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Research Paper

Gd-DTPA L-Cystine Bisamide Copolymers as Novel Biodegradable Macromolecular Contrast Agents for MR Blood Pool Imaging

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Purpose. The purpose of this study was to synthesize biodegradable Gd-DTPA L-cystine bisamide copolymers (GCAC) as safe and effective, macromolecular contrast agents for magnetic resonance imaging (MRI) and to evaluate their biodegradability and efficacy in MR blood pool imaging in an animal model.

Methods. Three new biodegradable GCAC with different substituents at the cystine bisamide [R = H (GCAC), $CH_2CH_2CH_3$ (Gd-DTPA L-cystine bispropyl amide copolymers, GCPC), and $CH(CH_3)_2$ (Gd-DTPA cystine bisisopropyl copolymers, GCIC)] were prepared by the condensation copolymerization of diethylenetriamine pentaacetic acid (DTPA) dianhydride with cystine bisamide or bisalkyl amides, followed by complexation with gadolinium triacetate. The degradability of the agents was studied *in vitro* by incubation in 15 μM cysteine and *in vivo* with Sprague-Dawley rats. The kinetics of *in vivo* contrast enhancement was investigated in Sprague-Dawley rats on a Siemens Trio 3 T scanner.

Results. The apparent molecular weight of the polydisulfide Gd(III) chelates ranged from 22 to 25 kDa. The longitudinal (T_1) relaxivities of GCAC, GCPC, and GCIC were 4.37, 5.28, and 5.56 $mM^{-1}\ s^{-1}$ at 3 T, respectively. The polymeric ligands and polymeric Gd(III) chelates readily degraded into smaller molecules in incubation with 15 μM cysteine via disulfide–thiol exchange reactions. The *in vitro* degradation rates of both the polymeric ligands and macromolecular Gd(III) chelates decreased as the steric effect around the disulfide bonds increased. The agents readily degraded *in vivo*, and the catabolic degradation products were detected in rat urine samples collected after intravenous injection. The agents showed strong contrast enhancement in the blood pool, major organs, and tissues at a dose of 0.1 mmol Gd/kg. The difference of their *in vitro* degradability did not significantly alter the kinetics of *in vivo* contrast enhancement of the agents.

Conclusion. These novel GCAC are promising contrast agents for cardiovascular and tumor MRI, which are later cleaved into low molecular weight Gd(III) chelates and rapidly cleared from the body.

KEY WORDS: biodegradable macromolecular contrast agent; blood pool imaging; Gd-DTPA L-cystine bisamide copolymers; magnetic resonance imaging; polydisulfide.

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ABBREVIATIONS: DCAC, DTPA L-cystine bisamide copolymers; DCC, dicyclohexylcarbodiimide; DCIC, DTPA L bisisopropyl amide copolymers; DCPC, DTPA L-cystine bispropyl amide copolymers; DCU, dicyclohexylurea; DI, deionized; DMSO, dimethylsulfoxide; DTPA dianhydride, diethylenetriamine penta acetic acid dianhydride; ESI-MS, electrospray ionization mass spectrometry; GCAC, Gd-DTPA L-cystine bisamide copolymers; GCIC, Gd-DTPA L-cystine bisisopropyl amide copolymers; GCPC, Gd-DTPA L-cystine bispropyl amide copolymers; GDCC, Gd-DTPA cystamine copolymers; GDCEP, Gd-DTPA cystine diethyl ester copolymers; GDCP, Gd-DTPA cystine copolymers; Gd-(DTPA-BMA), Gd-(DTPA-bismethyl amide); $Gd(OAc)_3$, gadolinium triacetate; HPMA, poly[*N*-(2-hydroxypropyl)methacrylamide]; ICP-OES, inductively coupled argon plasma optical emission spectrometer; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MRI, magnetic resonance imaging; MWCO, molecular weight cutoff; PBS, phosphate-buffered saline; r_1, longitudinal relaxivity; r_2, transverse relaxivity; SEC, size exclusion chromatography; T_1, longitudinal; T_2, transverse; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; $1/T_1$, proton longitudinal relaxation rate; $1/T_2$, proton transverse relaxation rate.

INTRODUCTION

Magnetic resonance imaging (MRI) is a medical imaging modality that is routinely used to identify abnormal tissues. The high spatial and temporal resolution of MRI provides both anatomical and physiological information (1,2). Paramagnetic gadolinium [Gd(III)] complexes, or Gd(III) chelates, can significantly increase the proton longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of surrounding water molecules (3,4). Stable Gd(III) chelates, e.g., Gd-(DTPA-bismethyl amide) [Gd-(DTPA-BMA)], are used as low molecular weight MRI contrast agents to improve the diagnostic accuracy by enhancing the image contrast between normal and diseased tissues. Approximately 30% of the MRI examinations utilize these contrast agents (3).

The most common clinical MRI contrast agents are Gd-diethylenetriamine pentaacetic acid (Gd-DTPA), Gd-DOTA, or their derivatives (4). However, their clinical application is limited because of their transient tissue retention and unfavorable pharmacokinetics, which includes rapid extravasation from the cardiovasculature and undiscriminable distribution into the extracellular space (5,6). As a result, macromolecular Gd(III) chelates have been developed as blood pool MRI contrast agents to modify *in vivo* pharmacokinetics (5–9). These blood pool contrast agents have a prolonged blood retention time or plasma half-life that permits a broader contrast enhancement time window for effective MRI of the vasculature. These agents also exhibit greater image contrast because they have higher proton relaxation rates, which is caused by the larger number of Gd(III) chelates in a single macromolecule. These high molecular weight agents are often prepared by conjugation of a Gd(III) chelate, such as Gd-DTPA, to polymers (7,9) dendrimers (5,8), or proteins (10) or by the copolymerization of Gd-DTPA with alkyldiamines (6,11). One limitation of macromolecular contrast agents for clinical applications, however, is their slow excretion, which may result in the release of toxic Gd^{3+} ions from the chelates by metabolism and accumulation of the ions in the bone (9,12).

To resolve the issue of macromolecular contrast agents limiting their clinical development, we have recently designed and developed polydisulfide-based macromolecular Gd(III) chelates as biodegradable MRI contrast agents (13–17). These agents provide superior blood pool contrast enhancement within vasculature and readily degrade into low molecular weight species that can rapidly excrete from the body (16). Because the polymeric backbone is constructed upon disulfide bonds, they can degrade via reduction with endogenous plasma free thiols, including glutathione and cysteine.

Polydisulfide Gd(III) chelates with different structural modifications around the disulfide bonds have been prepared and evaluated. The preliminary results have shown that the introduction of functional groups, like carboxylic acid in Gd-DTPA cystine copolymers (GDCP) and ethyl carboxylate Gd-DTPA cystine diethyl ester copolymers (GDCEP), improved *in vivo* contrast enhancement as compared with Gd-DTPA cystamine copolymers (GDCC) (14).

Here, we report the synthesis and evaluation of three Gd-DTPA L-cystine bisamide copolymers (GCAC) as biodegradable macromolecular MRI contrast agents. These novel macromolecular MRI contrast agents were prepared in high yield and purity. In addition, we investigated the effects that the amide and substituted alkyl amides have on the physicochemical properties and *in vivo* contrast enhancement of the agents. The macromolecular Gd(III) chelates with substituted cystine bisamides exhibited slightly higher relaxivities and a slower *in vitro* degradation rate than that of nonsubstituted cystine bisamide Gd(III) complexes. *In vivo* degradation was studied in rats and showed that the catabolic products, which were low molecular weight Gd(III) chelates, were detected in the urine samples. MRI studies in rats showed that these newly prepared biodegradable, high molecular weight contrast agents significantly improved contrast enhancement of the blood pool, liver, and kidneys.

MATERIALS AND METHODS

All reagents were used without further purification unless otherwise stated. All reactions were performed at room temperature under ambient pressure unless otherwise stated. Cystine was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Cystine bisamide was purchased from Peninsula Laboratories (San Carlos, CA, USA). Isopropyl amine, propyl amine, and *N*-hydroxysuccinimide (NHS) were purchased from Lancaster Synthesis Inc. (Pelham, NH, USA). Gadolinium triacetate ($Gd(OAc)_3$) and di-*tert*-butyl dicarbonate (*t*-BOC) were purchased from Alfa Aesar (Ward Hill, MA, USA). DTPA was purchased from J.T. Baker (Philipsburg, NJ, USA). Dicyclohexylcarbodiimide (DCC) was purchased from Pierce (Rockford, IL, USA). $(t\text{-BOC})_2$-cystine (18) and DTPA dianhydride (19) were synthesized according to the literature. Spectra/Pore regenerated cellulose membranes [molecular weight cutoff (MWCO) = 25 kDa] were purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). PD-10 desalting columns were purchased from Amersham Bioscience (Uppsala, Sweden).

^{1}H NMR spectra were acquired on a Varian INOVA 400 at 400 MHz at 25°C. Molecular weight was determined by size exclusion chromatography (SEC) on an AKTA FPLC system with a Superdex 200 (10/300 GL) column equipped with UV and refractive index detectors (Amersham Biosciences Corp., Piscataway, NJ, USA), calibrated with poly[*N*-(2-hydroxypropyl)methacrylamide] (HPMA) standards with a molecular weight range of 14–300 kDa. Electrospray ionization mass spectrometry (ESI-MS) spectra were acquired on a PE Sciex API III Mass Spectrometer at the University of Utah Mass Spectrometry and Proteonomic Core Facility (Salt Lake City, UT, USA). The Gd metal content was determined using an inductively coupled argon plasma optical emission spectrometer (ICP-OES) (Optima 3100XL; Perkin Elmer, Norwalk, CT, USA). The T_1 relaxivity measurements for the polymeric Gd(III) chelates were performed on a Siemens Trio 3 T MRI scanner utilizing an inversion recovery sequence; the T_2 relaxivity measurements for the macromolecular Gd(III) chelates were performed utilizing a spin-echo recovery sequence. T_1-weighted MRI was acquired on a Siemens Trio 3 T MRI scanner.

Synthesis of Cystine Bispropyl Amide

$(t\text{-BOC-L-Cys})_2$ (10.0 g, 22.8 mmol) and NHS (5.84 g, 50.7 mmol) were dissolved in tetrahydrofuran (THF; 200 mL). DCC (10.1 g, 49.1 mmol) was added to the solution, and the

mixture was stirred overnight at room temperature. *N*,*N*′-Dicyclohexylurea (DCU) precipitated out of the THF solution and was removed by filtration. Propyl amine (10.0 mL, 116 mmol) was added to the filtrate while stirring at −5°C. Additional DCU precipitated out within 5 min and was removed by vacuum filtration. The solvent was removed in vacuo to result in the crude product. The residue was dissolved in chloroform (100 mL) and washed three times with deionized (DI) H_2O at pH 10. The organic layer was dried over anhydrous Na_2SO_4, filtered, and then concentrated in vacuo to dryness. The crude product was washed with ethyl acetate (100 mL), filtered, and then washed again with diethyl ether to yield (*t*-BOC)$_2$-cystine bispropyl amide (71%) as a white solid. ^{1}H NMR ($CDCl_3$, ppm): 7.64 (d, 2H, *t*-BOC-NHCH), 5.55 (br, 2H, $CONHCH_2CH_2CH_3$), 4.79 [q, 2H, $NHCH(CO)CH_2$], 3.18–3.22 (m, 8H, $NHCH_2CH_2CH_3$ + SCH_2CH), 2.90 (m, 4H, $NHCH_2CH_2CH_3$), 1.40 [s, 18H, $OC(CH_3)_3$], 0.90 (t, 6H $NHCH_2CH_2CH_3$). ESI-MS (*m*/*z*, M + H^+): 523.3 (observed), 523.2 (calculated).

t-BOC protection was removed by dissolving the above product (1.54 g, 2.94 mmol) in trifluoroacetic acid (TFA) (3.0 mL) for 30 min while stirring at room temperature. The solution was then concentrated in vacuo to give a viscous oil. The product was precipitated out in diethyl ether and dissolved in DI H_2O. The aqueous phase was separated and concentrated to dryness in vacuo to yield L-cystine bispropyl amide (41%) as a colorless salt. ^{1}H NMR (D_2O, ppm): 4.15 [t, 2H, $CO(NH)CHCH_2$], 3.22–2.96 (m, 8H, SCH_2CH + $NHCH_2CH_2CH_3$), 1.40 (m, 4H, $CH_2CH_2CH_3$), 0.26 [t, 6H, $CH(CH_3)_2$]. ESI-MS (*m*/*z*, M + H^+): 323.3 (observed), 323.2 (calculated).

Synthesis of L-Cystine Bisisopropyl Amide

(*t*-BOC-L-Cys)$_2$-bisisopropyl amide was synthesized similarly as (*t*-BOC-L-Cys)$_2$-bispropyl amide. Yield: 35%. ^{1}H NMR ($CDCl_3$, ppm): 7.34 (d, 2H, *t*-BOC-NHCH), 5.57 [d, 2H, $CONHCH(CH_3)_2$], 4.70 [m, 2H, $NHCH(CO)CH_2$], 4.12 [m, 2H, $NHCH(CH_3)_2$], 2.90 (m, 4H, SCH_2CH), 1.48 [s, 18H, $OC(CH_3)_3$], 1.19 [d, 12H, $CH(CH_3)_2$]. ESI-MS (*m*/*z*, M + H^+): 523.3 (observed), 523.2 (calculated).

L-Cystine bisisopropyl amide was similarly prepared and purified as L-cystine bispropyl amide. Yield: 71%. ^{1}H-NMR (D_2O, ppm): 4.08 [t, 2H, $CO(NH)CHCH_2$], 3.83 [m, 2H, $NHCH(CH_3)_2$], 3.12 (m, 4H, SCH_2CH), 1.05 [d, 12H, $CH(CH_3)_2$]. ESI-MS (*m*/*z*, M + H^+): 323.3 (observed), 323.2 (calculated).

Synthesis of Biodegradable Paramagnetic Copolymers

The general procedure for the synthesis of Gd-DTPA L-cystine bisamide and substituted bisamide copolymers is described below using L-cystine bisisopropyl amide as an example. L-Cystine bisisopropyl amide (0.815 g, 2.53 mmol) was dissolved in 0.30 mL triethylamine (TEA) and 0.70 mL of anhydrous dimethyl sulfoxide (DMSO) while stirring in an ice-water bath at 5°C. DTPA dianhydride (0.904 g, 2.53 mmol) was added in portions over a 25 min period. The reaction mixture turned brown and viscous and then solidified after 40 min. The mixture was removed from the ice-water bath and allowed to settle to room temperature. An additional 0.50 mL of DMSO was added, and the reaction was stirred overnight at room temperature. The solution was diluted with methanol, and DTPA L-cystine bisisopropyl amide copolymers (DCIC) were precipitated in acetone. DCIC was dissolved in DI H_2O at pH 7 and dialyzed against DI H_2O for approximately 30 h using a regenerated cellulose membrane (MWCO = 25 kDa). The polymeric ligand was obtained after concentrating to dryness in vacuo. The molecular weight of DCIC was determined by SEC. DCIC was mixed with a large excess of $Gd(OAc)_3$ in 10 mL of DI H_2O at pH 5.5 while stirring at room temperature for 1 h. Free Gd^{3+} ions were removed by SEC using a PD-10 desalting column, eluted with DI H_2O. The solution was concentrated in vacuo to dryness to give Gd-DTPA L-cystine bisisopropyl copolymers (GCIC). The Gd content of GCIC was measured using ICP-OES; molecular weight was determined by SEC.

Gd-DTPA L-Cystine Bisamide Copolymers

DTPA L-cystine bisamide copolymers (DCAC): yield = 67.5%; M_n = 30.8 kDa, M_w = 34.5 kDa; ^{1}H NMR (D_2O, ppm): 4.20 (t, 2H, $NHCHCO$), 3.77 (s, 6H, NCH_2COOH), 3.58 (s, 4H, $COCH_2N$), 3.20 (d, 4H, SCH_2CH), 2.87, (d, 8H, NCH_2CH_2N). GCAC: yield = 81%; M_n = 14.4 kDa, M_w = 22.3 kDa. The Gd content in GCAC was 169 mg Gd/g copolymer.

Gd-DTPA L-Cystine Bispropyl Amide Copolymer

DTPA L-cystine bispropyl copolymers (DCPC): yield = 60.2%; M_n = 24.1 kDa, M_w = 33.2 kDa; ^{1}H NMR (D_2O, ppm): 4.55 (t, 2H, $NHCHCO$), 3.80 [m, 2H, $NHCH(CH_3)_2$], 3.66 (s, 6H, $COCH_2N$ + NCH_2COOH), 3.55 (s, 4H, NCH_2COOH), 3.22 (d, 4H, SCH_2CH), 3.03 (m, 2H, $NHCH_2CH_2CH_3$), 2.87, (d, 8H, NCH_2CH_2N), 1.37 (m, 4H, $NHCH_2CH_2CH_3$), 0.78 (t, 6H, $NHCH_2CH_2CH_3$). GCPC: yield = 73%; M_n = 17.1 kDa, M_w = 22.3 kDa. The Gd content in GCPC was 169 mg Gd/g copolymer.

Gd-DTPA L-Cystine Bisisopropyl Copolymers

DCIC: yield = 60.0 %; M_n = 26.4 kDa, M_w = 36.7 kDa; ^{1}H NMR (D_2O, ppm): 4.55 (t, 2H, $NHCHCO$), 3.80 [m, 2H, $NHCH(CH_3)_2$], 3.66 (s, 6H, $COCH_2N$ + NCH_2COOH), 3.54 (s, 4H, NCH_2COOH), 3.21 (d, 4H, SCH_2CH), 2.86 (m, 8H, NCH_2CH_2N), 1.01 [d, 12H, $CH(CH_3)_2$]. GCIC: yield = 71%; M_n = 16.6 kDa, M_w = 25.0 kDa. The Gd content was 162 mg Gd/g.

Relaxivity Measurement of Gd-Containing Copolymers

Longitudinal relaxivity (r_1) was determined by measuring the T_1 relaxation times for three different concentrations of GCAC (22.3 kDa), GCPC (22.3 kDa), and GCIC (25.0 kDa), as well as a water reference on a Siemens Trio 3T MRI Scanner at room temperature using an inversion recovery prepared turbo spin-echo imaging pulse sequence. The inversion times were as follows: inversion time (TI) = 24, 50, 75, 100, 150, 200, 300, 400, 600, 800, 1200, and 1600 ms; repetition time (TR) = 5000 ms; echo time (TE) = 15.0 ms.

The net magnetization amplitude data from the region of interest (ROI) of each sample were fit using a Marquardt-Levenberg algorithm for multiparametric nonlinear regression analysis (20). T_1 and M_0 were calculated from these data and r_1 was determined from the slope of $1/T_1$ *vs.* $[Gd^{3+}]$ plot. Transverse relaxivity (r_2) was determined by measuring the T_2 relaxation times for four different concentrations of GCAC, GCPC, and GCIC, as well as a ultrapure H_2O reference on a Siemens Trio 3T MRI scanner at room temperature using a turbo spin-echo imaging sequence with turbo factor 3. The following times were as follows: TE = 12, 24, 35, 47, 59, 71, 83, 94, and 106 ms; TR = 3000 ms. T_2 values were calculated on a semilog plot of the net magnetization amplitude data from the ROI; r_2 was determined from the slope of $1/T_2$ *vs.* $[Gd^{3+}]$ plot.

In Vitro Degradation of Copolymers

The degradation of each copolymer, with and without Gd(III), was studied in the presence of L-cysteine at 15 μM, which is the free thiol concentration in the plasma. The copolymers (420 μM, monomeric repeat unit concentration) and 15 μM L-cysteine in phosphate-buffered saline (PBS; pH 7.4) were incubated at 37°C. The molecular weight distributions were immediately evaluated by SEC. The molecular weights of the remaining copolymers were calculated using a time window, which was from the time point when the copolymers first appeared to 36 mins, when the monomeric degradation products began to elute. The molecular weight reduction of copolymers in the incubation was calculated as the percentage of the weight-average molecular weight of the remaining copolymers at various time points of the incubation to their original values.

In Vivo Metabolism

GCAC, GCPC, and GCIC with average molecular weights (M_w) of 22.3, 22.3, and 25.0 kDa, respectively, were prepared with similar methods described above for *in vivo* metabolism and MRI studies. The agents were intravenously injected into male Sprague-Dawley rats (200–260 g) at a dose of 0.1 mmol Gd/kg body weight via the tail vein. The rats were kept in metabolic cages, and their urine samples were collected at 8 and 24 h postinjection; samples were analyzed with both positive- and negative-charge-labeled MALDI-TOF mass spectrometry on an α-cyano-4-hydroxycinnamic acid matrix.

Contrast-Enhanced Magnetic Resonance Imaging in Rats

In vivo magnetic resonance imaging (MRI) contrast enhancement of the agents was evaluated in male Sprague-Dawley rats (200–260 g). The animals were cared for under an approved protocol by the University of Utah Institutional Animal Care and Use Committee. The rats were anesthetized by intraperitoneal administration of xylazine (12 mg/kg) and ketamine (80 mg/kg). The contrast agents were administered at a dose of 0.1 mmol Gd/kg via intravenous tail vein injection; each agent was investigated in a group of three rats. MR images were obtained on a Siemens Trio 3 T MRI scanner before injection and at 2, 5, 10, 15, and 30 min postinjection. Images were obtained on a wrist coil using a 3D FLASH pulse sequence. The imaging parameters were TE = 1.64 ms, TR = 4.30 ms, 19° flip angle, 3D acquisition with 80 slices/slab, and 0.5-mm coronal slice thickness. *In vivo* contrast enhancement in the major organs and tissues, including the heart, kidneys, liver, bladder, and muscle periphery, was analyzed using Osirix software package (http://homepage.mac.com/rossetantoine/osirix/). Signal intensities were measured in different ROIs at each time point and were averaged from three different Sprague-Dawley rats. A one-way ANOVA statistical analysis was performed to determine statistical significance ($p < 0.05$) of the differences in the signal intensities of the contrast agents in the ROIs.

RESULTS

Synthesis of Gd-DTPA L-Cystine Bisamide Copolymers

Three new polydisulfide Gd(III) chelates with different amide substituents at the cystine carboxylic groups were synthesized as novel biodegradable macromolecular MRI contrast agents. Alkyl-substituted cystine amides, L-cystine bispropyl amide and L-cystine bisisopropyl amide, were first prepared by the reaction of (*t*-BOC-Cys)$_2$ bis(*N*-hydroxysuccinimide) ester with the alkyl amines, followed by *t*-BOC deprotection with TFA (Fig. 1). The monomers were extensively purified to ensure high purity because monomer purity was crucial for the molecular weight of the copolymers.

Figure 2 shows the synthetic procedure for paramagnetic copolymers of Gd-DTPA and cystine bisamides. Macromolecular ligands, DCAC, DCPC, and DCIC, were first synthesized by condensation copolymerization of equimolar amounts of DTPA dianhydride and corresponding L-cystine bisamides. The reaction was performed in a mixture of TEA and anhydrous DMSO. TEA acted as a base to neutralize the salts of the monomers and as a solvent to increase the solubility of the copolymers. The macromolecular Gd(III) chelates were prepared in relatively high yield by complexation of the polymeric ligands with $Gd(OAc)_3$. The molecular weight of the paramagnetic Gd(III) chelates can be as high as 55 kDa. The molecular weights, Gd content, and T_1 and T_2 relaxivities of the paramagnetic copolymers used in this study are listed in Table I.

After complexation with Gd^{3+}, there was a significant decrease in the apparent molecular weights of the copolymers (Table I). The polymeric ligands were anionic and had a large hydrodynamic volume as a result of the free carboxylic groups of DTPA. The complexation with Gd^{3+} ions formed neutral macromolecular Gd(III) chelates and resulted in a significant reduction of the copolymers' hydrodynamic volume. The apparent molecular weights of the macromolecular Gd(III) chelates, GCAC, GCPC, and GCIC, decreased by approximately 30% as compared with the average molecular weight of the corresponding polymeric ligands.

The Gd content of GCAC, GCPC, and GCIC was 16.9%, 16.9%, and 16.2%, respectively, as calculated by ICP-OES. Elemental metal analysis showed that the Gd content for the polymeric chelates was lower than the calculated content, which was the theoretical Gd composition calculated from the

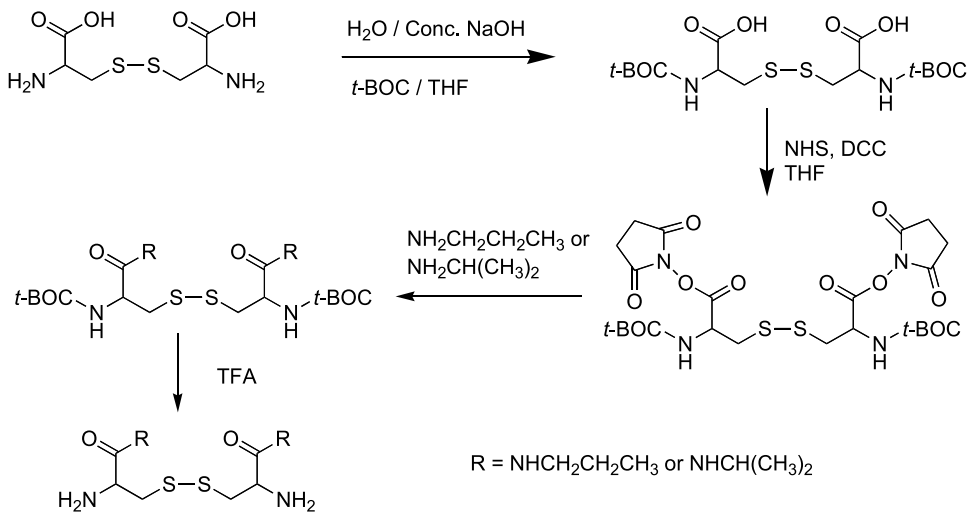

Fig. 1. Synthetic scheme of cystine bispropyl amide and L-cystine bisisopropyl amide.

structure of the repeat units of the copolymers. The low measured Gd content might be attributed to the association of water molecules to the hydrophilic polymers. The Gd cystine bisamide copolymers were stable in the solid state, and their molecular weight distributions did not change after approximately 6 months in cold storage.

T_1 and T_2 Relaxivities of the Paramagnetic Copolymers

The T_1 relaxivities of the macromolecular Gd(III) chelates GCAC, GCPC, and GCIC at 3 T were 4.37, 5.28, and 5.56 $mM^{-1}\ s^{-1}$, respectively. These values were similar to the clinical contrast agent Gd-(DTPA-BMA) (4.62 $mM^{-1}\ s^{-1}$) and the previously reported GDCP (5.43 $mM^{-1}\ s^{-1}$) and GDCEP (5.86 $mM^{-1}\ s^{-1}$) at 3 T (14). The T_2 relaxivities of GCAC, GCPC, and GCIC at 3 T were 6.21, 6.08, and 6.54 $mM^{-1}\ s^{-1}$, respectively, and were similar to our previously reported GDCC (13). The relaxivities of the agents were lower than those of other reported macromolecular Gd(III) chelates because of the local flexibility of the disulfide bonds.

In Vitro Degradation of the Copolymers

Figure 3 ($N = 3$, mean ± SD) shows the molecular weight reduction of the remaining polymeric ligands from their original values at various time points in the incubation with 15 μM cysteine in PBS at 37°C. The disulfide–thiol exchange reaction between the polydisulfide backbone and cysteine resulted in molecular weight decreases with increasing incubation time. The rates of molecular weight reduction varied among the copolymers. DCIC with bulky isopropyl groups degraded much slower than DCPC and DCAC. All polymeric ligands completely degraded into low molecular weight species within 24 h. The steric effect around the disulfide bonds significantly affected the degradation rate of the copolymers in the presence of cysteine at the endogenous free thiol concentration.

The polymeric Gd(III) chelates were more susceptible to the disulfide–thiol exchange reaction with cysteine than the corresponding polymeric ligands. Figure 4 ($N = 3$, mean ± SD) shows the percentage of the molecular weight reduction of the remaining polymeric Gd(III) chelates at various time points in the incubation with 15 μM cysteine at 37°C. The degradation rate of the polymeric chelates decreased in the order of GCAC, GCPC, and GCIC as the steric hindrance around the disulfide bonds increased. All macromolecular Gd(III) chelates completely degraded into low molecular weight oligomers after 75 min.

The degradation products, as a result of the disulfide–thiol exchange reaction with cysteine, were also identified in the MALDI-TOF mass spectra of the incubation mixtures,

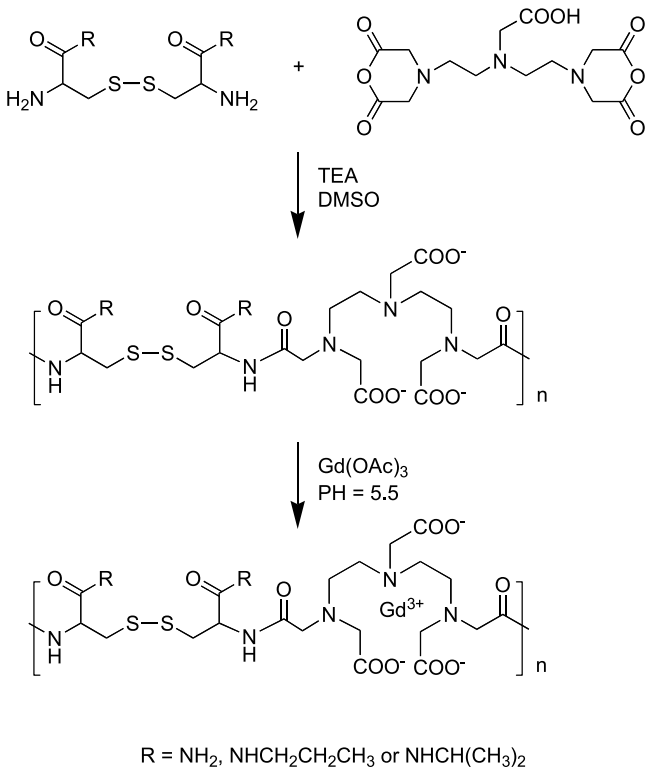

Fig. 2. Synthetic scheme for the Gd-DTPA L-cystine bisamide copolymers (GCAC) and alkyl-substituted GCAC.

Table I. Physicochemical Parameters of the Copolymers

Copolymers	M_n (kDa)	M_w (kDa)	Gd content (calculated)	r_1 (mM^{-1} s^{-1})	r_2 (mM^{-1} s^{-1})	Yield (%)
DCAC	30.8	34.5	–	–	–	67.5
GCAC	14.1	22.3	16.9% (20.4%)	4.37	6.21	81.0
DCPC	24.1	33.2	–	–	–	60.2
GCPC	17.1	22.3	16.9% (18.4%)	5.28	6.08	73.0
DCIC	26.4	36.7	–	–	–	60.0
GCIC	16.6	25.0	16.2% (18.4%)	5.56	6.53	71.0

Apparent M_n and M_w were determined by size exclusion chromatography, whereas Gd^{3+} content was determined by inductively coupled argon plasma optical emission spectrometer analysis, longitudinal and transverse relaxivities (determined at 3 T), and reaction yield.
DCAC = DTPA L-cystine bisamide copolymers; GCAC = Gd-DTPA L-cystine bisamide copolymers; DCPC = DTPA L-cystine bispropyl amide copolymers; GCPC = Gd-DTPA L-cystine bispropyl amide copolymers; DCIC = DTPA L-cystine bisisopropyl amide copolymers and GCIC = Gd-DTPA L-cystine bisisopropyl amide copolymers.

which verified the degradation reaction mechanism (Fig. 5). The degradation products had molecular weights that were indicative of a single repeat unit, Gd-DTPA bis(cysteine amide), and up to three repeat units. The mass of degradation products with one or two repeat units for GCAC was around 753.0 or 1501.2 (m/z) as observed in the mass spectrum (not shown). The *in vitro* degradation products of GCPC and GCIC had the same masses, and the degradation products with one, two, and three repeat units were identified with the mass of 835.2, 1668.4, and 2502.6 (m/z), respectively.

In Vivo Metabolism in Rats

Catabolic degradation products were identified by MALDI-TOF mass spectrometry in the urine samples collected 8 h postinjection with all three agents. No major metabolite was found in the 24 h urine samples. Figure 6 shows the positive- and negative-charge-labeled mass spectra of the 8-h urine samples of GCIC. The major metabolites for GCIC had a mass (m/z) of approximately 718.26, 875.15, 913.11, 988.02, 1589.36, and 1707.29 at 8 h postinjection in the positive-charge-labeled mass spectrum (Fig. 6a). Three unknown peaks were present with masses (m/z) of 895.09, 1215.02, and 1397.51. Less metabolites were identified in the positive-charge-labeled mass spectra of GCPC and GCAC (spectra not shown). The major metabolites for GCPC had a mass (m/z) of 718.25, 875.15, 913.12, and 988.02, whereas only one major metabolite with a mass (m/z) of 988.02 was identified for GCAC. One unknown peak with a mass (m/z) of 1214.04 was observed for GCPC.

The Gd isotope pattern was observed in every major peak except for a mass (m/z) at 718 for GCIC and GCPC. The peaks at approximately 718 for both GCPC and GCIC correspond to the ligands ($679.26 + K^+$) of the repeat units in these agents. The compounds might be formed by the dissociation of Gd^{3+} ions from the chelates at low pH in the process of sample preparation for mass spectrometry. α-Cyano-4-hydroxycinnamic acid was used as the MALDI-TOF matrix, and the pH of the samples was approximately 1.2.

The peaks around 875.15 (m/z) and 913.11 (m/z) for GCIC and GCPC represent their monomeric repeat units with a K^+ ion ($834.18 + 2H + K^+$) and two K^+ ions ($834.18 + H + 2K^+$), respectively. The metabolite with a mass 988.02 (m/z) was also observed in the mass spectra of all three agents. This metabolite was also observed in the urine samples of the other polydisulfide agents (14). For GCIC, the peak with a mass (m/z) of approximately 1589.36 corresponds to two GCIC monomeric repeat units with two of its isopropyl amide groups hydrolyzed [$834.18 \times 2 - 2$ C_3H_8N (58.07) + 2 OH + 2 H + H^+], and the peak at 1707.29 (m/z) corresponds to two GCIC monomeric repeat units plus a K^+ ion ($834.18 \times 2 + K^+$). These additional dimeric degradation products have been observed for GCIC probably because the steric hindrance of the isopropyl groups around the disulfide spacer slows polymer degradation. Further investigation of the unknown metabolites for both GCIC and GCPC is planned for future studies.

Metabolites were also identified in the negative charge labeled mass spectra of the 8 h urine samples with the agents. Both GCIC (Fig. 6b) and GCPC (spectrum not included) had major metabolites with similar masses (m/z) at 589.04, 734.08, and 911.04. The masses (m/z) of the major metabolites of GCAC (spectrum not included) were approximately 589.06, 707.09, and 734.10, which were all observed in our previous study on GDCP and GDCEP (14). Two unknown metabolites were observed for GCIC, 531.97 (m/z) and

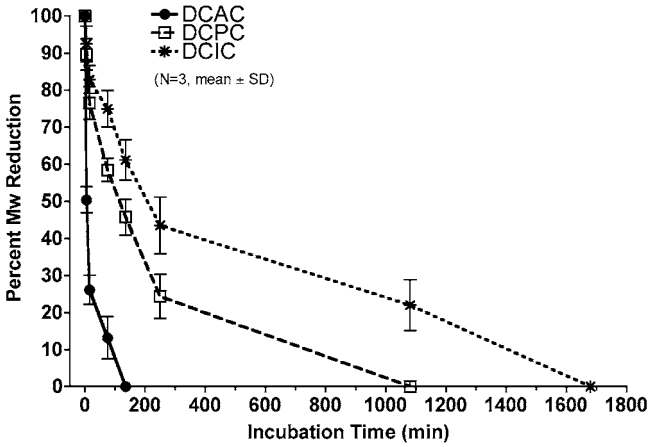

Fig. 3. Percent molecular weight reduction of the remaining polymeric ligands from their original values in 15 μM cysteine [phosphate-buffered saline (PBS) and 37°C] at varying incubation time (in minutes). M_w was determined by size exclusion chromatography (SEC) ($N = 3$, mean ± SD).

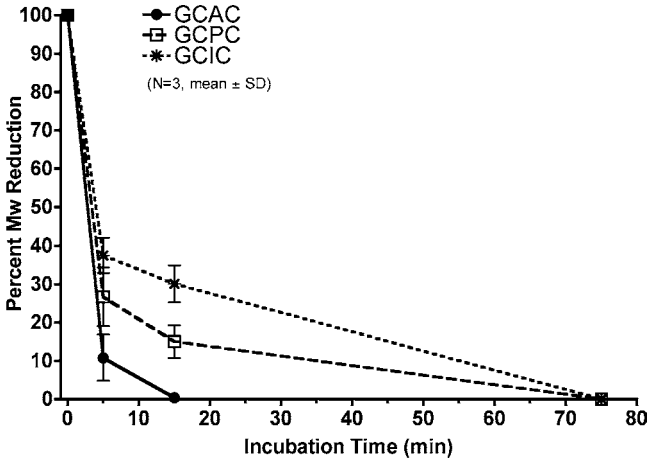

Fig. 4. Percent molecular weight reduction of the remaining macromolecular Gd(III) complexes from their original values in 15 μM cysteine (PBS and 37°C) at varying incubation time (in minutes). M_w was determined by SEC (N = 3, mean ± SD).

631.03 (m/z), and one unknown metabolite was found in GCPC, 531.95 (m/z).

The metabolite with the mass (m/z) of 911 for GCIC and GCPC could be the same metabolite with the mass (m/z) of 913 identified in the positive-charge-labeled mass spectra of the agents. The structures of other metabolites are unknown, and further study is on going for accurate characterization of these metabolites. Furthermore, no metabolites were observed in the negative charge labeled mass spectra for all three agents at 24 h postinjection.

In Vivo Contrast Enhancement in Rats

Figure 7 shows the 2D coronal rat MR images cross the heart and liver before and at 2, 5, 10, 15, and 30 min after injection of the agents at a dose of 0.1 mmol Gd/kg. Strong contrast enhancement was observed within the heart and blood vessels, differentiating these structures from the surrounding tissues at 2 min postinjection. The contrast enhancement then gradually decreased but was still visible at 30 min postinjection. Significant contrast enhancement was also observed in the liver and kidneys, with similar dynamic changes as observed in the heart. Contrast enhancement in the urinary bladder gradually increased over time, indicating the accumulation of low molecular weight Gd(III) chelates. Statistical analysis of MR signal intensities revealed no significant difference ($p > 0.05$) between the contrast enhancement in the organs and tissues (heart, liver, kidney, bladder, and muscle periphery) for GCAC, GCPC, and GCIC at all time points post injection.

DISCUSSION

Biodegradable, macromolecular MRI contrast agents were designed and prepared to facilitate the excretion of low molecular weight Gd(III) chelates after the polydisulfide backbone was cleaved via thiol–disulfide exchange reactions with the endogenous thiols in the body. These novel agents were readily degraded when incubated with 15 μM cysteine under physiological conditions (Fig. 4).

The degradation rates of the polymeric ligands were slower than the macromolecular Gd(III) chelates as a result of the ligands' negative charges; negative charges in the polymeric ligands may inhibit the reaction of disulfide bonds with negatively charged cysteine as a result of charge repulsion at pH 7.4 (21). The alkyl substituents had a similar steric effect on the degradation of the polymeric Gd(III) chelates as the ligands. GCIC, which contain isopropyl groups, degraded slower than GCPC and GCAC when incubated with cysteine (Fig. 4). The degradation rate of the Gd-DTPA copolymers with cystine bisamide and substituted bisamides was also much faster than the previously reported GDCC, GDCP, and GDCEP under the same reaction conditions (14). Apparently, substituting the carboxylic groups in L-cystine with amide groups renders the disulfide bonds more susceptible to degradation.

The identification of catabolic degradation products in rat urine samples demonstrated that the novel GCAC were cleaved into smaller Gd(III) chelate moieties, which were readily excreted via renal filtration. Analyses of degradation products within urine samples suggest that *in vivo* degradation processes are more complicated than *in vitro* disulfide–thiol exchange reactions. The complicated MALDI-TOF spectra of the *in vivo* metabolism of GCAC, GCPC, and GCIC may be the result of other degradative processes [e.g., enzymatic (22) reactions and oxidation] within the blood. Although there were a few unknown peaks, the mass of these catabolites is above the Gd-DTPA chelate (584.04 Da), indicating that the Gd(III)-DTPA complexes were intact and stable in the plasma. The absence of major metabolic peaks at 24 h postinjection for both the positive- and

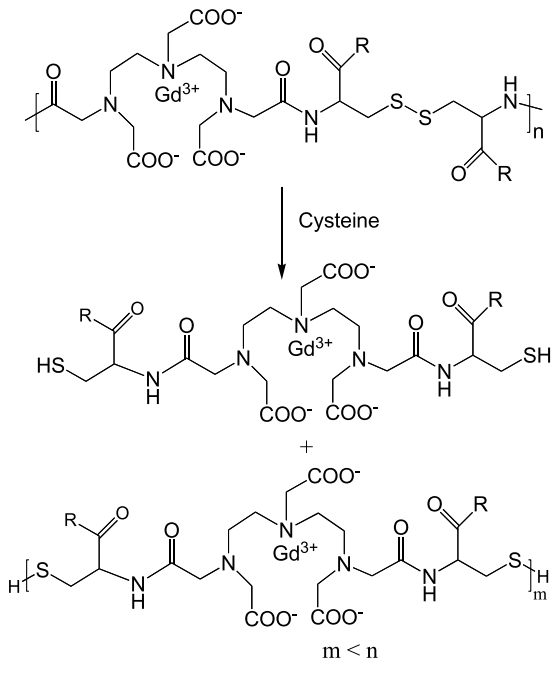

Fig. 5. Degradation reaction of GCAC with cysteine. *In vitro* degradation products were identified by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) analysis.

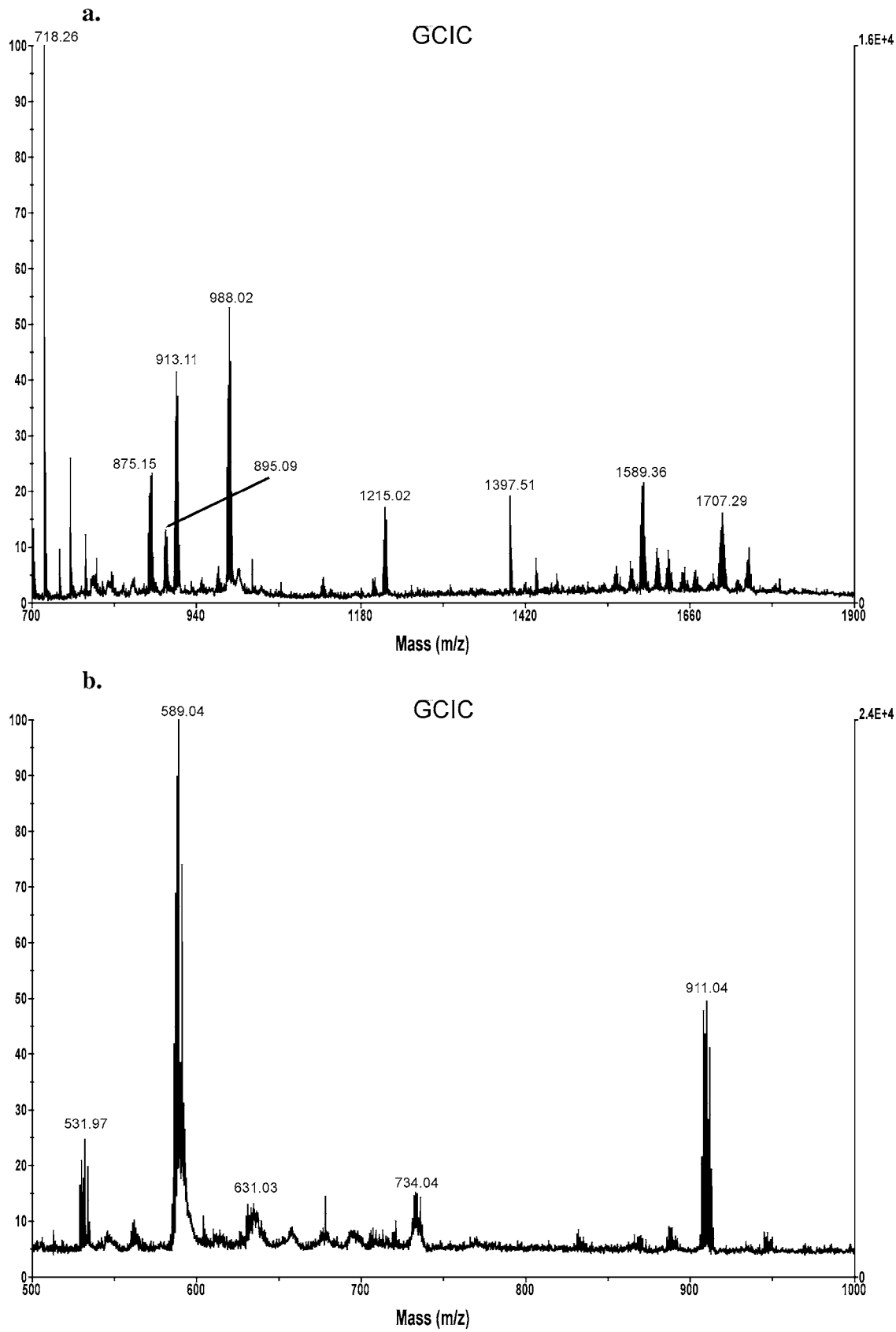

Fig. 6. Positive-charge-labeled (a, top) and negative-charge-labeled (b, bottom) MALDI-TOF spectra of rat urine samples collected at 8 h postinjection of Gd-DTPA cystine bisisopropyl amide copolymers (GCIC) at 0.10 mmol Gd/kg.

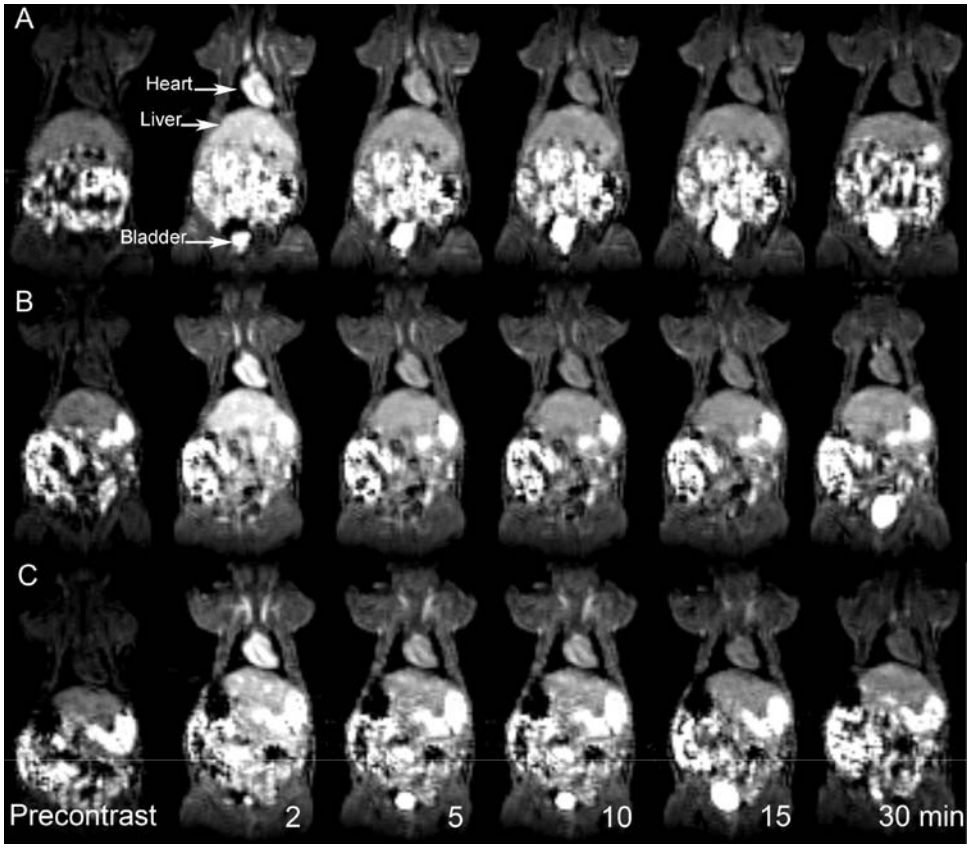

Fig. 7. 2D coronal magnetic resonance images at precontrast and 2, 5, 10, 15, and 30 min postinjection of GCAC (A), GCPC (B), and GCIC (C).

negative-charge-labeled MALDI-TOF spectra indicates that the majority of the macromolecular Gd(III) chelates were excreted and cleared from the body within a short time period. These results were similar to the clearance of GDCC, which showed that most of the agents were excreted in the urine sample within the first 8 h after injection (16).

All three biodegradable macromolecular agents of similar molecular weights resulted in more significant contrast enhancement in the blood pool of rats than a low molecular weight clinical agent, Gd-(DTPA-BMA), which was reported previously (16). The heart, liver, kidneys, and blood vessels were clearly visualized with these new contrast agents. As shown in the dynamic contrast-enhanced MR images, the enhancement in the heart and liver was still visible at 30 min postinjection (Fig. 7). The enhancement window should be long enough for most clinical MRI procedures of cancer imaging and angiography. All three agents resulted in similar enhancement kinetics in MR blood pool imaging in rats. Although the steric effect in the Gd-DTPA cystamine bisamide copolymers resulted in slight differences in the *in vitro* degradation rates among the agents, they all had similar enhancement kinetics. This is attributed to their rapid degradation, which was not able to differentiate the effect of the structural differences on *in vivo* contrast enhancement. A similar phenomenon was also observed for another rapid degrading polydisulfide agent, GDCC. GDCC with a high molecular weight (60 kDa) and a low molecular weight (18 kDa) resulted in similar enhancement kinetics in rats (16).

These newly synthesized agents have a relatively fast degradation rate and short enhancement window for vascular imaging as compared with our previously prepared MRI contrast agents, GDCP and PEG-*g*-GDCP (14,15,17). The steric effect of the alkyl groups was not observed during *in vivo* MRI because of their rapid degradation. On the other hand, GCAC, GCPC, and GCIC are neutral and are expected to have lower osmolality than the negatively charged GDCP and PEG-*g*-GDCP. Therefore, these neutral copolymers show potential as MRI contrast agents for patients with a history of renal malfunction.

CONCLUSION

Biodegradable macromolecular Gd(III) chelates, (Gd-DTPA) cystine bisamide copolymers, can be prepared in relatively high molecular weight. The paramagnetic copolymers rapidly degraded into low molecular weight species in the presence of cysteine via the disulfide–thiol exchange reaction. The *in vitro* degradation rate was reduced with the substitution of bulky alkyl groups in cystine bisamides. These agents were readily degraded and excreted via renal filtration, which was confirmed by the detection of catabolic products in rat urine samples. The agents resulted in significant MRI contrast enhancement of the heart, vasculature, liver, and kidneys in rats. No significant difference was observed in the dynamic contrast enhancement among the macromolecular Gd(III) chelates. These novel agents are

promising safe and effective, biodegradable macromolecular MRI contrast agents for blood pool imaging, including cancer imaging and angiography.

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