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Atherosclerotic Neovasculature MR Imaging with Mixed Manganese-Gadolinium Nanocolloids in Hyperlipidemic Rabbits

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Abstract

A high r1 relaxivity manganese-gadolinium nanocolloid ($\alpha_v\beta_3$ -MnOL-Gd NC) was developed and effectively detected atherosclerotic angiogenesis in rabbits fed cholesterol-rich diets for 12 months using a clinical MRI scanner (3T). 3D mapping of neovasculature signal intensity revealed the spatial coherence and intensity of plaque angiogenic expansion, which may, with other high risk MR bioindicators, help identify high-risk patients with moderate (40% to 60%) vascular stenosis. Microscopy confirmed the predominant media and plaque distribution of fluorescent $\alpha_v\beta_3$ -MnOL-Gd NC, mirroring the MR data. An expected close spatial association of $\alpha_v\beta_3$ -integrin neovasculature and macrophages was noted, particularly within plaque shoulder regions. Manganese oleate bioelimination occurred via the biliary system into feces. Gd-DOTA was eliminated through the bile-fecal and renal excretion routes. $\alpha_v\beta_3$ -MnOL-Gd NC offers an effective vehicle for T1w neovascular imaging in atherosclerosis.

Graphical abstract

Three-dimensional (3D) MR reconstruction (3T) of angiogenesis index within the descending thoracic aorta in hyperlipidemic rabbit (12 month) based on molecular imaging with $\alpha_v\beta_3$ -

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Conflict of Interest: Intellectual property surrounding the hybrid MnOL NC technology has been licensed nonexclusively by Washington University School of Medicine to Ocean NanoTech, LLC, San Diego, CA. Further, the gadolinium perfluorocarbon nanoparticles discussed in this manuscript were licensed from Barnes-Jewish Hospital Research Foundation to Kereos, Inc, St. Louis, MO, for which GL is a scientific co-founder.

MnOLGd NC illustrating marked neovascularity subjacent to intima. For example, such an approach in conjunction with current MR biomarkers of plaque instability could be used to risk-stratify therapeutic care in patients with moderate carotid disease.

**High
risk region
for Rupture?**

Keywords

MRI; contrast media; molecular imaging; atherosclerosis; angiogenesis; nanotechnology

Introduction

Strokes, or cerebrovascular accidents (CVA), occur suddenly with dramatic, life changing impact for the patient, the family, close colleagues and friends, and in the aggregate for society. Stroke is often heralded by transient neurological events that bring patients to medical attention, while others present with an equivocal recent medical history. Since the early work of Benson¹ and Constantinides² the acute formation of thrombus following atherosclerotic plaque rupture has been well recognized as the etiology of transient ischemic attacks and stroke. Although a myriad of medical advances in the detection and treatment of severe carotid stenosis have emerged, the most common source of thromboembolism remain rupturing vulnerable plaques that reside in vessels with only 40 to 60% residual stenosis.^{3, 4} Recent guideline changes have lowered the threshold for surgical intervention for patients with greater than 60% stenosis and low surgical risk. However, for lesions with 40% to 60%, even in the context of suspected ulcerations, carotid surgery remains contraindicated because those individuals at high risk who would benefit from prophylactic intervention cannot be identified with fidelity using current imaging techniques. We hypothesize that

intensive angiogenesis expansion, particularly localized subjacent to the intimal cap in plaques, is a biomarker for high vulnerability for impending plaque rupture, which could assist in improved risk-stratification.

Neovascularization is a key microanatomical feature of vulnerable atherosclerotic plaque.^{5, 6} T1-weighted (T1w) MR molecular imaging of angiogenesis using the $\alpha_v\beta_3$ -integrin, a heterodimeric transmembrane glycoprotein, to differentiate proliferating versus quiescent endothelium, has been achieved with high longitudinal relaxivity (r1) paramagnetic nanoparticles presenting high surface levels of lipid-anchored gadolinium chelates.^{7–15} To date, surface concentrations of gadolinium chelates with very high r1 relaxivity have been required to overcome partial volume dilution and detect sparse epitopes at low nanomolar per voxel densities, such as $\alpha_v\beta_3$ -integrin presented on the neovasculature. While concerns for gadolinium complex stability in vivo are greatly abrogated through the use of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-lipid conjugates,^{16, 17} the presentation of gadolinium-DOTA in high density on nanoparticles can cause acute complement activation.¹⁸

Manganese (Mn^{3+}/Mn^{2+}) by virtue of its high spin number, labile water exchange, long electronic relaxation time, natural prevalence and known human biochemistry has long been studied as a T1w MR contrast agent.^{19–24} We reported the first synthesis and MR characterization of a “soft” type manganese nanocolloid incorporating bivalent manganese oleate (MnOL), which had greater r1 relaxivity than a similar nanocolloid encapsulating 12 nm-sized manganese oxide particles on an equimolar metal basis.^{24, 25} The agent was effective for fibrin imaging in vitro, but not for angiogenesis molecular imaging in vivo, despite fluorescence microscopy corroborating effective targeting.²⁶ The development, optimization, and characterization of a gadolinium-manganese hybrid nanocolloid (MnOL-Gd NC) now provide higher r1 relaxivity, with minimal risk of transmetallation, and negligible acute complement activation.²⁶ Higher r1 particulate relaxivity was achieved by an unexpected synergism between the minute levels of Gd-DOTA-lipid in the encapsulating membrane and the manganese-rich core. MnOL-Gd NC had optimal T1-weighted (T1w) MR detectability at low nanomolar/voxel concentrations in vitro and lowered expected gadolinium exposure and transmetallation risk 300-fold relative to current approved dosages.

The objectives of the present research were: 1) to assess the efficacy of MnOL-Gd NC as a molecular imaging contrast agent for plaque neovasculature, 2) to consider the potential of 3D reconstruction of signal-intensity neovascular distribution as a plaque risk-stratification biomarker, and 3) to characterize the pharmacokinetics, biodistribution and bioelimination of the MnOL-Gd NC.

Methods

Materials

Solvents and reagents, including manganese chloride and sorbitan sesquioleate, were purchased and used as received from Aldrich Chemical Co (St. Louis, MO, USA). Anhydrous chloroform was distilled over calcium hydride prior to use. High purity egg yolk

phosphatidylcholine was purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA). Sodium oleate was used as received from TCI America. (Portland, OR, USA). Argon and nitrogen (UHP, 99.99%) were used for storage of materials. The Spectra/Por membrane (Cellulose MWCO: 20 000 Da) was used for dialysis (Spectrum Medical Industries, Inc., Laguna Hills, CA, USA).

Synthesis of manganese oleate nanocolloids with titrated gadolinium chelates

MnOL-Gd NC was synthesized and commixed with polysorbate to form the emulsion core, which was encapsulated with phospholipid via microfluidization to produce the base nanocolloid.²⁵ The surfactant mixtures were comprised of 0 or 1.25 mole% Gd-DOTA-PE with phosphatidylcholine (PC). Nanocolloids were extensively dialyzed against water using a 20,000 Da MWCO cellulose membrane, passed through a 0.45 μ m Acrodisc Syringe filter, and stored under argon atmosphere at 4°C to reduce oxidation and bacterial growth. Nominal particle sizes and zeta potentials were estimated with dynamic light scattering (DLS, Brookhaven Instrument Corp., Holtsville, NY): DLS (D_{av})=138 $\pm$ 10 nm; Zeta (ζ)=-27 $\pm$ 2 mV; PDI: 0.06. Manganese and gadolinium concentrations were confirmed by inductively coupled plasma optical emission spectrometry (ICP OES, Perkin Elmer, Waltham, MA, USA). The concentration of manganese was 20.2 $\pm$ 0.02 mM Mn²⁺/ml, equal to ~100,000 Mn²⁺ atoms per nanoparticle. The gadolinium (Gd³⁺) content was 0.36 $\pm$ 0.02 mM Gd³⁺/ml, equal to 1,700 Gd³⁺ atoms per particle. For competition treatments, $\alpha_v\beta_3$ -targeted perfluoro-octylbromide (PFOB) nanoparticles without gadolinium were prepared as previously described to compete with $\alpha_v\beta_3$ - MnOL-Gd NC.^{9, 13, 15} In selected preparations, the lipid surfactant included AlexaFluor 594 (Life Technologies, Thermo Fisher Scientific Company, St. Louis, MO, USA) coupled to caproyl-phosphatidylethanolamine at 0.5 mol % for microscopic fluorescence imaging.

Angiogenesis homing utilized a quinolone nonpeptide antagonist developed by Bristol-Myers Squibb Medical Imaging to $\alpha_v\beta_3$ -integrin (US patent 6,511,648 and related patents) that was initially reported and characterized as the ¹¹¹In-DOTA conjugate RP478 and cyan 5.5 homologue TA145.²⁷ The targeted particles presented ~300 ligands/particle with an IC₅₀ of 50 pM for the Mn²⁺-activated $\alpha_v\beta_3$ -integrin.¹⁴ Homing specificity to neovascular sprouts was previously demonstrated in a well defined Matrigel™ plug study using the *Rag1^{tm1Mom}* Tg(TIE-2-lacZ)182-Sato mouse (Jackson Labs, Bar Harbor, ME, USA).²⁸

Neovascular MR Imaging in Hyperlipidemic New Zealand White rabbits

Male New Zealand White rabbits (Harlan Laboratories, Indianapolis, IN, USA) were fed either 0.25% cholesterol (n=20, cholesterol from egg yolk) or standard rabbit chow (n=8) for 12 months. Cholesterol-fed rabbits were randomized into 2 groups and received ear vein injections of either $\alpha_v\beta_3$ -MnOL-Gd NC (1ml/kg; n=7) or nontargeted (NT) MnOL-Gd NC (1ml/kg; n=6). Rabbits fed the control diet received $\alpha_v\beta_3$ -MnOL-Gd NC (1ml/kg; n=8). In a second cohort, fat-fed rabbits (12 months⁺) were randomized to a competition group, receiving a mixture of $\alpha_v\beta_3$ -perfluorocarbon nanoparticles ($\alpha_v\beta_3$ -PFC NP): $\alpha_v\beta_3$ -MnOL-Gd NC (2:1ml/kg; n=6) or to a positive control group given $\alpha_v\beta_3$ -MnOL-Gd NC (1ml/kg; n=2). The Washington University Animal Studies Committee approved animal protocols.

In vivo imaging was performed at 3T with a custom-built 6-element transmit-receive open coil.²⁹ Rabbits were intubated, anesthetized with 2% isoflurane to effect, and dynamic MR molecular imaging of the rabbit thoracic aorta was performed at baseline and 30, 60, 90 and 120 minutes after IV (ear vein) treatment using a 3D T1w, fat-suppressed, black-blood, turbo spin echo imaging sequence (TSE factor=4, TR/TE =380/11ms echo train length, resolution=250um×250um×4 mm, 20 slices, number of signals averages=14, scan time≈15.6 min). Twenty image slices were acquired from immediately below the left subclavian artery to the diaphragm. In-flowing blood signal was nulled with saturation bands positioned superior and inferior to the imaging stack for each imaging plane. Fat suppression was achieved with a spectral selection attenuated inversion recovery technique. Rabbits were euthanized and aortas excised with surrounding adventia for microscopic analysis.

MRI signal enhancement from the aortic wall was averaged over all image slices using a custom, semiautomatic segmentation program^{9, 13, 15}. Briefly, the aortic lumen was defined in each 2D image with a seeded region-growing algorithm using MATLAB (The MathWorks, Inc., Natick, MA, USA). The aortic wall was defined by dilation of the luminal mask followed by automated threshold to obtain a consistent and objective region-of-interest (ROI) encompassing the entire aortic wall. The ROI was manually corrected when the MATLAB algorithm visually extended the ROI beyond the aortic wall. Typically 2–4 slices from each end of the image stack were excluded due to uneven fat suppression or signal drop off from the coil position. Image intensities of each slice were normalized relative to a fiduciary marker (25 mol/l gadolinium diethylene-triamine-pentaacetic acid in saline reference) placed within the field of view. Signal intensity at 2 hr was normalized to the baseline image by dividing by the average muscle signal intensity. An enhanced voxel threshold was equated to the baseline aorta signal average plus two standard deviations. The percent area of enhanced voxels for each slice was calculated and averaged over the length of the aorta. Groups of contiguous enhancing pixels in or through plane were identified as clusters. The angiogenesis index was calculated by multiplying the size (i.e. # pixels) of an enhancing cluster by the average percent enhancement of that cluster. These are summed over the entire aorta studied. Cholesterol fed rabbits without histologically confirmed plaque were excluded from the data analysis; the final treatment group replications were: $\alpha_v\beta_3$ -MnOL-Gd NC/cholesterol diet (n=7), NT-MnOL-Gd NC/cholesterol diet (n=6) and $\alpha_v\beta_3$ -MnOL-Gd NC/normal chow (n=8)

Histopathology and immunohistochemistry

Thoracic aorta segments aligned to the MR imaged region were excised, aliquoted into 5 mm segments, frozen in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA), and sectioned (8 μ m). Sections were stained with hematoxylin/eosin for white-light microscopy. The expression of $\alpha_v\beta_3$ -integrin in the aortic wall was confirmed by immunohistochemistry of selected acetone-fixed sections with use of LM609 (Chemicon, Temecula, CA, USA). Co-staining of $\alpha_v\beta_3$ -integrin in animals receiving $\alpha_v\beta_3$ -MnOL-Gd NC was precluded due to competitive inhibition by the nanoparticles. Ram 11 (Dako, Carpinteria, CA, USA) was used to identify macrophages. Immunohistochemistry stains were developed using the ABC Elite kit and VIP Substrate Kit (Vector Laboratories, Burlingame, CA, USA) with methyl green nuclear counterstaining. Sections were acquired

and processed with an Olympus BX61 microscope (Olympus, Tokyo, Japan) and digital software.

Pharmacokinetic and biodistribution in New Zealand White rabbits

In a third cohort, New Zealand White rabbits (n=3, approximately 3kg) were anesthetized using ketamine and xylazine (30/5 mg/kg). Catheters were placed in marginal ear vein catheter for drug and fluid administration and in the ear artery for serial blood sampling. Thirty minutes following a 20ml bolus of heparinized normal saline, rabbits received MnOL-Gd NC (2ml/kg). The intravenous dose was doubled relative to that used for imaging to improve the detectability of manganese and gadolinium in blood and tissues at later time points. Blood samples (1mL) were taken 1, 5, 10, 15, 30, 45, 60, 90, 120, 150, 180 min. and at 24hr (i.e., sacrifice). Organs were excised, weighed and submitted for ICP OES metal analysis (Perkin Elmer, Santa Clara, USA) including: liver, lung, spleen, kidney, heart, muscle, lymph, bile, feces, urine, brain, bone marrow, stomach, lower and upper GI.

Biological fluids and tissues samples of 1g or less were weighed and placed in digestion vessels with a nitric acid (5ml): HCl (2ml) mixture into which hydrogen peroxide (1 ml) was added drop-wise. The sealed contents were microwaved (Multiwave 3000, Perkin Elmer, Santa Clara, CA, USA) in a two-step digestion technique with microwave power ramping to 1000 W over 20 min, which was held constant for an additional 10 min. After 30 min of venting and cooling, the vessels were opened and the contents transferred with deionized water washing (3×). Additional free deionized water was added to a total dilution volume of 50 ml. Metal content of the samples was measured by ICP OES.

Statistical analysis

Data were statistically analyzed using the Statistical Analysis System (SAS, Cary, NC, USA) procedures for ANOVA and General Linear Models. Differences between the 3 groups with probabilities less than $p < 0.05$ considered significant based on a significant F-test; mean separation utilized Tukey's range test. Quantitative data are reported as the mean $\pm$ the standard error of the mean.

Results

In vivo angiogenesis imaging in hyperlipidemic rabbits

Figures 1A–E show the progressive $\alpha_v\beta_3$ -MnOL-Gd NC contrast signal enhancement observed in a representative cholesterol-fed rabbit, illustrating the dynamic changes in the intramural spatial distribution and neovascular signal intensity. The signal enhancement over the 120 min imaging period (Figure 1F) indicates the consistent monotonic aortic signal increase depicted by the T1w aortic signal enhancement maps. Of note, signal within the aortic lumen was negligible at baseline and all time points thereafter, indicating that contrast changes in aortic wall were not confounded by signal arising from particles within the circulating blood pool.

Average neovascular response to hyperlipidemic dietary treatment at 2 hours post injection was assessed as the overall percent area of angiogenesis (i.e., area of enhancement) and as

an angiogenesis index, a cross product of the number of enhanced voxels and their percent enhancement within the aorta. The percent area of angiogenesis enhancement among fat-fed animals receiving $\alpha_v\beta_3$ -MnOL-Gd NC was higher ($1.7 \pm 0.6\%$, $p < 0.05$) than the NT-MnOL-Gd NC ($0.1 \pm 0.1\%$), and $\alpha_v\beta_3$ -MnOL-Gd NC control diet ($0.1 \pm 0.1\%$) groups. (Figure 2A) In contradistinction to the adventitial distribution of neovasculature expansion observed in hyperlipidemic rabbits fed cholesterol rich diets for four months^{9, 13, 15}, angiogenesis signal at 12 months of dietary treatment was localized mostly within the inner half of the aortic wall, which encompassed the plaque and part of the media. Less contrast signal was appreciated in the outer aortic wall, predominately the adventitia. In the aortic plaque region area of enhancement was greater than 7-fold higher ($p < 0.05$) in cholesterol-fed rabbits imaged after $\alpha_v\beta_3$ -MnOL-Gd NC ($4.0 \pm 1.6\%$) than those animals receiving NT-MnOL-Gd NC ($0.5 \pm 0.5\%$). Animals fed the control chow and injected with $\alpha_v\beta_3$ -MnOL-Gd NC ($0.3 \pm 0.2\%$) had negligible contrast enhancement. In the outer aorta, the relative relationship of angiogenesis signal among the treatment groups was similar ($p < 0.05$), but the magnitude of contrast signal was considerably lower. Using an angiogenesis index (AI), which additionally accounts for contrast intensity, the same treatment group relationships were noted but the magnitude of response among cholesterol fed rabbits receiving $\alpha_v\beta_3$ -MnOL-Gd NC (4389 ± 1920 , $p < 0.05$) increased to 12-fold as compared with the signal observed with NT-MnOL-Gd NC (247 ± 247) or the $\alpha_v\beta_3$ -MnOL-Gd NC (369 ± 138) in control rabbits. (Figure 2B). The majority of the AI response was again within the inner half of the aortic wall ($\alpha_v\beta_3$ -MnOL-Gd NC/cholesterol-fed: 3737 ± 1917 ; $p < 0.05$ versus NT-MnOL-Gd NC/cholesterol-fed: 140 ± 140 and $\alpha_v\beta_3$ -MnOL-Gd NC/control-diet: 276 ± 123). For the outer half of the aortic wall, no differences ($p > 0.05$) in AI between treatments were measured. In the second cohort of hyperlipidemic rabbits, the AI signal was clearly diminished by in vivo competitive blockade of the integrin receptor with $\alpha_v\beta_3$ -PFOB nanoparticles within the inner and outer aspects of the aorta. (Supplemental Figure 1)

Three-dimensional (3D) mapping of angiogenic voxel signal intensity in fat-fed rabbits imaged with $\alpha_v\beta_3$ -MnOL-Gd NC provided highly informative insight into the spatial coherence and intensity of plaque neovascular expansion in the fat-fed targeted animal aorta. (Figure 3ABC) 3D angiogenesis mapping using two levels of color-encoding, blue and red for lower intensity (2 + standard deviation) and higher intensity (4+ standard deviation) over baseline respectively, provided a unique perspective into the neovascular expansion patterns. Lower intensity voxels were more prevalent and distributed in large coherent regions throughout the plaque, typically along the media and plaque border. Intense areas of angiogenesis were less coherently scattered among the lower AI regions, but in several instances focal regions of high AI were detected beneath the intima. Some of these intense neovascular regions extended transmurally into the media. (Figure 3D)

Light and fluorescent microscopy

Light microscopy of the descending thoracic aorta revealed moderate intimal thickening and expanded vasa vasorum in the cholesterol-fed rabbits, which was not appreciated in control animals fed normal chow. (Figure 4AB) Control rabbit aortas revealed no intimal thickening, a well-ordered media, and a thin adventitia with sparse vasa vasorum. (Figure 4C)

Fluorescent microscopy of $\alpha_v\beta_3$ -MnOL-Gd-AlexaFluor 594 NC in the hyperlipidemic rabbits showed some accumulation in the adventia, as observed in earlier studies, but the nanocolloids were most prominently observed from the disrupted media to the intimal border with high intensity staining often observed subjacent to the intima. (Figure 4D) NT-MnOL-Gd AlexaFluor 594-labeled NC administered to fat-fed rabbits animals were also found within the aortas but to a lesser extent than in their $\alpha_v\beta_3$ -MnOL-Gd-AlexaFluor 594 NC counterparts. NT-MnOL-Gd AlexaFluor 594 NC was predominately near the border region between the base of the plaque and the media. (Figure 4E) In rabbits fed normal chow diet, $\alpha_v\beta_3$ -MnOL-Gd-AlexaFluor 594 NC was detected at low frequency in the adventia, consistent with a constitutive level of angiogenesis required to maintain the vasa vasorum. (Figure 4F) These results mirrored and corroborated the MR molecular imaging results.

Specific examination of high MR signal intensity regions in two rabbits similarly revealed proliferation of $\alpha_v\beta_3$ -integrin positive neovasculature in close proximity to dense macrophage accumulation (RAM 11) immediately below the intima. (Figure 5) Although angiogenesis was very common within the inner half of the aortic wall, such high intensity regions subjacent to the intima were only intermittently discovered over the thoracic aorta segments, precluding robust quantitation. These data suggest that noninvasive 3D MR angiogenesis mapping may reveal neovascular “hot spots” that might specifically identify the subset of patients with moderate carotid stenosis and regions of high focal sub-intimal carotid plaque vulnerability affording for more aggressive clinical care.

Pharmacokinetic, biodistribution and bioelimination of MnOL-Gd NC

The simultaneous pharmacokinetics and biodistribution of manganese and gadolinium were assessed in serial blood samples over 3 hours. The dosage of MnOL-Gd was doubled over that used in the efficacy studies to improve metal detection. The pharmacokinetic half-lives of both metals were relatively short compared to prior experience with perfluorocarbon nanoparticles in rabbits. The gadolinium time-course profile fit a monoexponential model (i.e., $C_p = Ae^{-\alpha t}$) with an estimated half-life of around 80 minutes (Figures 6AB), whereas, the Mn^{+2} pharmacokinetics fit a biexponential model (i.e., $C_p = Ae^{-\alpha t} + Be^{-\beta t}$) with short beta elimination half-life (~25 min) (Figures 6CD). These data suggested MnOL loss during circulation, likely diffusional into plasma, relative to the lipid-anchored gadolinium in the outer membrane.

At 24 hours, organs were excised, weighed and analyzed for metal content including: liver, lung, spleen, kidney, heart, muscle, lymph, bile, feces, urine, brain, bone marrow, stomach, lower and upper GI. Manganese was predominantly recovered in bile and feces, with nearly nondetectable concentrations of the metal in all other tissues. (Figure 6E) Gadolinium was bioeliminated into the urine as well as through feces. (Figure 6F) These data suggest that Gd-DOTA was cleaved from the lipid anchor by phospholipase metabolism allowing the water-soluble complex to pass through the renal system. Another portion of the lipophilic gadolinium chelate complex was excreted into the bile and eliminated through the feces. Low residual levels of gadolinium remained in the liver and spleen, key organs of the monocyte phagocytic system. The concentrations of gadolinium detected in all other organs was very low, and may reflect particles in circulation since a persistent low level of the

lanthanide was measured in blood at 24 hours post-injection. These biodistribution-bioelimination data suggest that the exogenous metals have a rather short residence time in vivo, as is intended for “soft” nanoparticles utilizing small molecular weight organometallic complexes to generate contrast.

Discussion

The enhanced r_1 relaxivity and improved low nanomolar detectability of MnOL-Gd NC measured previously in vitro²⁶ was effective in the present study for neovascular imaging. $\alpha_v\beta_3$ -MnOL-Gd NC was shown to enhance the MR T1w molecular imaging of angiogenesis within the descending thoracic aorta in rabbits fed cholesterol-rich diets for 12 months. Hyperlipidemic rabbits imaged with $\alpha_v\beta_3$ -MnOL-Gd NC had higher ($p<0.05$) intramural signal localized heavily within the plaque in comparison to similar animals receiving the nontargeted MnOL-Gd NC. Rabbits fed a normal chow given $\alpha_v\beta_3$ -MnOL-Gd NC had negligible aortic MR contrast enhancement. In vivo competition of the $\alpha_v\beta_3$ -MnOL-Gd NC signal with $\alpha_v\beta_3$ -PFOB nanoparticles substantially diminished intramural T1w-contrast enhancement, reflecting the high specificity of angiogenesis molecular imaging. In previous studies using rabbits fat-fed for four months, angiogenesis was predominantly appreciated as vascular expansion within the adventitia associated with very nascent plaque development.^{13, 15} In the present study animals fed for 12 months presented with much greater disease burden and neovascular contrast enhancement localized within the plaque itself.

Three-dimensional mapping of the neovasculature signal intensity provided informative insight into the spatial coherence and intensity of plaque angiogenic expansion. Lower intensity (blue) voxels were prevalent and distributed in large coherent regions throughout the plaque and media. Focal coherent regions of high intensity angiogenesis (red voxels) were detected beneath the intima and extended transmurally in some instances. These occasional regions of intense angiogenesis immediately beneath the intima reflect high macrophage activity with concurrent release of proangiogenic cytokines as well as matrix digesting enzymes. Microscopic study revealed a predominant media and plaque distribution of fluorescent $\alpha_v\beta_3$ -MnOL-Gd NC that mirrored the MR molecular imaging data. As expected, a close spatial association of $\alpha_v\beta_3$ -integrin neovasculature and macrophages was observed within plaque using immunohistochemistry staining.

3D visualization of neovascular expansion could be applied to patients with moderate luminal carotid plaque encroachment as a component of risk stratification algorithm. The most common source of carotid thromboembolism remains rupturing vulnerable plaque that reside in vessels with only 40 to 60% residual stenosis.^{3, 4} Despite recent guideline changes³⁰ carotid intervention for patients with 40% to 60% stenosis, even with suspected ulcerations, is contraindicated. With stroke costs in the US around \$54 billion/year and climbing, perhaps $\alpha_v\beta_3$ -MnOL-Gd NC with 3D neovascular mapping of sub-intimal plaque vulnerability in conjunction with other high risk MR bioindicators, such as intraplaque hemorrhage, would compose a useful strategy to address this unmet need.^{31–33}

The biodistribution and bioelimination of MR contrast metals are important safety considerations. $\alpha_v\beta_3$ -MnOL-Gd NC at experimental doses employed in this study reduced total gadolinium exposure by 300-fold relative to current clinically approved blood pool dosages. Moreover, the high stability of the lanthanide DOTA complex would be expected to minimize the risk of transmetallation^{16, 17} and nephrogenic systemic fibrosis.^{34–36} The gadolinium employed with $\alpha_v\beta_3$ -MnOL-Gd NC was detected in the urine, bile and feces, suggesting that much of the Gd-DOTA-lipid conjugate was excreted through the bile into feces intact. Another portion of the lipid conjugate was likely metabolized by lipases, such as phospholipase D, releasing Gd-DOTA from its lipid anchor, which then cleared through the renal system into urine. Low levels of gadolinium were appreciated in the liver and spleen, which are primary clearance organs of the monocyte phagocytic system, and minimal metal levels remained in blood and other organs at 24 hours. Manganese at 24 hours post administration was richly present in bile and feces but not in urine or other organs and tissues, suggesting that the lipophilic MnOL conjugate was bioeliminated intact. Manganese was one of the earliest reported examples of paramagnetic contrast material for MRI because of its efficient positive contrast enhancement.³⁷ Unlike the lanthanides, it is a natural cellular constituent resembling Ca^{2+} that often acts as a regulatory cofactor for enzymes and receptors. However, ionic manganese is not without toxicity issues. The brain is particularly vulnerable to high concentration of manganese exposure, which can cause a neurodegenerative disorder known as ‘manganism’ with characteristics akin to Parkinson’s disease.³⁸ However, chelated manganese, such as mangascan-ethylene-diamine -tetraacetic acid (Mn-EDTA, 10 ml/kg) or pentamang-diethylene-triamine-pentaacetic acid (Mn-DTPA, 5 mL/kg) showed MR contrast effectiveness with no indication of toxicity in mice.³⁹ In the present concept, the organometallic manganese complex was rapidly bioeliminated through the biliary system with no indications of acute toxicity. (Supplemental Data Table 1)

In summary, we have reported the development of a novel ultralow gadolinium-manganese nanocolloid construct that is effective for rapid T1w MR neovascular imaging of sparse epitopes, such as $\alpha_v\beta_3$ -integrin. High r_1 relaxivity was achieved by augmenting the T1w contrast effect of a “soft” manganese oleate-based nanocolloid with minute levels of gadolinium. In vivo, this MR molecular imaging agent afforded effective detection of atherosclerotic angiogenesis in rabbits fed cholesterol-rich diets for 12 months, with the majority of contrast enhancement appreciated within the plaque and media of the diseased aorta. Three-dimensional mapping of neovasculature signal intensity provided informative insight into the spatial coherence and intensity of plaque angiogenic expansion, and could provide an effective tool to identify a highest risk subset of patients presenting with moderate carotid stenosis, which remains an unaddressed clinical need. Bioelimination of manganese occurred via the biliary system into feces; whereas, gadolinium was eliminated through the bile-fecal route or via renal excretion. Collectively, $\alpha_v\beta_3$ -MnOL-Gd NC offers an effective, safer alternative to high surface density gadolinium nanoparticles for T1w neovascular molecular imaging in atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Benson R. Present status of coronary artery disease. *Arch Pathol Lab Med.* 1926; 2:876–916.
2. Constantinides P. Plaque fissures in human coronary thrombosis. *J Atheroscler Res.* 1966; 6:1–17.
3. Ambrose JA, Winters SL, Stern A, Eng A, Teichholz LE, Gorlin R, et al. Angiographic morphology and pathogenesis of unstable angina pectoris. *J Am Coll Cardiol.* 1985; 5:609–616. [PubMed: 3973257]
4. Brown BG, Gallery CA, Badger RS, Kennedy JW, Mathey D, Bolson EL, et al. Incomplete lysis of thrombus in the moderate underlying atherosclerotic lesion during intracoronary infusion of streptokinase for acute myocardial infarction: Quantitative angiographic observations. *Circulation.* 1986; 73:653–661. [PubMed: 3948368]
5. Moreno PR, Purushothaman KR, Fuster V, Echeverri D, Truszczyńska H, Sharma SK, et al. Plaque neovascularization is increased in ruptured atherosclerotic lesions of human aorta: Implications for plaque vulnerability. *Circulation.* 2004; 110:2032–2038. [PubMed: 15451780]
6. Moreno PR, Purushothaman KR, Sirol M, Levy AP, Fuster V. Neovascularization in human atherosclerosis. *Circulation.* 2006; 113:2245–2252. [PubMed: 16684874]
7. Sipkins DA, Cheresch DA, Kazemi MR, Nevin LM, Bednarski MD, Li KC. Detection of tumor angiogenesis in vivo by alphavbeta3-targeted magnetic resonance imaging. *Nat Med.* 1998; 4:623–626. [PubMed: 9585240]
8. Flacke S, Fischer S, Scott MJ, Fuhrhop RJ, Allen JS, McLean M, et al. Novel mri contrast agent for molecular imaging of fibrin: Implications for detecting vulnerable plaques. *Circulation.* 2001; 104:1280–1285. [PubMed: 11551880]
9. Winter PM, Morawski AM, Caruthers SD, Fuhrhop RW, Zhang H, Williams TA, et al. Molecular imaging of angiogenesis in early-stage atherosclerosis with alpha(v)beta3-integrin-targeted nanoparticles. *Circulation.* 2003; 108:2270–2274. [PubMed: 14557370]
10. Schmieder AH, Winter PM, Caruthers SD, Harris TD, Williams TA, Allen JS, et al. Molecular mr imaging of melanoma angiogenesis with alpha (v) beta (3)-targeted paramagnetic nanoparticles. *Magn Reson Med.* 2005; 53:621–627. [PubMed: 15723405]
11. Mulder WJ, Strijkers GJ, Habets JW, Bleeker EJ, van der Schaft DW, Storm G, et al. Mr molecular imaging and fluorescence microscopy for identification of activated tumor endothelium using a bimodal lipidic nanoparticle. *Faseb J.* 2005; 19:2008–2010. [PubMed: 16204353]
12. Strijkers GJ, Mulder WJ, van Heeswijk RB, Frederik PM, Bomans P, Magusin PC, et al. Relaxivity of liposomal paramagnetic mri contrast agents. *Magma.* 2005; 18:186–192. [PubMed: 16155762]
13. Winter P, Neubauer A, Caruthers S, Harris T, Robertson J, Williams T, et al. Endothelial alpha(nu)beta(3)-integrin targeted fumagillin nanoparticles inhibit angiogenesis in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2006; 26:2103–2109. [PubMed: 16825592]
14. Schmieder AH, Caruthers SD, Zhang H, Williams TA, Robertson JD, Wickline SA, et al. Three-dimensional mr mapping of angiogenesis with {alpha}5{beta}1({alpha}{nu}{beta}3)-targeted theranostic nanoparticles in the mda-mb-435 xenograft mouse model. *FASEB J.* 2008; 22:4179–4189. [PubMed: 18697838]

15. Winter P, Caruthers S, Zhang H, Williams T, Wickline S, Lanza G. Antiangiogenic synergism of integrin-targeted fumagillin nanoparticles and atorvastatin in atherosclerosis. *J Am Coll Cardiol Img.* 2008; 1:624–634.
16. Sherry A, Cacheris W, Kuan K. Stability constants for gd3+ binding to model dtpa-conjugates and dtpa-proteins: Implications for their use as magnetic resonance contrast agents. *Magn Reson Med.* 1988; 8:180–190. [PubMed: 3210955]
17. Winter P, Athey P, Kiefer G, Gulyas G, Frank K, Fuhrhop R, et al. Improved paramagnetic chelate for molecular imaging with mri. *J Magn Magn Mater.* 2005; 293:540–545.
18. Pham CT, Mitchell LM, Huang JL, Lubniewski CM, Schall OF, Killgore JK, et al. Variable antibody-dependent activation of complement by functionalized phospholipid nanoparticle surfaces. *J Biol Chem.* 2011; 286:123–130. [PubMed: 21047788]
19. Pflugfelder PW, Wendland MF, Holt WW, Quay SC, Worah D, Derugin N, et al. Acute myocardial ischemia: Mr imaging with mn-tp. *Radiology.* 1988; 167:129–133. [PubMed: 3347711]
20. Pomeroy OH, Wendland M, Wagner S, Derugin N, Holt WW, Rocklage SM, et al. Magnetic resonance imaging of acute myocardial ischemia using a manganese chelate, mn-dpdp. *Invest Radiol.* 1989; 24:531–536. [PubMed: 2502502]
21. Saeed M, Wagner S, Wendland MF, Derugin N, Finkbeiner WE, Higgins CB. Occlusive and reperfused myocardial infarcts: Differentiation with mn-dpdp-enhanced mr imaging. *Radiology.* 1989; 172:59–64. [PubMed: 2500678]
22. Saeed M, Higgins CB, Geschwind JF, Wendland MF. T1-relaxation kinetics of extracellular, intracellular and intravascular mr contrast agents in normal and acutely reperfused infarcted myocardium using echo-planar mr imaging. *Eur Radiol.* 2000; 10:310–318. [PubMed: 10663763]
23. Troughton JS, Greenfield MT, Greenwood JM, Dumas S, Wiethoff AJ, Wang J, et al. Synthesis and evaluation of a high relaxivity manganese(ii)-based mri contrast agent. *Inorg Chem.* 2004; 43:6313–6323. [PubMed: 15446878]
24. Pan D, Caruthers SD, Senpan A, Schmieder AH, Wickline SA, Lanza GM. Revisiting an old friend: Manganese-based mri contrast agents. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2010; 10:1002/wnan.116
25. Pan D, Senpan A, Caruthers SD, Williams TA, Scott MJ, Gaffney PJ, et al. Sensitive and efficient detection of thrombus with fibrin-specific manganese nanocolloids. *Chem Commun (Camb).* 2009; 22:3234–3236. [PubMed: 19587924]
26. Wang K, Pan D, Schmieder A, Senpan A, Hourcade D, Pham C, et al. Synergy between surface and core entrapped metals in a mixed manganese-gadolinium nanobeacon affords safer mr imaging of sparse biomarkers. *Nanomed.* 2014
27. Meoli DF, Sadeghi MM, Krassilnikova S, Bourke BN, Giordano FJ, Dione DP, et al. Noninvasive imaging of myocardial angiogenesis following experimental myocardial infarction. *J Clin Invest.* 2004; 113:1684–1691. [PubMed: 15199403]
28. Pan D, Pramanik M, Senpan A, Allen JS, Zhang H, Wickline SA, et al. Molecular photoacoustic imaging of angiogenesis with integrin-targeted gold nanobecons. *FASEB J.* 2011; 25:875–882. [PubMed: 21097518]
29. Hockett FD, Wallace KD, Schmieder AH, Caruthers SD, Pham CT, Wickline SA, et al. Simultaneous dual frequency 1h and 19f open coil imaging of arthritic rabbit knee at 3t. *IEEE Trans Med Imaging.* 2011; 30:22–27. [PubMed: 20699209]
30. Brott TG, Halperin JL, Abbara S, Bacharach JM, Barr JD, Bush RL, et al. 2011 asa/accf/aha/aann/aans/acr/asnr/cns/saip/scai/sir/snis/svm/svs guideline on the management of patients with extracranial carotid and vertebral artery disease: Executive summary. *Stroke.* 2011; 42:e420–463. [PubMed: 21282494]
31. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics–2013 update: A report from the american heart association. *Circulation.* 2013; 127:e6–e245. [PubMed: 23239837]
32. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, et al. Executive summary: Heart disease and stroke statistics–2012 update: A report from the american heart association. *Circulation.* 2012; 125:188–197. [PubMed: 22215894]

33. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics–2012 update: A report from the american heart association. *Circulation*. 2012; 125:e2–e220. [PubMed: 22179539]
34. Grobner T. Gadolinium—a specific trigger for the development of nephrogenic fibrosing dermopathy and nephrogenic systemic fibrosis? *Nephrol Dial Transplant*. 2006; 21:1104–1108. [PubMed: 16431890]
35. Ersoy H, Rybicki FJ. Biochemical safety profiles of gadolinium-based extracellular contrast agents and nephrogenic systemic fibrosis. *Journal of Magnetic Resonance Imaging*. 2007; 26:1190–1197. [PubMed: 17969161]
36. Perkins JD. Warning regarding gadolinium and nephrogenic systemic fibrosis. *Liver Transpl*. 2007; 13:1752–1753. [PubMed: 18044785]
37. Mendonca-Dias MH, Gaggelli E, Lauterbur PC. Paramagnetic contrast agents in nuclear magnetic resonance medical imaging. *Seminars in nuclear medicine*. 1983; 13:364–376. [PubMed: 6359418]
38. Calne DB, Chu NS, Huang CC, Lu CS, Olanow W. Manganism and idiopathic parkinsonism: Similarities and differences. *Neurology*. 1994; 44:1583–1586. [PubMed: 7936278]
39. Ye Z, Jeong EK, Wu X, Tan M, Yin S, Lu ZR. Polydisulfide manganese(ii) complexes as non-gadolinium biodegradable macromolecular mri contrast agents. *J Magn Reson Imaging*. 2012; 35:737–744. [PubMed: 22031457]

Figure 1.

A–E. $\alpha_v\beta_3$ -MnOL-Gd NC contrast signal in the descending thoracic aorta of a cholesterol-fed rabbit evolved progressively from baseline through 120 minutes post-injection (3D T1w, fat-suppressed, black-blood, turbo spin echo imaging sequence). **F.** Neovascular signal intensity increased monotonically within the aortic wall.

Figure 2.

Average neovascular response to hyperlipidemic dietary treatment at 2 hours post injection was assessed as the overall percent area of angiogenesis (*i.e.*, area of enhancement, **A**) and as an angiogenesis index (**B**), a cross product of the number of enhanced voxels and their percent enhancement within the aorta. (* $p < 0.05$)

Figure 3.

Three-dimensional (3D) MR reconstruction (3T) of descending thoracic aorta (DA, chain modeled) in hyperlipidemic rabbit (12 month) with high angiogenesis index based on molecular imaging with $\alpha_v\beta_3$ -MnOL-Gd NC. **A.** DA with angiogenesis rich color enhanced voxels. Red voxels represent signal intensity greater than four standard deviations over the mean signal of hyperlipidemic animals treated with nontargeted nanoparticles. The blue voxels represent signal increases greater than two standard deviations over the NT group mean. Top images are transaxial 3D views of the lower aortic slices. Bottom images are the entire 3D reconstructed vessel. **B.** Neovascular enhancement appreciated within the inner half of the aortic wall (plaque). **C.** Neovascular enhancement appreciated within the outer half of the aortic wall (adventia and media) **D.** Cut-away reconstruction with example of a focal “hot spot” region of neovascularity.

Figure 4.

A. H&E of atherosclerotic rabbit aorta depicted in **D** following dosing with Alexafluor 594 labeled $\alpha_v\beta_3$ -integrin MnOL-Gd NC. NCs are appreciated in the adventitia, media and expanded intima. **B.** H&E of atherosclerotic rabbit aorta depicted in **E** following nontargeted MnOL-Gd NC in atherosclerotic rabbits. The general distribution of fluorescent NC is similar to that in **C** but a far lower prevalence is appreciated within each layer of the aorta, particularly the plaque beyond the medial border. (White arrows) **C.** H&E of normal rabbit aorta with preserved adventitia. Very sparse accumulation of AlexaFluor 594 labeled $\alpha_v\beta_3$ -integrin MnOL-Gd NC is observed in the media with far fewer NC noted in media or thin intima layers. **(F)** Brown arrows demarcate neointima in panels **A**, **B**, **D** and **E**, and the normal endothelial lining in panels **C** and **F**. (—200 μ m)

Figure 5.

A. H&E of atherosclerotic rabbit aorta (4 $\times$) with inset (20 $\times$) showing plaque shoulder near subclavian artery. **B.** Immunohistochemical labeling of $\alpha_v\beta_3$ -integrin of neovessels (e.g., black arrow) in shoulder region in very close proximity to dense macrophage accumulation (e.g., orange arrow, **C**). These data suggest that noninvasive 3D MR angiogenesis mapping may reveal focal neovascular “hot spots” intimately associated with macrophage-driven inflammation that are prognostic of enhanced carotid plaque vulnerability and supportive of decisions for more aggressive clinical care. Solid bar equals 500 μm and 100 μm for 4 $\times$ and 20 $\times$ images, respectively. Black arrows demarcate region of thickened plaque neointima.

Figure 6.

The simultaneous pharmacokinetics and biodistribution of manganese and gadolinium were assessed in serial blood samples over 3 hours. **A–D.** The gadolinium time-course profile fit a monoexponential model (i.e., $C_p = Ae^{-\alpha t}$) with an estimated half-life of around 80 minutes. **B.** The Mn^{+2} pharmacokinetics fit a biexponential model (i.e., $C_p = Ae^{-\alpha t} + Be^{-\beta t}$) with short beta elimination half-life (~25 min). **D.** Biodistribution of Mn (**E**) and gadolinium (**F**) at 24 hours in: liver (LVR), lung, spleen (SPLN), kidney (L KID), heart (HRT), muscle (MUS), lymph (LYM), bile, feces (FCS), urine (URN), brain (BRN), bone marrow (BM), stomach (STOM), lower (L GI) and upper GI (U GI).