Effect of size and charge on pharmacokinetics and in vivo MRI contrast enhancement of biodegradable polydisulfide Gd(III) complexes

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Abstract

The purpose of this study is to investigate how the structures of polydisulfide Gd(III) complexes affect their pharmacokinetics and in vivo contrast enhancement as biodegradable macromolecular MRI contrast agents. A negatively charged polydisulfide Gd(III) complex, (Gd-DTPA)–cystine copolymers (GDCP), and a neutral agent, (Gd-DTPA)–cystine diethyl ester copolymers (GDCEP), with different molecular weights were prepared and characterized. The MRI contrast enhancement of the agents was studied in mice. Neutral GDCEP showed more rapid degradation than negatively charged GDCP in the blood plasma. Consequently, GDCP resulted in more significant and prolonged contrast enhancement in the blood pool and liver than GDCEP. The size of GDCEP did not significantly affect its in vivo contrast enhancement due to rapid degradation and clearance from the blood circulation. The increase in the molecular weight of GDCP resulted in prolonged in vivo contrast enhancement in the blood pool. The structural modification of polydisulfide Gd(III) complexes resulted in biodegradable macromolecular MRI contrast agents with different degradability and in vivo contrast enhancement.

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1. Introduction

Paramagnetic gadolinium(III) chelates, such as Gd-DTPA (diethylenetriaminepentaacetate) and Gd(DTPA-BMA) (diethylenetriaminepentaacetic bismethyl amide), are routinely used as contrast agents for magnetic resonance imaging (MRI) in clinical practice. These agents are extracellular contrast agents, extravasate rapidly into extracellular fluid space and have short tissue retention time due to their small sizes, leading to some disadvantages including short imaging time window and low signal to noise ratio. In order to prolong the circulation and retention of contrast agents, significant amounts of efforts have been made on the increase in the sizes of the contrast agents using different carrier systems, such as proteins [1], dendrimers [2], linear polymers [3] and liposomes [4]. Macromolecular contrast agents have exhibited improved pharmacokinetics and image contrast efficacy as compared to low molecular weight contrast agents. For example, the half-life of albumin–(Gd-DTPA) conjugate is approximately 3 h in the blood [5]. Gd-DTPA-labeled dextran (MW ~ 75 kDa) has a long half-life of 6.1 h as compared to 13 min of Gd-DTPA in rats [6]. Polymeric contrast agents also possess increased proton T1 relaxivity resulting from long rotational time compared to small size molecules [7].

The potential toxicity of macromolecular contrast agents related to slow excretion and long-term tissue Gd accumulation, however, hinders their further development. (Gd-DTPA)–albumin conjugate, a prototype of macromolecular MRI contrast agent, showed the high accumulation of Gd in the bone and liver consequential of its slow excretion [5], which increased possibility of cellular uptake of the agent through endocytosis and dissociation of Gd-DTPA complexes in the lysosome due to low pH and enzymatic degradation [8,9]. High long-term Gd tissue accumulation was also observed for other macromolecular Gd(III) complexes. It has been reported that the conjugation of Gd-DO3A to carboxymethyl hydroxylethyl starch resulted in a macromolecular agent (72 kDa) that had about 47% of injected dose detected in rat body seven days after the injection.
A Gd-DTPA polypropyleneimine dendrimer (generation 2) conjugate (7 kDa) resulted in the retention of 45% of injected dose in rats 14 days after injection [11]. High long-term in vivo accumulation of the contrast agents significantly increases the possibility of metabolic release of toxic Gd(III) ions from the chelates.

We have recently designed and developed the biodegradable macromolecular MRI contrast agents based on polydisulfide Gd (III) complexes to facilitate excretion of Gd chelates via in vivo degradation of the macromolecular agents [12]. Disulfide bonds can be rapidly reduced by the free plasma thiols including glutathione, cysteine and etc. [13,14]. We have shown that (Gd-DTPA)–cystamine copolymers (GDCC), the first polydisulfide MRI contrast agent, produced significant blood pool contrast enhancement in rats than a clinically available MRI contrast agent, Gd-(DTPA-BMA), and then cleared rapidly from the blood pool. GDCC exhibited minimal long-term tissue accumulation of Gd comparable to the clinically used Gd-(DTPA-BMA). We have hypothesized that the structure of the polydisulfide agents can be modified to design and develop biodegradable macromolecular MRI contrast agents with different degradability and pharmacokinetics for different applications. For example, a relatively long blood circulation time is necessary for more accurate diagnostic imaging of diseases in the cardiovascular systems and for detection and staging of tumor.

We have introduced functional groups around the disulfide bonds to tune the degradation rate of the paramagnetic polydisulfide agents and to prepare biodegradable macromolecular agents with various pharmacokinetic properties. Previously, we reported two new modified polydisulfide MRI contrast agents, (Gd-DTPA)–cystamine copolymers (GDCP) and (Gd-DTPA)–cystine diethyl ester copolymers (GDCEP), and preliminary results of these agents in contrast enhanced tumor MR imaging [15]. Further detailed evaluation of these agents has been performed on how the structure and size affect their degradation in the blood plasma and in vivo MRI contrast enhancement in mice. It was observed that GDCP and GDCEP had different degradation rate in the plasma and significantly different blood pool pharmacokinetics and contrast enhancement. Here we report the effect of structural differences in GDCP and GDCEP on their pharmacokinetics and in vivo MRI contrast enhancement.

2. Materials and experiments

2.1. Materials

DTPA was purchased from J.K. Baker (Philipsburg, NJ). Cystine diethyl ester was purchased from Bachem (Torrance, CA). Cysteine (99% purity) and Gd(OAc)₃ were purchased from Alfa Aesar (Ward Hill, MA). DTPA dianhydride was prepared according to literature [16].

2.2. DTPA–cystine copolymers

Cystine (10 mmol, 2.403 g) was dissolved in 5 ml of aqueous NaOH at pH 11 at room temperature and then the mixture was cooled in an ice water bath. DTPA dianhydride (10 mmol, 3.573 g) was then added in portions within 1 h, maintaining at pH 11 with NaOH aqueous solution. After the reaction, HCl was added to adjust pH to 7 and the solution was dialyzed against deionized water using membrane with molecular weight cutoff of 6–8000 Da for 24 h. The copolymer solution was lyophilized giving 3.2 g colorless solid product (54%). The number ($M_n$) and weight ($M_w$) average molecular weights of the copolymers were 18 and 33 kDa as determined by size exclusion chromatography (SEC) using AKTA FPLC system (Amersham Bioscience Corp., Piscataway, NJ) with a Superose 12 column. The system was calibrated with standard poly[N-(2-hydroxypropyl)methacrylamide].

2.3. DTPA–cystine diethyl ester copolymers

A mixture of cystine diethyl ester hydrochloride (3.18 mmol, 1.18 g), DTPA dianhydride (3.18 mmol, 1.14 g), triethylamine (2.6 g) in 2 ml anhydrous DMSO was stirred in ice-water bath in an atmosphere of nitrogen for 1 h. The mixture was then added to acetone with stirring. The precipitate was collected and dialyzed against deionized water using membrane with molecular weight cutoff of 6–8000 Da for 24 h at 4 °C. The copolymer solution was evaporated at 30 °C giving 1.4 g colorless product (61%). The number ($M_n$) and weight ($M_w$) average molecular weights of the copolymers were 20 and 49 kDa as determined by size exclusion chromatography (SEC).

2.4. GDCP and GDCEP

The paramagnetic complexes GDCP and GDCEP were prepared by the complexation of DTPA–cystine diethyl ester copolymers and DTPA–cystine copolymers with Gd(OAc)₃ at pH 5.5. GDCP and GDCEP were then fractionated to prepare the agents with different molecular weights using size exclusion chromatography with 26/60 Sphacryl S-300 column (Amersham Bioscience Corp., Piscataway, NJ). The flow rate was 80 ml/h and the mobile phase was deionized water. The weight ($M_w$) average molecular weights of the fractions were determined by SEC eluted with 20 mM Tris buffer with 0.1 M NaCl (pH 7.4). The Gd contents of GDCP and GDCEP were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Perkin Elmer, Norwalk, CT, Optima 3100XL). Their $T_1$ relaxivities were measured on a Siemens Trio 3T scanner at room temperature using an inversion recovery (IR)-prepared turbo spin echo (TSE) imaging pulse sequence and a birdcage human head coil.

2.5. Degradation of GDCP and GDCEP in rat plasma

Fresh blood from Sprague–Dawley rats (Charles River Laboratories) was centrifuged at 4 °C for 5 min at 4000 rpm. The plasma was then isolated, and plasma solutions of GDCP (46 kDa) and GDCEP (48 kDa) with 0.042 mM Gd were incubated at 37 °C in a G24 Environmental incubator shaker (New Brunswick Scientific Co. Inc., Edison, NJ, USA) with a medium shaking speed. Aliquots (300 μl) were taken after
incubation for 5, 10, 20 and 30 min and 1, 2, 3, 4, 5 and 6 h, and diluted with 300 μl of deionized water. The mixtures were separated by centrifuge ultrafiltration using Centricon® YM-3 centrifugal filters (molecular weight cutoff 3000 Da, Millipore, USA) at 4 °C and 4000 rpm for 20 min. The degradation products were collected in the filtrates and the Gd content was determined by ICP-OES.

2.6. MR imaging

Female athymic nude mice (5–6 weeks old, 20–25 g, Charles River Laboratories) were cared for under an approved protocol and the guidelines of the University of Utah Institutional Animal Care and Use Committee. The mice were anesthetized by the intramuscular administration of a mixture of ketamine (80 mg/kg) and xylazine (12 mg/kg). Contrast enhanced MR images of the mice were acquired on a Siemens Trio 3T scanner using a 3D fast low-angle shot (3D FLASH) pulse sequence with a human wrist coil before and at 2, 5, 10, 15, 30 and 60 min after injection of the contrast agents at a dose of 0.1 mmol Gd/kg via a tail vein. Imaging parameters were 2.44 ms TE, 7.36 ms TR, 25° RF tip angle, 120 mm field of view, 0.5 mm coronal slice thickness. A group of three mice were used for each polymeric contrast agent. The MR images were analyzed using a free software package OSIRIX. Regions of interest (ROIs) were set on the right ventricle of the heart, liver and left kidney of each mouse. Signal intensities in these ROIs were measured at different imaging time points.

2.7. Data analysis

Each data point for the contrast enhancement pattern is the average from three different mice. Error bars are ± the standard deviation. Statistical analysis was performed to compare the polymeric contrast agents at each time point in each tissue using a one-way ANOVA with a Tukey post-test. A statistical difference was considered to be \( p < 0.05 \).

3. Results

3.1. Properties of GDCP and GDCEP

The chemical structures of Gd-DTPA cystine copolymers (GDCP) and Gd-DTPA cystine diethyl ester copolymers (GDCEP) are shown in Fig. 1. GDCP is negatively charged and GDCEP is a neutral agent. Both agents had a broad molecular weight distribution after condensation copolymerization. In order to understand better how the size of the biodegradable macromolecular agents affects their pharmacokinetics and in vivo contrast enhancement, the paramagnetic polydisulfides with narrower molecular weight distribution were prepared by fractionation with size exclusion chromatography. Three fractions from each agent were selected in this study. The apparent molecular weights, polydispersity index (PDI) and \( T_1 \) relaxivity of the agents are listed in Table 1. The fractionated polymeric relaxivities were ca. 1.4). Same fractions were collected for GDCP and GDCEP with deionized water as the eluent. However, GDCEP had relatively higher average molecular weights than GDCP, because the molecular weight distribution of the copolymers in the fractions was not uniform as shown in the chromatograms. The molecular weight distribution of GDCEP had more portions of high molecular weight polymers than that of GDCP in the same fractions, particularly for the fractions of high and low molecular weights.

3.2. Degradation of GDCP and GDCEP in the plasma

GDCP and GDCEP had different degradation rates in the rat blood plasma. In order to avoid any potential complication caused by size difference of the agents, GDCP (46 kDa) and GDCEP (48 kDa) with similar average molecular weights or hydrodynamic volume was used in the degradation study. Fig. 2 shows the percentage of the contrast agents that had molecular weight larger than the molecular weight cutoff of the filter (3000 Da). It appears that the GDCEP degraded more rapidly into small oligomeric Gd(III) complexes in the plasma than GDCP. Approximately 10% of GDCP degraded into Gd(III)

![Fig. 1. The chemical structures of GDCEP and GDCP.](image)

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Physicochemical parameters of GDCEP and GDCP</th>
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<tr>
<td></td>
<td>GDCEP</td>
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<tr>
<td>( M_w ) (kDa)</td>
<td>73</td>
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<tr>
<td>PDI</td>
<td>1.2</td>
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<tr>
<td>Gd content (by wt.%)</td>
<td>16.9</td>
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<tr>
<td>Relaxivity ( (\text{mM}^{-1} \cdot \text{s}^{-1}) )</td>
<td>4.5</td>
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Fig. 2. The percentage–time profile of copolymers with molecular weight larger than 3000 Da in the degradation of GDCEP (48 kDa, ▲) and GDCP (46 kDa, △) in plasma at 37 °C.
complexes with molecular weight less than 3000 Da in 6-h incubation, while over 60% of GDCEP degraded and passed through the filter within the first hour of the incubation. No further significant degradation was observed thereafter, possibly due to the exhaustion of free thiols in the limited volume of the plasma. Degradation product with a mass of the repeat units after disulfide–thiols exchange reaction (m/z = 808.6) was identified in the filtrate of GDCEP with MALDI-TOF mass spectrometry. No degradation products were detected in the filtrate of GDCP, possibly due to their low concentration.

3.3. Contrast enhanced MRI of GDCEP and GDCP

Three-dimensional maximum intensity project (MIP) MR images of mice before and at various time points after injection of GDCP and GDCEP with different molecular weights are shown in Fig. 3. Strong contrast enhancement was observed in the liver, kidneys and blood in the heart and vasculature with all agents at 2 min post-injection and the signal intensity gradually decreased thereafter. Neutral GDCEP resulted in more rapid decrease of contrast enhancement in the heart than the negatively charged GDCP. GDCP with high molecular weights (46 and 62 kDa) exhibited the most prolonged blood enhancement.

![Fig. 3. Three-dimensional maximum intensity projection MR images of mice before injection (a) and 2 (b), 5 (c), 10 (d), 15 (e), 30 (f) and 60 (g) min after injection of GDCEP (A: 34 kDa, B: 48 kDa, C: 73 kDa) and GDCP (D: 22 kDa, E: 46 kDa, F: 62 kDa) at a dose of 0.1 mmol Gd/kg via a tail vein.](image)

The difference between GDCP of molecular weight 46 and 62 kDa was not significant possibly because their molecular weight difference was not large enough. The contrast enhancement of the agents in the liver showed similar dynamic patterns as in the heart. Gradual enhancement in the urinary bladder was observed for all compounds, indicating urinary clearance of the agents. The analysis of the dynamic change of the signal intensity ratios of the liver, kidneys and blood in the heart to muscle, which represented the contrast enhancement in the organs to the surrounding tissues, revealed the significant difference between GDCP and GDCEP in MRI contrast enhancement (Fig. 4). All three GDCEP with different molecular weight resulted in similar contrast enhancement in the blood (p > 0.05), while GDCP of 46 and 62 kDa provided significantly stronger and more prolonged contrast enhancement than that of 22 kDa (p < 0.05) (Fig. 4(a)). The size difference between GDCP of 46 and 62 kDa was not big enough to result in significant difference in contrast enhancement. GDCP of 22 kDa demonstrated significantly stronger contrast enhancement than GDCEP of all molecular weights in the blood and liver (p < 0.05) in the first 15 min post-injection, while GDCP of 46 and 62 kDa showed significantly stronger contrast enhancement in the entire experimental period (60 min, p < 0.05). The dynamic change of signal intensity of the agents in the liver followed similar pattern as the heart (Fig. 4(b)).

![Fig. 4. Relative MR signal intensities in the blood (a) and liver (b) of mice before and at various time points after the intravenous injection of GDCEP (34 kDa, ▼; 48 kDa, ▲; 73 kDa, ■) and GDCP (22 kDa, ▽; 46 kDa, △; 62 kDa, □).](image)
The signal intensity of the contrast agents in the kidneys decreased over time similarly as that in the blood and liver (Fig. 5). Relatively higher signal intensities were measured in the kidney medulla than in the cortex with the same agent. GDCEP showed higher contrast enhancement than GDCEP in both cortex and medulla all the time post-injection. However, the differences among the agents were not significant. Corresponding to the signal decrease in the kidneys, the signal intensity in the bladder gradually increased (Fig. 3), indicating the agents were excreted via renal filtration.

4. Discussion

Macromolecular Gd(III) complexes have demonstrated superior contrast enhancement in MR imaging than the clinically available low molecular Gd(III) chelates in animal models. The long blood circulation of macromolecular agents provides a sufficient imaging time window for more accurate diagnostic MR imaging with various techniques. They provide better delineation of the heart and blood vessels in the cardiovascular imaging [5] and are able to grade tumor based on the hyperpermeability of solid tumors [17]. They are also advantageous in contrast enhanced lymphatic imaging and renal imaging as shown in animal models [18,19]. These agents are currently not available for clinical applications because of the safety concerns related to their slow excretion and consequent tissue deposition of toxic Gd(III) ions. Polydisulfide Gd(III) complexes are designed as biodegradable macromolecular MRI contrast agents to alleviate the safety concerns by accelerating the excretion of Gd(III) chelates after in vivo breakdown of the macromolecules. We have shown previously that the polydisulfide Gd(III) complexes including GDCP and GDCEP can be readily degraded both in vitro and in vivo at the disulfide bonds in the polymer backbone by free thiols via disulfide–thiol exchange reaction [15,20,21]. The preliminary studies showed that these agents provide more significant in vivo contrast enhancement than the clinically available low molecular weight agent, Gd(DTPA-BMA). In order to design optimal biodegradable macromolecular MRI contrast agents for various applications, we need to understand how the structure including charge and size of the agents influences their in vivo degradation rate, pharmacokinetics and contrast enhancement.

The results have demonstrated that the charge of polydisulfide Gd(III) complexes have a dominant effect on in vivo degradation of the agents in the plasma. Significant size effect on pharmacokinetics and MR contrast enhancement was observed only when the agents degraded slowly. Negatively charged GDCP degraded slowly in the plasma, had a relatively long blood circulation and resulted in prolonged contrast enhancement in the blood pool. In contrast, neutral GDCEP degraded rapidly and resulted in rapid clearance from the blood pool and relatively short contrast enhancement. The size of the GDCEP did not significantly affect the pharmacokinetics and in vivo contrast enhancement in the blood pool, while the increase in the size of GDCP resulted in significantly prolonged in vivo contrast enhancement.

The degradability of GDCP and GDCEP in the blood plasma (Fig. 2) was similar to that observed in the incubation with cysteine [15]. GDCEP degraded rapidly in the plasma and in the presence of cysteine, while GDCP was relatively stable. It would be ideal if the kinetics of the molecular weight reduction due to the degradation can be determined in the plasma. However, the change of molecular weight distribution of the polydisulfide agents could not be accurately measured with size exclusion chromatography because of the interference of the biomacromolecules in the plasma. With ultrafiltration, the degradation products were readily separated and then accurately determined by ICP-OES. Negative charges in GDCP slow down the reaction of disulfide bonds with free thiols in the plasma due to electrostatic repulsion [22,23] of GDCP and endogenous free thiols, such as cysteine, glutathione and homocysteine, which present in the negatively charged form at physiological pH. In contrast, the neutral GDCEP is more susceptible to the chain cleavage by the plasma thiols.

The differences in the degradation rates of the agents in the plasma significantly affected the pharmacokinetics and in vivo contrast enhancement. The dynamic changes of signal intensity in the regions of interest can qualitatively reflect the pharmacokinetic properties of the contrast agents albeit the signal intensity is not linearly proportional to the concentration of the agents. The rapid degradation of GDCEP in the plasma resulted in rapid clearance of the agent in the blood pool and short...
contrast enhancement window. The increase in molecular weight of GDCEP did not significantly prolong its blood circulation possibly because the random cleavage of the polymer chains produced oligomers with molecular sizes smaller than the renal filtration threshold, which were rapidly eliminated thereafter. Similar result was observed for another neutral polydisulfide agent, Gd-DTPA cystamine copolymers (GDCC), whose size did not significantly affect its pharmacokinetics and in vivo contrast enhancement [12]. The slow degradation of GDCP resulted in the prolonged blood circulation and differentiated the size effect on in vivo contrast enhancement.

GDCP resulted in more significant contrast enhancement than GDCEP in the blood, vasculature and the liver because of its prolonged blood circulation. The increase in the size of GDCP further enhances the contrast in these organs and tissues. GDCEP of three molecular weights only provided strong contrast in first 5 min post-injection. Strong contrast enhancement was observed in the kidneys for the agents with different molecular weights and differences were not significant possibly because most of degradation products were eliminated via the kidneys. The signal intensity in the kidneys gradually decreased and the signal in the bladder gradually increased (Fig. 3), indicating the excretion of the agents in the bladder.

This study has shown that the structures of the polydisulfide Gd(III) complexes significantly affect their biodegradability, pharmacokinetics and in vivo contrast enhancement. This implies that optimal polydisulfide Gd(III) complexes can be designed and prepared by structural optimization as safe and effective biodegradable macromolecular MRI contrast agents for clinical development. The contrast agents with different pharmacokinetic properties may have different applications. Generally, the agents with an acceptably long blood circulation are more effective in contrast enhanced cardiovascular imaging and cancer imaging.

5. Conclusions

The negative charges in polydisulfide Gd(III) complexes have significant impact on the degradation in the plasma, pharmacokinetics and in vivo contrast enhancement as shown in mice. The negatively charged GDCP degraded slowly in the blood plasma than the neutral agent GDCEP. The increase in the size of GDCP significantly prolonged the blood circulation and in vivo contrast enhancement. The size of GDCEP did not significantly affect its pharmacokinetics and contrast enhancement because of rapid breakdown of the polymer backbones. Biodegradable macromolecular MRI contrast agents with optimal pharmacokinetics and in vivo contrast enhancement can be developed by structural modification of polydisulfide Gd(III) complexes.

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References

comparison of macromolecular and small-molecular contrast media-


