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PAPER

Facile synthesis and *in vivo* evaluation of biodegradable dendritic MRI contrast agents†

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Dendritic gadolinium chelates as magnetic resonance imaging (MRI) contrast agents (CAs) have shown great potential in blood pool and tumor imaging due to their enhanced relaxivities (R_1s), prolonged blood circulation time, and homogeneous pharmaceutical properties. However, the non-biodegradability of the currently used dendrimers causes long-term retention of toxic gadolinium ions, limiting their clinical applications. Herein, we report a facile synthesis of biodegradable polyester dendrimer-based CAs. The prepared dendritic CAs have nano-sized structures with the hydrodynamic diameters ranging from 2.8 to 8.6 nm, and show high R_1s up to 11.7 mM^{-1} s^{-1}, approximately 2.7 times that of a clinically used small molecule CA (Magnevist). They are slowly hydrolyzed at an acidic pH, but rapidly hydrolyzed at pH 7.4, especially in the presence of esterase. The *in vivo* evaluation indicates they not only provide much better MRI contrast enhancement than Magnevist, but also show minimal tissue retention of Gd, which is much lower than those of non-biodegradable CAs.

1. Introduction

Magnetic resonance imaging (MRI) is one of the most important medical imaging techniques in clinics owing to its high spatial resolution and no radiation risk.[1] In many cases contrast agents (CAs) are needed to improve diagnostic sensitivity and accuracy of MRI.[2,3] Currently, clinically used CAs are mainly small molecule paramagnetic gadolinium (Gd) chelates such as diethylenetriaminepentaacetic acid–Gd (DTPA–Gd, Magnevist)[4] and Gd–diethylenetriaminepentaacetic acid bismethylamide (Gd–DTPA-BMA, Omniscan).[5] These small molecule CAs suffer many drawbacks including non-specificity, rapid renal excretion and low relaxivity.[1]

Conjugation of Gd-chelates onto polymers forming macromolecular CAs can enhance their relaxivities and prolong their blood circulation times.[6–8] Among the many reported systems, the conjugates of Gd-chelates onto dendrimers are of particular interest.[9] Such dendritic CAs (DCAs) have not only higher relaxivities but also well controlled three-dimensional structures and sizes, which are essential for homogeneous pharmacological properties.[10] They have excellent potential for use in the MRI of blood pools,[11] the liver,[12] kidneys,[13,14] and tumors.[15]

However, DCAs developed to date are primarily based on non-biodegradable dendrimers such as polyamidoamine (PAMAM) and polypropyleneimine (PPI).[14] The large DCAs are bigger than the renal threshold and thus they cannot be excreted effectively from the body. They may subsequently be taken up by cells and metabolized, releasing toxic Gd^{3+} ions.[16–18] For instance, 80% of the injected PAMAM-G6 based DCA remained in the body after 2 days, and more than 10% of the dose remained after 10 days.[19,20] To speed up the excretion of Gd ions, Lu *et al.* conjugated Gd chelates onto PAMAM *via* a cleavable disulfide linker.[20] The Gd-chelates could be intracellularly cleaved from the conjugate by intracellular glythione (GSH) and thus rapidly excreted as small molecules *via* renal filtration. Unfortunately, this DCA was highly toxic due to the intrinsic toxicity of the PAMAM dendrimer.[20] Biodegradable polylysine dendrimers were also used as carriers to prepare DCAs.[21,22] The resultant DCAs showed improved biocompatibility.[23,24] Hence, a polylysine dendrimer-based CA (Gadomer 17) is currently undergoing clinical trials.[25] However, the complicated syntheses of these dendrimers, involving multiple protection and deprotection steps, hindered wider application.

Recently, we developed a facile method to synthesize novel polyester dendrimers by "sequentially clicking" asymmetrical monomers.[26] The synthesized dendrimers were biodegradable *via* the ester bonds, and had a large number of surface amino groups useful for conjugation of small molecule CAs. Herein, we developed a further simplified dendrimer synthesis using β-cyclodextrin (β-CD). β-CD has 21 hydroxyl groups and enabled us to use one-pot per generation method to synthesize sufficiently large dendrimers for fabricating DCAs in much fewer steps. The DCAs prepared from these dendrimers showed much

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higher relaxivities and blood pool contrast enhancement than the standard small molecule CA Magnevist. Most significantly, the degradability of the dendrimer led to minimal long-term tissue retention of Gd in mice.

2. Materials and methods

2.1 Materials

All the organic solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Dichloromethane (CH_2Cl_2), pyridine and dimethyl sulfoxide (DMSO) were purified by distillation under nitrogen (N_2) or vacuum over calcium hydride. Acetonitrile was purified with phosphorus pentoxide. β-Cyclodextrin purchased from Sinopharm was recrystallized three times from ultrapure water and dried at 105 °C for 24 h before used. 2-Hydroxyethylmetharylate, 2,6-di-*tert*-butyl-4-methylphenol (BHT), cysteamine and gadolinium chloride were purchased from Alfa Aesar (Ward Hill, MA, USA). Acryloyl chloride was from Zouping Mingxing Chemical Reagent Co., Ltd. (Zouping, China). Diethylenetriaminepentaacetic acid (DTPA), *N*,*N*-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were bought from Shanghai Jingchun Chemical Reagent Co., Ltd. (Shanghai, China). 2-Chloro-1,3,2-dioxaphospholane-2-oxide (COP) was purchased from Joy-Nature Technology Institute (Nanjing, China). Deuterated solvents for NMR measurements such as deuterium oxide (D_2O) and deuterated chloroform ($CDCl_3$) were purchased from Energy Chemical Co., Ltd. (Shanghai, China). All other chemicals were used as received. 2-[(Methacryloyl)oxy]ethyl acrylate (MAEA) was synthesized as reported.[26]

2.2 Synthesis of (2,3-di-*o*-methacrylated-6-methacrylated)-CD (β-CDMA, G0)

β-CD (5.00 g, 4.41 mmol, 92.6 mmol of hydroxyl group), 2,6-di-*tert*-butyl-4-methylphenol (BHT) (0.04 g, 0.18 mmol), and pyridine (20 mL) were mixed in a 100 mL flask. Methacrylic anhydride (20.5 mL, 138 mmol, 1.48 eq.) in pyridine (20 mL) was added dropwise into the flask. The mixture was stirred at room temperature for 2 h and at 50 °C for an additional 5 h. The resulting solution was poured into cold water (0 °C, 500 mL) to precipitate out the product. The product was collected by filtration, and purified by dissolving in methanol (10 mL) and reprecipitation in ice water (500 mL) three times. The product was isolated and dried under vacuum with a yield of 72%. ^{1}H-NMR ($CDCl_3$, 400 Hz), δ_{ppm}: 6.17 (s, 20H, ***H***CH=C(CH_3)–CO–), 5.62 (s, 20.8H, HC***H***=C(CH_3)–CO–), 5.18 (7H, C(1)***H*** β-CD), 4.80 (7H, C(2)***H*** of β-CD), 4.60 (7H, C(3)***H*** of β-CD), 3.95 4.37 (7H, C(5)***H*** and 14H, C(6)***H*** of β-CD), 3.58 (7H, C(4)***H*** of β-CD), 1.95 (61.2H, C***H***$_3$).

2.3 Synthesis of the first-generation dendrimer (G1)

β-CDMA (0.400 g, 0.156 mmol, 3.18 mmol methacrylate group), cysteamine (0.303 g, 3.94 mmol, 1.2 eq.), BHT (0.372 g, 1.69 mmol) and DMSO (5 mL) were charged into a 25 mL flask under N_2. The mixture was stirred at room temperature for 1 h. The reaction was monitored by ^{1}H-NMR. After all the methacrylate groups reacted with cysteamine, MAEA (2.05 g, 11.1 mmol, 3.38 eq.) was added to the solution and stirred at room temperature for 24 h. The solution was further heated at 50 °C for 72 h. The mixture was diluted with dichloromethane (100 mL), washed with water (3 × 100 mL), and then dried with anhydrous magnesium sulfate. The solution was concentrated and poured into 100 mL hexane to precipitate out the product. The product was then purified by reprecipitation three times and dried under vacuum. A light-yellow oily product G1 was obtained with a yield of 1.63 g (91%). ^{1}H-NMR ($CDCl_3$, 400 Hz), δ_{ppm}: 6.02 (s, 40H, ***H***CH=C(CH_3)–CO–), 5.49 (s, 40H, HC***H***=C(CH_3)–CO–), 4.25 (s, 160H, –CO–O–C***H***$_2$–C***H***$_2$–O–CO–), 2.67 (m, 80H, –CH_2–N–C***H***$_2$–CH_2–CO–), 2.52 (m, 60H, –CO–C***H***(CH_3)–CH_2– & –C***H***$_2$–N–CH_2–CH_2–CO–), 2.50 (m, 40H, –S–C***H***$_2$–CH_2–N–), 2.46 (m, 40H, –CH(CH_3)–C***H***$_2$–S–), 2.36 (m, 80H, –CH_2–C***H***$_2$–CO–), 1.84 (s, 120H, –CO–C(C***H***$_3$)=CH_2), 1.12 (s, 60H, –CO–CH(C***H***$_3$)–CH_2–).

2.4 Synthesis of the second-generation dendrimer (G2)

G1 (1.0 g, 0.087 mmol, 3.51 mmol methacrylate group), cysteamine (0.324 g, 4.21 mmol, 1.2 eq.), BHT (0.390 g 1.77 mmol) and DMSO (10 mL) were added to a 25 mL flask and stirred for 1 h. MAEA (1.94 g, 10.5 mmol, 3.0 eq.) was then added to the solution. The subsequent reaction and purification followed the procedure used in the G1 synthesis, yielding a light-yellow oil at 89%. ^{1}H-NMR ($CDCl_3$, 400 Hz), δ_{ppm}: 6.02 (s, 80H, ***H***CH=C(CH_3)–CO–), 5.49 (s, 80H, HC***H***=C(CH_3)–CO–), 4.25 (d, 480H, –CO–O–C***H***$_2$–C***H***$_2$–O–CO–), 2.67 (m, 240H, –CH_2–N–C***H***$_2$–CH_2–CO–), 2.52 (m, 180H, –CO–C***H***(CH_3)–CH_2– & –C***H***$_2$–N–CH_2–CH_2–CO–), 2.50 (m, 120H, –S–C***H***$_2$–CH_2–N–), 2.46 (m, 120H, –CH(CH_3)–C***H***$_2$–S–), 2.36 (m, 240H, –CH_2–C***H***$_2$–CO–), 1.84 (s, 240H, –CO–C(C***H***$_3$)=CH_2), 1.12 (d, 180H, –CO–CH(C***H***$_3$)–CH_2–).

2.5 Synthesis of the third-generation dendrimer (G3)

G2 (0.61 g, 0.021 mmol, 1.70 mmol methacrylate group), cysteamine (0.160 g, 2.07 mmol, 1.2 eq.), BHT (0.19 g, 0.86 mmol) and DMSO (6 mL) were added to a 25 mL flask and stirred for 1 h. MAEA (0.94 g, 5.1 mmol, 3.0 eq.) was then added dropwise to the solution. The subsequent reaction and purification followed the procedure used in the G1 synthesis, yielding a light-yellow oil at 88%. ^{1}H-NMR ($CDCl_3$, 400 Hz), δ_{ppm}: 6.02 (s, 160H, HC***H***=C(CH_3)–CO–), 5.49 (s, 160H, HC***H***=C(CH_3)–CO–), 4.25 (d, 1120H, –CO–O–C***H***$_2$–C***H***$_2$–O–CO–), 2.67 (m, 560H, –CH_2–N–C***H***$_2$–CH_2–CO–), 2.52 (m, 420H, –CO–C***H***(CH_3)–CH_2– & –C***H***$_2$–N–CH_2–CH_2–CO–), 2.50 (m, 280H, –S–C***H***$_2$–CH_2–N–), 2.46 (m, 280H, –CH(CH_3)–C***H***$_2$–S–), 2.36 (m, 560H, –CH_2–C***H***$_2$–CO–), 1.86 (s, 480H, –CO–C(CH_3)=CH_2), 1.12 (d, 420H, –CO–CH(C***H***$_3$)–CH_2–).

2.6. Synthesis of DTPA *N*-hydroxysuccinimide ester (DTPA–NHS)

DTPA (8.00 g, 20.4 mmol) and triethylamine (11.7 mL, 81.1 mmol) were dissolved in 200 mL acetonitrile and stirred at 50 °C for 1 h. The solution was cooled to 0 °C. NHS (1.65 g, 14.3 mmol) and DCC (2.96 g, 14.4 mmol) were then added to the solution, and the resulting mixture was stirred at room temperature for 8 h. The insoluble dicyclohexylurea (DCU) was

removed by filtration. The filtrate was dried using a rotary evaporator, and the residue was dissolved in DMSO to obtain 20 mL DTPA–NHS solution at a concentration of 0.72 mmol mL^{-1}.

2.7 Synthesis of G0–DTPA

β-CDMA (0.400 g, 0.156 mmol, 3.18 mmol methacrylate group), cysteamine (0.288 g, 3.75 mmol, 1.2 eq.) and DMSO (4 mL) were charged into a 25 mL flask under N_2 and stirred at room temperature for 1 h. The reaction was monitored by ^{1}H-NMR. After all the methacrylate groups reacted with cysteamine, the DTPA–NHS (13.1 mL, containing 9.4 mmol DTPA–NHS, 3 eq.) was added to the reaction solution and stirred at room temperature for 12 h. Methanol (100 mL) was then added into the mixture to precipitate out the product. The precipitate was redissolved in 3 mL H_2O and reprecipitated in 30 mL methanol three times. The product was then collected and dried under vacuum, yielding a white powder at 64%. ^{1}H-NMR (D_2O, 400 Hz), δ_{ppm}: 3.84 (s, 160H, –N–C***H***$_2$–COOH), 3.54 (s, 40H, –N–C***H***$_2$–CO–NH–), 3.40 (t, 80H, –N–C***H***$_2$–CH_2–N–CH_2–C***H***$_2$–N–), 3.12 (t, 40H, –C***H***$_2$–NH–CO–), 3.11 (t, 80H, –N–CH_2–C***H***$_2$–N–C***H***$_2$–CH_2–N–), 2.91 (t, 20H, –CO–C***H***(CH_3)–CH_2–), 2.79 (m, 40H, –S–C***H***$_2$–CH_2–NH–), 2.73 (m, 40H, –$CH(CH_3)$–C***H***$_2$–S–), 1.17 (t, 60H, –CO–CH(C***H***$_3$)–CH_2–).

2.8 Synthesis of G1–DTPA

G1 (0.400 g, 0.0349 mmol, 1.40 mmol methacrylate group) and cysteamine (0.129 g, 1.67 mmol, 1.2 eq.) reacted in DMSO (3 mL) at room temperature for 1 h and the solution of DTPA–NHS (5.9 mL, containing 4.2 mmol DTPA–NHS, 3 eq.) was added to the solution. The subsequent reaction and purification followed the procedure used in the G0–DTPA synthesis, yielding a white powder at 52%. ^{1}H-NMR (D_2O, 400 Hz), δ_{ppm}: 4.38 (s, 160H, –CO–O–C***H***$_2$–C***H***$_2$–O–CO–), 3.84 (s, 282H, –N–C***H***$_2$–COOH), 3.54 (s, 72, –N–C***H***$_2$–CO–NH–), 3.44 (t, 145H, –N–C***H***$_2$–CH_2–N–CH_2–C***H***$_2$–N–), 3.18 (t, 72H, –C***H***$_2$–NH–CO–), 3.14 (t, 144H, –N–CH_2–C***H***$_2$–N–C***H***$_2$–CH_2–N–), 2.97 (t, 100H, –CO–C***H***(CH_3)–CH_2– & –C***H***$_2$–N–CH_2–CH_2–CO–), 2.82 (m, 80H, –CH_2–N–C***H***$_2$–CH_2–CO–), 2.78 (m, 120H, –S–C***H***$_2$–CH_2–N–), 2.76 (m, 120H, –$CH(CH_3)$–C***H***$_2$–S–), 2.74 (m, 80H, –CH_2–C***H***$_2$–CO–), 1.21 (s, 180H, –CO–CH(C***H***$_3$)–CH_2–).

2.9 Synthesis of G2–DTPA

G2 (0.400 g, 0.0137 mmol, 1.09 mmol methacrylate group) and cysteamine (0.101 g, 1.31 mmol, 1.2 eq.) were reacted in DMSO (3 mL) at room temperature for 1 h and the solution of DTPA–NHS (4.6 mL, containing 3.3 mmol DTPA–NHS, 3 eq.) was added to the solution. The subsequent reaction and purification followed the procedure used in the G0–DTPA synthesis, yielding a white powder at 51%. ^{1}H-NMR (D_2O, 400 Hz), δ_{ppm}: 4.39 (s, 480H, –CO–O–C***H***$_2$–C***H***$_2$–O–CO–), 3.84 (s, 596H, –N–C***H***$_2$–COOH), 3.54 (s, 149H, –N–C***H***$_2$–CO–NH–), 3.44 (t, 298H, –N–C***H***$_2$–CH_2–N–CH_2–C***H***$_2$–N–), 3.19 (t, 149H, –C***H***$_2$–NH–CO–), 3.14 (t, 298H, –N–CH_2–C***H***$_2$–N–C***H***$_2$–CH_2–N–), 2.97 (t, 260H, –CO–C***H***(CH_3)–CH_2– & –C***H***$_2$–N–CH_2–CH_2–CO–), 2.82 (m, 240H, –CH_2–N–C***H***$_2$–CH_2–CO–), 2.78 (m, 280H, –S–C***H***$_2$–CH_2–N–), 2.76 (m, 280H, –$CH(CH_3)$–C***H***$_2$–S–), 2.74 (m, 240H, –CH_2–C***H***$_2$–CO–), 1.22 (s, 420H, –CO–CH(C***H***$_3$)–CH_2–).

2.10 Synthesis of G3–DTPA

G3 (0.400 g, 0.00617 mmol, 0.987 mmol methacrylate group) and cysteamine (0.091 g, 1.18 mmol, 1.2 eq.) were reacted in DMSO (3 mL) at room temperature for 1 h and the solution of DTPA–NHS (4.1 mL, containing 2.9 mmol DTPA–NHS, 3 eq.) was added to the solution, The subsequent reaction and purification followed the procedure used in the G0–DTPA synthesis, yielding a white powder at 53%. ^{1}H-NMR (D_2O, 400 Hz), δ_{ppm}: 4.39 (s, 1120H, –CO–O–C***H***$_2$–C***H***$_2$–O–CO–), 3.84 (s, 1202H, –N–C***H***$_2$–COOH), 3.56 (s, 300H, –N–C***H***$_2$–CO–NH–), 3.44 (t, 602H, –N–C***H***$_2$–CH_2–N–CH_2–C***H***$_2$–N–), 3.20 (t, 300H, –C***H***$_2$–NH–CO–), 3.13 (t, 601H, –N–CH_2–C***H***$_2$–N–C***H***$_2$–CH_2–N–), 3.00 (t, 580H, –CO–C***H***(CH_3)–CH_2– & –C***H***$_2$–N–CH_2–CH_2–CO–), 2.87 (m, 560H, –CH_2–N–C***H***$_2$–CH_2–CO–), 2.78 (m, 600H, –S–C***H***$_2$–CH_2–N–), 2.76 (m, 600H, –$CH(CH_3)$–C***H***$_2$–S–), 2.74 (m, 560H, –CH_2–C***H***$_2$–CO–), 1.22 (s, 900H, –CO–CH(C***H***$_3$)–CH_2–).

2.11 Synthesis of zwitterionized G2–DTPA (G2/MOP–DTPA)

2-Chloro-1,3,2-dioxaphospholane-2-oxide (COP) (0.645 mL, 7.02 mmol) was dissolved in 6 mL of dry THF. Anhydrous methanol (0.567 mL, 14.0 mmol, 2 eq.) and anhydrous triethylamine (1.01 mL, 7.02 mmol) were mixed and added dropwise into the COP solution at −20 °C. The mixture was allowed to warm up to ambient temperature over 4 h. The reaction mixture was then cooled to 0 °C and filtered. The filtrate was concentrated by rotary evaporation and then dissolved in 5 mL anhydrous DMSO to get a 2-methoxy-1,3,2-dioxaphospholane-2-oxide (MOP) solution (1.4 mol L^{-1}). G2–DTPA (0.800 g, 0.0120 mmol) was added to the resultant DMSO solution, and the solution was stirred at 70 °C under N_2 for 24 h. The product was reprecipitated in ethyl ether/acetone three times. The precipitate was then collected and dried under vacuum, yielding a yellow powder (55%).

2.12 Synthesis of dendritic CAs

As an example, G1–DTPA (0.100 g, 0.00352 mmol, 0.124 mmol DTPA) was added into a solution of gadolinium chloride hexahydrate (0.055 g, 0.15 mmol, 1.2 eq.) in 0.1 M sodium acetate buffer (pH = 7). The solution was stirred at room temperature for 2 h. The excess Gd^{3+} was removed by diafiltration four times using Amicon® Ultra-4, and then the solution was lyophilized, yielding G1–DTPA–Gd as a white floccule of 0.064 g (54%). The syntheses of other dendrimer–DTPA–Gd samples with higher generations followed the same procedures. G1–DTPA–Gd, G2–DTPA–Gd, G3–DTPA–Gd and G2/MOP–DTPA–Gd were obtained with yields of 50%, 55%, 58% and 52%, respectively. The Gd content of the DCAs was determined by ICP-MS Thermo Element XSeries II.

2.13 *In vitro* degradation of the dendritic ligand

G3–DTPA (7 mg mL^{-1}) was incubated in water at different pH or in 7.4 PBS buffer with or without esterase (EC 3.1.1.1,

15 U mL^{-1}) at 37 °C on a shaker. Samples were taken at timed intervals and lyophilized. The degradation of the DCA was calculated from the integrations of the ester peak of –C$\boldsymbol{H}_2$–O–CO– at 4.31 ppm and the peak from DTPA at 3.74 ppm in the ^{1}H-NMR spectra.

2.14 Relaxivity measurement

The relaxivities were measured with a 0.52-T MicroMR Imaging & Analyzing System (Shanghai Niumag Corporation, China) at 32 °C. The solutions of CAs with Gd^{3+} concentrations between 0.5 and 2 mM in water were prepared and their longitudinal relaxation times (T_1) were determined *in vitro* using the inversion recovery method. The longitudinal relaxivities (R_1s) of the CAs were defined by the formula (1).

$$(1/T_1)_{obs} = (1/T_1)_d + R_1[Gd] \quad (1)$$

where [Gd] is the concentration of Gd, $(1/T_1)_{obs}$ is the observed relaxation rate, and $(1/T_1)_d$ is the relaxation rate of water protons. The plot of $(1/T_1)_{obs}$ *vs.* [Gd] gives the R_1 as its slope.

2.15 *In vitro* MRI

In vitro MRI was also performed on the 0.52-T MicroMR Imaging & Analyzing System. The solutions of DCAs at different generations and Magnevist for comparison were prepared at a Gd concentration of 0.1 mM in 4 mm diameter test tubes. The samples were measured using a multi-slice spin echo (MSE) sequence. The parameters were as fellows: TR = 300 ms, effective TE = 1.5 ms and the matrix dimensions = 512 × 512. Signals were recorded by a resonant coil with an inner diameter of 2 cm.

2.16 *In vivo* MR imaging

In vivo contrast-enhanced MR imaging using the dendritic agents was performed using female ICR mice (4 weeks old). The animals were purchased from the Animal Center of Zhejiang University and used in strict accordance with the guidelines established by the Institute for Experimental Animals of Zhejiang University. The mice were anesthetized by pelltobarbitalum natricum at a dose of 80 mg kg^{-1}. Contrast-enhanced images of mice were obtained on a GE signa HDxt 3-T MRI system equipped with a mice coil using a 3D Fast SPGR sequence. The imaging parameters were as follows: TE = 2.3 ms, TR = 11 ms, flip angle = 12°, bandwidth = 31.25 kHz, FOV = 8 cm and slice thickness = 1.4 mm. The CAs were injected *via* the tail vein into the anesthetized mice and the images were acquired before injection and at 5, 15, 30 and 60 min post injection. Contrast to noise ratio (CNR) was calculated using the following equation: $CNR = (S - S_0)/\sigma_n$, where S (post-injection) and S_0 (pre-injection) are the signals within the regions of interest (about 1.92 mm^2) and σ_n is the standard deviation of noise estimated from the background air. The pharmacokinetics were evaluated semiquantitatively by plotting the CNR within the blood *versus* time.

2.17 Tissue retention of Gd in mice

A group of 3 mice were used to study the long-term Gd retention of G2/MOP–DTPA–Gd in the major organs and tissues. The mice were injected with the CA at a dose of 0.1 mmol Gd kg^{-1} *via* the tail vein, and then terminated by cervical dislocation at 10 days post injection. The organ and tissue samples, including femur, heart, lungs, liver, muscles, spleen and kidneys, were collected and weighed. The tissue samples were mixed with 1.0 mL ultra-pure nitric acid and liquefied at 100 °C. The solution was fixed to a volume of 5 mL by adding water, and then transferred to a centrifuge tube and centrifuged at 13 000 rpm for 10 min. The Gd concentration in the supernatant was measured by ICP-MS. The average Gd content in each organ or tissue was calculated from the measured Gd concentration. The Gd content in muscle was calculated with an estimate that the muscle was 40% of body weight.[27]

2.18 Equipment and measurements

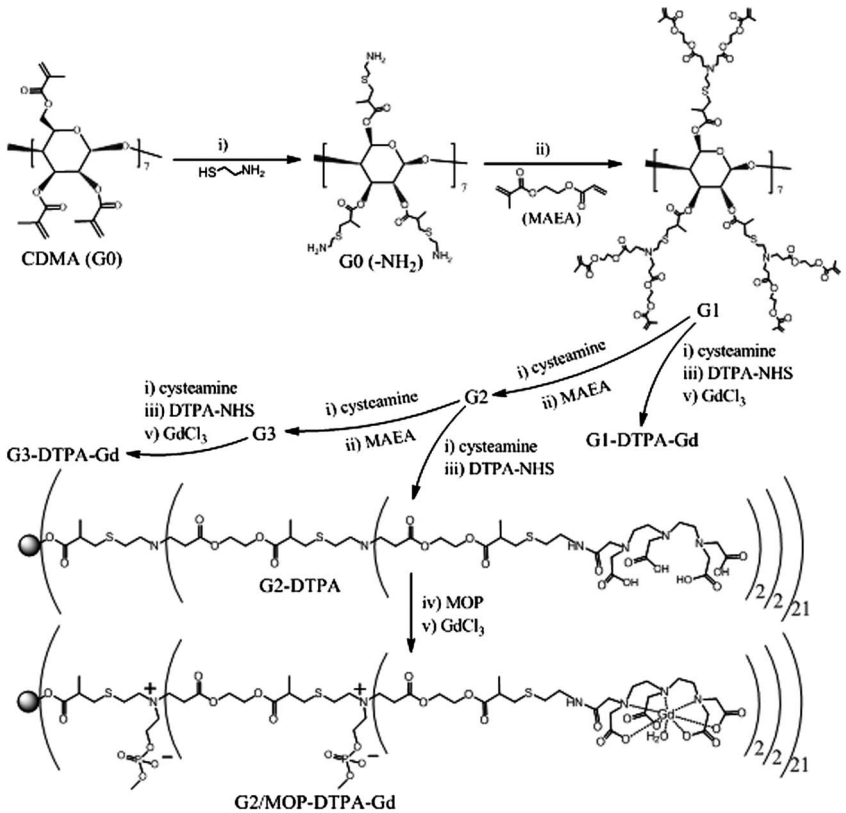
^{1}H-NMR spectra were recorded on a Bruker Advance DRX-400 NMR spectrometer using D_2O or $CDCl_3$ as solvents at room temperature. Molecular weights and their distribution (polydispersity index) of dendrimers were measured on a gel permeation chromatography (GPC) system equipped with Schambeck SFD GmbH RI2000 detector and a pair of Shodex KF-402.5 HQ and KF-404 HQ columns. THF was used as the eluent at a flow rate of 0.3 mL min^{-1} at 40 °C. A series of polystyrene standard samples were used to generate calibration curves. The sizes (hydrodynamic diameters) of the dendrimers were measured using a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, UK). FT-IR spectra were recorded on a Bruker vector 22 FT-IR spectrometer (Germany).

3. Results and discussion

3.1 Synthesis and characterization of the dendrimers

The synthesis of polyester dendrimers with a one-pot per generation method using β-CD as the core-forming compound is shown in Scheme 1. β-CD first reacted with methacrylic anhydride or methacryloyl chloride to prepare methacrylated CD (β-CDMA, G0). Methacryloyl chloride was more active but the obtained product had poor purity due to the inevitable side reactions.[28] Methacrylation using methacrylic anhydride was found to be more effective and produced pure β-CDMA. β-CDMA was characterized by ^{1}H-NMR spectroscopy (ESI, Fig. S1†). The number of methacrylate groups in β-CDMA calculated from the integration ratio of the C(7)H peaks at 5.62–6.17 to the C(1)H–C(6)H peaks at 3.58–5.18 was 20.3 per β-CD on average. The GPC profile shown in Fig. 1 indicates that β-CDMA had a M_n of 1800 with a polydispersity index (PDI) of 1.01.

The one-pot per generation method was based on the sequential click coupling of cysteamine and MAEA. The acrylate in MAEA reacted with the $–NH_2$ group and the resulting secondary amine group in cysteamine. In contrast, the methacrylate could not react with $–NH_2$ but reacted with the thiol group in cysteamine.[29,30] β-CDMA first reacted with cysteamine. A slight excess of cysteamine (SH/methacrylate ratio of 1.2) was used to ensure a complete reaction of the methacrylate groups. The reaction was monitored using ^{1}H-NMR spectrum by observing the disappearance of the methacrylate signals. Without any purification to remove the redundant cysteamine,



Scheme 1 Synthesis of the dendritic contrast agents. (i) Cysteamine, 6-di-*tert*-butyl-4-methylphenol, DMSO, room temperature, 1 h; (ii) MAEA, DMSO, at room temperature for 24 h and 50 °C for additional 72 h; (iii) DTPA *N*-hydroxysuccinimide ester (DTPA–NHS) in acetonitrile, DMSO, room temperature, 12 h; (iv) 2-methoxy-1,3,2-dioxaphospholane-2-oxide (MOP), DMSO, 70 °C, N_2 protection, 24 h; (v) $GdCl_3$, sodium acetate buffer (pH = 7.0), 2 h.

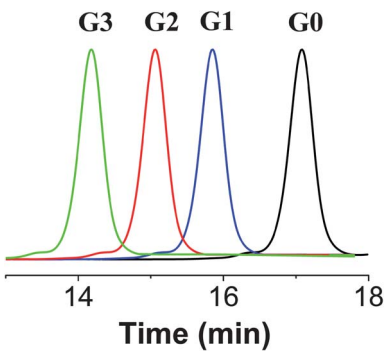


Fig. 1 Molecular weight progress of the dendrimers measured by GPC.

an excess of MAEA in terms of the primary amine and the unreacted cysteamine was then added to the solution. The terminal amino groups on the core reacted with MAEA and produced the first generation dendrimer (G1) with methacrylate terminal groups, while the slight excess of cysteamine reacted with MAEA and produced small molecule impurities, which could be easily removed by a simple precipitation. Thus, the sequential addition of cysteamine and MAEA followed by precipitation produced pure G1 dendrimer with a yield of 91%. Repeating these steps produced the dendrimers of higher generations. For instance, a G3 dendrimer with 160 peripheral methacrylates and 64.9 kDa molecular weight was synthesized in 3 steps with a yield of higher than 88%. We found that the dendrimers with terminal methacrylate groups were more stable than those with primary amine terminal groups, and hence the dendrimers should be stored in methacrylated form.

The dendrimers were characterized by ^{1}H-NMR spectra (Fig. S2†). The integral ratio of the peaks from the terminal methacrylate groups (5.49 ppm and 6.02 ppm), the esters (4.25 ppm) and methyl groups (1.12 ppm) of the reacted methacrylate were very consistent with the calculated values, which indicates the excellent definition of the dendrimer structure. For example, the G2 dendrimer should have 240 ester bonds and 80 terminal methacrylate groups. The theoretical integral ratio of the ester bonds to methacrylate groups in the ^{1}H-NMR spectra was 3.0. The calculated ratio from the ^{1}H-NMR spectrum in Fig. S2† was exactly 3.0. The dendrimers of G1, G2, and G3 had theoretical molecular weights (M_n) of 11.5 kDa, 29.3 kDa, 64.9 kDa with 40, 80, 160 terminal functional groups, respectively. Their molecular weights were also characterized by GPC (Fig. 1). The molecular weights increased with increasing generations and their distributions were very narrow with a polydispersity index (M_w/M_n) of less than 1.02. The compact and branched dendrimers had smaller hydrodynamic volumes than their linear counterparts and thus showed smaller molecular weights when measured by GPC using linear PS as standards (Table S1†).

3.2. Synthesis of the DCAs (G*x*–DTPA–Gd)

The DCAs were prepared from the dendrimers as shown in Scheme 1. The terminal methacrylate groups of the dendrimers were easily converted into amino groups by reacting with a slight excess of cysteamine. The amino groups were then reacted with DTPA–NHS to form dendritic ligand (G*x*–DTPA). It was important to use a large excess of DTPA–NHS to ensure the complete reaction of the terminal amino groups and prevent cross-linking. The excess DTPA can be easily removed by precipitating the dendrimer in methanol. The dendrimer–DTPA was characterized by ^{1}H-NMR, shown in Fig. 2. The appearance of DTPA signals at 3.84, 3.44 and 3.18 ppm and the signal of –CO–NH–C$\boldsymbol{H}_2$– from the formed amide at 3.54 ppm indicated that DTPA was successfully conjugated onto the dendrimers. The number of conjugated DTPA per dendrimer molecule was calculated from the integral ratio of a DTPA peak at 3.84 ppm to the ester peak at 4.38 ppm or the methyl peak at 1.17 ppm in the dendrimers. The conjugation degrees of G0–DTPA, G1–DTPA, G2–DTPA and G3–DTPA were 95%, 88%, 93% and 94%, respectively.

G*x*–DTPA chelated with excess gadolinium and formed the DCAs G*x*–DTPA–Gd. The resultant DCAs were soluble and had nano-sized structures in water. The sizes of G*x*–DTPA–Gd measured by a dynamic light scattering instrument (DLS) (Fig. S3†) increased with increasing generations. The volume-averaged hydrodynamic diameters of G0–DTPA–Gd, G1–DTPA–Gd, G2–DTPA–Gd and G3–DTPA–Gd were 2.8, 3.9, 5.8, and 8.6 nm, respectively. However, the solubility of G*x*–DTPA–Gd in water became poor at pH 7.4 or higher because of the hydrophobicity of the core. This low solubility may cause rapid clearance of the DCAs from the circulatory system by the liver, and cause *in vivo* toxicity. Thus, zwitterions were further introduced into the dendrimers to increase their solubility. 2-Methoxy-1,3,2-dioxaphospholane-2-oxide (MOP) is known to readily react with tertiary amines *via* the ring-opening reaction to produce nontoxic zwitterions.[31] Excess MOP was thus used to react with the tertiary amines in the G*x*–DTPA and the reaction was confirmed by FT-IR spectroscopy (Fig. S4†). The strong absorptions of P═O at 1220 cm^{-1} and P–O at 1050 cm^{-1} appeared after reacting with MOP, indicating that the zwitterions had formed.[32] The zwitterionized DCAs became highly soluble in water at pH 7.4.

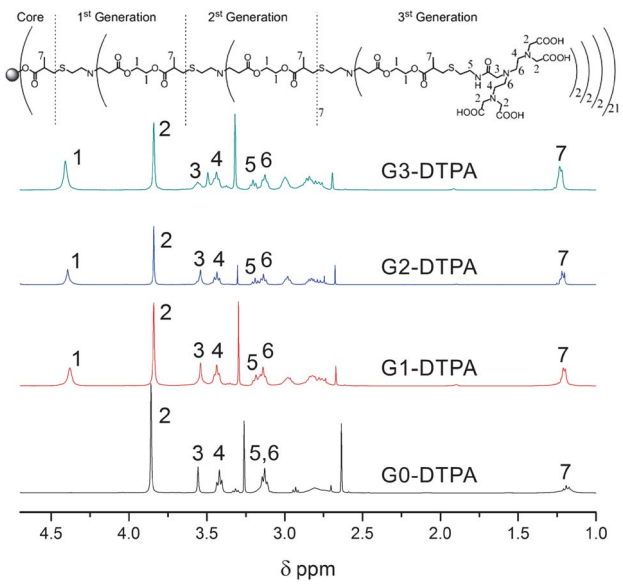


Fig. 2 ^{1}H-NMR spectra of the DCAs.

3.3 *In vitro* degradation of the DCAs

The degradability of the DCAs was evaluated by monitoring the hydrolysis of their ester bonds. As shown in Fig. 3, the hydrolysis kinetics of the dendritic ligand (G3) was pH-dependant. The dendrimer was stable under acidic conditions, but hydrolyzed quickly as the pH increased. In pH 7.4 PBS for 20 h, only 70% of the ester bonds remained. The hydrolysis was further accelerated in the presence of an esterase, which is abundant in cytoplasm. Under the same conditions, 50% of the ester bonds were hydrolyzed within 6 h with the catalysis of 15 U mL^{-1} esterase. These results suggest that the DCAs can be degraded into small pieces in the blood and cells, and easily excreted from the body, thereby avoiding the systemic toxicity that results from the long-term retention of Gd^{3+}.

3.4 *In vitro* relaxivities and MRI

The longitudinal relaxation rates $(1/T_1)_{obs}$ of the DCAs, and a commercial CA (DTPA–Gd, Magnevist) for comparison were measured at 0.52 T and 32 °C. As shown in Fig. 4a, the $(1/T_1)_{obs}$ linearly increased with the concentration of Gd^{3+}. The R_1 of DTPA–Gd calculated from the slope was 4.3 mM^{-1} s^{-1}, consistent to that reported in the literature.[33] Using the same method, G0–DTPA–Gd was found to have an R_1 of 10.6 mM^{-1} s^{-1}, 2.5 times higher than that of DTPA–Gd. G1–DTPA–Gd, G2–DTPA–Gd, G3–DTPA–Gd and G2/MOP–DTPA–Gd had similar R_1 values of 11.7 mM^{-1} s^{-1}, about 2.7 times higher than that of DTPA–Gd. The high R_1s of the DCAs were mainly due to the increased rotational correlation time (τ_R) after conjugation of DTPA–Gd onto the rigid dendrimers.[34,35] The R_1s of the DCAs initially increased with increasing dendrimer generation, but the R_1s leveled off at higher generations of the DCAs. The internal flexibility of the DCAs was responsible for the phenomenon.[36] The flexiblity of linker groups between the Gd chelate and dendrimer scaffold prevented the continuous growth of τ_R and

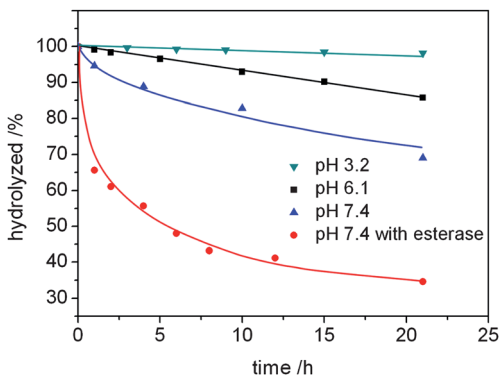


Fig. 3 Hydrolysis kinetics of G3–DTPA at different pH values with and without esterase (esterase concentration, 15 U mL^{-1}).

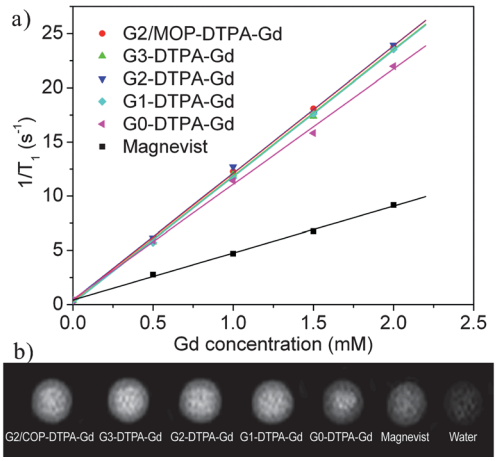


Fig. 4 *In vitro* relaxivities (a) and T_1-weighted MRI (b) of the DCAs in water at 0.52 T and 32 °C.

then hindered the rise of the relaxivity. Fig. 4b shows the T_1-weighted MR images of the DCA solutions and Magnevist solution for comparison at the same Gd concentration (0.1 mM). All three DCAs produced much brighter images than Magnevist, indicating that the DCAs had enhanced contrast efficiency over Magnevist.

3.5 Contrast-enhanced MRI in mice

Fig. 5a and b show the MR images of the mice injected with a zwitterionized DCA (G2/MOP–DTPA–Gd) or Magnevist at a dose of 0.1 mmol Gd kg^{-1} at a level which includes the heart. The mouse with G2/MOP–DTPA–Gd had a much brighter image of the heart than that with Magnevist due to its higher R_1 value and longer blood retention of the DCA. The signal intensity decreased gradually as time increased. After 60 min, the mouse injected with G2/MOP–DTPA–Gd still had a significant image enhancement of the heart, while that injected with Magnevist had negligible enhancement, indicating G2/MOP–DTPA–Gd had longer retention in the blood. Fig. 5c and d show the MR images of the mice at a level which includes the kidneys. Clearly, G2/MOP–DTPA–Gd had a stronger enhancement in kidney imaging than Magnevist at 15 min post injection. The signal intensity in the kidneys decreased considerably after 30 min. This result was consistent with the observations in Fig. 5a and b that the signal intensity in the heart decreased gradually over time. Very significantly, the mouse injected with G2/MOP–DTPA–Gd displayed a very low signal enhancement in the liver at all time points, indicating that it would not accumulate in the liver and thus would not cause liver toxicity from the retention of Gd^{3+}.

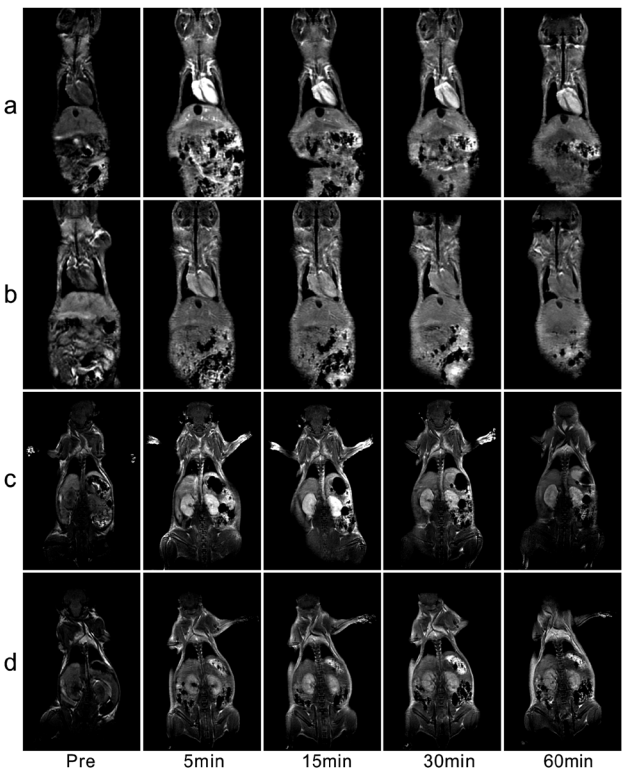


Fig. 5 T_1 weighted images of the mice injected with G2/MOP–DTPA–Gd (a and c) or Magnevist (b and d) at a dose of 0.1 mmol Gd kg^{-1} at the layers with heart (a and b) and kidneys (c and d).

The contrast-to-noise ratios (CNRs) in the heart, inferior vena cava, kidneys and liver of the mice injected with G2/MOP–DTPA–Gd or Magnevist at various time points are shown in Fig. 6. There was a significant increase in the CNR within the heart and inferior vena cava soon after the injection of G2/MOP–DTPA–Gd, and the CNR of G2/MOP–DTPA–Gd was much higher than that of Magnevist. The CNR reached about 16 at 5 min post injection and then gradually decayed over time. G2/MOP–DTPA–Gd also provided higher renal enhancement in the 15 min post injection. The signal in the kidney gradually faded away while Gd was excreted to the bladder. The signal intensity of G2/MOP–DTPA–Gd in the liver was very low at all time points. The low liver enhancement indicated that the DCA was hardly absorbed by hepatocytes, likely because the zwitterionization conferred low fouling properties to the DCA.[37]

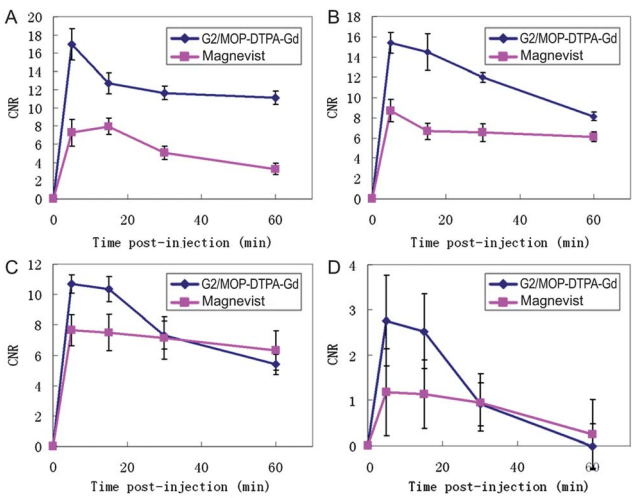


Fig. 6 Contrast-to-noiseratio (CNR) from the heart (A), inferior vena cava (B), kidneys (C) and liver (D) of the mice before and at various time points after injection of G2/MOP–DTPA–Gd (◆) or Magnevist (■) at a dose of 0.1 mmol Gd kg^{-1}.

3.6 Tissue retention of Gd

Fig. 7 shows the tissue retention of Gd in the major organs and tissues, including the femur, heart, kidneys, liver, lungs, muscles, and spleen of mice 10 days after a single injection of G2/MOP–DTPA–Gd at a dose of 0.1 mmol Gd kg^{-1}. One can see that the G2/MOP–DTPA–Gd had much lower long-term Gd retentions in all the organs or tissues than the non-degradable dendritic MRI agent PAMAM-G6-(Gd-DO3A).[20] The long-term Gd retentions of G2/MOP–DTPA–Gd were at the same minimal level as the clinically used small molecule CA Gd–(DTPA-BMA) and the polydisulfide-based biodegradable macromolecular CAs, except that the DCA had a slightly higher Gd accumulation in the liver.[18,38] Such low Gd retentions confirmed that G2/MOP–DTPA–Gd could be degraded into small pieces *in vivo* and excreted through renal glomerular filtration. In addition, the remaining Gd was mainly distributed in the liver and muscle, with the least accumulation in the heart and lungs.

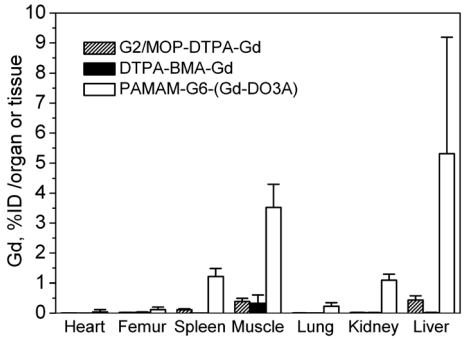


Fig. 7 Retention of Gd in the mice 10 days after intravenous injection of G2/MOP–DTPA–Gd, Gd–(DTPA-BMA)* and PAMAM-G6-(Gd-DO3A)* at a dose of 0.1 mmol Gd kg^{-1}. * The data of Gd–(DTPA-BMA) and PAMAM-G6-(Gd-DO3A) are from the literature.[20,38]

4. Conclusions

Polyester dendrimers with β-cyclodextrin core were synthesized by the sequential clicking reaction of cysteamine and 2-[(meth-acryloyl)oxy]ethyl acrylate and used to prepare biodegradable DCAs. The GPC and ^{1}H-NMR characterization indicated that the dendrimers had narrow molecular weight distributions and well-defined structures. The corresponding DCAs were synthesized by conjugation with DTPA–Gd. The volume-averaged hydrodynamic diameters of the DCAs were tunable from 2.8 to 8.6 nm by changing the generation of the dendrimers. While the DCAs showed good stability in acidic solution, they could be hydrolyzed in pH 7.4 PBS buffer, and the hydrolysis rate could be greatly accelerated by esterase. The relaxivities of the DCAs were up to 11.7 mM^{-1} s^{-1}, approximately 2.7 times that of the small molecule CA DTPA–Gd (Magnevist). The *in vivo* evaluation indicated that the zwitterionized DCA had significant MRI contrast enhancement in blood pools and the kidneys. Very importantly, the biodegradable DCAs (G2/MOP–DTPA–Gd) showed minimal tissue retentions, which were much lower than those of non-degradable DCAs. These properties made the DCAs very promising as CAs for blood pool and kidney imaging.

Acknowledgements

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