Published in final edited form as:

Contrast Media Mol Imaging. 2016 January ; 11(1): 32–40. doi:10.1002/cmmi.1655.

A neutral polydisulfide containing Gd(III) DOTA monoamide as a redox-sensitive biodegradable macromolecular MRI contrast agent

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Abstract

This work aims to develop safe and effective gadolinium (III)-based biodegradable macromolecular MRI contrast agents for blood pool and cancer imaging. A neutral polydisulfide containing macrocyclic Gd-DOTA monoamide (GOLS) was synthesized and characterized. In addition to studying the *in vitro* degradation of GOLS, its kinetic stability was also investigated in an *in vivo* model. The efficacy of GOLS for contrast enhanced MRI was examined with female BALB/c mice bearing 4T1 breast cancer xenografts. The pharmacokinetics, biodistribution and metabolism of GOLS were also determined in mice. GOLS has an apparent molecular weight of 23.0 kDa with T_1 relaxivities of $7.20 \text{ mM}^{-1}\text{s}^{-1}$ per Gd at 1.5 T, and $6.62 \text{ mM}^{-1}\text{s}^{-1}$ at 7.0T. GOLS had high kinetic inertness against transmetallation with Zn^{2+} ions and its polymer backbone was readily cleaved by *L*-cysteine. The agent showed improved efficacy for blood pool and tumor MR imaging. The structural effect on biodistribution and *in vivo* chelation stability was assessed by comparing GOLS with Gd(HP-DO3A), a negatively charged polydisulfide containing Gd-DOTA monoamide GODC, and a polydisulfide containing Gd-DTPA-bisamide (GDCC). GOLS showed high *in vivo* chelation stability and minimal tissue deposition of gadolinium. The biodegradable macromolecular contrast agent GOLS is a promising polymeric contrast agent for clinical MR cardiovascular imaging and cancer imaging.

Graphical Abstract

A neutral polydisulfide containing macrocyclic Gd-DOTA monoamide (GOLS) was synthesized to optimize pharmacokinetic properties of biodegradable macromolecular MRI contrast agent. GOLS has shown high chelation stability against transmetallation *in vitro* and *in vivo* and improved minimal tissue deposition of gadolinium. It produces robust blood pool and tumor enhancement in MRI.

Keywords

gadolinium contrast agents; biodegradable macromolecular contrast agents; blood pool MRI; high kinetic stability; polydisulfide

INTRODUCTION

Gadolinium chelates with high thermodynamic stability are predominantly used as clinical MRI contrast agents for the diagnosis of life-threatening diseases, including cancer and cardiovascular diseases [1, 2]. However, most of the clinical Gd(III) contrast agents are non-specific small molecules with relatively low relaxivities and rapid vascular extravasation rates [3]. In order to overcome these limitations, macromolecular Gd(III) contrast agents have been developed with improved relaxivities, specificity, and stability [4–6].

Macromolecular Gd(III) MRI contrast agents are normally synthesized through the covalent incorporation of small molecular Gd(III) chelates into biocompatible macromolecules such as proteins, dendrimers, and natural and synthetic polymers [7–9]. This allows the agents to have significantly improved physicochemical and biological properties, including a 2–4 fold relaxivity increase due to the reduced molecular tumbling of Gd(III) chelates [10, 11].

Additionally, macromolecular MRI agents have been shown to have an enhanced tumor accumulation by the enhanced permeability and retention (EPR) effect as well as a prolonged blood pool circulation [12, 13]. While macromolecular agents have been successful in preclinical studies, their translation into clinical application has been hindered due to safety concerns arising from their ineffective excretion, which can cause the release and long term deposition of the toxic Gd^{3+} ions [14, 15].

To facilitate the rapid excretion of Gd(III) chelates, environmentally labile chemical bonds have been incorporated into polymeric systems. These bonds allow for the breakdown of polymer backbones or the release of Gd(III) chelates from macromolecules [16, 17]. Examples of these cleavable bonds include acid labile bonds [18, 19], redox sensitive bonds [20–22] and enzymatic degradable bonds [23–25]. These biodegradable macromolecular contrast agents have maintained the advantages of macromolecular contrast agents for blood pool and tumor imaging and shown minimal long-term tissue accumulation. Previously, we have designed and synthesized redox sensitive polydisulfide Gd-DTPA complexes as extracellular degradable macromolecular contrast agents [7, 26–28]. These complexes have a backbone consisting of disulfide bonds, which are environmentally labile *in vivo*. The disulfide bonds are gradually reduced by the plasma thiols to produce monomeric or oligomeric Gd(III) complexes, which are rapidly excreted via renal filtration [29]. The

polydisulfide contrast agents produced significant tumor contrast enhancement. However, it was found that these Gd-DTPA complexes exhibit low kinetic inertness and release toxic Gd(III) ions by transmetallation with endogenous metal ions, mainly Zn(II) ions [30]. Agents with low kinetic stability are likely to cause nephrogenic systemic fibrosis (NSF) in a small portion of the patients with compromised renal function [31, 32]. Macrocyclic Gd(III) chelates have shown high kinetic stability with no transmetallation with endogenous metal ions and appeared safe from NSF [31–33].

A new generation of polydisulfides containing macrocyclic Gd(III) chelates have been designed and synthesized to further improve the kinetic stability of the Gd(III) chelates in the redox sensitive biodegradable macromolecular MRI contrast agents. (N₆-lysyl)lysine-(Gd-DOTA) monoamide and 3-(2-carboxyethylidysulfanyl)propanoic acid copolymers (GODC) demonstrated high kinetic inertness and superior image enhancement [21]. GODC has a free carboxylic group on each monomer unit, and is anionic under physiological pH. This has a significant impact on its *in vivo* properties, including pharmacokinetics and biodistribution.

In this study, we synthesized a neutral macrocyclic Gd(III) chelate containing polydisulfide N¹-lysylethylenediamine Gd-DOTA monoamide and dithiobispropionic acid copolymers (GOLS). This agent is a neutral, redox sensitive, and biodegradable macromolecular contrast agent that was prepared by the condensation copolymerization of N¹-lysylethylenediamine DOTA monoamide and dithiobis(succinimidylpropionate), followed by complexation with Gd(OAc)₃. In addition to analyzing the physiochemical properties, the chelation stability, pharmacokinetics, and biodistribution of different polydisulfide Gd(III) complexes were analyzed *in vivo*. These studies were performed in comparison to the clinical contrast agent Gd(HP-DO3A) and a previously reported polydisulfide Gd-DTPA complexes, GDCC [27], in a mouse model. The effectiveness of GOLS for contrast enhanced blood pool and tumor MRI was also evaluated.

METHODS

Reagents and Materials

2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and benzotriazol-1-yl-oxytripyrrolidino-phosphonium hexafluorophosphate (PyBOP) 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 TCI America (Portland, OR). *N,N*-Diisopropylethylamine (DIPEA) and trifluoroacetic acid (TFA) were purchased from Alfa Aesar (Ward Hill, MA). Lysine methyl ester dihydrochloride, di-*tert*-butyl dicarbonate (Boc₂O), sodium bicarbonate, ethylenediamine, 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTTSP) and Gd(OAc)₃ were purchased from Sigma-Aldrich, Inc. (Louis, MO). GODC was synthesized as previously described [21]. All reagents were used without further purification unless otherwise stated.

Synthesis of N¹-lysylethylenediamine DOTA monoamide

N^ε-Boc-N^α-Boc-L-lysine methyl ester [34] and N^ε-Boc-N^α-Boc-L-lysine N-(2-aminoethyl) amide [35] were synthesized based on reported methods. N^ε-Boc-N^α-Boc-L-lysine N-(2-aminoethyl)amide (2.6 g, 6.8 mmol) and DOTA-*tris*(*t*-Bu) (3.2 g, 5.7 mmol) was dissolved in DMF (8 ml). The coupling agent ,PyBOP (3.5 g, 6.8 mmol), as well as HBTU (1.3 g, 6.8 mmol) and DIPEA (1.2 ml, 6.8 mmol) were added into the mixture. The reaction solution was stirred at room temperature overnight. After the reaction, EtOAc (80 ml) was added, followed by removal of the solvent via rotary evaporation. The residue was purified through preparative silica TLC (EtOAc : DCM = 2:3). The product was obtained as colorless crystalline compound (yield = 56%). The purified product was de-protected by mixing with TFA (10 ml) and stirred overnight at room temperature. The desired compound N¹-lysylethylenediamine Gd-DOTA monoamide was precipitated out in ether to give a colorless compound (yield = 92%). ¹H NMR (300 Hz, D₂O): 1.6 (m, 3H), 1.9 (m, 3H), 2.1 (m, 3H), 3.1 (m, 5H), 3.4 (m, 18H), 4.0 (m, 7H). MALDI-TOF-MS: *m/z* = 574.4 (M⁺).

Synthesis of N¹-lysylethylenediamine Gd-DOTA monoamide and dithiobispropionic acid copolymer (OLS) and its Gd(III) complex (GOLS)

GOLS was synthesized using a similar method that was previously reported [28]. Briefly, the polymerization was carried out by slowly adding 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTTSP) (0.6 g, 1.1 mmol) portion by portion to the concentrated aqueous solution of N¹-lysylethylenediamine DOTA monoamide (0.8 g, 1.1 mmol in 0.7 ml de-ionized water) while stirring. The mixture was stirred at room temperature for 4 hours after the addition of DTTSP. The product was purified via dialysis against de-ionized water using dialysis membrane with molecular weight cut-off 6000–8000 Da, and freeze-dried to bring the white polymer ligand (OLS). The yield of the reaction was 16% after purification. Gd(III) complexation was then carried out by stirring the mixture of the polymeric ligand OLS and two-fold excess of Gd(OAc)₃ in de-ionized water for 48 hours at pH 6 under room temperature. The polymeric Gd(III) complexes was purified through size exclusion chromatography using a Sephadex[®] G-50 column. Xylenol Orange was added as an indicator to monitor the complexation and purification. The final product GOLS was obtained after lyophilization as white solid. The purity and apparent molecular weight of OLS and GOLS were determined using size exclusion chromatography on an AKTA FPLC system with a Superose[™] 12 column (GE Healthcare Life Sciences). The column was calibrated using water soluble poly[N-(2-hydroxypropyl)methacrylamide] (HPMA) standards with a series of different molecular weight (*M_n* = 14, 23, 32 and 47 kDa). The Gd(III) content of GOLS was determined using inductive coupled plasma-optical emission spectrometry (ICP-OES, Perkin-Elmer). The hydrodynamic size of GOLS in solution was determined using dynamic light scattering (DLS). The DLS experiment was carried out using a Brookhaven BI-200SM goniometer and BI-9000AT digital correlator equipped with a He-Ne laser (*λ*=633 nm) at room temperature. The aqueous solution of GOLS (1.33 mg/ml) was filtered through a 0.22 μm filter, and then measured at a scattering angle of 90. A Nanosphere[™] polystyrene size standard (diameter = 102 nm ± 3 nm) (Thermo Scientific, Waltham, MA) was analyzed in line to confirm accuracy.

Relaxivity measurements

The T_1 relaxivity of GOLS was determined on a Bruker Minispec® Relaxometer (1.5T, 60 Hz) at 37°C, and a Bruker 7.0 T MR Biospec small animal scanner. GOLS solutions containing a gradient of 0.2, 0.4, 0.6 and 0.8 mM of Gd(III) were prepared. At 1.5T, the T_1 values of these solutions were measured by the relaxometer using an inversion-recovery pulse sequence. Similarly, Bruker's inversion recovery sequence with an echo planar (EPI) readout was used to determine the T_1 values at 7.0T. The longitudinal relaxivity (r_1) was calculated as the slope of $1/T_1$ versus Gd(III) concentration plot. The transverse relaxivity (r_2) was measured similarly; the T_2 values were determined using a Carr-Purcell-Meiboom-Gill (CPMG) spin echo sequence at 1.5T. Additionally, Bruker's standard multi-slice-multi-echo (MSME) sequence was used to measure the T_2 values at 7.0T (TE = variable, TR = 2000 ms, flip angle = 180°, slice thickness = 1 mm, slice number = 5, average = 2, matrix = 128 × 64, field of view = 3 cm × 3 cm, resolution = 0.0234 × 0.3 mm). The Gd(III) content of the sample was re-confirmed using ICP-OES after the relaxivity measurement.

In vitro Degradation of GOLS

GOLS (0.42 mM-Gd) and cysteine (15 μM) were incubated together in PBS buffer (pH=7.4) at room temperature for 6 hours. The change of molecular weight of GOLS over time during the incubation was monitored using size exclusion chromatography on an AKTA® FPLC system with a Superose® 12 column.

In vitro Kinetic Stability of GOLS

GOLS (0.42 mM-Gd) was incubated with ZnCl₂ (50 μM) in PBS buffer at physiological pH under 37°C for 2 hours. Samples before and at 30, 60 and 120 minutes of incubation were collected, then went through the PD-10 column (GE Health Science) to separate the polymer bound and free Gd(III) species. The Gd(III) content in the polymers after separation was determined using ICP-OES (Perkin-Elmer). The percentage of polymer bound Gd(III) post-incubation versus pre-incubation was calculated. The value was compared with that of our previously reported linear chelates based polymeric agent, Gd-DTPA cystamine copolymers (GDCC, 0.42 mM-Gd). In addition, GOLS and GDCC incubated with Zn²⁺ free PBS buffer were used as negative controls.

MRI experiment

Female BALB/c mice (18–22 g) were purchased from Charles River (Wilmington, MA, USA). The animals were cared under an animal protocol approved by the IACUC of Case Western Reserve University. 4T1 mouse breast cancer cells (5×10^4 cells) were inoculated into the inguinal mammary fat pads of each mouse, generating an orthotopic mouse model of malignant breast cancer. Contrast-enhanced MR examinations were performed when the tumor size reached 0.5–1.0 cm in diameter as measured by a caliper. Anaesthetization of the tumor bearing mice was initially conducted using 2.5%–3.5% of isoflurane in oxygen, and then maintained with 1.5%–2.5% of isoflurane during the MR examination. The animals were kept warm at 34°C using a temperature controlling air conditioning system. Parameters such as temperature, respiration and electrocardiograms (ECGs) of the mice were monitored during image acquisition to ensure steady physical conditions. The contrast agent was

intravenously administered via a tail vein at the standard clinical dose of 0.1 mmol-Gd/kg. A clinical agent Gd(HP-DO3A) (Bracco, Milan, Italy) was delivered at the same dose as a control. MR diagnosis was performed before and at 1, 5, 10, 15 and 20 minutes post injection using a Bruker 7.0 T MR Biospec small animal scanner with rat-size coil to cover whole body of mice. A 3D FLASH pulse sequence (TE = 2.6, TR = 8.5, flip angle = 15°, slice thickness = 0.688 mm, slice number = 32, average = 3, matrix = 100 × 512, field of view = 10 cm × 3 cm, resolution = 0.195 mm × 0.3 mm) and 2D spin echo sequence (TE = 8.06 ms, TR = 500 ms, flip angle = 90°, slice thickness = 1.2 mm, slice number = 16, average = 2, matrix = 128 × 128, FOV = 3 cm × 3 cm, resolution = 0.234 mm × 0.234 mm) were used to acquire the MR images. Three animals were used for each contrast agent.

MRI data analysis

MR image analysis was performed using Bruker Biospec Topspin software. Regions of interest (ROI) were set in the heart (signal from blood), tumor and thigh muscle. Contrast to noise ratios (CNR) were calculated at each time point and averaged from different mice (n = 3) for the organ/tissue. The CNR in the tumor was calculated using the following equation: $CNR = (S_t - S_m) / (\sigma_n)$, where S_t and S_m denote the signal in tumor and thigh muscle and σ_n is the standard deviation of noise estimated from the background air. The CNR in the blood was calculated using the same equation, where S_t and S_m denote the signal in blood and thigh muscle. The p values were calculated using the student's two-tailed t -test, assuming statistical significance at $p < 0.05$.

Biodistribution study

The biodistribution of GOLS was determined at the 2-day and 10-day post-injection in comparison with GODC, GDCC, and Gd(HP-DO3A). At each time point, a group of BALB/c (male and female) mice was used for each agent. The contrast agents were administered at a dose of 0.1 mmol Gd/kg via tail vein injection. The animals were sacrificed with an overdose of isoflurane, and the tissue samples (heart, spleen, lung, muscle, femur, skin, brain, liver and kidney) were collected and weighed. All the samples were dried in the 50°C oven for 3 days before dissolving in 1.0 ml of concentrated nitric acid (70%). The samples were then ten-fold diluted with de-ionized water, followed by centrifugation at 4000 rpm for 25 min. The supernatant was collected for the determination of Gd content using ICP-OES.

In vivo chelation stability

The *in vivo* chelation stability of GOLS was determined in BALB/c mice in comparison with GODC, GDCC and Gd(HP-DO3A). A total of sixteen mice (male and female) was randomly divided into four groups (n=4 each) for four contrast agents. The mice were anesthetized with 1.5–2.5% of isoflurane in oxygen, and administered with the contrast agents at a dose of 0.1 mmol-Gd/kg via tail vein injection. The mice were immediately placed into metabolic cages after the injection. Urine samples were collected during the period of 8 hours pre-injection and 0–8, 8–16, 16–24 and 24–48 hours post-injection from the metabolic cages. The urine samples were diluted using de-ionized water, then filtered to

collect the filtrate. The concentration of Gd(III), Zn(II), Cu(II) and Ca(II) was determined by ICP-OES.

RESULTS

Synthesis of N¹-lysylethylenediamine Gd-DOTA monoamide and dithiobispropionic acid copolymers (GOLS)

The DOTA contained monomer, N¹-lysylethylenediamine DOTA monoamide, was first synthesized through the carboxyl-amine coupling reaction as shown in Fig. 1. The neutral biodegradable macromolecular MRI contrast agent, N¹-lysylethylenediamine Gd-DOTA monoamide and dithiobispropionic acid copolymers (GOLS), was then synthesized through condensation polymerization of the DOTA monoamide and 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTTSP), followed by Gd(III) complexation (Fig. 2). The structure of the monomer N¹-lysylethylenediamine Gd-DOTA monoamide was verified using ¹H NMR and MALDI-TOF mass spectrometry. The number average and weight average molecular weights of the polymeric ligand were 18.4 and 29.3 kDa, respectively. After complexation, the final product of GOLS was purified and fractionated through size exclusion chromatography using a Sephadex[®] G-50 column. GOLS with number average and weight average molecular weights of 23.0 and 24.6 kDa were collected for *in vivo* animal studies. The hydrodynamic diameter of GOLS was around 3 nm as determined by DLS. The Gd(III) content in the agent was 16.1 % (w/w) as determined by ICP-OES, which corresponds to complexation efficiency of 94.7 % as compared to the calculated Gd content (17.0 %, w/w). The longitudinal (r_1) and transverse relaxivities (r_2) of GOLS were 7.20 mM⁻¹s⁻¹ and 9.70 mM⁻¹s⁻¹ respectively at 1.5 T, 37°C, approximately 2-fold compared to that of Gd(HP-DO3A) (ProHance[®], 4.1 mM⁻¹s⁻¹ at 1.5T, 37°C). Similarly, the longitudinal and transverse relaxivities of GOLS at 7.0T were r_1 = 6.62 mM⁻¹s⁻¹ and r_2 = 8.92 mM⁻¹s⁻¹.

Degradability of GOLS

The polydisulfide structure of GOLS was designed so that the polymers could be readily degraded and excreted via renal filtration *in vivo* through the disulfide-thiol exchange reaction. The degradability of GOLS was demonstrated via an *in vitro* incubation study with L-cysteine, the most abundant free thiols in plasma. As shown in Fig. 3, the number average molecular weight of GOLS changed from 23.0 kDa before incubation to 23.0, 22.5, 21.2 and 19.0 kDa at 30, 60, 120 and 240 min during the incubation. Consequently, an increase of small molecular fractions (peaks from 30–40 min) was observed over time, indicating the process of degradation via incubation with free thiols.

In vitro chelation stability of GOLS

The kinetic inertness of GOLS against transmetallation was evaluated *in vitro* by incubation with ZnCl₂ at a plasma concentration mimicking the *in vivo* environment. Zn(II) was the major metal species involved in the transmetallation of Gd(III) based contrast agents *in vivo*. As shown in Fig. 4, a gradual loss of Gd(III) in the polymers was observed with the Gd-DTPA containing polydisulfide GDCC, from the initial 100 % to 92% at 30 min, 88 % at 90 min and 82 % at 120 min during the incubation with Zn²⁺. In contrast, negligible loss of

Gd(III) was observed with GOLS in the presence of Zn^{2+} , similar to what was observed with the control samples of GDCC and GOLS incubated with Zn(II)-free PBS buffer. The results demonstrated the polydisulfide containing macrocyclic Gd(III) chelate (GOLS) possessed high kinetic inertness against transmetallation.

***In vivo* MR imaging**

Fig. 5 showed the T_1 -weighted 3D maximum intensity projection MR images of mice bearing orthotopic 4T1 mouse malignant tumor before and after intravenous injection of GOLS and Gd(HP-DO3A). The blood pool enhancement generated by GOLS was gradually decreased, but still visible at 10 minutes post-injection. In comparison, the Gd(HP-DO3A) generated blood pool signals that decreased sharply in 1 minute post injection, and become negligible at 5 minutes post-injection. Signal intensity in the kidneys decreased over time, while that in the urinary bladder increased for both agents, indicating the elimination of both agents through kidney filtration. The bladder enhancement was first observed at 1 minute post injection for Gd(HP-DO3A), and 5 minutes for GOLS, indicating slightly slower excretion of the macromolecular agent. Fig.6 showed the axial T_1 -weighted 2D spin-echo tumor images before and at various time points after injection of GOLS and Gd(HP-DO3A). In comparison, GOLS generated significant and prolonged signal enhancement in the tumor than Gd(HP-DO3A). As shown in Fig. 7, GOLS generated significantly higher CNR in blood than Gd(HP-DO3A) in the first 10 minutes post injection. GOLS also produced more than 2-fold CNR increase in the tumor than Gd(HP-DO3A) for at least 30 minutes.

Biodistribution

Fig. 8 shows the biodistribution of Gd(III) in the major organs and tissues, including the heart, spleen, lung, muscle, femur, skin, brain, liver and kidney of mice, at 2 days and 10 days after intravenous injection of GOLS, GODC, GDCC and Gd(HP-DO3A) at 0.1 mmol-Gd/kg. The polydisulfide agents GOLS, GODC, and GDCC had similar low Gd(III) distribution in the heart, spleen, lung, muscle, femur, skin and brain at both time points as Gd(HP-DO3A). The agents showed higher Gd(III) concentration in the liver and kidney than that in the other organs. The neutral polydisulfide agent GOLS showed much lower accumulation in the liver and kidneys than the negatively charged agent GODC and polydisulfide with linear Gd(III) chelates GDCC ($p < 0.05$) at 2 days post-injection, which further decreased at 10 day post-injection. Approximately 0.3 % and 0.1 % of injected GOLS was measured in the liver and kidneys 10 days post-injection, lower than that of GDCC (0.5 % of injected dose/liver, 0.3 % of injected dose/kidney) and GODC (0.9 % of injected dose/liver, 0.3% of injected dose/ kidney) ($p < 0.05$). Overall, the neutral agent GOLS had much lower long-term body retention than GODC and GDCC.

***In vivo* chelation stability**

Fig. 9 shows the concentrations of Gd(III), Zn(II), Ca(II), and Cu(II) in the urine samples collected before and during different time period of 0–8 , 8–16 , 16–24 and 24–48 hours after intravenous administration of GOLS, GODC, GDCC, and Gd(HP-DO3A). Most of the contrast agents were excreted through renal filtration within the first 8 hours post-injection. The Gd(III) urine levels were comparable among GOLS, GODC and Gd(HP-DO3A). The linear agent GDCC had relatively lower Gd(III) concentrations than the other agents. In

correlation, there was an significant increase of Zn(II) concentration in the urine samples collected in the first 8 hours post-injection of GDCC ($p < 0.05$), indicating transmetallation of GDCC with endogenous Zn(II) ions. There was no change in Zn(II) concentration in the urine samples from the mice injected with the macrocyclic agents GOLS, GODC, and Gd(HP-DO3A). No significant change of the urine Cu(II) and Ca(II) content was observed during different time period after injection of the contrast agents, indicating the chelation stability of the tested agents. The result demonstrated high chelation stability of the neutral polydisulfide containing macrocyclic Gd(III) chelates against transmetallation with endogenous metal ions.

DISCUSSION

GOLS is a neutral alternative of the previously developed macrocyclic polydisulfide GODC. We have shown that negatively charged GODC is readily degraded *in vivo* and effective for blood pool and cancer MRI. However, the negative charges on GODC might complicate biodistribution and elimination of the agent due to non-specific charge interaction with tissue. The neutral agent GOLS was designed to eliminate potential non-specific tissue interaction. The T_1 and T_2 relaxivities of GOLS were slightly smaller than those of GODC ($r_1 = 8.25 \text{ mM}^{-1}\text{s}^{-1}$ and $r_2 = 10.08 \text{ mM}^{-1}\text{s}^{-1}$), but much higher than the clinical agent Gd(HP-DO3A). Similar to GODC, the polymer chains of GOLS were readily reduced by L-cysteine, the most abundant free thiol in plasma [29, 36]. The *in vivo* study validated the degradation mechanism through analysis of the urine metabolites of GODC using mass spectrometry. It was shown in both *in vitro* and *in vivo* studies that GOLS and GODC demonstrated high kinetic inertness against transmetallation with endogenous metal ions, particularly Zn(II) ions. Since both agents contained Gd-DOTA monoamide chelates, their *in vivo* kinetic inertness are similar to the clinical macrocyclic agent Gd(HP-DO3A). They have a much higher kinetic inertness than that of GDCC, a polydisulfide agent based on linear chelates. High kinetic inertness of Gd(III) based MRI contrast agents is a critical safety parameter for complete and intact excretion of the agents from the body.

GOLS demonstrated similar blood pool and tumor contrast enhancement as GODC, which was superior over the clinical agent Gd(HP-DO3A). The strong and prolonged blood pool enhancement of GOLS was a result of increased relaxivity and relatively extended blood circulation. Strong bladder enhancement was observed at 5 min post administration of GOLS, which was slower than that of Gd(HP-DO3A) but faster than that of GODC (10 min). This implies the macromolecular feature and strong degradability of GOLS. The gradual increase of bladder signals indicated that GOLS could be readily excreted via renal filtration. The neutral macrocyclic chelate based biodegradable macromolecular MR contrast agent GOLS was advantageous for contrast enhanced blood pool and tumor imaging over the clinical agent Gd(HP-DO3A), and was readily excreted via renal filtration.

GOLS exhibited similar retention in most organs and tissues as the clinical agent Gd(HP-DO3A), only slightly higher retention in the liver and kidneys. However, the retention of GOLS in the liver and kidneys was much lower than that of GODC and GDCC. The negatively charged GODC had significantly higher liver retention than GOLS, which could be attributed to the relatively slower *in vivo* excretion of GODC and non-specific interaction

of the liver with negative charges of the agent. Nevertheless, the polydisulfide MRI contrast agents, including GDCC, had much lower long-term tissue retention than other non-degradable macromolecular MRI contrast agents as shown in our previously publications [37, 38]. In addition, the liver and kidney retentions of the three polydisulfides agents were dramatically lower than that of some other reported macromolecular Gd(III) agents such as the second-generation-Gd-DTPA polypropyleneimine dendrimer conjugate (7 kDa) (45 % of injected dose in rats 14 days after injection) [39] and carboxymethyl hydroxyethyl starch-(Gd-DO3A) (47 % of injected dose remained in the body 7 days post injection) [40]. The result validated our design hypothesis that the neutral GOLS would minimize non-specific tissue interaction and result in more complete elimination than negatively charged GODC. The comparable long-term tissue retention of GOLS with the clinical agent Gd(HP-DO3A) is an advantageous safety feature of macromolecular blood pool contrast agent for further clinical development.

CONCLUSIONS

A neutral polydisulfide polydisulfide containing Gd(III) DOTA monoamide, GOLS, was synthesized and evaluated as a new generation of biodegradable macromolecular MRI contrast agent with high chelation stability. GOLS was readily reduced by endogenous thiols into smaller oligomers that were excreted from renal filtration. It possessed high kinetic inertness against transmetallation with endogenous metal ions both *in vitro* and *in vivo*. GOLS produced superior blood pool and tumor contrast enhancement as compared to small molecular clinical contrast agents. Most importantly, GOLS resulted in minimal long-term tissue retention comparable to a macrocyclic clinical contrast agent Gd(HP-DO3A). The neutral biodegradable macromolecular contrast agent GOLS has the potential to be developed as a safe and effective macromolecular blood pool MRI contrast agent for clinical cardiovascular imaging and cancer imaging.

ACKNOWLEDGMENTS

The research work is supported in part by the NIH grant R01 EB00489.

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Figure 1.
Synthetic scheme of N¹-lysylethylenediamine Gd-DOTA monoamide.

Figure 2.
Synthetic scheme of N¹-lysylethylenediamine Gd-DOTA monoamide and
dithiobispropionic acid copolymers (GOLS).

Figure 3.

The apparent molecular weight (MW) distribution of GOLs (0.42 mM-Gd) before (0 min) and after incubation with L-cysteine (15 μ M) in PBS buffer for 30, 60, 120 and 240 min at 37°C. The apparent molecular weight distribution was measured by size exclusion chromatograms. Before incubation (0 min), only polymer peak with 23.0 kDa (peak at 23 min) was observed. With incubation time at 30, 60, 120 and 240 min, the MW of polymer decreased to 23.0 kDa (peak at 22 min), 22.5 kDa (peak at 23 min), 21.2 kDa (peak at 24 min), and 19.0 kDa (peak at 25 min), with an increase of their corresponding small molecular fractions (peaks from 30–40 min).

Figure 4.

The Gd(III) content in GDCC (0.42 mM-Gd, square) and GOLS (0.42 mM-Gd, circle) before and in the incubation in PBS buffer with ZnCl₂ (50 μ M, filled) and without ZnCl₂ (open) for 30, 60, and 120 min (* p <0.05). Data presented as mean $\pm$ SD.

Figure 5.

Three-dimensional maximum intensity projection images (coronal view) of mice bearing 4T1 breast cancer before and at 1, 5, 10, 20 and 30 min post injection of Gd(HP-DO3A) (A) and GOLS (B) at a dose of 0.1 mmol-Gd/kg. The major organs were pointed out as h (heart), l (liver), k (kidney), b (bladder) and t (tumor).

Figure 6.

Axial 2D spin echo images of mice bearing 4T1 orthotopic breast tumor before and at 1, 3, 5, 10, 15 and 30 min post injection of Gd(HP-DO3A) (A) and GOLs (B) at a dose of 0.1 mmol-Gd/kg. Arrow points to the tumor.

Figure 7. Contrast-to-noise ratio (CNR) of blood pool (A), and tumor (B) of mice bearing malignant breast cancer before and at different time points after administration of Gd(HP-DO3A) (open) and GOLS (filled) (* $p < 0.05$). Data presented as mean $\pm$ SD.

Figure 8. Biodistribution of Gd(III) in Balb/c mice 2 days (A) and 10 days (B) after intravenous injection of GOLS (black bar), GODC (white bar), GDCC (strip bar) and Gd(HP-DO3A) (star bar) at a dose of 0.1 mmol-Gd/kg. Data presented as mean $\pm$ SD.

Figure 9.

Concentrations of Gd(III) (A), Zn(II) (B), Ca(II) (C), and Cu(II) (D) measured in urine before and after administration of GOLS (black bar), GODC (white bar), GDCC (strip bar) and Gd(HP-DO3A) (star bar) at a dose of 0.1 mmol-Gd/kg. Data presented as mean $\pm$ SD.