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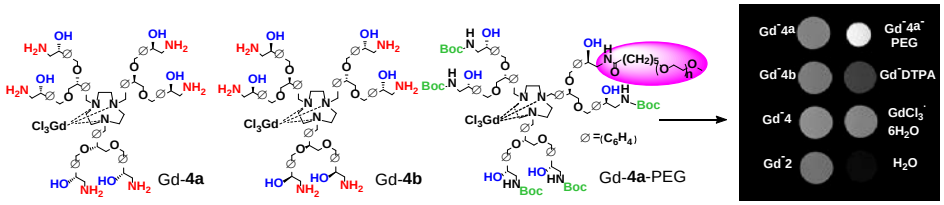

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Synthesis and functional evaluation of chiral dendrimer-triamine-coordinated Gd complexes with polyaminoalcohol end groups as highly sensitive MRI contrast agents

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ABSTRACT

Novel chiral dendrimer-triamine-coordinated Gd complexes with polyaminoalcohol end groups were synthesized and shown to have longitudinal relaxivity (r_1) values 5 times higher than that of clinically used Gd-DTPA. The affinities of Gd-**4a** and Gd-**4b** for bovine serum albumin (BSA), respectively, were estimated by a quartz crystal microbalance (QCM) measurement. The amino groups of the dendrimer were then conjugated with PEG. This conjugation with PEG strongly affected its ability to attenuate signals in T1-weighted MRI.

1. Introduction

Magnetic resonance imaging (MRI) has become a prominent non- or low-invasive imaging technique for disease diagnosis.[1,2] Low-molecular-weight contrast agents (CAs), such as Gd-DTPA (DTPA = diethylenetriaminepentaacetic acid) and Gd-DOTA (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), are widely used in the clinical diagnosis of tumors (Figure 1).[3-8]

However, the low contrast efficiency, non-specific retention and rapid clearance of these low-molecular-weight CAs in the body necessitate a high dosage (*ca*. 0.5 M), which imposes a great physical strain and can lead to side effects in the patient, such as osmotic shock.[9,10] The main reason for their low contrast efficiency is that up to eight of the nine coordination sites of Gd are firmly occupied with ionic chelating ligands, and only one site remains available for coordination with free water molecules, which can be observed by MRI. In addition, Gd metal could rotate quite rapidly at the center of small ligands, and enhancement of the image contrast would be reduced.

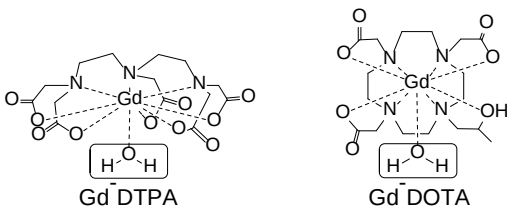

Figure 1. Structures of Gd-DTPA and Gd-DOTA.

Therefore, there is a strong need for the development of highly sensitive Gd-MRI CAs, and recently there has been growing worldwide interest in the development of Gd-MRI CAs that consist of Gd-functionalized dendrimers[11-15] and other macromolecules.[16-19] Dendrimers[20-23] are a unique category of macromolecules with well-controlled sizes, nanoscopic dimensions, and numerous peripheral chemical groups to which Gd chelates can be coupled. Gd-functionalized poly(amido-amine) (PAMAM)[24-27] and poly(propyleneimine) (PPI)[28-30] dendrimers have been reported and evaluated in animal models for high-resolution MRI, in which dendrimers were used as a core and Gd chelates were positioned in the periphery.[31-33]

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Unfortunately, among the Gd-functionalized dendrimers that have been reported for use as contrast agents, dendrimers were only used to slow the molecular tumbling and rotation of Gd. The intravascular retention time is prolonged, but the principle of reducing the ^{1}H relaxation time of a water molecule is the same as that with low-molecular-weight contrast agents.

We previously reported the synthesis and functional evaluation of a chiral 2nd-generation dendrimer-triamine-coordinated Gd complex with polyol end groups as a highly sensitive CA for MRI (Figure 2).[34, 35]

In that report, we synthesized chiral dendrimer-triamine-coordinated Gd-MRI CAs and clarified the pharmacokinetic differences between the optical isomers, Gd-**2a** and Gd-**2b**. The synthesized Gd-MRI CAs were shown to have longitudinal relaxivity (r_1) values 3 times higher than that of clinically used Gd-DTPA. The pharmacokinetic differences between optical isomers were estimated based on T1-weighted MR images of mice before and after the intravenous injection of Gd-**2a** and Gd-**2b**. As a result, Gd-**2a** is retained in the vasculature for a longer time after administration.

Although the conjugation of polyethylene glycol (PEG) and/or antibodies with CAs might be needed to achieve specific targetability *in vivo*, such as for cancers, polyol end groups in Gd-**2a** or Gd-**2b** did not seem to be suitable for conjugation under physiological conditions. In this paper, based on this preparation of 2nd-generation dendrimer-triamine-coordinated CAs, we tried to replace these hydroxyl end groups with amino end groups for the easy introduction of PEG and antibodies. We synthesized novel highly sensitive chiral and racemic dendrimer-triamine-coordinated Gd complexes with polyaminoalcohol end groups, Gd-**4a**, Gd-**4b** and Gd-**4** (racemic) (Figure 3), and evaluated their function as Gd-MRI CAs. The amino end groups of the dendrimer CAs were conjugated with a PEG chain, and the functions of the resulting complex were evaluated *in vitro*.

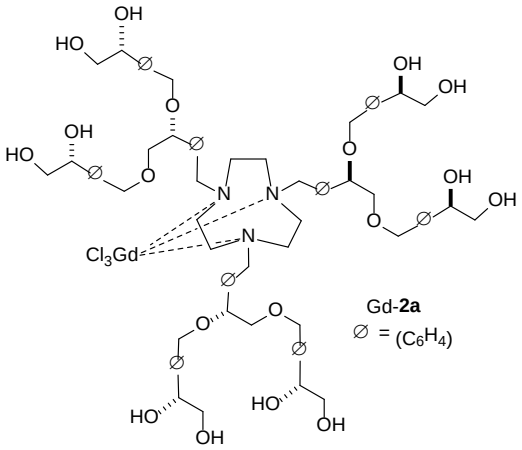


Figure 2. Structure of a chiral dendrimer-triamine-coordinated Gd-MRI CA with polyol end groups, Gd-**2a**.

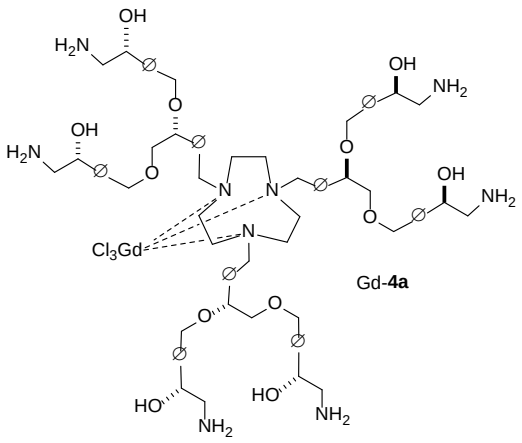


Figure 3. Structure of novel chiral dendrimer-triamine-coordinated Gd-MRI CA with polyaminoalcohol end groups, Gd-**4a**.

2. Results and discussions

*2.1. Synthesis of chiral dendrimer-triamine-coordinated Gd-MRI CAs with polyaminoalcohol end groups, Gd-**4a** and Gd-**4b***

(*R*)- and (*S*)-chiral dendrimer-triamine ligands with polyol end groups were synthesized by the previously reported method.[34, 35] According to Scheme 1, 2nd-generation dendrimer Gd-MRI CAs with polyaminoalcohol end groups, Gd-**4a** and Gd-**4b**, were synthesized. (*R*)- and (*S*)-chiral dendrimer-triamine ligands with polyol end groups, **2a** and **2b**, were epoxidated with trimethylsilyl chloride and trimethyl orthoacetate.[38] A ring-opening reaction of the epoxide groups with ammonium hydroxide was performed and novel chiral dendrimer-triamine-coordinated Gd complexes, Gd-**4a** and Gd-**4b**, were obtained after complexation with gadolinium trichloride ($GdCl_3 \cdot 6H_2O$).

*2.2. Synthesis of a racemic dendrimer-triamine-coordinated Gd-MRI CA with polyaminoalcohol end groups, Gd-**4** (racemic)*

According to Scheme 2, a racemic dendrimer Gd-MRI CA with polyaminoalcohol end groups Gd-**4** (racemic) was synthesized. The dendron with olefin ends **8** was synthesized and the olefin was epoxidated with *meta*-chloroperoxybenzoic acid (*m*CPBA) to give **9**. After the conjugation of dendron with epoxide end groups **9** was conjugated with 1,4,7-triazacyclononane (TACN) to give **10**, a ring-opening reaction of the epoxide groups with ammonium hydroxide was performed to give **4** (racemic), and novel chiral dendrimer-triamine-coordinated Gd complexes Gd-**4** (racemic) were obtained after complexation with $GdCl_3 \cdot 6H_2O$.

*2.3. Synthesis of Boc-protected dendrimer-triamine-coordinated Gd-MRI CA,Gd-**4**-Boc*

The six amino groups of dendrimer ligand **4** (racemic) were protected with *tert*-butoxycarbonyl (Boc) groups and subsequent complexation with $GdCl_3 \cdot 6H_2O$ gave Gd-**4**-Boc.

*2.4. Synthesis of PEG-dendrimer-triamine-coordinated Gd-MRI CA, Gd-**4a**-PEG*

The conjugation of **4a** with PEG bearing an activated ester group (average molecular weight: 5,000) was carried out, and the reactant was purified by GPC. Subsequent complexation with $GdCl_3 \cdot 6H_2O$ gave Gd-**4a**-PEG.

*2.5. Evaluation of synthesized Gd-MRI CAs, Gd-**4** (racemic), Gd-**4a**, Gd-**4b** and Gd-**4a**-PEG*

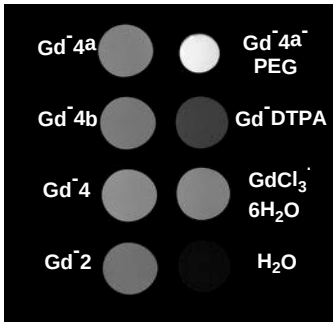


Figure 4. T1-weighted MR images of Gd-DTPA, $GdCl_3 \cdot 6H_2O$, Gd-**2a**, Gd-**4** (racemic), Gd-**4a**, Gd-**4b**, Gd-**4a**-PEG (0.25 mM) and water (Spin echo from TR/TE 200/6.2 ms, 7 T, 20 ºC).

The longitudinal relaxivity (r_1) values of dendrimer CAs, $GdCl_3{\cdot}6H_2O$, and Gd-DTPA were measured (Figure 4, Table 1).

Table 1. Longitudinal relaxivities (r_1) of Gd-DTPA, $GdCl_3{\cdot}6H_2O$, Gd-**4** (racemic), Gd-**4a**, Gd-**4b**, Gd-**2a** and Gd-**4a**-PEG.

Entry	r_1 / $mM^{-1}s^{-1}$
Gd-DTPA	3.9
$GdCl_3 \cdot 6H_2O$	15.3
Gd-**4** (racemic)	19.4
Gd-**4a**	21.5
Gd-**4b**	20.1
Gd-**2a**	11.4
Gd-**4a**-PEG	52.6

The r_1 values of 2nd-generation dendrimer CAs with polyaminoalcohol end groups Gd-**4** (racemic), Gd-**4a** and Gd-**4b** were 19.4 $mM^{-1}s^{-1}$, 21.5 $mM^{-1}s^{-1}$, and 20.1 $mM^{-1}s^{-1}$ respectively, which were approximately 5 times larger than that of Gd-DTPA (r_1 = 3.9 $mM^{-1}s^{-1}$). Moreover, the r_1 values of 2nd-generation dendrimer CAs with polyaminoalcohol end groups Gd-**4** (racemic), Gd-**4a** and Gd-**4b** were higher than that of 2nd-generation dendrimer CA with polyol end groups Gd-**2a** (r_1 = 11.4 $mM^{-1}s^{-1}$). As mentioned previously, established low-molecular-weight Gd contrast agents such as Gd-DTPA and Gd-DOTA have ionic chelating ligands that strongly suppress eight of the nine coordination sites of Gd. On the other hand, the novel Gd complexes have a triamine ligand and three chloride ligands, which stably occupy six coordination sites of Gd. Accordingly, three coordination sites remain for water molecules, and thus the present Gd complexes show longitudinal relaxivity values that are 5 times higher than that of Gd-DTPA.

Figure 5 shows the cytotoxicities of Gd-**4** (racemic), Gd-**2a**, and Gd-DTPA toward L929 cells.[39] The result indicated that dendrimer CAs with polyaminoalcohol end groups Gd-**4** (racemic) were cytotoxic at a concentration at which dendrimer CA with polyol end groups Gd-**2a** and Gd-DTPA were not cytotoxic (0.25 mM). This cytotoxicity would be due to the primary amino end groups of the dendrimer. Therefore, Boc-protected 2nd-generation dendrimer CA Gd-**4**-Boc was synthesized and its cytotoxicity was evaluated. As a result, Boc-protected dendrimer CA Gd-**4**-Boc was not cytotoxic, similar to Gd-**2a** and Gd-DTPA. These observations suggest that the CAs conjugated with PEG would have low toxicity and high longitudinal relaxivity.

To anticipate the pharmacokinetic differences between optical isomers, the affinities of Gd-**4a** and Gd-**4b** for bovine serum albumin (BSA), which is a model of plasma protein, were estimated by a quartz crystal microbalance (QCM) measurement. In sharp contrast to the results of Gd-**2a** and Gd-**2b**[35] the association constant K_a of Gd-**4a** (1.08×10^6 M^{-1}) was about 2 times higher than that of Gd-**4b** (5.21×10^5 M^{-1}), which means that Gd-**4a** is retained in the vasculature for a longer time after administration in a mouse body. This result was different than that with polyol CAs, probably due to the stronger interaction of amino groups in Gd-**4a** and Gd-**4b** with albumin, which also explain the cytotoxicity of Gd-**4** (racemic) (Figure 5, *vide infra*).

2.6. Evaluation of synthesized PEG-dendrimer Gd-MRI CA, Gd-4-PEG

The longitudinal relaxivity (r_1) value of PEG-dendrimer CA Gd-**4**-PEG was 52.6 $mM^{-1}s^{-1}$, which is approximately 13 times higher than that of Gd-DTPA and 2.5 times higher than that of Gd-**4a** (Table 1). In the case of the present PEG-dendrimer CAs, the slower molecular tumbling with an increase in molecular weight as a result of conjugation with PEG may be responsible for the stronger contrast enhancement.

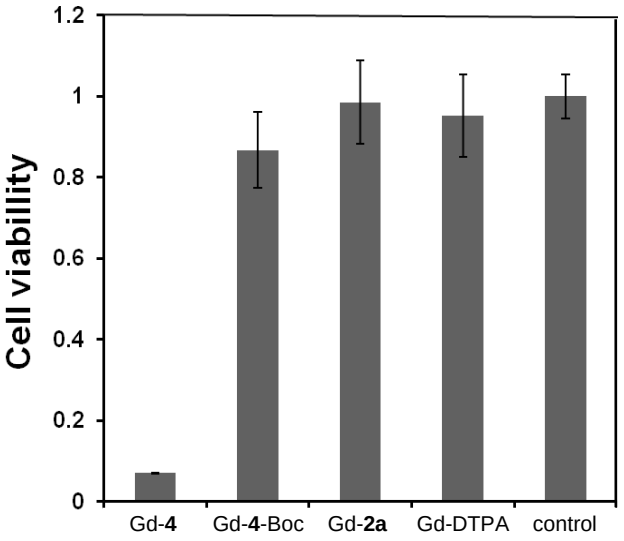
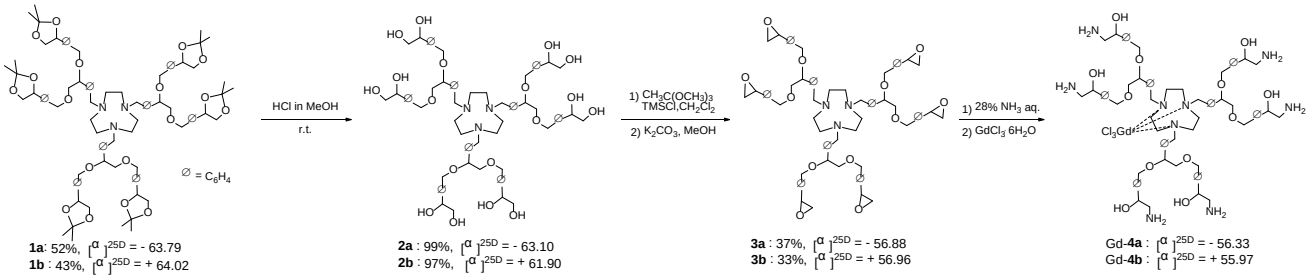


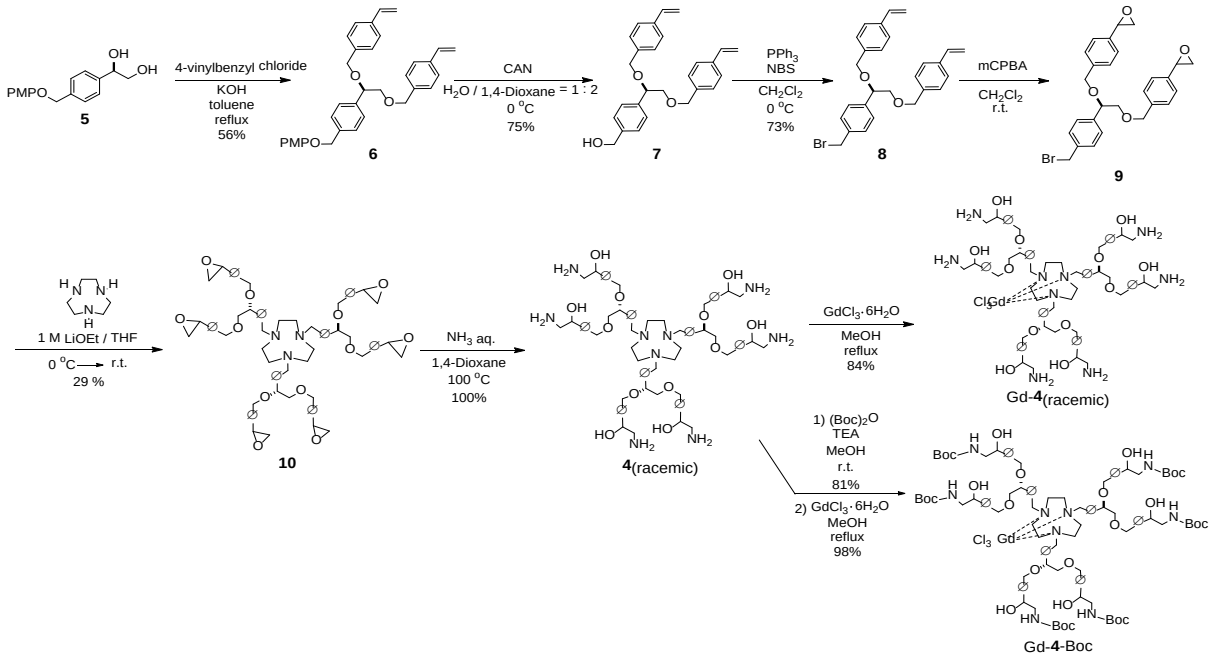
Figure 5. Viabilities of L929 cells exposed Gd-DTPA, Gd-**1a**, Gd-**4** (racemic) and Gd-**4**-Boc at 0.25 mM.

3. Conclusions

In conclusion, we have synthesized the first chiral dendrimer-triamine-coordinated Gd contrast agents with polyaminoalcohol end groups, and then conjugated these contrast agents with PEG. The effects of the molecular weight of PEG on contrast and tumor targeting ability in MRI through the EPR (Enhanced and Permeability Retention) effect[36,37] are now under investigation.



Scheme 1. Synthesis of chiral dendrimer-triamine-coordinated Gd complexes with polyaminoalcohol end groups, Gd-**4a** and Gd-**4b**



Scheme 2. Synthesis of racemic dendrimer-triamine-coordinated Gd complexes with polyaminoalcohol end groups, Gd-**4** (racemic) and Boc-protected Gd complex, Gd-**4**-Boc

4. Experimental section

4.1. General Methods

4.1.1. Materials

All reagents were obtained commercially and used without further purification unless otherwise noted. CH_3CN (99.8%), $CHCl_3$ (99.9%), distilled water, acetone (99%), PPh_3 (97.0%), *N*-bromosuccinimide (NBS, 99.9%), *meta*-chloroperoxybenzoic acid (*m*CPBA, 75%), $Ce(NH_4)_2(NO_3)_6$ (CAN, 95%), $GdCl_3{\cdot}6H_2O$, 1,4-dioxane (99.5%), hexane (99%), KOH (85%), K_2CO_3 (99.5%), $MgSO_4$ (99.0%), $NaHCO_3$ (99.5%), methanol (MeOH, 99.8%), Na_2CO_3 (99.8%), Na_2SO_3 (97.0%), Na_2SO_4 (99.0%), tetrahydrofuran (THF), triethylamine (TEA) (98.0%), and BSA were purchased from Nacalai Tesque Inc. (Kyoto, Japan). 1.0 M Lithium ethoxide in tetrahydrofuran (LiOEt in THF) and 4-vinylbenzyl chloride (90%) were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). 1,4,7-Triazacyclononane (TACN, 98%), trimethylsilyl chloride (98%) and trimethyl orthoacetate (98%) were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Hydrochloric acid methanolic solution (HCl in MeOH, 0.50 mM), 28% NH_3 aqueous solution, and dimethyl sulfoxide dehydrated (DMSO; 99.0%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methylene chloride (CH_2Cl_2) was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Gd-DTPA was purchased from Bayer Holding Ltd. (Tokyo, Japan). Aluminum oxide 90 active neutral was purchased from Merck Chemical Inc. (Darmstadt, Germany). Silica gel 60N spherical for flash chromatography was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). The filter aid Celite Standard Super-gel was purchased from Alfa Aesar (Lancashire, UK). Di-*tert*-butyl dicarbonate was purchased from Peptide Institute (Osaka, Japan). NHS-PEG (SUNBRIGHT ME 050 AS, Mw 5000) was purchased from NOF Corporation (Tokyo, Japan).

^{1}H-NMR spectra were recorded at 400 MHz, and 13C-NMR spectra were recorded at 100 MHz with a JEOL EX400. ESI TOF mass spectra were obtained by micrOTOF focus (Bruker Daltonics Co., Billerica, MA). The average molecular weight of PEG-conjugated CA was determined by gel permeation chromatography (ChromNAV, JASCO Co., Tokyo Japan). The concentration of gadolinium was determined by atomic absorption spectrometry (AAS Z-2710, Hitachi Ltd., Tokyo, Japan) using a gadolinium standard solution (1,000 ppm, analytical grade, Wako). Clinically-used Gd-DTPA (gadopentetate meglumine) was used as a control for MR measurements of the synthesized CAs.

4.1.2. Relaxation Time Measurements

Longitudinal relaxivity (r_1) was calculated from the relaxation time of water proton (T_1) with four different concentrations of CAs at 20 °C on a Biospec 7.0 T/ 20 USR (Bruker Biospin Inc., Billerica, MA) with a 72 mm i.d. Quadrature resonator.

4.1.3. Cytotoxicity Assay

Murine L929 fibroblast cells were plated into each well of 96-multiwell cell culture plates (Corning Inc., Lowell, MA) with growth medium (Dulbecco's modified minimum essential medium (DMEM; Life Technologies Japan Ltd., Tokyo, Japan) containing 10% of fetal bovine serum (FBS; Sanko Pure Chemical Co., Tokyo, Japan), 100 units/ml of penicillin and 0.1 mg/ml of streptomycin (Sigma-Aldrich Japan K.K., Tokyo, Japan) at a density of 1×10^4 cells/cm^2. After incubation for 24 h at 37 °C and 5% CO_2 - 95% air at atmospheric pressure, the medium was replaced by fresh growth medium containing 0.25 mM of CAs. After incubation for 48 h under the same condition, the cell number was evaluated. After Cell Count Reagent SF (10 μL, Nacalai Tesque, Kyoto, Japan) was added to each well, plates were incubated for 60 min. The absorbance at 450 nm was then measured by a spectrophotometer (Versa max, Molecular Devices Inc., Union City, CA). The absorbance was normalized by that in cells incubated with growth medium without CA and expressed as a cell viability ratio.

4.1.4. Quartz Crystal Microbalance Analysis

The ability of CAs to bind to BSA was evaluated with an Affinix-QN Pro® system (Initium Inc., Tokyo Japan) according to the manufacturer's instructions. The dissociation rate constant (K_d) was estimated by a global fitting analysis of the saturated adsorption amount at each concentration, and the association rate constant (K_a) was calculated as $1/K_d$.

4.2. Synthesis

4.2.1. Synthesis of 2nd-Generation Dendrimer-triamine-coordinated Gd-MRI CA with Polyaminoalcohol End Groups (Scheme 1)

*4.2.1.1. 2nd-Generation chiral acetonide-triamine dendrimer (**1a**)*

A solution of a LiOEt in THF (1.0 M, 6.0 mL) was added dropwise to a solution of TACN (80 mg, 0.62 mmol) in THF (10 mL) and stirred at 0 °C under Ar. After 10 min, a solution of 2nd-generation dendron (1.00 g, 1.64 mmol) was added dropwise at 0 °C, and the mixture was stirred at room temperature for 12 h. The mixture was then diluted with 2 × 50 mL of ethyl acetate and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, $CHCl_3$: MeOH = 9 : 1) to give 560 mg (0.325 mmol, 52%) of **1a** as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.48, 1.54 (d, 36H); 2.90 (br, 12H); 3.53-3.56 (q, 3H); 3.65-3.74 (m, 15H); 4.26-4.30 (m, 6H); 4.35, 4.38 (d, 3H); 4.45-4.61 (m, 12H); 5.03-5.07 (t, 6H); 7.24-7.33 (m, 36H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 25.6, 26.2, 70.0, 71.2, 72.5, 74.4, 77.3, 80.0, 109.2, 125.8, 127.2, 127.4, 129.0, 137.8, 137.8, 138.0, 138.1. ESI TOF MS: m/z $[M+H]^+$ calcd. for 1721.9377, found 1721.9919. Elemental analysis calcd. for $C_{105}H_{129}N_3O_{18}\cdot 6H_2O$: C 68.94, H 7.77, N 2.30, O 20.99; found: C 69.24, H 7.53, N 2.45, O 20.78.

*4.2.1.2. 2nd-Generation chiral 12-ol-triamine dendrimer (**2a**)*

A solution of **1a** (559 mg, 0.325 mmol) in 0.50 mM HCl/MeOH solution (20 mL) was stirred at room temperature for 4 h. The reaction was monitored by ESI TOF MS. After the reaction was completed, the solvent was removed under reduced pressure to give 478 mg (0.324 mmol, 99%) of **2a** as a yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 2.81 (br, 12H); 3.53-3.74 (m 12H); 3.68 (s, 6H); 4.31-4.43 (m, 8H); 4.62-4.64 (m, 6H); 7.14-7.46 (m, 36H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm): 50.2, 60.6, 68.7, 71.8, 74.0, 75.4, 75.6, 81.4, 127.4, 128.6, 128.9, 131.6, 136.1, 138.7, 141.6, 142.7. ESI TOF MS: m/z $[M+H]^+$ calcd. for 1480.7466, found 1480.7818.

*4.2.1.3 2nd-Generation chiral epoxide-triamine dendrimer (**3a**)*

Trimethylsilyl chloride (5.0 mL, 40 mmol) was added to a solution of **2a** (300 mg, 0.203 mmol) and trimethyl orthoacetate (3.0 mL, 24 mmol) in CH_2C1_2 (3.0 mL) at 0 °C. The solution was stirred for 60 min, and then evaporated to obtain crude product. It was dissolved in dry methanol (10 mL), and K_2CO_3 (1.00 g, 7.25 mmol) was added. The suspension was stirred vigorously for 2 h, and then filtered and the residue was washed with CH_2Cl_2. The filtrate was extracted with CH_2Cl_2 and the organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated by an evaporator. The residue was purified by flash column chromatography (Al_2O_3, $CHCl_3$: MeOH = 30 : 1) to obtain **3a** as a yellow oil (103.4 mg, 75 μmol, 37 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.76-2.79 (q, 6H); 2.83(s, 12H); 3.12-3.14 (q, 6H); 3.53-3.56(q, 3H); 3.57 (s, 6H); 3.61-3.75 (q, 3H); 3.83-3.85 (q, 6H); 4.35-4.38 (d, 2H); 4.47-4.59 (m, 12H); 7.21-7.32 (m, 36H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 51.2, 52.2, 55.4, 62.7, 70.3, 73.0, 74.9, 80.6, 125.5, 126.9, 127.7, 127.8, 129.1, 136.8, 137.6, 138.5, 138.6, 140.3. ESI TOF MS: m/z $[M+H]^+$ calcd. for 1372.6832, found 1372.6850.

*4.2.1.4. 2nd-Generation chiral aminoalcohol-triamine dendrimer (**4a**)*

A mixture of 28% NH_3 aqueous solution (15.0 mL), 1,4-dioxane (5.0 mL), and **3a** (50.0 mg, 36.5 μmol) was placed in a 20 mL reaction vial in an Initiator 2.50 microwave reactor at 200 W, 100 °C, and 14 bar for 60 min. The solvent was removed under reduced pressure to give 30 mg (20.3 μmol, 56%) of **4a** as a yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 2.79-3.01 (m, 18H); 3.03-3.05 (m, 6H); 3.44-3.89 (m, 15H); 4.19-4.57 (m, 18H); 7.03-7.38 (m, 36H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm): 47.3, 58.1, 64.4, 70.7, 71.7, 73.9, 75.5, 81.5, 127.0, 128.5, 129.0, 131.7, 136.3, 139.6, 141.3, 141.8. ESI TOF MS: m/z $[M+H]^+$ calcd. for 1474.8425, found 1474.8333.

*4.2.1.5. 2nd-Generation chiral aminoalcohol-triamine dendrimer gadolinium complex (Gd-**4a**)*

A solution of $GdCl_3\cdot 6H_2O$ (82 mg, 225 μmol) in MeOH (2.0 mL) was added dropwise to a solution of **4a** (130 mg, 226 μmol) in MeOH (3.0 mL), and the mixture was refluxed under argon for 48 h. The solvent was removed under reduced pressure to give 160 mg (190 μmol) of Gd-**4a** as a yellow solid. ESI TOF MS: m/z $[M-Cl]^+$ 1701.7327.

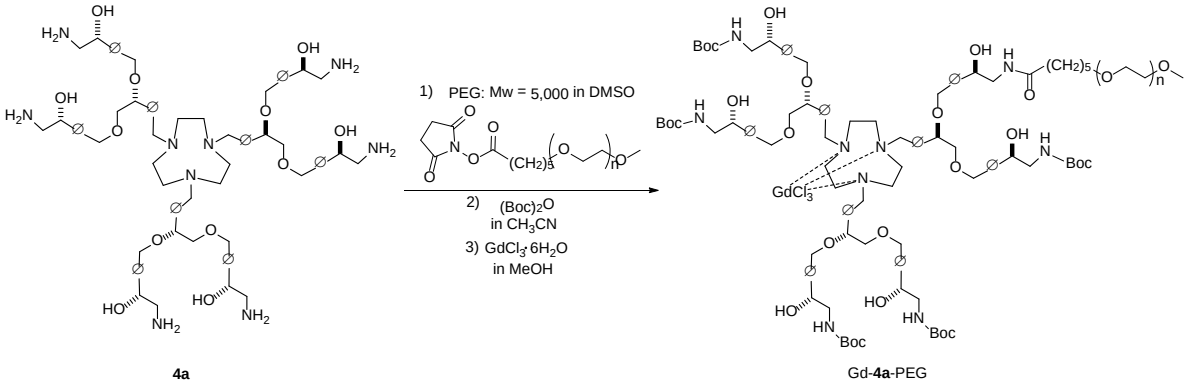
4.2.2. Synthesis of 2nd-generation Dendrimer-triamine-coordinated Gd-MRI CA with Polyaminoalcohol End Groups (Scheme 2)

*4.2.2.1. 2nd-Generation dendron of bis(olefin)ether (**6**)*

To a reaction apparatus equipped with a Dean-Stark tube and a reflux cooling tube was added **5** (5.00 g, 18.2 mmol), and the inside air was replaced with argon. Subsequently, 4-vinylbenzyl chloride (7.5 mL, 56.3 mmol) and 250 mL of toluene were added to the reactor under a flow of argon. The mixture was heated and stirred for approximately 30 min to make a uniform solution. To the resulting uniform solution was added 6.38 g of KOH to make an orange-colored cloudy solution. Thereafter, the solution was heated under reflux for 5 h, and then stirred at 100 °C for 12 h. After the reaction was completed, the solvent was removed under reduced pressure. The crude product was dissolved in CH_2Cl_2 and suction filtration to give a yellow-colored uniform solution. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, $CHCl_3$) to give 5.13 g (10.2 mmol, 56%) of **6** as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.43-3.47 (m, 1H); 3.57-3.65 (m, 4H); 4.24, 4.27 (d, 1H); 4.36-4.52 (m, 4H); 4.87 (s, 2H); 5.09, 5.11 (d, 2H); 5.59, 5.63 (d, 2H); 6.54-6.62 (m, 2H); 6.69-6.81 (dd, 4H); 7.12-7.31 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 55.5, 70.3, 72.9, 74.6, 80.2, 113.5, 114.5, 115.7, 126.0, 127.2, 127.6, 127.8, 136.5, 136.7, 137.0, 137.8, 138.9, 152.8, 153.9. ESI TOF MS: m/z $[M+Na]^+$ calcd. for 529.2349, found 529.2450.

*4.2.2.2. 2nd-Generation dendron of bis(olefin)benzyl alcohol (**7**)*

A solution of **6** (5.13g, 10.2 mmol) in 1,4-dioxane (40 mL) was stirred at 0 °C, and an aqueous solution of CAN (7.35 g, 13.4 mmol) in 20 mL of water was added dropwise. After the addition was completed, the resulting mixture was stirred for 30 min and cooled. 150 mL of water was added to stop the reaction. The resulting mixture was transferred to a separating funnel and the water layer was extracted three times with ethyl acetate. The organic layer was collected and washed twice with a 5% K_2CO_3 aqueous solution. The washing solution was again washed twice with ethyl acetate. The organic layers were all collected and washed with a 10% Na_2SO_3 aqueous solution and then with a 5% K_2CO_3 aqueous solution, dehydrated with brine and $MgSO_4$, and concentrated by an evaporator. The residue was purified by column chromatography (SiO_2, 100% $CHCl_3$ to 100% ethyl acetate) to give 3.06 g (7.65 mmol, 75%) of **7** as an orange oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.27 (br, 1H); 3.42-3.46 (m, 1H); 3.59-3.88 (m, 1H); 4.23, 4.26 (d, 1H); 4.36-4.48 (m, 4H); 4.51 (s, 2H); 5.10, 5.12 (d, 2H); 5.59, 5.64 (d, 2H); 6.54-6.62 (m,



Scheme 3. Synthesis of PEG-conjugated Gd complex, Gd-**4a**-PEG

2H); 7.13-7.26 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 64.7, 70.3, 72.9, 74.6, 80.2, 113.6, 126.0, 127.2, 127.6, 127.8, 136.4, 136.7, 137.8, 138.4, 140.7. ESI TOF MS: m/z $[M+Na]^+$ calcd. for 423.1931, found 423.2087.

*4.2.2.3. 2nd-Generation dendron of bis(olefin)benzyl bromide (**8**)*

7 (3.06 g, 7.65 mmol) and 10 mL of CH_2Cl_2 were added to a reactor filled with argon. The reactor was immersed in a cooling bath and cooled to 0 °C. PPh_3 (2.21 g, 8.42 mmol) and NBS (1.50 g, 8.42 mmol) were added under a flow of argon, and then the mixture was stirred for 12 h. After the reaction was completed, to the reaction mixture were added 10 mL of diethyl ether and 20 mL of saturated Na_2CO_3 aqueous solution. After the solution was separated, the organic layer was dehydrated with brine and $MgSO_4$. The resulting organic layer was concentrated by an evaporator, and the residue was purified by column chromatography (SiO_2, ethyl acetate: hexane = 1:3) to give 2.58 g (5.58 mmol, 73%) of **8** as an orange oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.45-3.48 (m, 1H); 3.63-3.68 (m, 1H); 4.27-4.30 (d, 1H); 4.41-4.53 (m, 6H); 5.13, 5.16 (d, 2H); 5.63, 5.67 (d, 2H); 6.58-6.66 (m, 2H); 7.14-7.31 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 33.2, 70.5, 73.0, 74.5, 80.1, 113.7, 126.1, 127.5, 127.7, 129.1, 136.5, 136.9, 137.4, 137.8, 139.7. ESI TOF MS: m/z $[M+Na]^+$ calcd. for 485.1087, 487.1070, found 485.1381, 487.1363.

*4.2.2.4. 2nd-Generation dendron of bis(epoxide)benzyl bromide (**9**)*

A solution of *m*CPBA (2.41 g, 14.0 mmol) in CH_2Cl_2 (2 mL) was added dropwise to a solution of **8** (2.58 g, 5.58 mmol) in CH_2Cl_2 (10 mL) at 0 °C, and the mixture was stirred at room temperature for 12 h. A saturated Na_2SO_2 aqueous solution (10 mL) and a saturated $NaHCO_3$ aqueous solution (10 mL) were added to stop the reaction. The mixture was then diluted with 2 × 10 mL of CH_2Cl_2 and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, $CHCl_3$) to give 772 mg (1.56 mmol, 28%) of **9** as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.77-2.79 (m, 2H); 3.12-3.15 (m, 2H); 3.52-3.56 (m, 1H); 3.69-3.73 (m, 1H); 3.83-3.86 (m, 2H); 4.36, 4.39 (d, 1H); 4.48-4.61 (m, 6H); 7.23-7.39 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 33.2, 51.2, 52.2, 70.5, 73.0, 74.6, 80.3, 125.5, 127.5, 127.9, 129.2, 136.9, 137.5, 138.3, 139.7. ESI TOF MS: m/z $[M+Na]^+$ calcd. for 517.0985, 519.0969, found 517.0993, 519.0976.

*4.2.2.5. 2nd-Generation epoxide-triamine dendrimer (**10**)*

A solution of a LiOEt in THF (1.0 M, 2.0 mL) was added dropwise to a solution of TACN (201 mg, 1.56 mmol) in THF (20 mL), and stirred at 0 °C under argon. After 10 min, a solution of **9** (772 mg, 1.56 mmol) was added dropwise at 0 °C, and the mixture was stirred at room temperature for 12 h. The mixture was then diluted with 2 × 50 mL of ethyl acetate and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (Al_2O_3, $CHCl_3$) to give 620 mg (0.452 mmol, 29%) of **10** as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.76-2.79 (q, 6H); 2.83 (s, 12H); 3.12-3.14 (q, 6H); 3.53-3.56 (q, 3H); 3.57 (s, 6H); 3.61-3.75 (q, 3H); 3.83-3.85 (q, 6H); 4.35-4.38 (d, 2H); 4.47-4.59 (m, 12H); 7.21-7.32 (m, 36H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 51.2, 52.2, 55.4, 62.7, 70.3, 73.0, 74.9, 80.6, 125.5, 126.9, 127.7, 127.8, 129.1, 136.8, 137.6, 138.5, 138.6, 140.3. ESI TOF MS: m/z $[M+H]^+$ calcd. for 1372.6832, found 1372.6834.

*4.2.2.6. 2nd-Generation- aminoalcohol-triamine dendrimer (**4** (racemic))*

A mixture of 28% NH_3 aqueous solution (3.0 mL), 1,4-dioxane (1.0 mL) and **10** (119 mg, 225 μmol) was placed in a 2 mL reaction vial in an Initiator 2.50 microwave reactor at 200 W, 120 °C, and 12 bar for 60 min. The solvent was removed under reduced pressure to give 130 mg (225 μmol, 99%) of **4** (racemic) as a yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 2.79-3.01 (m, 18H); 3.03-3.05 (m, 6H); 3.44-3.89 (m, 15H); 4.19-4.57 (m, 18H); 7.03-7.38 (m, 36H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm): 47.3, 58.1, 64.4, 70.7, 71.7, 73.9, 75.5, 81.5, 127.0, 128.5, 129.0, 131.7, 136.3, 139.6, 141.3, 141.8. ESI TOF MS: m/z $[M+H]^+$ calcd. for 1474.8425, found 1475.7655.

*4.2.2.7. 2nd Generation aminoalcohol-triamine dendrimer gadolinium complex (Gd-**4** (racemic))*

A solution of $GdCl_3 \cdot 6H_2O$ (82 mg, 225 μmol) in MeOH (2.0 mL) was added dropwise to a solution of **4** (racemic) (130 mg, 226 μmol) in MeOH (3.0 mL), and the mixture was refluxed under argon for 48 h. The solvent was removed under reduced pressure to give 160 mg (190 μmol) of Gd-**4** (racemic) as a yellow solid.

*4.2.3. Synthesis of Boc-Protected Dendrimer-triamine-coordinated Gd-MRI CA (Gd-**4**-Boc) (Scheme 3)*

$(Boc)_2O$ (39.8 mg, 42 μL, 181 μmol) and TEA (14.5 mg, 20 μL, 143 μmol) was added dropwise to a solution of **4** (racemic) (47.0 mg, 31.9 μmol) in MeOH (10 mL), and the mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, $CHCl_3$: MeOH = 10 : 1) to give 54.0 mg (26.0 μmol, 81%) of **4**-Boc (racemic) as a yellow solid. ESI TOF MS: m/z $[M+H]^+$ calcd. for 2075.5980, found 2076.7795. A solution of $GdCl_3 \cdot 6H_2O$ (9.60 mg, 26.0 μmol) in MeOH (2.0 mL) was added dropwise to a solution of **4**-Boc (54.0 mg, 26.0 μmol) in MeOH (3.0 mL), and the mixture was refluxed

under argon for 48 h. The solvent was removed under reduced pressure to give 60.4 mg (25.6 μmol, 98%) of Gd-**4**-Boc as a yellow solid. ESI TOF MS: m/z $[M+H]^+$ calcd. for 2339.1980, found 2339.6185.

*4.2.4. Synthesis of PEG-Dendrimer-triamine-coordinated Gd-MRI CA (Gd-**4a**-PEG) (Scheme 3)*

A solution of **4a** (30.0 mg, 20.4 μmol) and NHS-PEG (Mw 5,000, 10 mg, 2.0 μmol) in DMSO (1 mL) was stirred at room temperature for 24 h. The residue was subjected to GPC (SB-803HQ, eluent: ultrapure water, current speed: 1.0 mL/min, column temperature: 40 °C). The solvent was removed by lyophilization to give a white solid. This white solid and $(Boc)_2O$ (1.3 mg, 6.0 µmol) in CH_3CN (5.0 mL) were stirred at room temperature for 24 h. The solvent was removed under reduced pressure, ultrapure water was added and the precipitate was removed by filter. To filtrate, $GdCl_3 \cdot 6H_2O$ (4.00 mg, 11.0 μmol) and MeOH (2.0 mL) were added and refluxed under argon for 48 h. The solvent was removed under reduced pressure to give 6.9 mg (0.63 μmol) of Gd-**4a**-PEG as a white solid.

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