Selective sensing of zinc ions with a novel magnetic resonance imaging contrast agent †

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Light-based microscope imaging techniques using fluorescence sensor molecules suffer from photobleaching and light scattering, but magnetic resonance imaging (MRI) can provide three-dimensional imaging without these problems. Recently, "smart" MRI contrast agents which modulate the access of water to a chelated gadolinium (Gd^{3+}) ion in the presence or absence of a specific trigger have been reported. Zinc (Zn^{2+}) is an essential component of many enzymes, transcription factors and synaptic vesicles in excitatory nerve terminals, so imaging of chelatable Zn^{2+} is of interest. We have designed and synthesized the Gd^{3+} DTPA bisamide complex **7a** as a Zn^{2+}-sensitive MRI contrast agent. Compound **7a** shows a dose-dependent change in the R_1 relaxivity in the presence of Zn^{2+}. We investigated this relaxation behavior, and for this purpose we also synthesized the Gd^{3+} DTPA amide ethyl ester complex **7b**. It was shown that binding between **7a** and Zn^{2+} caused a change in the relaxation time. Moreover, **7a** had high selectivity for Zn^{2+} against Ca^{2+} and Mg^{2+}. Compound **7a** may have practical problems for *in vivo* usage, since the R_1 relaxivity is reduced with increased Zn^{2+} concentration. However, this report demonstrates new approaches to the design and synthesis of Gd^{3+} complexes with R_1 values that change with variation in Zn^{2+} concentration.

Introduction

Zinc (Zn^{2+}), which is a key component of many enzymes, transcription factors[1] and synaptic vesicles in excitatory nerve terminals,[2] is present in serum at a concentration of *ca.* 12 μM (total Zn^{2+}).[3] Light-based microscope imaging of chelatable Zn^{2+} in the extra- and intra-cellular environments or tissues with fluorescent dyes that respond dose-dependently to Zn^{2+} has contributed to our understanding the roles of Zn^{2+} in cells and tissues.[4] However, light-based microscope imaging techniques with fluorescent dyes can be limited by photobleaching of the dyes and also by light scattering to cells within 100 μm of the surface. Magnetic resonance imaging (MRI) can overcome these problems and seems to be suitable for three-dimensional imaging.[5]

Gadolinium (Gd^{3+}) complexes are frequently chosen as MRI contrast agents.[6] These complexes enhance the T_1 (spin-lattice) and T_2 (spin–spin) relaxation rates of water protons by rapid exchange of inner-sphere water molecules with bulk solvent.[7] The enhancement is, in part, due to the direct interaction of water molecules (inner sphere) with the unpaired electrons of the paramagnetic metal ion, Gd^{3+}. Recently, some Gd^{3+}-based "smart" MRI contrast agents have been reported for monitoring enzyme activity, Ca^{2+} or pH.[8–10] These MRI agents show a change in the relaxation rate in the presence of enzyme, Ca^{2+} or pH owing to modulation of the accessibility of water molecules to the chelated Gd^{3+} ion. Here, we report the Gd^{3+} diethylenetriaminepentaacetic acid (DTPA) bisamide complex **7a** as a Zn^{2+}-sensitive MRI contrast agent. This complex was designed on the basis that *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) readily complexes with Zn^{2+}, but hardly complexes with Ca^{2+} and Mg^{2+}.[11]

† Electronic supplementary information (ESI) available: Fig. S1 and Fig S2 showing HPLC confirmation of complexes **7a** and **7b**. See http://www.rsc.org/suppdata/p2/b1/b100994j/

Experimental

Materials

DTPA bisanhydride was purchased from Aldrich Chemical Co. Inc., USA. DTPA anhydride ethyl ester was kindly donated by Nihon Schering K.K., Japan. All other reagents were purchased from either Tokyo Kasei Kogyo Co., Ltd., Japan or Wako Pure Chemical Industries, Ltd., Japan. Ethanol, triethylamine, dichloromethane and *N,N*-dimethylformamide were used after distillation.

Instruments

^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-LA300. Mass spectra (MS) were measured with a JEOL JMS-DX 300 and a JEOL JMS-SX 102A mass spectrometer. UV-visible spectra were obtained on a Shimadzu UV-1600. HPLC purification was performed on a reversed-phase column (Gl Sciences, Inertsil Prep-ODS 30 mm × 250 mm) fitted on a Jasco PU-1587 System.

Synthesis

***N*-*tert*-Butoxycarbonylethylenediamine (2).** To a solution of ethylenediamine (**1**) (32 ml, 479 mmol) in a mixture of ethanol (30 ml) and triethylamine (2.8 ml) was added dropwise a solution of di-*tert*-butyl dicarbonate (4.7 ml, 20.4 mmol) in ethanol (10 ml) at 0 °C. The mixture was stirred for 1 h at room temperature and evaporated to dryness. The residue was dissolved in dichloromethane (50 ml) and extracted with 1 M aqueous acetic acid three times. The solution was basified with 2 M aqueous NaOH and extracted with dichloromethane four times. The combined dichloromethane extract was dried over anhydrous potassium carbonate and evaporated to dryness, to afford **2** (2.83 g, 86.7%) as a colorless oil. ^{1}H-NMR (300 MHz; $CDCl_3$; Me_4Si): δ 1.25 (2H, br s, NH_2), 1.45 (9H, s, Bu^t), 2.80 (2H, t, J = 5.7 Hz, NCH_2), 3.17 (2H, q, J = 6.0, 5.7 Hz, $CNCH_2$), 5.02

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(1H, br s, NH). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 28.43, 41.92, 43.49, 79.11, 156.30. MS (EI^+): *m/z* 160 (M^+).

***N*-*tert*-Butoxycarbonyl-*N*′,*N*′-bis(2-pyridylmethyl)ethylenediamine (3).** To a solution of **2** (2.50 g, 15.6 mmol) in ethanol (100 ml) were added anhydrous sodium carbonate (7.30 g, 68.8 mmol) and 2-(chloromethyl)pyridine hydrochloride (5.60 g, 34.4 mmol). The resulting suspension was heated under reflux under Ar overnight (21 h) and evaporated to dryness. The residue was suspended in 2 M aqueous NaOH (100 ml) and extracted with dichloromethane three times. The combined dichloromethane extract was washed with brine, dried over anhydrous potassium carbonate and evaporated to dryness. The residue was purified by aluminum oxide column chromatography (n-hexane–dichloromethane, 1 : 1) to give **3** (4.34 g, 81.2%) as an orange oil. ^{1}H-NMR (300 MHz; $CDCl_3$; Me_4Si): δ 1.45 (9H, s, Bu^t), 2.71 (2H, t, J = 5.7 Hz, C_2NCH_2), 3.23 (2H, m, $CONCH_2$), 3.87 (4H, s, $NCCNCH_2$), 5.79 (1H, br s, NH), 7.16 (2H, dd, J = 6.4, 4.1 Hz, 5-H), 7.42 (2H, d, J = 7.7 Hz, 3-H), 7.64 (2H, dd, J = 7.7, 6.4 Hz, 4-H), 8.55 (2H, d, J = 4.1 Hz, 6-H). ^{13}C-NMR (75 MHz; $CDCl_3$): δ 28.48, 38.53, 53.52, 60.22, 78.65, 122.02, 123.03, 136.39, 149.10, 156.16, 159.32. MS (EI^+): *m/z* 342 (M^+).

***N*,*N*-Bis(2-pyridylmethyl)ethylenediamine (4).** A solution of **3** (4.30 g, 12.6 mmol) in dichloromethane (40 ml) was added dropwise to TFA (100 ml) at 0 °C. The mixture was stirred for 1 h at room temperature and evaporated to dryness. The residue was dissolved in 2 M aqueous NaOH (75 ml) and extracted with dichloromethane. The combined dichloromethane extract was dried over anhydrous potassium carbonate and evaporated to dryness. The residue was purified by aluminum oxide column chromatography (dichloromethane–methanol; 95 : 5) to give **4** (1.95 g, 64.1%) as an orange oil. ^{1}H-NMR (300 MHz; $CDCl_3$; Me_4Si): δ 1.60 (2H, br s, NH_2), 2.67 (2H, t, J = 5.7 Hz, CH_2), 2.80 (2H, t, J = 5.7 Hz, CH_2), 3.85 (4H, s, $CCNCH_2$), 7.15 (2H, dd, J = 7.3, 5.0 Hz, 5-H), 7.49 (2H, d, J = 7.5 Hz, 3-H), 7.65 (2H, dd, J = 7.5, 7.3 Hz, 4-H), 8.53 (2H, m, 6-H). ^{13}C-NMR (75 MHz; $CDCl_3$): δ 39.57, 57.34, 60.71, 122.02, 123.05, 136.43, 149.06, 159.58. MS (EI^+): *m/z* 242 (M^+).

DTPA bisamide (6a). Compounds **6a** and **6b** were synthesized according to the literature.[12] Briefly, DTPA bisanhydride (**5a**, 148 mg, 0.48 mmol) was slowly added to a solution of **4** (200 mg, 0.83 mmol) in DMF (1.3 ml) at 50 °C and the mixture was heated under Ar for 4 h at 70 °C. The solution was cooled down and was evaporated to dryness to give a colorless oil. Compound **6a** was precipitated from ethanol by the addition of acetone, washed with acetone and dried under reduced pressure to afford a colorless solid (127 mg, 36.5%). ^{1}H-NMR (300 MHz; D_2O): δ 2.95 (4H, t, J = 6.2 Hz), 3.04 (4H, t, J = 5.7 Hz), 3.08 (4H, s), 3.17 (4H, t, J = 6.2 Hz), 3.23 (4H, s), 3.38 (4H, t, J = 5.7 Hz), 3.64 (2H, s), 4.12 (8H, s), 7.37 (4H, t, J = 7.7, 5.3 Hz), 7.42 (4H, d, J = 7.9 Hz), 7.85 (4H, t, J = 7.9, 7.7 Hz), 8.40 (4H, d, J = 5.3 Hz). ^{13}C-NMR (75 MHz; D_2O): δ 37.10, 51.48, 53.97, 55.47, 55.56, 58.72, 59.71, 59.91, 125.64, 126.52, 142.17, 146.92, 153.61, 171.35, 174.76, 179.19. MS (FAB^+): *m/z* 842 ($[M + H]^+$).

DTPA amide ethyl ester (6b). DTPA amide ethyl ester **6b** was prepared by reacting DTPA anhydride ethyl ester **5b** with **4** (1 equiv.) using the same method as described for DTPA bisamide **6a**. Yield 49.6%. ^{1}H-NMR (300 MHz; D_2O): δ 1.14 (3H, t, J = 7.3 Hz), 2.96 (2H, t, J = 5.3 Hz), 3.08 (2H, t, J = 6.0 Hz), 3.15 (2H, t, J = 6.6 Hz), 3.25–3.30 (6H, m), 3.40 (2H, t, 5.7 Hz), 3.42 (2H, s), 3.43 (2H, s), 3.65 (2H, s), 3.70 (2H, s), 4.09 (2H, q, J = 7.3 Hz), 4.17 (4H, s), 7.59 (2H, dd, J = 7.5, 5.7 Hz), 7.64 (2H, d, J = 8.0 Hz), 8.10 (2H, dd, J = 8.0, 7.5 Hz), 8.52 (2H, d, J = 5.7 Hz). ^{13}C-NMR (75 MHz; D_2O): δ 14.17, 37.32, 50.57, 51.90, 53.34, 53.60, 55.51, 55.99, 56.26, 57.83, 57.92, 58.96, 59.52, 63.11, 126.22, 127.23, 144.19, 145.43, 153.33, 172.13, 172.67, 173.77, 176.70, 177.95. MS (FAB^+): *m/z* 646 ($[M + H]^+$).

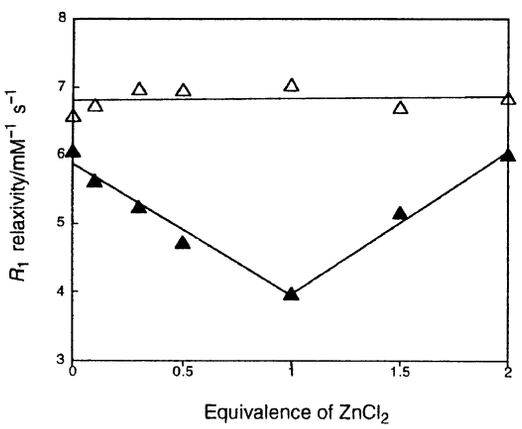


Fig. 1 The water proton relaxivity of **7a** (closed triangle) and **7b** (open triangle) at pH 8.0, 25 °C in the presence of various concentrations of Zn^{2+}: 0, 0.1, 0.3, 0.5, 1.0, 1.5 and 2.0 equiv.

Gd^{3+} DTPA bisamide complex (7a). Compounds **7a** and **7b** were synthesized according to the literature.[13] Briefly, to a solution of **6a** (120 mg, 0.143 mmol) in Tris buffer (1 M; pH 8.0, 5 ml) was added 100 mM aqueous $GdCl_3$ (1.57 ml). The resulting mixture was stirred at room temperature for 30 min and purified by HPLC with methanol–H_2O (3 : 2) as eluent to give **7a** (71.0 mg, 50.0%) as a colorless solid. Mp > 276 °C (decomp.). IR (KBr): ν_{max}/cm^{-1} 3391 (H_2O), 3260, 3090, 2953, 1624, 1435, 1400, 769. MS (FAB^+): *m/z* 997 ($[M + H]^+$). Anal. Calcd. (found) for $C_{42}H_{52}N_{11}O_8Gd\cdot 7.5H_2O$: C, 44.59 (44.65); H, 5.97 (6.02); N, 13.62% (13.40%).

Gd^{3+} DTPA amide ethyl ester complex (7b). The reaction mixture was purified by HPLC with methanol–H_2O (2 : 3) as eluent. Yield 27.9%. Mp > 237 °C (decomp.). IR (KBr): ν_{max}/cm^{-1} 3398 (H_2O), 3260, 3120, 2980, 1597, 1435, 1404, 1207, 769. MS (FAB^+): *m/z* 801 ($[M + H]^+$). Anal. Calcd. (found) for $C_{30}H_{40}N_7O_9Gd\cdot 4.0H_2O$: C, 41.32 (41.31); H, 5.55 (5.49); N, 11.24% (11.02%).

Relaxation time measurement

Relaxation time, T_1, of aqueous solutions of the Gd^{3+} complex **7a** or **7b** was measured in Tris buffer (0.1 M; pH 8.0) by using the standard inversion–recovery procedure (JEOL JNM-LA300, 25 °C). The relaxivity, R_1, of **7a** or **7b** was determined from the slope of the plot of $1/T_1$ *vs.* [**7a**] or [**7b**] (0.3, 0.5, 0.7, 1.0 mM). The buffered Gd^{3+} complex (**7a** or **7b**) solution was allowed to equilibrate for at least 10 min after addition of $ZnCl_2$, $CaCl_2$ or $MgCl_2$ aqueous stock solution.

Results and discussion

It is known that *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) complexes strongly with Zn^{2+}, but hardly at all with Ca^{2+} or Mg^{2+}. Reactions between primary amines and DTPA bisanhydride have been widely used in the synthesis of DTPA bisamide chelaters.[12] Therefore, we designed the Gd^{3+} DTPA bisamide complex **7a**, which contains the TPEN moiety, as a Zn^{2+}-sensitive MRI contrast agent. Compound **7a** was obtained according to the reaction Scheme 1. In characterizing the Gd^{3+} DTPA bisamide complex **7a**, we observed that the water proton R_1 relaxivity of **7a** had an unusual Zn^{2+} dependence. The R_1 decreased dose-dependently between 0 and 1.0 equiv. Zn^{2+}, reaching a minimum at 1.0 equiv. Zn^{2+} to **7a**, then R_1 increased dose-dependently between 1.0 and 2.0 equiv. (Fig. 1). The R_1 relaxivity of the complex **7a** decreased approximately 33% when Zn^{2+} (1.0 equiv. to **7a**) was added to a Zn^{2+}-free

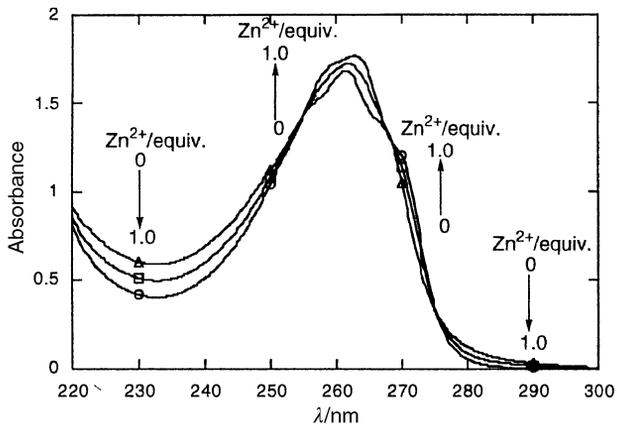


Fig. 2 UV-visible spectra of **7a** (150 μM) in Tris buffer (0.1 M; pH 8.0) in the presence of various concentrations of Zn^{2+}: 0 equiv. (open triangle), 0.5 equiv. (open square), 1.0 equiv. (open circle). For the addition of Zn^{2+} between 1.0 and 3.0 equiv., the absorbance spectra remained at a plateau.

solution. To provide further insight into the unusual relaxation behavior of **7a**, we examined the UV-visible spectra of a **7a** solution (150 μM) at pH 8.0 (0.1 M Tris buffer) in the presence of various concentrations of Zn^{2+} (Fig. 2). The absorbance between 220 and 300 nm changed linearly with increase in Zn^{2+} concentration up to 1 : 1 Zn^{2+}–**7a** molar ratio, with isosbestic points at 217.5, 255.0, 268.0, 275.5 nm and remained at a plateau with further increase of Zn^{2+}. The reason for these results is considered to be as follows. When the Zn^{2+}–**7a** molar ratio is between 0 : 1 and 1 : 1, Zn^{2+} and **7a** form a 1 : 1 complex. On the other hand, when the Zn^{2+}–**7a** molar ratio exceeds one, Zn^{2+} and **7a** form a 2 : 1 complex (Fig. 3). We hypothesize that **7a** in the Zn^{2+}–**7a**; 1 : 1 complex has fewer water molecules binding directly to Gd^{3+} than **7a** in a Zn^{2+}-free solution. This can be understood in terms of the Zn^{2+} coordination geometry, which is proposed to be as shown in Fig. 3. Complex **7a**

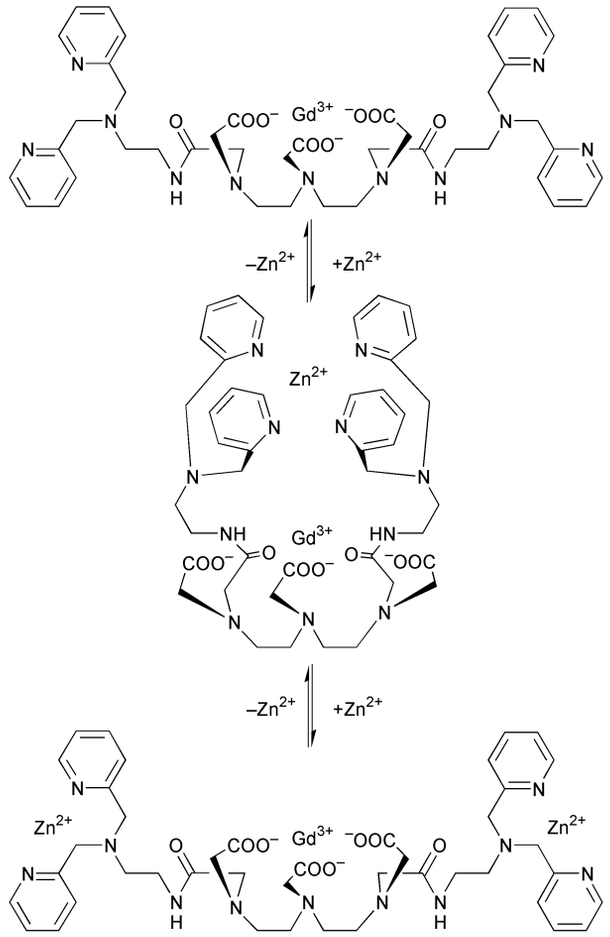
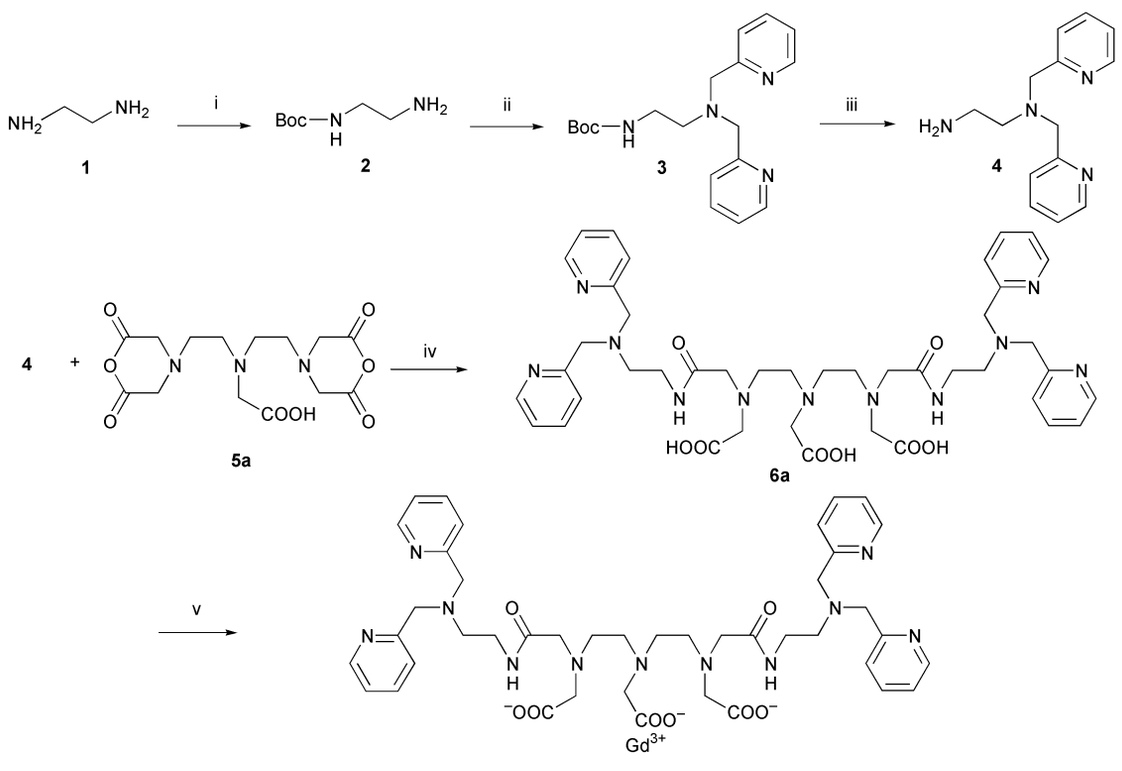
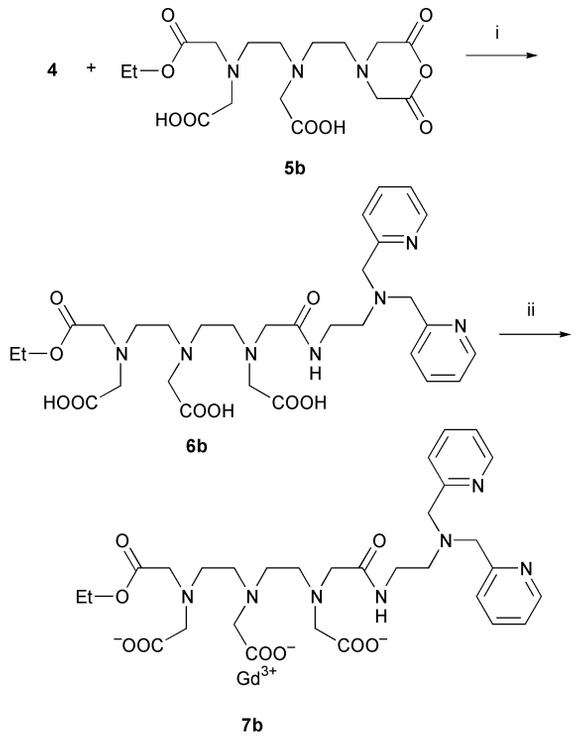


Fig. 3 Schematic representation of Gd^{3+} DTPA bisamide complex **7a** for the proposed conformational dependence of the structure in the presence and absence of Zn^{2+}.



Scheme 1 Synthesis of Gd^{3+} DTPA bisamide complex. *Reagents and conditions*: (i) di-*tert*-butyl dicarbonate, EtOH, rt; (ii) 2-(chloromethyl)-pyridine hydrochloride, Na_2CO_3, EtOH, reflux, under Ar; (iii) TFA, rt; (iv) DTPA bisanhydride, DMF, 70 °C, under Ar; (v) $GdCl_3 \cdot 6H_2O$, Tris buffer (pH 8.0), rt.



Scheme 2 Synthesis of Gd^{3+} DTPA amide ethyl ester complex **7b**. *Reagents and conditions*: (i) DTPA anhydride ethyl ester, DMF, 70 °C, under Ar; (ii) $GdCl_3 \cdot 6H_2O$ Tris buffer (pH 8.0), rt.

contains two amides, which have weak chelating ability, and there is probably a steric repulsion effect of the four pyridines. In the Zn^{2+}–**7a** 2 : 1 complex, **7a** has the same number of water molecules binding directly to Gd^{3+} as **7a** in a Zn^{2+}-free solution, because the second Zn^{2+} opens up the water-accessible space on the surface of Gd^{3+} (Fig. 3). These Zn^{2+}-chelating characteristics of **7a** therefore modulate access of water to the Gd^{3+} ion.

To understand further the chelating characteristics of such compounds, we examined the Gd^{3+} DTPA amide ethyl ester complex **7b**. Compound **7b** has a different Zn^{2+}-chelating segment from **7a**, with the same Gd^{3+} chelating moiety. Compound **7b** was synthesized according to reaction Scheme 2. The water proton R_1 relaxivity of **7b** at pH 8.0 (0.1 M Tris buffer) in the presence of various concentrations of Zn^{2+} did not change with Zn^{2+} concentration (Fig. 1). We also measured the UV-visible spectra of a **7b** solution (300 μM) at pH 8.0 (0.1 M Tris buffer) upon addition of Zn^{2+} (data not shown). The absorbance spectra of a **7b** solution changed similarly to that of a **7a** solution. The absorbance between 220 and 300 nm changed linearly with Zn^{2+} concentration up to 1 : 1 Zn^{2+}–**7b** molar ratio and remained at a plateau with further increase in Zn^{2+} concentration. It can be considered from these results that **7b** and Zn^{2+} form a 1 : 1 complex, and that the water-accessibility of the chelated Gd^{3+} ion is not changed by this complexation. These results are consistent with the hypothesis in the case of **7a**.

For a Zn^{2+}-sensitive MRI contrast agent, high selectivity against Mg^{2+} and Ca^{2+} is crucial. Therefore, we also examined the effects of Ca^{2+}, Mg^{2+} and H^+ on the binding of **7a** to Zn^{2+}. No effect of H^+ on the R_1 relaxivity of **7a** was observed between pH 4 and 10, in the absence of Zn^{2+} (data not shown). The R_1 relaxivity of **7a** solution on the addition of one equivalent of Ca^{2+} or Mg^{2+} at pH 8.0 (0.1 M Tris buffer) is shown in Table 1: there was no change. The Gd^{3+} DTPA bisamide complex **7a** thus showed high selectivity for binding Zn^{2+} against Ca^{2+} and Mg^{2+}.

Conclusion

We designed and synthesized the Gd^{3+} DTPA bisamide complex **7a** as a Zn^{2+}-sensitive MRI contrast agent. The **7a** solution showed characteristic changes of the R_1 relaxivity in the presence of various concentrations of Zn^{2+} from 0 : 1 to 1 : 1 Zn^{2+}–**7a** molar ratio, and increased gradually from 1 : 1 to 2 : 1 Zn^{2+}–**7a** molar ratio. We hypothesize that upon the addition of Zn^{2+} between 0 : 1 and 1 : 1 Zn^{2+}–**7a** molar ratio, water molecules bound directly to Gd^{3+} are displaced. However, upon the addition of Zn^{2+} between 1 : 1 and 2 : 1 Zn^{2+}–**7a** molar ratio, **7a** and Zn^{2+} form a 1 : 2 complex which bears the same number of water molecules as **7a**. The R_1 relaxivity and UV-visible spectra of the Gd^{3+} DTPA amide ethyl ester complex **7b** are also consistent with this idea. Moreover, compound **7a** had high selectivity for Zn^{2+} against Ca^{2+} and Mg^{2+}. This agent is the first non-ionic Zn^{2+}-sensitive MRI contrast agent,[14] and a decrease of the R_1 relaxivity upon addition of Zn^{2+} has never previously been reported.

Table 1 R_1 relaxivity[a] for solutions of the Gd^{3+} DTPA bisamide complex **7a** upon addition[b] of Zn^{2+}, Ca^{2+} or Mg^{2+}

No addition	Zn^{2+}	Ca^{2+}	Mg^{2+}
6.06	3.98	6.14	6.41

[a] R_1 relaxivity/$mM^{-1}\,s^{-1}$ was measured at 25 °C and 100 mM Tris buffer, pH 8.0. [b] All cations were added as $ZnCl_2$, $CaCl_2$ or $MgCl_2$ (1 equiv. to **7a**).

As a means for measuring Zn^{2+}, this new derivative **7a** may not differentiate the concentration in the likely desirable range of 10–15 μM. However, this compound should be an excellent candidate for incorporation into sensors designed for the selective detection of Zn^{2+} in biological applications. Such studies are in progress. Compound **7a** may also be a candidate for the formation of responsive luminescent lanthanide complexes (Eu, Tb).[15]

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