Published in final edited form as:

Bioconjug Chem. 2011 May 18; 22(5): 931–937. doi:10.1021/bc100573t.

Synthesis and Evaluation of Nanoglobular Macrocyclic Mn(II) Chelate Conjugates As Non-Gadolinium(III) MRI Contrast Agents

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Abstract

Because of the recent observation of the toxic side effects of Gd(III) based MRI contrast agents in the patients with impaired renal functions, there is a strong interest on developing alternative contrast agents for MRI. In this study, lysine dendrimers with a silsesquioxane core acted as nanoglobular carriers that were conjugated to Mn(II)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate monoamide (Generation 2, 3, 4-DOTA-Mn) to synthesize non-Gd(III) based contrast agents for magnetic resonance imaging (MRI). A generation 3 nanoglobular conjugate of Mn(II)-1,4,7-triaazacyclononane-1,4,7-triacetate-GA amide (G3-NOTA-Mn) was also synthesized and evaluated. The per ion T_1 and T_2 relaxivities of G2, G3, G4 nanoglobular Mn(II)-DOTA monoamide conjugates decreased with increasing generation of the carriers. The T_1 relaxivity of G2, G3, G4 nanoglobular Mn(II)-DOTA conjugates was 3.3, 2.8, 2.4 $\text{mM}^{-1}\text{sec}^{-1}$ per Mn(II) chelate at 3 T, respectively. The T_1 relaxivity of G3-NOTA-Mn was 3.80 $\text{mM}^{-1}\text{sec}^{-1}$ per Mn(II) chelate at 3 T. The nanoglobular macrocyclic Mn(II) chelate conjugates showed good in vivo stability and were readily excreted via renal filtration. The conjugates resulted in much less non-specific liver enhancement than MnCl_2 and were effective for contrast enhanced tumor imaging in nude mice bearing MDA-MB-231 breast tumor xenografts at a dose of 0.03 mmol Mn/kg. The nanoglobular macrocyclic Mn(II) chelate conjugates are promising non-gadolinium based MRI contrast agents.

Introduction

Gadolinium-based chelates are the most commonly used contrast agents for clinical magnetic resonance imaging (MRI). However, the recently reported nephrogenic system fibrosis associated with Gd(III) based contrast agents cause serious safety concerns, especially for the use in the patients with abnormal kidney functions^{1–3}. There is a continual interest in developing alternative MRI contrast agents with much less toxicity. Manganese(II) is an essential element for humans, and has relatively high electronic spins (5/2) and fast water exchange rate⁴. It is less toxic than other paramagnetic metal ions at low concentrations. Mn(II) chelates have been investigated as MRI contrast agents over the past decades. Two Mn(II) based contrast agents, hepatocyte-specific Mn(II) dipyradoxal diphosphate (Mn-DPDP) and an oral formulation containing MnCl_2 , have been clinically

used for liver imaging and gastro-intestinal imaging⁵. As compared to Gd(III) based agents, Mn(II)-based MRI contrast agents remain less investigated.

The main challenges for Mn(II) based MRI contrast agents are the low chelation stability and low relaxivity of Mn(II) chelates. Low stability of Mn(II) chelates can result in the release of free Mn(II) ions in vivo. Overexposure to free Mn(II) ions could lead to neurodegenerative damage, such as Parkinson's disease⁶. The design of Mn(II) chelates with high stability is essential to minimize their potential toxicity as MRI contrast agents. Macrocyclic chelates of transition metal ions have shown higher kinetic stability than linear chelates. Mn(II) forms thermodynamically and kinetically stable chelates with macrocyclic ligands, such as DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), DO3A (1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid), and NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid)^{4,7,8}. Stability constants (logK) of Mn(II)-DOTA, Mn(II)-DO3A (7) and Mn(II)-NOTA (8) are 19.89, 19.4 and 14.9, respectively. The macrocyclic Mn(II) chelates with high kinetic and thermodynamic stability can minimize in vivo release of free Mn(II) ions from the contrast agents.

Low relaxivity of Mn(II) based contrast agents could be overcome by conjugating multiple stable Mn(II) chelates to biocompatible polymers. Dendrimers are a class of highly functionalized macromolecules with precisely defined structures⁹. A large number of Mn(II) chelates can be conjugated to the surface of dendrimers, resulting in nanosized contrast agents with high overall relaxivity and improved contrast enhancement at reduced doses. Lysine dendrimers with a symmetric silsesquioxane cubic core were recently reported as a nanoglobular platform for the delivery of therapeutics and diagnostics^{10–12}. The generation 1 through 3 nanoglobular Gd-DOTA monoamide conjugates has shown increased relaxivity and size dependent contrast enhancement for magnetic resonance angiography and cancer imaging¹¹. The conjugation of Mn(II)-DOTA into a CLT1 peptide targeted G3 nanoglobular lysine dendrimer resulted in a tumor specific contrast agent with high overall relaxivity and significant tumor enhancement at a low dose¹³.

In this study, we synthesized Mn(II)-DOTA monoamide conjugates of generation 2 through 4 nanoglobules to study size effect on the Mn(II) based nanoglobular MRI contrast agents. A generation 3 nanoglobular Mn(II)-NOTA-GA conjugate was also synthesized to study ligand effect on the relaxivity and in vivo contrast enhancement of the agents. The nanoglobular Mn(II)-based MRI contrast agents were characterized by mass spectrometry and inductively coupled plasma-optical emission spectroscopy (ICP-OES). The relaxivities of the nanoglobular Mn(II) agents were measured. The effectiveness of the Mn(II) based contrast agents in contrast enhanced MRI were evaluated in mice bearing breast cancer xenografts at a low dose.

Materials and Methods

4-(4,7-Bis(2-tert-butoxy-2-oxoethyl)-1,4,7-triazonan-1-yl)-5-tert-butoxy-5-oxopentanoic acid [NODA-GA(tBu)₃] was purchased from CheMatech (Dijon, France). 1-Hydroxybenzotriazole (HOBt), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N_α,N_ε-di-*t*-BOC-L-lysine dicyclohexylammonium salt [(di-*t*-BOC)₂-L-lys-OH·DCHA] were purchased from Nova Biochem (Darmstadt, Germany). 1,4,7,10-Tetraazacyclododecane-1,4,7-tris-*tert*-butyl acetate-10-acetic acid [DOTA-tris(*t*-Bu)] was purchased from Macrocyclics (Dallas, TX). N,N-Diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA) and N,N-dimethylformamide anhydrous (DMF) were purchased from Alfa Aesar (Ward Hill, MA). Kaiser test kit was purchased from Sigma-Aldrich, Inc. (Louis, MO). All reagents were used without further purification unless otherwise stated. Dendrimers and their conjugates were purified by ultrafiltration with

Millipore's Amicon® Ultra-15 centrifugal filter of 3 kDa molecular weight cutoff in deionized water. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra were acquired on a Voyager DE-STR spectrometer (PerSeptive BioSystems) in linear mode with α -cyano-4-hydroxycinnamic acid as a matrix. The Mn(II) content was measured by inductively coupled plasma-optical emission spectroscopy (ICP-OES Optima 3100XL, Perkin Elmer, Norwalk, CT).

Synthesis of nanoglobule-(Mn-DOTA) monoamide conjugates

Generation 2–4 (G2, G3 and G4) nanoglobules with an OAS core were synthesized using standard solution phase peptide synthesis chemistry^{11,14}. Octa(3-aminopropyl)silsesquioxane hydrochloride (OctaAmmonium POSS®-HCl) (0.70 g, 0.597 mmol), HOBt (2.70 g, 20.0 mmol), and (di-*t*-BOC)₂-L-lys-OH-DCHA (10.4 g, 20.0 mmol) were dissolved in 30 ml DMF. To the above solution was added 10 ml of DIPEA and the mixture was stirred at room temperature for 48 h. The reaction mixture was added to 400 ml of 0.5 M citric acid aqueous solution giving a white, sticky precipitate. The precipitate was then treated with acetonitrile giving a colorless solid. The solid product was collected by filtration and dried under vacuum. Yield of [(*t*-BOC)₂-L-lysine]₈-OAS was 1.67 g, 80 %. [(*t*-BOC)₂-L-lysine]₈-OAS (1.67 g) was dissolved in 7 ml ice-cold trifluoroacetic acid (TFA) and stirred at room temperature for one hour to remove *t*-BOC protection. The residue was treated with anhydrous diethyl ether giving a colorless solid product. The yield of (L-lysine)₈-OAS trifluoroacetate (G1) was 1.32 grams (59.6 %). The G2 and G3 nanoglobules were synthesized following the general procedure of G1 giving the product of G2 (2.23 g, 82.4%) and G3 (2.72 g, 64.2%).

The synthesis of G2 nanoglobular Mn(II)-DOTA monoamide was used as an example. G2 nanoglobule (50 mg, 6.57 μ mol), DOTA-tris(*t*-Bu) (240 mg, 420 μ mol), HBTU (159 mg, 420 μ mol), HOBt (57 mg, 420 μ mol) and DIPEA were dissolved in DMF (2.5 ml) and stirred at room temperature for 24 h. The product was precipitated by adding diethyl ether into the reaction mixture. The oily precipitate was washed several times with diethyl ether and the *t*-butyl groups were removed by dissolving the precipitate in a mixture of trifluoroacetic acid and methylene chloride (6 mL, volume/volume = 1:1) for 4 h at room temperature. The residue was treated with ice-cold diethyl ether and purified with ultracentrifugation and then by dialysis to give a white solid precipitate after lyophilization. Yield of G2-DOTA monoamide conjugate was 65 mg, 70.6%, based on the G2 dendrimer. MALDI-TOF (*m/z*, [M+H]⁺): 12,855 (calculated for G2 with 23 DOTA on average), 12,848 (observed). A mixture of G2 nanoglobule-DOTA conjugate (31 mg, 2.2 μ mol) and Mn(OAc)₂ (70 mg, 282 μ mol) in 1 ml 0.5 M ammonium acetate solution were refluxed for 50 min. The final product G2 nanoglobule-(Mn-DOTA) monoamide conjugate was further purified by dialysis with a membrane of 1000 Da molecule weight cutoff to remove the excess free Mn(II) ions. The yield of purified G2 nanoglobule-(Mn-DOTA) monoamide conjugate was 14 mg, 41%. The G3 and G4 nanoglobule-(Mn-DOTA) monoamide conjugates were similarly synthesized. Yield of G3-DOTA conjugate was 85 mg, 91%. MALDI-TOF (*m/z*, [M+H]⁺): 28,560 (calculated for G3 with 53 DOTA on average), 28,785 (observed). Yield of G4-DOTA conjugate was 82 mg, 76.7%. MALDI-TOF (*m/z*, [M+H]⁺): 54,465 (calculated for G4 with 99 DOTA on average), 54,399 (observed).

Synthesis of G3 nanoglobule-(Mn-NOTA-GA) conjugate

G3 nanoglobule (30 mg, 1.95 μ mol), NODA-GA(*t*Bu)₃ (100 mg, 184 μ mol), HBTU (71 mg, 184 μ mol), HOBt (25 mg, 184 μ mol) with DIPEA were reacted according to the above procedure to give G3 nanoglobular NOTA-GA conjugate. MALDI-TOF (*m/z*, [M+H]⁺): 27,584 (calculated for G3 with 55 NOTA); 27,637 (observed). Yield of G3-NOTA conjugate was 55 mg, 92%. The complexation of G3 nanoglobule-(NOTA)₅₅ conjugate (40

mg, 1.45 μmol) with 4-fold excess $\text{Mn}(\text{OAc})_2$ gave G3 nanoglobule-(Mn-NOTA)₅₅ as colorless solid (44 mg, 97%).

Measurement of relaxivities of the nanoglobular contrast agents

The T_1 and T_2 values of the aqueous solutions containing contrast agents were determined on a Siemens 3T MRI scanner at room temperature. The water proton T_1 relaxation times without and with the agents at different concentrations were measured using an inversion recovery prepared turbo spin echo imaging pulse sequence. The inversion times (TI) were 25, 35, 50, 75, 100, 200, 400, 800, 1600, and 3200 ms with repetition time (TR) = 5000 ms and echo time (TE) = 16.0 ms. The net magnetization of each sample was fit for multiparametric nonlinear regression analysis using a Marquardt-Levenberg algorithm and a MATLAB program to calculate T_1 and M_0 . Relaxivity r_1 was determined from the slope of $1/T_1$ versus $[\text{Mn}^{2+}]$ plot. The T_2 relaxation times of the samples were measured using a turbo spin echo sequence with turbo factor 3. The parameters were TE = 12, 24, 35, 47, 59, 71, 83, 94 and 106 ms, and TR = 3000 ms. T_2 values were calculated from $M_{TE} = M_0 e^{-(TE/T_2)}$ after non-linear regression with various TE. The r_2 relaxivity was determined from the slope of $1/T_2$ versus $[\text{Mn}^{2+}]$ plot. The Mn(II) concentrations of the samples were measured by ICP-OES for r_1 and r_2 calculation.

Animal Tumor Model

Human breast cancer MDA-MB-231 cells were grown in L-15 medium supplemented with 10% fetal bovine serum and 1% penicillin at 37 °C in 5% CO_2 . Female athymic nu/nu mice (6 weeks, 20–25 g) were purchased from the National Cancer Institute (Frederick, MD). Each mouse was subcutaneously implanted in the both flanks with 2×10^6 MDA-MB-231 cells mixed with an equal volume of in a mixture of Matrigel (50 μL culture medium and 50 μL Matrigel). Three to four weeks after inoculation, tumors reached an average size of 0.5 cm in diameter. The mice were randomly divided into groups with three mice per group for MRI study. All animal experiments were performed in accordance with an animal protocol approved by the IACUC of the University of Utah.

Contrast Enhanced MRI

The mice were anesthetized by injecting a mixture of 12 mg/kg xylazine and 80 mg/kg ketamine. MRI contrast agents were administered via a tail vein at a dose of 0.03 mmol-Mn/kg. The contrast agent MnCl_2 was injected at 0.05 mmol/kg. The mice were placed in a human wrist coil and scanned in a Siemens Trio 3 T MRI scanner before injection and at 2, 5, 10, 15, 20 and 25 min post-injection using a fat suppression 3D FLASH sequence (TR = 7.8 ms, TE = 2.74 ms, 25° flip angle, 0.4 mm slice thickness, $128 \times 256 \times 48$ matrix size, $50 \times 100 \times 24 \text{ mm}^3$ field of view, $0.39 \times 0.39 \times 0.5 \text{ mm}^3$ spatial resolution, 4 averages, 47.3 s acquisition time for one image set, 25 min for full set of dynamic images). Axial tumor images were also acquired using a 2D spin-echo sequence (400 ms TR, 8.9 ms TE, 90° flip angle, 2.0 mm slice thickness, $0.125 \times 0.5 \text{ mm}^2$ in-plane view, 384×192 matrix size, $48 \times 96 \text{ mm}^2$ field of view, 76.8 s acquisition time for one image set, 2 averages, 25 min for full set of dynamic images) immediately after the acquisition of the 3D FLASH images. Signal intensity of the regions of interest (ROIs) was measured using Osirix software. Signal enhancement ratios (ER) in the liver, kidneys and tumor at each time point were calculated using the equation $\text{ER} = S_{\text{post}}/S_{\text{pre}}$, where S_{post} (post-injection) and S_{pre} (pre-injection) denote the signal within the ROIs, and averaged from three different mice (N=3). Statistical analysis was performed using a two-way ANOVA with Bonferroni's, assuming statistical significance at $p < 0.05$.

Results and discussion

Chemistry and characterization

The nanoglobular Mn(II)-DOTA monoamide and Mn(II)-NOTA conjugates was designed as the Mn(II) based macromolecular MRI contrast agents to improve their relaxivities. Macrocyclic ligands NOTA and DOTA monoamide were used to chelate paramagnetic Mn(II) ions. NOTA had a coordination number of 6 and DOTA monoamide had a coordination number of 8. The ligands with different coordination number were used to investigate the ligand effect on relaxivities of the agents. The synthetic procedure of nanoglobular Mn(II)-DOTA and Mn(II)-NOTA conjugates is depicted in scheme 1. One-fold excess of DOTA-tris(*t*-Bu) and 50% excess of NODA-GA(*t*Bu)₃ to the number of amines on the dendrimers were used for the conjugation of the ligands to the nanoglobules. The Boc protection was removed with TFA to yield nanoglobule-DOTA-monoamide or nanoglobule-(NOTA-GA) conjugates. Nanoglobular Mn(II) complexes were prepared by reacting the nanoglobular ligand conjugates with 4-fold excess Mn(OAc)₂ to each ligand molecule. High temperature (100 °C) was used to achieve complete complexation of Mn(II) with the ligands¹⁵. Both ultrafiltration and dialysis were effective to remove low molecular weight impurities before and after complexation with Mn(II) ions. Approximately 70 – 86% of the surface amino groups of the nanoglobules were conjugated with macrocyclic Mn(II) chelates (Table 1). Complete conjugation of the surface amino groups of nanoglobules was difficult to achieve due to steric hindrance. Higher conjugation degree might be achieved if a much larger excess of the protected ligands was used in the conjugation.

The physicochemical parameters of the nanoglobular Mn(II) complexes are summarized in Table 1. The manganese content of agents was 1.45 – 1.89 mmol/g nanoglobules, as determined by ICP-OES. Interestingly, the r_1 and r_2 relaxivities per Mn(II) chelate of the nanoglobular Mn(II)-DOTA conjugates decreased with increasing generation of the agents from G2 to G4, contrary to the observation of the nanoglobular Gd-DOTA monoamide conjugates¹¹. The G2 nanoglobular conjugate, G2-DOTA-Mn, showed the highest relaxivities per Mn(II) chelate among the three nanoglobular Mn(II)-DOTA monoamide conjugates. The reverse size effect of nanoglobular Mn(II)-DOTA monoamide conjugates could be attributed to the saturated complexation of Mn(II) ions. Since Mn(II) has a maximum coordination of 7 with macrocyclic chelators^{7,16}, the coordination of Mn(II) in Mn(II)-DOTA monoamide is saturated by the ligand without water molecule directly bound to it in the inner sphere. The water molecules immediately surrounding the Mn(II) macrocyclic chelates form a relatively ordered secondary solvation sphere due to hydrogen bonding between to the water molecules and the metal complexes. Water molecules beyond the secondary sphere is less organized and referred to as the outer solvation sphere where water diffuses freely¹⁷. For the paramagnetic metal chelates of saturated coordination, the contribution to their overall relaxivities could be mainly from the relaxivities of the secondary and outer solvation spheres, which were determined by the relaxation time (T'_{m}) and residency time (τ'_{m}) of secondary and outer sphere water molecules¹⁷. With the increased number of Mn(II) chelates on the surface and amino groups of larger nanoglobules, the residency time (τ'_{m}) of water molecules in the secondary sphere might increase due to the increased probability of hydrogen bonding of the water molecules. The increased residency time might outweigh the reduced relaxation times of water molecules in the secondary and outer sphere due to increased rotational correlation time (τ_{R}) of larger nanoglobular complexes. In addition, it may be possible that the DOTA chelates are buried in the G3 or G4 dendrimers and less accessible to solvation as compared to the G2 dendrimer. Consequently, the relaxivities per Mn(II) chelate of the nanoglobular Mn(II)-DOTA conjugates would decrease with the increased size and number of chelates of the agents.

The r_1 relaxivity of G2-DOTA-Mn was $3.32 \text{ mM}^{-1}\text{s}^{-1}$ per Mn(II) chelate, about 9-fold higher than that ($0.37 \text{ mM}^{-1}\text{s}^{-1}$) of MnO nanoparticle-based MRI contrast agent with a size of 7 nm^{18} . The r_1 relaxivity of the nanoglobular Mn(II)-NOTA conjugate was $3.8 \text{ mM}^{-1}\text{s}^{-1}$ per Mn(II) chelate, 35% higher than that of the corresponding G3-DOTA-Mn agent. NOTA is a hexadentate ligand with three oxygen and three nitrogen as donor atoms¹⁹. Mn(II)-NOTA chelate is a not saturated complex due to the ability of Mn(II) to form seven-coordinated complexes. A water molecule may complex to Mn(II) in the inner sphere of the Mn(II)-NOTA chelate to complete seven-coordination. Higher relaxivity G3-NOTA-Mn might be due to a bound water molecule in the inner solvation site. It was shown that the higher coordination number resulted in the lower relaxivity for the Mn(II) complexes of cyclen derivatives at 20 MHz^4 . The result in this study was consistent to what was previously observed. Most of the nanoglobular Mn(II) complexes had higher per-ion r_1 relaxivity than MnCl_2 except for G4-DOTA-Mn. The overall relaxivities the nanoglobular Mn(II)-DOTA conjugates increased with size of the agents because of increased number of the chelates in higher generation of nanoglobules. The overall r_1 relaxivity of G4-DOTA-Mn was as high as $233 \text{ mM}^{-1}\text{s}^{-1}$ per molecule.

The ratio of the r_1 to r_2 relaxivities of the nanoglobular Mn(II) chelate conjugates was much lower than that of the corresponding nanoglobular Gd(III) chelates. High r_2 relaxivity could result in significant reduction signal intensity in T_1 -weighted contrast enhancement. The ratio of the r_1 to r_2 relaxivities of the nanoglobular Mn(II) chelate conjugates was much higher than that of MnCl_2 . As reported in the literature, MnCl_2 had r_2 relaxivity as high as $103.5 \text{ s}^{-1}\text{mM}^{-1}$ at 3 T^{20} and very high r_2 relaxivity at high magnetic field strength¹⁷. The complexation of Mn(II) resulted in significantly reduced r_2 relaxivity.

In Vivo MR Imaging

The effectiveness of the Mn(II) based nanoglobular MRI contrast agents, G2, G3, G4-DOTA-Mn and G3-NOTA-Mn, were evaluated in female athymic nu/nu mice bearing MDA-MB-231 human breast carcinoma xenografts with MnCl_2 as a control. Figure 1 shows the dynamic 2D coronal images of mice before and at various time points after the injection of MnCl_2 at a dose of 0.05 mmol-Mn/kg and the nanoglobular agents at a dose of 0.03 mmol-Mn/kg . Little enhancement was observed in the blood pool for all agents, which could be attributed to rapid vascular clearance of the agents. Strong contrast enhancement was observed for all of the nanoglobular agents for the fluid in the urinary bladder 10 – 15 min after the injection, indicating rapid excretion of the nanosized contrast agents via renal filtration. Little enhancement was observed inside the bladder for the mice injected with MnCl_2 , indicating the uptake of Mn^{2+} ions in the body. MnCl_2 resulted in stronger liver enhancement than the chelated nanoglobular contrast agents. The nanoglobular Mn(II)-DOTA monoamide conjugates showed generation-dependent liver enhancement. G2-DOTA-Mn and G3-NOTA-Mn resulted in slightly stronger signal enhancement in the liver than the other nanoglobular agents and G4-DOTA-Mn produced the least liver enhancement. MnCl_2 also resulted in stronger and more prolonged enhancement in the myocardium than the nanoglobular chelates. It is known that free Mn(II) ions can preferentially accumulate in the liver, myocardium and other normal tissue through Ca^{2+} channels^{21,22}, resulting in prolonged strong liver and myocardium enhancement. The relatively weak liver and myocardium enhancement of the nanoglobular agents suggested that these agents with macrocyclic Mn(II) chelates were stable within the body within the time frame of the MRI study. Further detailed studies are required to further demonstrate the in vivo stability of these agents.

The signal intensity of the liver, kidneys and the fluid in the bladder was measured before and at various time points after injecting the contrast agents. Figure 2 shows the enhancement ratio, the ratio of the signal intensity post-injection to that before injection.

MnCl₂ resulted in at least 2.8-fold signal increase in the liver due to high liver accumulation of free Mn²⁺, which was much higher than the 1.2- to 1.8-fold signal increase generated by the nanoglobular complexes although the dose of the former was only 67% higher than the latter. The difference of the enhancement ratios in the liver among the nanoglobular agents was not significant. MnCl₂ also resulted in higher signal increase in the kidneys than the nanoglobular chelates, but the difference was not as significant as in the liver ($p > 0.05$), especially in the first 2 minutes post-injection. The enhancement ratio in the kidneys for the nanoglobular complexes decreased gradually during the period of 25 min post injection and the enhancement ratio inside the bladder correspondingly increased (Figure 1), indicating renal excretion of the agents. In comparison, the enhancement ratio in the kidneys for MnCl₂ did not change during the period and no enhancement was shown in the bladder, suggesting retention of Mn²⁺ ions in the tissue within the 30-minute time frame of this study.

Figure 3 shows the axial 2D T_1 -weighted spin-echo images of tumor tissue contrast enhanced by MnCl₂ and the nanoglobular MRI contrast agents. Substantial contrast enhancement was observed in the tumor after the injection of the nanoglobular contrast agents. It appears that G3-NOTA-Mn generated more tumor enhancement than the nanoglobular Mn(II)-DOTA monoamide conjugates. Quantitative analysis of the contrast enhancement ratio (ER) in the tumor tissue also suggested that G3-NOTA-Mn resulted in significantly higher enhancement ratio in tumor than the other tested agents after 2 minutes post-injection ($p < 0.05$), Figure 4, possibly because of its larger r_1 relaxivity, but the difference was not significant.

This study showed the conjugation of macrocyclic Mn(II) chelates on the highly functionalized nanoglobular dendrimers resulted in non-gadolinium(III) based macromolecular MRI contrast agents with high overall relaxivities. The data of macrocyclic Mn(II) chelates suggested good in vivo stability against transmetallation with the endogenous metal ions, including Ca²⁺, Cu²⁺ and Zn²⁺, which was similar to macrocyclic Gd chelates²³. Consequently, the agents could readily clear from the major organs, particularly from the liver, and excrete via renal filtration. As compared to the corresponding nanoglobular Gd(III)-DOTA monoamide conjugates¹¹, G2-DOTA-Mn had similar in vivo enhancement as the G2-DOTA-Gd because both agents were rapidly excreted via renal filtration. G3-DOTA-Mn and G3-NOTA-Mn were less effective than the corresponding G3 Gd-DOTA monoamide conjugate for both blood pool and cancer imaging at the same dose, even though both Mn(II)-based agents had high overall T_1 relaxivity. It is plausible to assume that much higher T_2 relaxivity of nanoglobular Mn(II) complexes may reduce MR signal in the T_1 -weighted contrast enhancement. The use of smaller macrocyclic ligand NOTA improved the relaxivity and in vivo contrast enhancement of the Mn(II)-based contrast agent. Nevertheless, we have shown in this study that it is possible to design macromolecular Mn(II) complexes to improve their relaxivity and for in vivo MR imaging. Future studies on the design and development effective Mn(II) based MRI contrast agents should focus on further improvement of the stability and T_1 relaxivity of Mn(II) chelates and reduction of the T_2 effect.

Conclusions

Nanoglobular Mn(II)-DOTA-monoamide and Mn-NOTA conjugates were synthesized as non-Gd(III) based macromolecular contrast agents for magnetic resonance imaging. The relaxivities per ion of the nanoglobular macrocyclic Mn(II) chelates decreased with the increasing generation of the dendrimers. The G3 nanoglobular Mn(II)-NOTA conjugate had higher relaxivities than the corresponding Mn(II)-DOTA conjugate. The nanoglobular macrocyclic Mn(II) chelates readily excreted via renal filtration. They showed less non-specific liver enhancement than MnCl₂. The Mn(II)-based nanoglobular agents were

effective for contrast enhanced cancer imaging in the mouse tumor model at a low dose. The nanoglobular macrocyclic Mn(II) chelate conjugates are promising for MR cancer imaging as non-gadolinium(III) based contrast agents.

Acknowledgments

This work was supported in part by the NIH R01 CA097465. We greatly appreciate Dr. Yongen Sun and Dr. Xianfeng Shi for their technical assistance in animal handling and MRI data acquisition.

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Figure 1. Representative 2D coronal T₁-weighted MR images of tumor bearing mice injected with Mn(II)-based nanoglobular MRI contrast agents G3-NOTA-Mn and G2, G3, G4-DOTA-Mn at 0.03 mmol-Mn/kg and MnCl₂ at 0.05 mmol/kg. Arrows 1, 2 and 3 point to the heart, liver and bladder, respectively.

Figure 2. The enhancement ratio (ER) of the MR signal post-injection to that of pre-injection in the liver, kidneys and inside the bladder of the nude mice with nanoglobular macrocyclic Mn(II) chelate conjugates G2, G3, G4-DOTA-Mn and G3-NOTA-Mn at 0.03 mmol-Mn/kg and MnCl₂ at 0.05 mmol/kg.

Figure 3.

2D axial T_1 -weighted spin-echo MR images of MDA-MB-231 tumor xenografts in nu/nu female mice before and after the tail-vein injection of $MnCl_2$ at 0.05 mmol-Mn/kg and nanoglobular macrocyclic Mn(II) chelate conjugates G2, G3, G4-DOTA-Mn and G3-NOTA-Mn at 0.03 mmol-Mn/kg. Arrows point to the tumors.

Figure 4.

The enhancement ratio (ER) of the MR signal post-injection to that of pre-injection in the tumor before and at various time points after injection of the nanoglobular MRI contrast agents G2, G3, G4-DOTA-Mn and G3-NOTA-Mn at 0.03 mmol Mn/kg and MnCl₂ at 0.05 mmol/kg in nu/nu female mice bearing MDA-MB-231 tumor xenografts.

Scheme 1.
Synthesis of G3 nanoglobular macrocyclic Mn(II) chelate conjugates.

Table 1

Physicochemical properties of the nanoglobular MRI contrast agents

Contrast agents	Mn content (mmol Mn/g nanoglobule)	Macrocyclic Mn chelates/amino groups	Molecular Weight	r_1/r_2 [$\text{mM}^{-1}(\text{Mn})\text{s}^{-1}$] at 3T	r_1/r_2 [$\text{mM}^{-1}(\text{nanoglobule})\text{s}^{-1}$] at 3T
MnCl_2	-	-	125.8	2.4/103.5 [*]	-
G2-DOTA-Mn	1.45	23/32 (72%)	14.1 kDa	3.3/14.7	76/340
G3-DOTA-Mn	1.71	54/64 (84%)	31.8 kDa	2.8/12.6	153/681
G4-DOTA-Mn	1.51	99/128 (77%)	59.8 kDa	2.4/8.7	233/863
G3-NOTA-Mn	1.89	55/64 (86%)	30.6 kDa	3.8/24.5	209/1345

^{*} Note: The value is from reference 20.