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Gadolinium-labeled peptide dendrimers with controlled structures as potential magnetic resonance imaging contrast agents

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ABSTRACT

Gadolinium (Gd^{3+}) based dendrimers with precise and tunable nanoscopic sizes are excellent candidates as magnetic resonance imaging (MRI) contrast agents. Control of agents' sensitivity, biosafety and functionality is key to the successful applications. We report the synthesis of Gd(III)-based peptide dendrimers possessing highly controlled and precise structures, and their potential applications as MRI contrast agents. These agents have no obvious cytotoxicity as verified by *in vitro* studies. One of the dendrimer formulations with mPEG modification showed a 9-fold increase in T_1 relaxivity to 39.2 Gd(III) mM^{-1} s^{-1} comparing to Gd-DTPA. *In vivo* studies have shown that the mPEGylated Gd(III)-based dendrimer provided much higher signal intensity enhancement (SI) in mouse kidney, especially at 60 min post-injection, with 54.8% relatively enhanced SI. The accumulations of mPEGylated dendrimer in mouse liver and kidney were confirmed through measurement of gadolinium by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Meanwhile, mPEGylated dendrimer showed much higher Gd(III) concentration in blood with 38 μg Gd(III)/g blood at 1 h post-injection comparing to other dendrimer formulations. These findings provide an attractive alternative strategy to the design of multifunctional gadolinium-based dendrimers with controlled structures, and open up possibilities of using the Gd(III)-based peptide dendrimers as MRI probes.

1. Introduction

The quest for highly efficient and safe contrast agents has never stopped since the clinical introduction of gadolinium (Gd^{3+}) based MRI contrast agents [1–5]. Paramagnetic Gd(III)-based complexes have been widely used as contrast agents for their predominant positive signal enhancement [6]. The limitations in the clinical use of Gd(III)-chelates, such as Gd(III)-diethylenetriaminepentaacetic acid (Gd-DTPA) and Gd(III)-N,N',N'',N'''-tetracarboxymethyl-1,4,7,10-tetraazacyclododecane (Gd-DOTA), include non-specificity, low relatively signal enhancing effect (or longitudinal relaxivity r_1, mM^{-1} s^{-1}), and fast elimination from the circulatory system. The fast clearance is largely due to the small molecular sizes of Gd(III)-chelates because of their low molecular weights [7]. Currently, nanoscale particles [8–13], such as magnetic nanoparticles [14–18], have been designed as the alternatives for contrast enhancement, showing promising results in both diagnostic and therapeutic applications.

In addition to magnetic nanoparticles, Gd(III)-labeled dendrimer have also been investigated as MRI probes for soft tissue imaging [7]. Dendrimers are one unique category of macromolecules with well controlled sizes [19], nanoscopic dimensions and numerous peripheral chemical groups to which MRI probes can be coupled, and their Gd(III)-conjugates have shown superb image enhancement and relatively long blood circulation [20]. Gd(III)-functionalized poly(propyleneimine) (PPI) or poly(amidoamine) (PAMAM) dendrimers have been evaluated in animal models for high-resolution MRI [21]. The long blood circulation *in vivo* and the high longitudinal relaxivities are essential requirement for efficient MRI contrast agents, including the dendritic Gd(III)-chelates. PPI and PAMAM based Gd(III)-chelates with higher generations can enhance the half-life in the blood and the longitudinal relaxivities [22]. However, long-term *in vivo* circulation of the non-degradable contrast agents significantly increases the possibility of release of toxic Gd(III) ions from the macromolecular, which may prevent their clinical use [23]. In addition, *in vitro* and *in vivo* studies have shown that amine-

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terminated dendrimers, such as PPI and PAMAM, may be cytotoxic, due to their hemolytic effects [24]. It has been reported that lysine-based dendrimers showed no significant cytotoxicity [19]. Recently, Gd(III)-based peptide dendrimers such as lysine-based ones have been used as MRI contrast agents both *in vitro* and *in vivo* studies in experimental animals [25], showing good biocompatibility due to biodegradable property of the peptide branching units [26]. These dendritic complexes are considered as good candidates for blood pool MRI agents. However, for most reported peptide dendritic agents, the blood circulation and longitudinal relaxivities are still far from satisfaction, which limit their *in vivo* applications.

The potential use of Gd(III)-based peptide dendrimers is coupled with synthetic challenges [7]. An obstacle in designing and synthesis of effective dendritic MRI contrast agents is to tune and control exactly the ratio between MRI labels and other functional groups on a single dendritic scaffold [7,27–29]. In addition, the challenge of peptide dendritic MRI probes is the characterization of these complicated architectures. The longitudinal relaxivities and blood circulation *in vivo* of dendrimer-based Gd(III)-chelates are molecular weight dependent: the higher molecular weight, the longer circulation and higher longitudinal relaxivities [30]. The molecular weights and sizes of the dendrimers can be increased along with the higher number of generations. However, it's not easy to prepare higher generation dendrimers due to the steric hindrance to chemical reactions, especially for generations beyond five. In order to enhance the blood circulation of dendrimers, polyethylene glycol (PEG), which can extend retention time in blood by decreasing non-specific interactions with endogenous components and macrophages, has been conjugated to dendrimers to form a new kind of dendritic structures [31,32]. The PEGylated dendrimers have demonstrated better retention in blood, low accumulation in organs and high accumulation in tumor tissue via the enhanced permeability and retention (EPR) effect [32]. Based on above observations, an alternative strategy to the development of safe and efficient MRI contrast agents is the combination of biodegradable lysine-based dendritic Gd(III)-chelates and stealthy polymer PEG into one system.

In this study, we described the controllable synthesis and characterization of a new series of L-lysine-based dendrimer with Gd(III)-labeling. Their biosafety and MRI contrast enhancement capability were evaluated. The third generation (**G3**) peptide dendrimers with highly controlled structures and single molecular weights were synthesized (Fig. 1), as shown in Scheme 1–3. The number of Gd(III)-chelates at the surface of dendrimer was controlled exactly via appropriate molecular design, resulting in different functional Gd(III)-labeled peptide dendrimers: G3-24Gd-DTPA, G3-12Gd-DTPA, G3-6Gd-DTPA and G3-12Gd-DTPA-10mPEG. *In vitro* cytotoxicity of the dendrimers was evaluated via MTT cell proliferation assay. The T_1 relaxivities of the peptide dendrimers were measured under clinical MRI scanners, and the imaging contrast effect was compared with the small molecule agent Gd-DTPA. All agents were evaluated and compared for *in vivo* imaging performance. The accumulation of Gd(III)-chelates in liver, kidney and blood at different time points were measured by ICP-AES.

2. Materials and methods

2.1. Materials and measurements

N,*N*-Diisopropylethylamine (DIPEA), 1-hydroxybenzotriazole (HOBt), *N*,*N*,*N*′,*N*′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), 1-hydroxy-7-azabenzotriazole (HOAt), O-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), trifluoroacetic acid (TFA) and gadolinium chloride hexahydrate ($GdCl_3 \cdot 6H_2O$) were purchased from Sigma Aldrich Chemical Co. and used without further purification. Boc-L-Lys(Cbz)–OH was purchased from GL Biochem (Shanghai) Ltd. *N*,*N*-Bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl]-amino]ethyl]-L-glutamic acid 1-(1,1-dimethylethyl) ester (*t*-Bu esters protected DTPA) [33], activated mPEG (2 kDa) [34], dendritic compounds **1** (G1-6NHBoc), dendrimer **7** (G3-12NHBoc-12Cbz) and **11** (G3-24NHBoc) were synthesized according to the literatures [35].

The structures of dendritic intermediates and products were confirmed by ^{1}H NMR (600 MHz Bruker Advanced 600 Spectrometer), electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS, Waters Q-TOF Premier) and matrix assisted laser desorption ionization time-of-flight (MALDI-TOF, Autoflex MALDI-TOF/TOF) mass spectrometry. ICP-AES (IRIS Advantage, Thermo Electron, USA) method was used for determining the content of Gd(III) on dendritic MRI agents and the quantitative assessment of Gd(III) uptaken by different tissues *in vivo*.

2.2. Synthesis of dendritic compound **2** (G2-6NHBoc-6Cbz)

Compound **1** (566 mg, 0.5 mmol) was dissolved in 10 mL anhydrous solvents of dichloromethane (CH_2Cl_2) and TFA (1: 1). The solution was stirred at room temperature for 2 h. After the solvent was removed by rotary evaporation, anhydrous diethyl ether was added and precipitate appeared. The precipitate was collected by centrifugation and washed 3 times with anhydrous diethyl ether. The deprotected product was treated with DIPEA (969 mg, 7.5 mmol) in anhydrous *N*,*N*-dimethylformamide (DMF, 10 mL) under nitrogen. Boc-L-Lys(Cbz)–OH (1.52 g, 4 mmol), HBTU (1.52 g, 4 mmol) and HOBt (540 mg, 4 mmol) were added to the solution. The solution was stirred under nitrogen in ice bath for 30 min and at room temperature for another 12 h. After work-up, the solvent was removed. The residue was dissolved in ethyl acetate (EtOAc, 150 mL) and washed with $NaHCO_3$ aq. (satd.), $NaHSO_4$ aq. (0.1 M), $NaHCO_3$ aq. (satd.) and brine. The solution was dried (Na_2SO_4) and the solvent was removed by rotary evaporation to produce a white solid. This crude product was purified by column chromatography (silica gel, $CHCl_3$/EtOAc/EtOH = 6: 2: 1), giving white solid in 84.2% yield. ^{1}H NMR (600 MHz, DMSO-d_6): δ = 1.15–1.78 (m, CH_2-Lys and CH_3-Boc), 2.38 (m, NCH_2CH$_2$NHCO), 2.79 (m, CH_2NH-Lys), 3.13 (m, NCH$_2$CH_2NHCO), 3.84 (m, COC*H*(R)NH), 4.29 (m, COC*H*(R)NH), 5.10 (s, Ph-CH_2), 6.86 (s, N*H*CO), 7.19 (m, Ph-*H*), 7.63 (s, N*H*CO); ESI-TOF MS: *m*/*z* 2706.41 [$(M + H)^+$, $C_{138}H_{211}N_{22}O_{33}$]; 2728.34 [$(M + Na)^+$, $C_{138}H_{210}N_{22}O_{33}Na$].

2.3. Synthesis of dendritic compound **3** (G2.5-6NHBoc-12Cbz)

Compound **2** (190 mg, 0.1 mmol) was dissolved in CH_3OH (15 mL), 400 mg Pd/C (10%) as a suspension was added to the solution. Hydrogenation was conducted in a Parr apparatus at 50 psi H_2 for 48 h. The catalyst was removed by filtration through Celite, and the solvent was evaporated to afford colorless solid in 99% yield. The colorless solid was dissolved in anhydrous DMF (15 mL). DIPEA (155 mg, 1.2 mmol),

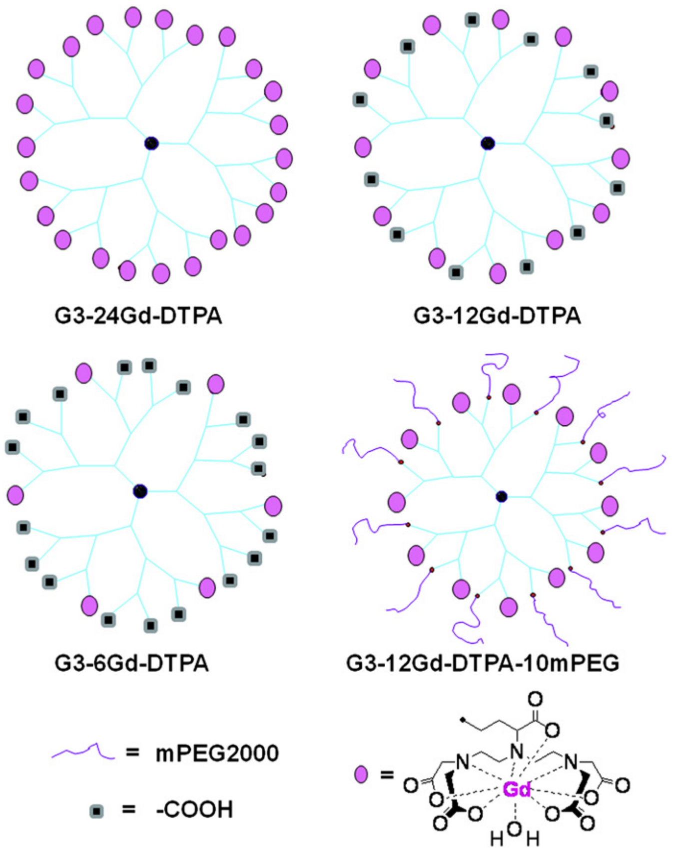

Fig. 1. The structures for gadolinium-based peptide dendrimers.