophage-rich atherosclerotic lesions may allow optimization of imaging characteristics on this approach.

Poly(amido amine) (PAMAM) dendrimers are synthetic macromolecules composed of repeating beta-alanine subunits. The rationale for using dendrimers is related to the ability to load large amounts of Mn while keeping the particle size small enough (<25 nm) to allow for luminal diffusion and intraplaque macrophage uptake. Reported studies show that dendrimers can be easily modified to allow for the addition of both paramagnetic ions (such as GdDTPA) and antibodies for targeted delivery (17,18). Since in vivo MR efficacy is directly related to the delivery of Mn payloads, it was hypothesized that dendrimers would be ideal carriers since generation eight dendrimers (G8) contain 1024 primary amine groups that may be modified and labeled with MnDTPA. This would be a significant improvement over the reported GdDTPA and MnDTPA micelles where there are only ≈ 50 gadolinium or manganese ions per micelle (14).

The use of DTPA as a chelating agent for Mn was not random. DTPA will completely bind Mn so that there are no exchange sites available for interaction with water protons ($q=0$). Initial studies using MnDTPA show adequate stability in the vascular phase and complete de-chelation after intracellular macrophage uptake (12). Although cyclic chelating agents, such as DOTA, may increase the vascular stability (thermodynamic stability constant of MnDOTA = 19.89) it is uncertain if the high affinity to Mn would limit intra-cellular de-chelation. Additionally, since the thermodynamic stability of MnDTPA ($K_s=15.6$) is comparable to that of Omniscan (GdDTPABMA, GE Health Care, $K_s=16.8$), the use of DTPA as a chelating agent was considered adequate for the current study (30). The $q=0$ along with the thermodynamic stability is advantageous from both a safety and efficacy point of view. A strong chelator is required to keep Mn bound to the particle in the vascular/bolus phase so that: 1) cardiotoxicity is nominal in the bolus phase and (2) the MR signal from background blood is low due to the limited MR efficacy of $q=0$ materials (r_1 values are low). Once delivered into the target cell, Mn is released and strong MR enhancement will be observed due to both increasing q values ($q \gg 1$) and the interaction with intracellular components and metalloproteins.

Therefore, the aim of the current study is to synthesize, characterize, and evaluate the MR efficacy of Mn dendrimers targeted to oxidation-specific epitopes in murine models of atherosclerosis.

Materials and Methods

Synthesis

G8 PAMAM Dendrimers (Sigma-Aldrich) were concentrated to 10 mg/ml and diafiltrated against 0.1 M sodium phosphate buffer (pH 9) as described previously (19). A 256-fold molar excess of isothiocyanatobenzyl diethylenetriaminepentaacetic acid (ITC-Bz-DTPA, (Macrocyclics) was added, and the solution was incubated at 40°C and for 48 hours (maintained at pH 9). Note that an excess of ITC-Bz-DTPA was added in order to ensure optimal labeling, as described previously (19). The solution was then purified by diafiltration using a Centricon 30 (Amicon Co., Beverly, MA). Stoichiometric amounts of $MnCl_2$ were then added and a 1 mole equivalent of tetramethyl rhodamine-ITC (TRITC) was added drop wise to the G8-MnDTPA in DMSO. The resultant G8 MnDTPA-TRITC-dendrimer was then purified (diafiltration), lyophilized, and stored at -40°C under N_2 to

prevent oxidation of the Mn. Based upon elemental analysis, 75 % (768 MnDTPA/1024 surface amines) of the available amines on the dendrimer surface covalently attached to MnDTPA. In addition, 10 % (102 TRITC/1024) of the surface amines were covalently attached to the fluorescent probe.

The remaining 154 surface amine groups associated with the G8 dendrimer were used to covalently link the targeting antibody to the dendrimer via interaction between thiol and maleimide. In short, this was performed by adding sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido) hexanoate (sulfo-LC-SPDP, 0.13 mg/ml, Fisher Scientific) to 5 mg/ml of the modified dendrimer solution in HEPES buffer. The sample was then placed on a bench top mixer for 3 hours at room temperature. Following incubation, excess sulfo-LC-SPDP was removed via ultra-centrifugation (4000 rpm, 4°C) using 10 mM EDTA added to HEPES buffer at pH 7. Filters with a molecular weight cut off (MWCO) of 10,000 were used. To cleave the disulfide bond and create a thiol group for binding, 75ml of 10 mM dithiothreitol (DDT) was added to the solution and allowed to react for 45 minutes at room temperature.

The murine antibody (MDA2) was covalently attached to the surface of the dendrimers to allow the specific targeting of oxidation-specific epitopes present in vulnerable plaque. (12-14). Briefly, MDA2, is a murine IgG monoclonal antibody that binds to malondialdehyde (MDA)-lysine epitopes present on OxLDL. The antibody was >99% pure and binding specificity was confirmed using in vitro chemiluminescent enzyme-linked immunosorbent assay (ELISA). MDA2 was modified by mixing 1.8 mg antibody with Sulfo-SMCC solution (108 µL; 15mM, Fisher Scientific) in HEPES buffer pH 7. The sample was incubated at room temperature for one hour with periodical stirring. Excess Sulfo-SMCC was removed via ultra-centrifugation using HEPES buffer and filters with MWCO of 10,000 MWCO. The antibody intermediate was then immediately added to the G8 MnDTPA-TRITC-dendrimer and the conjugation was allowed to proceed overnight at 4 °C. The reaction was quenched using a 50 molar excess of N-ethylmaleimide (505 µL; 245.3 mM, Fisher Scientific). The resulting antibody-dendrimer conjugate was washed twice with HEPES-EDTA buffer solution and concentrated down to 10 mM Mn. To remove any small amounts of antibody that may have aggregated during the binding process, the product solution was passed through a 220 nm Fisherbrand syringe filter. The solution was then stored under nitrogen at 4 °C to avoid oxidation of Mn. A schematic illustration of the synthesis is shown in Figures 1A-B.

Characterization

The untargeted and targeted (i.e. containing MDA2) dendrimers were characterized with respect to Mn content, hydrated particle size, in vitro MR efficacy, binding specificity and in vitro macrophage uptake.

Total Mn Content—The total Mn content of all formulations was determined using inductively coupled plasma mass spectrometry (ICP-MS, Cantest Ltd, Burnaby, British Columbia)(12).

Particle size—Dynamic light scattering was used to determine the mean hydrated particle diameter at 25° C in HEPES buffer (Malvern Instruments, Malvern, UK). All particle diameters were reported as the number weighted average.

In vitro MR efficacy—The longitudinal (r_1) and transverse (r_2) relaxivities were determined in HEPES buffer, wild type mouse blood and apoE^{-/-} mouse blood at 60 MHz and 40° C (Bruker Minispec). The longitudinal (R_1) and transverse (R_2) relaxation rates were determined at the following concentration levels using an inversion recovery sequence (15 different inversion times) or CPMG spin echo sequence (echo time of 1 ms), respectively: 0.01 mM, 0.015 mM, 0.02 mM, 0.03 mM and 0.04 mM. All relaxivity values were obtained as the slope associated with a linear fit of Mn concentration (mM Mn) versus R_1 (s^{-1}) or R_2 (s^{-1}).

In addition, the effect of the applied magnetic field strength on the longitudinal relaxivity, as defined by the nuclear magnetic relaxation dispersion (NMRD) profile, was determined for the untargeted material in water at 25°C. All NMRD profiles were obtained over a frequency range of 2 MHz to 61 MHz using the relaxometer at the National Institutes of Health (Bethesda, MD, USA), as described previously (19).

Binding Efficacy—The attachment of antibodies to the surface of the dendrimers was evaluated using a chemiluminescent enzyme-linked immunosorbent assay and reported as relative light units (RLU) per 100 milliseconds. Untargeted and targeted MDA2 dendrimers were plated directly to microtiter wells and the presence of MDA2 was determined with alkaline phosphatase-labeled goat anti-mouse IgG antibodies. The binding affinity obtained for the targeted dendrimers was then compared to the results reported for the unconjugated MDA2 antibody (12,14).

In vitro macrophage uptake—Previous in vitro studies have shown that oxidation-specific epitope targeted micelles exhibit increased macrophage uptake, particularly when pre-incubated with the MDA-LDL (12-14). Since the dendrimers do not contain a surfactant, such as PEG, unspecific untargeted uptake may occur. For this study, 8th generation J774A. 1 macrophages were plated in a medium of 10% fetal bovine serum (FBS) in PBS containing antibiotics penicillin and streptomycin. When confluent, the cells were activated with oxidized-LDL overnight, according to reported methods (12-14). The cells were then washed with fresh medium, scraped from the plates, and divided equally into three plates (six wells) plates. One plate of cells was aliquoted as a control, one for targeted material, and one for untargeted dendrimer. The targeted and untargeted dendrimers (83.3 μ L; 12mM) were added to the wells so that the concentration reached 1mM Mn. The cells were then allowed to incubate overnight at 37°C. Following incubation, the media was removed and the cells washed with PBS. After washing, 1-ml of PBS was added and the cells scrapped, transferred to centrifuge tubes, and centrifuged at 1500 rpm (room temperature) for ten minutes. The cells were then re-suspended PBS and counted using a hemocytometer.

Cell viability tests were performed using trypan blue dye. Cells suspended in PBS were mixed with 0.04% trypan blue and incubated for three minutes before counting on a hemocytometer. The percentage of viable cells, relative to the total number of cells, was

then determined. Mn content in the counted cells was determined by ICP-MS and the total concentration normalized to cell number.

Animal Model

Eight to ten month old apoE^{-/-} mice on a C57BL/6 background were used for all studies. All apoE^{-/-} mice were placed on a high-cholesterol diet (0.2% total cholesterol, Harlan Teklad, Madison, Wisconsin) *ad libitum* beginning at 6 weeks until 32-40 weeks of age. The ethics committee at Mount Sinai approved all of the animal experiments. All mice were randomized into each of the groups upon arrival from the vendor.

Pharmacokinetics and Biodistribution

The blood half-lives were determined in age matched apoE^{-/-} and WT mice. Animals were administered 0.05 mmol Mn/Kg via tail vein injection and blood was drawn via femoral vein puncture (into heparinized tubes) over a 24 hour time period post injection (n=3 mice per group at 5 minutes, 15 minutes, 30 minutes, 1 hour, 3 hours, and 24 hours post injection). ICP-MS was used to determine the amount Mn present in the blood as function of time post injection. The blood half-life was calculated from the resultant manganese concentration versus time curves using standard non-compartmental bi-exponential pharmacokinetic analysis.

The uptake into the liver, spleen, kidney and aorta was also determined in apoE^{-/-} mice (n=3 mice per group at 24 hours, 48 hours and 72 hours post injection). Mice were sacrificed using compressed CO₂, saline perfused, and the tissue excised, cleaned and weighed. The percent-injected dose was determined based upon the Mn concentration per gram wet organ weight.

MR Imaging

All MRI was performed using a clinical 3 Tesla hybrid time-of-flight PET/MR system (Philips Gemini MRI) and a specialized mouse coil in the prone position, as recently described (12-14). All mice were sedated using isoflurane anesthesia (1-2%) for the duration of the scans. Mice underwent a pre-injection MR scan within 24 hours prior to the administration of the untargeted (n=3) or targeted dendrimers (n=4). MR imaging was then performed over a 72-hour time interval after tail vein injection of a 0.050 mmol Mn/Kg dose. No adverse clinical signs indicative of toxicity (with respect to survival and behavior) were observed during or after injection.

MR imaging of the abdominal aorta was performed using a T1-weighted black blood spin-echo sequence (TR/TE = 667 ms/9.9 ms, number of averages =10, FOV=2.5 cm × 2.5 cm, slice thickness = 0.5 mm, 30 slices, and total scan time of 59 minutes) with a micro-scale in-plane resolution of 0.15 mm². Black blood sequences were required in order to allow for delineation of the arterial wall, as reported earlier (12,14). At each time point post injection, the slices were matched to the baseline pre-injection scans by using the unique vertebral anatomy and paraspinal muscular anatomy as anatomic landmarks. In order to evaluate the MR data, signal intensity (SI) measurements were obtained using regions of interest (ROIs) within the aortic wall on slices (n>3) exhibiting signal modulation post contrast using Osirix

software (Pixmeo Geneva, Switzerland). SI measurements of adjacent muscle and the standard deviation associated with noise were also obtained. The percent-normalized enhancement (%NENH), relative to muscle, was then determined according to reported methods. The %NENH values reflect the percent relative change in the contrast to noise ratios (CNR) as a function of time post injection.

Statistics

Student t-tests (two-tailed, paired, 95% confidence interval) were used to determine significant differences between paired variables and one-way ANOVA was used to evaluate differences between the untargeted and targeted dendrimers. $P < 0.05$ was considered significant.

Results

Characterization

The hydrated particle diameters associated with the untargeted and targeted were 11.6 ± 0.1 nm 13.34 ± 1.2 nm, as shown in Table 1. Schematic representations of the untargeted and targeted formulations are shown in Figure 2. The hydrated diameter associated with these formations is similar to that of endogenous low density lipoproteins. Reported studies have indicated that rigid particles exhibiting sizes less than 25 nm are able to easily diffuse through the aortic endothelial tight junctions associated with plaque (13). This is critical in pre-clinical studies, since apoE^{-/-} mice exhibit limited plaque neo-vascularization so that intraplaque macrophage uptake is primarily dependent upon arterial wall diffusion. The size of the targeted dendrimers is also similar to the size of the gadolinium micelles that have been used to target oxidation-specific epitopes. Unlike the micelles, however, the dendrimers are able to deliver a much greater payload of paramagnetic metal ions (300 for micelles, 768 for G8 dendrimers).

The in vitro MR efficacy of the dendrimer formulations is shown in Table 1. No significant differences in the relaxation properties were observed between the untargeted and targeted dendrimers in buffer and wild type mouse blood. However, in apoE^{-/-} mouse blood significant differences ($p > 0.05$) were observed. This data is consistent with reported data that shows interaction between MDA2 micelles and endogenous oxidation-specific epitopes in ApoE₂ blood (12-14). The NMRD profile (Figure 3) is indicative of a coordinatively saturated outer-sphere relaxivity at pH=7. As a result, the observed r_1 values are higher in the dendrimer ($3.4 \text{ mM}^{-1}\text{s}^{-1}$, Table 1) than uncoordinated MnDTPA solutions ($1.1 \text{ mM}^{-1}\text{s}^{-1}$ (28)) at 60 MHz).

Enzyme-linked immunosorbent assay (ELISA) showed that MDA2 was successfully conjugated to the dendrimers without effecting OxLDL binding specificity (untargeted dendrimers 50 ± 10 RLU and MDA2 dendrimers 955 ± 150 RLU, $P < 0.001$). ICP-MS results showed limited uptake of the untargeted dendrimers in J744A.1 macrophages (below detection limits of the method). However, significant uptake was observed for the targeted dendrimers with concentrations ranging from 0.8 to 1 pg Mn/cell. Cell viability test showed no significant variation in viable cell numbers after incubation with untargeted dendrimers

(77.8%), targeted dendrimers (74.3%), or control saline (62.6%). This finding is consistent with reported studies that show macrophages are able to effectively metabolize and exocytose Mn without significant changes in cell viability or function (phagocytosis of bacteria)(12,20,21).

Pharmacokinetics and Biodistribution

The blood half-life was determined in wild-type and apoE^{-/-} mice injected with targeted and untargeted materials. For the wild type mice, the half-life was determined to be 52 ± 6 and 51 ± 5 minutes for the untargeted and targeted dendrimers, respectively. In apoE^{-/-} mice, the targeted dendrimers exhibited extended circulation times relative to the untargeted material (176 ± 10 minutes and 50 ± 7 minutes, respectively). This data is consistent with previously published studies that show extended circulation times for OxLDL targeted gadolinium micelles in apoE^{-/-} mice (12). Although the exact mechanism responsible for extended circulation times is not known, it is hypothesized that the targeted particles bind oxidation-specific epitopes present in the plasma thereby extending the half-life. The in vivo data correlates well with the in vitro data that strongly suggests interaction between circulating OxLDL and the MDA2 dendrimers.

Biodistribution studies conducted on the aorta, kidney, spleen, and liver following administration to apoE^{-/-} mice show strong uptake of the targeted dendrimers within the arterial wall, as shown in Figure 4. The variation in the uptake and excretion kinetics between the untargeted and targeted materials within the kidney, liver and spleen are indicative of the effect of the antibody on the pk and excretion kinetics of the dendrimers.

MR Imaging

Representative MR images obtained prior to and after the administration of 0.05 mmol Mn/Kg are shown in Figure 5. Although limited enhancement was observed at 48 hours following administration of the untargeted material ($16 \pm 2\%$), no significant enhancement was observed 72 hours post injection of the untargeted materials. The targeted dendrimers exhibited significantly greater arterial wall enhancement at all time points post injection, relative to the untargeted material (Figure 6). The maximum arterial wall enhancement was observed 48 hours post injection ($62 \pm 3\%$), with residual signal present 72 hours post injection. Reported MDA2-Gd-micelles show maximum arterial wall enhancement at 72 hours with the signal persisting for over 3 weeks post injection (5,12). The variation in the wall enhancement is likely due to both differences in the pharmacokinetics (half-life of MDA2-Gd-micelles in apoE^{-/-} mice is 15 hours), as well as the rate of intraplaque macrophage metabolism and excretion. Reported studies have shown that intracellular metabolism of the gadolinium micelles results in the formation of insoluble gadolinium salts that are present within the cell even after the lipid components are excreted by the cell (5,12). As a result, prolonged MR enhancement was observed. Since Mn is endogenous and easily metabolized, prolonged arterial wall enhancement was not observed.

The presence of Mn within atherosclerotic lesions was confirmed using spectroscopic methods ($>8 \mu\text{g Mn/g}$ vs. $< 1 \mu\text{g Mn/g}$, $P<0.001$).

Discussion

The current study demonstrates the development of novel, biocompatible paramagnetic Mn based dendrimer probes targeted to OSE and their successful application of serial, non-invasive MR imaging of atherosclerotic plaques. The targeted Mn dendrimers accumulated within the arterial wall, presumably due to binding of endogenous OSE and subsequent uptake by macrophages, as suggested in other reported studies (12-14). Furthermore, specific targeting of OSE was demonstrated by the fact that untargeted control dendrimers provided limited lesion enhancement. Since Mn is a relatively non-toxic cation and non-cardiovascular formulations are FDA approved, these data provide a framework for the potential clinical translation of this approach to imaging human atherosclerosis and potentially vulnerable coronary and carotid human plaques that are enriched in OSE (10).

The key factor for successful imaging atherosclerotic plaques using Mn based dendrimers relates the ability of the particles to: 1) home to intraplaque macrophages and (2) to release Mn ions upon intracellular uptake of the particles, as shown by the dramatic increase in the MR efficacy of Mn upon intracellular uptake when these particles are taken up by intraplaque macrophages and foam cells (12). The MDA2 antibody used in the current study has been previously used to target MDA-lysine OSE for both MRI and SPECT imaging (12-14,22) and represents proof-of-principle in using other human antibodies fragments or peptides (23) for imaging other OSE. Although intracellular r_1 values were not determined in the current study, the *in vitro* cell studies show strong macrophage uptake of the targeted dendrimers with limited uptake of untargeted material. This is further demonstrated *in vivo* with strong MR enhancement observed for targeted material and nominal unspecific enhancement following untargeted dendrimer administration. In future studies, non-specific uptake may be further reduced by the addition of PEG to the dendrimer formulation. It is well appreciated that PEG reduces non-specific macrophage uptake, increases circulation times, and limits the potential *in vivo* aggregation of certain dendrimer formulations (29).

It should be noted that Mn is typically not used as a contrast agent, due to its low magnetic moment that limits the efficacy, relative to equivalent Gd formulations. However, several groups have reported that de-chelation and endogenous interaction with native metalloproteins and cellular components greatly increases the MR efficacy, as reflected by increased longitudinal relaxivity (r_1) values (24,25). Intracellular r_1 values within the range of 60–90 s⁻¹mM⁻¹ have been reported in cardiomyocytes. Although the exact mechanism of uptake into these cells is not known, our prior studies suggest that the targeted particles interact with endogenous OSE and are subsequently selectively taken up by macrophages. Since macrophages are able to safely excrete intracellular Mn, toxicity issues associated with bio-retention and transmetallation are expected to be nominal as long as the Mn remains chelated in the bolus/vascular phase. Once intracellular de-chelation occurs, the Mn enters the endogenous Mn pool and will be either taken up by metalloproteinase or interact with the cell membrane (12).

In the current study, Mn was chosen as the paramagnetic metal ion. Although gadolinium is a more effective T1 agent, reported studies have clearly shown that intracellular uptake of gadolinium induces cell apoptosis (26). Since we are targeting cells (intraplaque

macrophages/foam cells), alternative endogenous paramagnetic metal ions such as Mn or iron are required. The results of the current study show that macrophage uptake of the Mn dendrimers did not affect *in vitro* cell viability, when compared to saline controls. These findings are consistent with reported studies that show most cells are able to metabolize and excrete Mn without any effect on viability (12,20,21). Studies even indicate that the intracellular delivery of Mn may protect cardiomyocytes from injury during re-perfusion following an ischemic event (27). Studies have shown that intracellular uptake of PAMAM dendrimers causes degradation of the particles, via conformational changes caused by protonation of the surface amines within the endosomal environment (pH=5)(27). As a result, intracellular uptake promotes the degradation of the dendrimers and release of Mn thereby causing strong MR signal enhancement. Whereas the presence of the dendrimers did not induce macrophage apoptosis, studies have shown that *in vivo* distribution and toxicity is directly related to the surface chemistry and charge, with cationic materials inducing most cellular toxicity (29,31,32). However, further studies need to be performed in order to evaluate the safety of the current formulation.

When compared to targeted Gd and Mn micelles, the *in vivo* MR efficacy of Mn-dendrimers was reduced. At the maximum time point post injection, prior studies show %NENH values of $125 \pm 22\%$ and $141 \pm 20\%$ for targeted MDA2 Gd and Mn micelles, respectively (12,14). In the current study, however, a %NENH value of $62 \pm 3\%$ was observed for the targeted Mn dendrimers. The reasons for the decreased efficacy of the dendrimers relative to the micelles may be summarized as: (1) differences in dose, (2) differences in pKa values, (3) and differences in the imaging methodologies. Targeted Gd micelles were administered to apoE^{-/-} mice at a dose of 0.075 mmol Gd/Kg whereas targeted Mn dendrimers were administered at a dose of 0.050 mmol Mn/Kg. The dose of 0.050 mmol Mn/Kg was chosen based upon preliminary studies with targeted PEG Mn micelles that showed high *in vivo* efficacy at this dose (%NENH = $141 \pm 20\%$, 48 hours p.i., 9.4 T,)(12). Although the targeted dendrimers exhibited hydrated diameters similar to that of the targeted Gd and Mn micelles, significant differences in the vascular half-lives were observed. Whereas the targeted MDA2 Gd and Mn micelles circulated with a half-life of 15 hours and 16.4 hours, respectively, a half-life of only 2.9 hours was observed for the targeted dendrimers (12,14). The difference in circulation times is likely due to the presence of PEG (50% wt/wt) present on the targeted Gd micelles. Studies have indicated that prolonged circulation times allow for greater accumulation of molecular probes within or on a target. As a result, the shorter circulation times exhibited by the dendrimers may limit the efficacy of this platform to some extent. Finally, MR imaging of the targeted Gd and Mn micelles was performed using a high-resolution mouse MR scanner at 9.4T with a micro-scale in-plane resolution of 0.098 mm². In the current study a clinical imaging system (3T) was used with an in-plane resolution of 0.15 mm². The arterial wall of the mouse is very small (typically 4 pixels) so that the larger the volume imaged (larger voxel size) the greater the partial volume effects. It should be noted that on pre imaging scans the arterial wall is visible at 9.4T (using black blood T1-weighted sequences). At 3T, however, the arterial wall is not visible (due to the lower resolution) when similar pulse sequences are applied. Despite the limitations related to spatial resolution and partial voluming at 3T, significant MR enhancement was still observed following administration of the targeted dendrimers. Relaxation theory, however,

predicts that the imaging efficacy of the Gd and Mn micelles will be reduced at 9.4T, relative 3T (28). In order to allow for direct comparison, future studies will be performed to evaluate the efficacy of targeted Mn dendrimers and micelles at both 9.4T and 3T.

Limitations

This study did not quantitatively correlate the observed MR imaging signal with the histological presence of OSE, although this was done extensively in prior radionuclide imaging studies using the MDA2 and other targeting antibodies with Gd, Mn and Fe probes. Additionally, the current formulation of the dendrimers is likely sub-optimal as reflected by the blood half-life. Formulation studies that include PEG to increase circulation times as well as optimization of ratio between MDA2, Mn and fluorescent label will likely improve the imaging efficacy of the dendrimer platform.

Conclusions

The current study demonstrates the use of novel targeted Mn dendrimers for the *in vivo* detection of OSE within atherosclerotic lesions. The results from the current study support the concept that active targeting of Mn to OSE will allow for sensitive *in vivo* detection and monitoring of macrophage-rich rich atherosclerotic lesions.

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Figure 1A.

Sulfo-LC-SPDP modification of the dendrimer to allow for MDA2 attachment.

Approximately 1000 surface amines (1000 of 1024) are theoretically able to covalently attach MnDTPA. Based upon elemental analysis, 75 % (768 MnDTPA/1024 surface amines) were bound.

Figure 1B.
Antibody Modification and conjugation to the Sulfo-LC-SPDP modified dendrimer.

Figure 2. Schematic representations of the untargeted and targeted formulations showing the theoretical amount of MnDTPA (approximately 1000) conjugated to the dendrimer surface.

Figure 3.
 r_1 NMRD profile of the untargeted formulation in water at 25°C. The NMRD profile was obtained over a frequency range of 2 MHz to 61.

Figure 4.

Biodistribution of the targeted and untargeted dendrimers in apoE^{-/-} mice. The error bars reflect the standard deviation of three mice per time point for the untargeted and targeted formulations. “*” denotes significant differences between the untargeted and targeted formulations at any given time point post injection. “+” denotes significant differences for the untargeted formulation between time points and “#” reflects significant differences for the targeted material between time points.

Figure 5.

Representative MR images obtained on a human clinical system operating at 3 Tesla. For the targeted material, the results from two mice are shown (M1=mouse1 and M2=mouse2). The arrows point to regions within the arterial wall that contain plaque. Signal increase relative to the pre image is indicative to Mn dendrimer uptake. Note that it is the smaller vessel that is the abdominal aorta. The larger adjacent vein some time appears bright due to the relatively slow flow of blood.

Figure 6.

The percent normalized enhancement (%NENH, defined as the percent relative change in the contrast to noise ratios (CNR), relative to muscle) are shown as a function of time post injection for the untargeted and targeted formulations. The error bars reflect the standard deviation associated the %ENH values obtained for the untargeted (n=3) or targeted formulations (n=4). “*” denotes significant differences between the untargeted and targeted formulations at any given time point post injection. “+” denotes significant differences for the untargeted formulation between time points and “#” reflects significant differences for the targeted material between time points. No significant change ($p>0.1$) was observed in the muscle following administration of either formulation.

Table 1

Physical and chemical properties of the dendrimer formulations. WT = wild type mouse plasma. ApoE^{-/-} = apolipoprotein deficient mouse plasma. All longitudinal relaxivities, r_1 , determined at 60 MHz and 40 ° C.

Formulation	Size (nm± SD)	r_1 (s ⁻¹ mM ⁻¹) Buffer	r_1 (s ⁻¹ mM ⁻¹) WT plasma	r_1 (s ⁻¹ mM ⁻¹) ApoE ^{-/-} plasma
Untargeted	11.6 ± 0.1	3.4 ± 0.1	3.8 ± 0.1	4.0 ± 0.3
MDA2	13.3 ± 1.2	3.5 ± 0.1	4.0 ± 0.2	5.5 ± 0.4