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Design and Synthesis of a Novel Magnetic Resonance Imaging Contrast Agent for Selective Sensing of Zinc Ion

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Summary

A series of new diethylenetriaminepentaacetic acid (DTPA)-bisamide chelators has been prepared and characterized for application as zinc sensors. We have designed and synthesized $(GdL^a)^{2-}$, which contains a DTPA-bisamide moiety. The R_1 relaxivity of $(GdL^a)^{2-}$ solution decreased monotonically on the addition of Zn^{2+}. Moreover, $(GdL^a)^{2-}$ showed high selectivity for Zn^{2+} against Ca^{2+} and Mg^{2+}. We also measured the UV-visible spectra and the coldspray ionization (CSI) MS spectra and concluded that the 1-to-1 Zn^{2+} complex of $(GdL^a)^{2-}$ is stable at higher concentrations of Zn^{2+}. These complexes should provide the basis for creating a superior Zn^{2+}-sensitive MRI contrast agent and are excellent candidates for incorporation into sensors designed for selective detection of Zn^{2+} in biological applications.

Introduction

Zinc (Zn^{2+}) is the second most abundant heavy metal ion after iron in the human body. It is an essential component of many enzymes, transcription factors [1, 2], and synaptic vesicles in excitatory nerve terminals [3] and is present in serum at a concentration of ~12 μM (total Zn^{2+}) [4]. Recently, zinc has been reported to play important roles in regulating synaptic transmission and cell death [5]. Therefore, imaging of chelatable Zn^{2+} in the extra- and intracellular environments or in tissues is of interest.

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Light-based microscope imaging techniques using fluorescence sensor molecules sometimes suffer from photobleaching and light scattering, but magnetic resonance imaging (MRI) can allow imaging of intact, opaque organisms in three dimensions without these problems [6]. While nuclear spins are excited with RF pulses, the MRI apparatus typically imposes one or more magnetic field gradients upon a specimen. The MRI image is based upon the NMR signal from the protons of water, and the signal intensity depends upon the water concentration and relaxation times (T_1 and T_2). Nowadays, there is great interest in MRI contrast agents, which can improve the resolution of MR images [7]. Gadolinium (Gd^{3+}) complexes are widely used as contrast agents for MRI. Most Gd^{3+} 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. This enhancement is mediated, in part, by the direct interaction of water molecules (inner sphere) with the unpaired electrons of the paramagnetic metal ion Gd^{3+} [8]. The longitudinal and transverse relaxivity values R_1 and R_2 refer to the amount of increase in $1/T_1$ and $1/T_2$, respectively, per millimolar concentration of agent (often given as per mM Gd). Many MRI contrast agents for mapping of a particular tissue or organ have been reported. When the agents are distributed to a particular tissue, that tissue shows a brighter appearance in MRI [9, 10]. More 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. For example, MRI contrast agents which change the relaxation rate in the presence of an enzyme activity or Ca^{2+} or pH dependently have been reported [11–15]. On the basis of these reports, we have already developed the Gd^{3+} DTPA-bisamide complex GdL^c as a Zn^{2+}-sensitive MRI contrast agent (Figure 1). 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+} [16, 17]. In this report, we designed and synthesized a novel Zn^{2+}-sensitive MRI contrast agent: the Gd^{3+} DTPA-bisamide complex $(GdL^a)^{2-}$, which is a derivative of GdL^c (Figure 1), and measured the relaxation time T_1 of aqueous solutions of this compound.

Results and Discussion

The Gd^{3+} DTPA-bisamide complex GdL^c and the Gd^{3+} DTPA-amide-ethylester GdL^d have been reported previously (Figure 1) [18]. Solutions of GdL^c showed a dose-dependent change of the water proton R_1 relaxivity at pH 8.0 (0.1 M Tris buffer) in the presence of Zn^{2+} and had high selectivity for Zn^{2+} against Ca^{2+} and Mg^{2+} (Figure 2). The R_1 decreased dose dependently between 0 and 1.0 equivalents Zn^{2+}, reaching a minimum at 1.0 equivalent Zn^{2+}, then R_1 increased dose dependently between 1.0 and 2.0 equivalents. This complex was designed on the

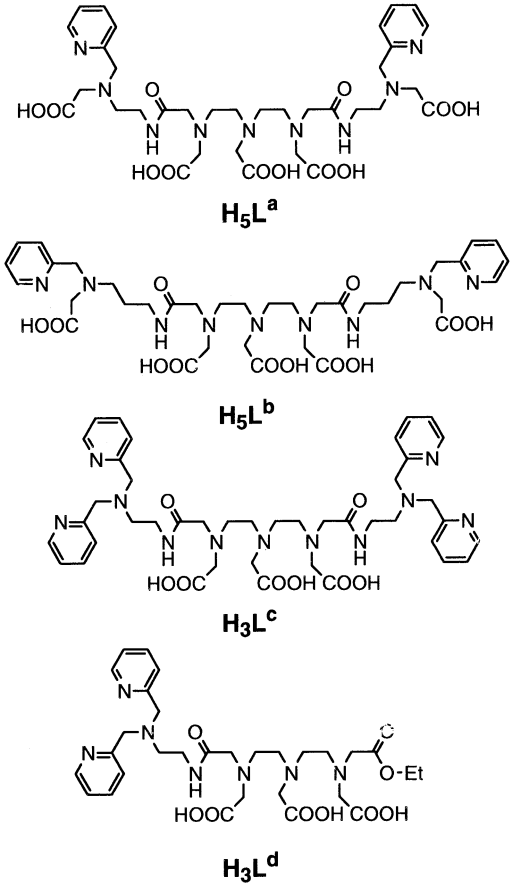

Figure 1. Structure of Synthesized DTPA-Bisamide Chelators

H_3L^c and H_3L^d were reported previously. H_5L^a and H_5L^b were newly synthesized.

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+} [16, 17], and reactions between primary amines and DTPA-bisanhydride have been widely used in the synthesis of DTPA-bisamide chelators [19]. On the other hand, the R_1 relaxivity of GdL^d solution in the presence of various concentrations of Zn^{2+} did not change with Zn^{2+} concentration. We previously suggested that, upon addition of Zn^{2+} between 0:1 and 1:1 Zn^{2+}/GdL^c molar ratio, water molecules bound directly to Gd^{3+} of GdL^c are displaced. However, upon the addition of Zn^{2+} between 1:1 and 2:1 Zn^{2+}/GdL^c molar ratio, Zn^{2+} and GdL^c form a 2:1 complex which bears the same number of water molecules as GdL^c only. This can be understood in terms of the Zn^{2+} coordination geometry, which is proposed to be as shown in Figure 3. Previously, this hypothesis was supported by measuring the UV-visible spectra and the R_1 relaxivity of GdL^d aqueous solution. In this report, we also measured the coldspray ionization (CSI) mass spectra of the 1:1 and 2:1 Zn^{2+}/GdL^c complexes [20–22]. In the mass spectrum of the 1:1 complex, $ZnGdL^c$ in H_2O:2% DMF (1 mM), three major ion peaks, m/z 531, 540, and 568, were clearly observed. The ions m/z 531, 540, and 568 obtained from $ZnGdL^c$ were assigned as

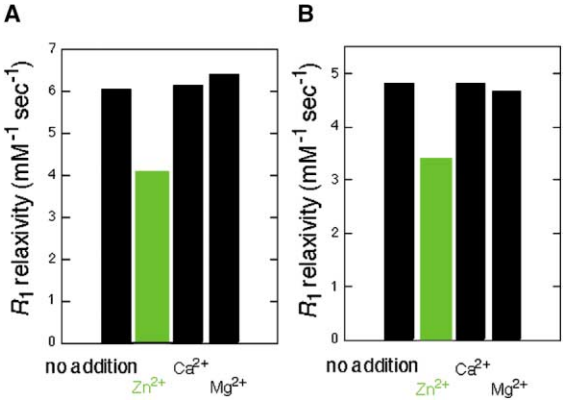

Figure 2. R_1 Relaxivity for Solutions of Gd^{3+} DTPA-Bisamide Complexes GdL^c and $(GdL^a)^{2-}$ upon Addition of Zn^{2+}, Ca^{2+}, or Mg^{2+}

R_1 relaxivity (mM^{-1} s^{-1}) of GdL^c (A) or $(GdL^a)^{2-}$ (B) was measured in 100 mM Tris buffer (pH 8.0) or 25 mM KMOPS buffer (pH 7.20), respectively, at 25°C. Cations were added as $ZnCl_2$, $CaCl_2$, or $MgCl_2$ (1 equivalent to Gd^{3+} DTPA-bisamide complexes, respectively).

$[ZnGdL^c]^{2+}$, $[ZnGdL^c(H_2O)]^{2+}$, and $[ZnGdL^c(DMF)]^{2+}$, respectively. The mass spectrum of the 2:1 complex, Zn_2GdL^c in H_2O:2% DMF (4 mM) displayed five major ion peaks at m/z = 433, 457, 481, 663, and 917, corresponding to the cations $[Zn_2GdL^c(ClO_4)(DMF)]^{3+}$, $[Zn_2GdL^c(ClO_4)(DMF)_2]^{3+}$, $[Zn_2GdL^c(ClO_4)(DMF)_3]^{3+}$, $[Zn_2GdL^c(ClO_4)_2]^{2+}$, and $[(Zn_2GdL^c)_2(ClO_4)_5]^{3+}$, respectively. These results support the above hypothesis on Zn^{2+} chelation stoichiometry.

The behavior of the R_1 relaxivity of GdL^c solution is problematic because the response peaks above 1 equivalent of Zn^{2+} are augmented and hence not monotonic. Briefly, for example, one cannot distinguish the R_1 values of solutions of GdL^c containing 0.3 and 1.5 equivalents of Zn^{2+}. Complex GdL^c contains two amides, which have weak chelating ability, and there is probably a steric repulsion effect of the four pyridines. On the assumption that this is so, we have designed and synthesized the Gd^{3+} DTPA-bisamide complex $(GdL^a)^{2-}$ as a Zn^{2+}-sensitive MRI contrast agent (Figure 1). The reasoning behind the design of $(GdL^a)^{2-}$ is as follows. The carboxylate substituent chelates Zn^{2+} strongly [23] and has a smaller steric repulsion than pyridyl. So, we hoped to obtain a monotonic change with this stronger chelator. Compound $(GdL^a)^{2-}$ was synthesized according to the reaction scheme shown in the Supplemental Data.

In characterizing $(GdL^a)^{2-}$, we observed that the water proton R_1 relaxivity of $(GdL^a)^{2-}$ solution had a Zn^{2+} dependence (Figure 4). The R_1 decreased dose dependently between 0 and 1.0 equivalents Zn^{2+}, reaching a minimum at 1.0 equivalent Zn^{2+}, then R_1 remained at a plateau with further increase of Zn^{2+}. The R_1 relaxivity of $(GdL^a)^{2-}$ solution decreased ~30% when 1.0 equivalent of Zn^{2+} was added. To provide further insight into this relaxation behavior, we examined the UV-visible spectra of $(GdL^a)^{2-}$ solution (300 μM) at pH 7.20 (25 mM KMOPS buffer) in the presence of various concentrations of Zn^{2+} (Figure 5). The absorbance between 220 and 300 nm changed linearly with increase of Zn^{2+} concentration up to 1:1 $Zn^{2+}/(GdL^a)^{2-}$ molar ratio, with iso-

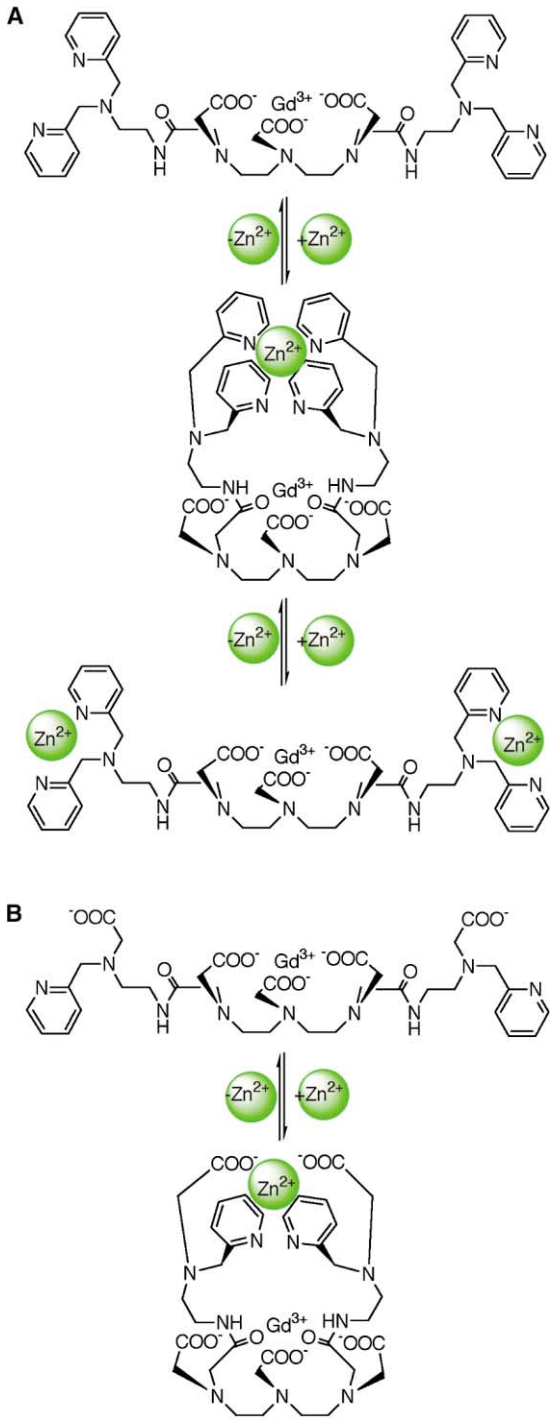

Figure 3. Proposed Mechanism for the Decrement of R_1 Relaxivity of Gd^{3+} DTPA-Bisamide Complex GdL^c and $(GdL^a)^{2-}$ in the Presence of Zn^{2+}

Schematic representation of GdL^c (A) and $(GdL^a)^{2-}$ (B) for the proposed conformational dependence of the structure in the presence and absence of Zn^{2+} is shown.

sbestic points at 254.5 and 272.5 nm, and remained at a plateau with further increase of Zn^{2+}. Moreover, in the CSI mass spectrum of $ZnGdL^a$ in H_2O/MeOH = 1/1 (2.5 mM), three major ion peaks, m/z 1033, 1102, and 1171,

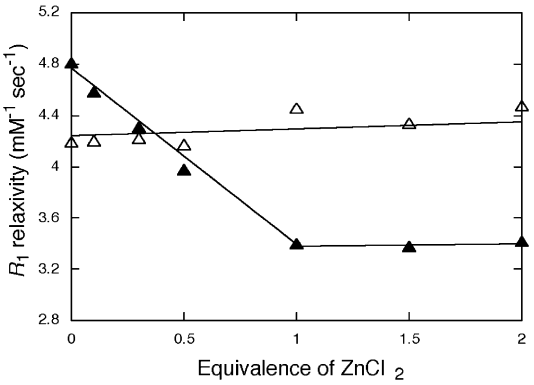

Figure 4. The Water Proton R_1 Relaxivity of $(GdL^a)^{2-}$ and $(GdL^b)^{2-}$

The water proton R_1 relaxivity of $(GdL^a)^{2-}$ (closed triangle) and $(GdL^b)^{2-}$ (open triangle) (B) are measured in the presence of various concentrations of Zn^{2+}: 0, 0.1, 0.3, 0.5, 1.0, 1.5, and 2.0 equivalents. The R_1 relaxivity of $(GdL^a)^{2-}$ and $(GdL^b)^{2-}$ were measured in 25 mM KMOPS buffer (pH 7.20).

were clearly observed (data not shown). These ions, m/z 1033, 1102, and 1171, were assigned as $[ZnGdL^a\text{-}K]^+$, $[(ZnGdL^a)_2K(ClO_4)\text{-}K_2]^{2+}$, and $[ZnGdL^aK(ClO_4)\text{-}K]^+$, respectively, supporting the idea that $(GdL^a)^{2-}$ forms a 1:1 complex with Zn^{2+}. From the above data, the behavior of the R_1 relaxivity of $(GdL^a)^{2-}$ solution can be rationalized as follows. When the $Zn^{2+}/(GdL^a)^{2-}$ molar ratio is between 0:1 and 1:1, Zn^{2+} and $(GdL^a)^{2-}$ form a 1:1 complex. However, even when the $Zn^{2+}/(GdL^a)^{2-}$ molar ratio exceeds one, Zn^{2+} and $(GdL^a)^{2-}$ do not form a 2:1 complex. We estimated that $(GdL^a)^{2-}$ in the $Zn^{2+}/(GdL^a)^{2-}$ 1:1 complex has fewer water molecules binding directly to Gd^{3+} than $(GdL^a)^{2-}$ in a Zn^{2+}-free solution, and this can be understood in terms of the Zn^{2+} coordination geometry, which is proposed to be as shown in Figure 3. The reason why Zn^{2+} and $(GdL^a)^{2-}$ do not form a 2:1 complex, whereas Zn^{2+} and GdL^c do form a 2:1 complex, may be as follows. Both $(GdL^a)^{2-}$ and GdL^c contain two amides,

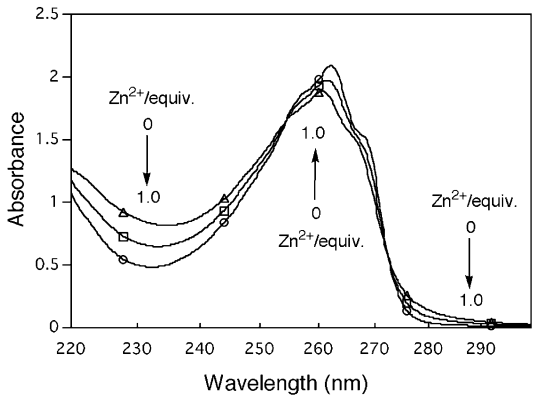

Figure 5. UV-Visible Spectra of $(GdL^a)^{2-}$ in the Presence of Various Concentrations of Zn^{2+}

UV-visible spectra of $(GdL^a)^{2-}$ (300 μM) in KMOPS (25 mM; pH 7.20) in the presence of various concentrations of Zn^{2+}: 0 equivalent (open triangle), 0.5 equivalent (open square), 1.0 equivalent (open circle). On the addition of Zn^{2+} between 1.0 and 3.0 equivalents, the absorbance spectra remained at a plateau.

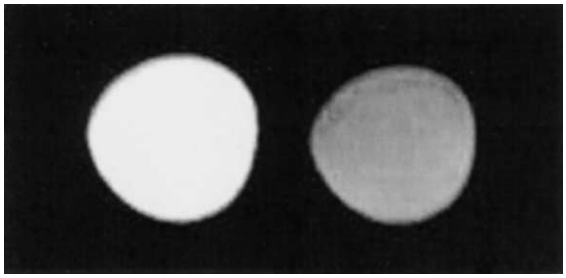

Figure 6. T_1-Weighted MR Images of $(GdL^a)^{2-}$ Solutions in the Presence and Absence of Zn^{2+}

T_1-weighted MRI contrast decreased as a result of Zn^{2+} chelating of $(GdL^a)^{2-}$. Images of a solution of $(GdL^a)^{2-}$ in the presence (right) and absence (left) of Zn^{2+} [right: 0.25 mM $(GdL^a)^{2-}$ and 0.25 mM Zn^{2+}, left: 0.25 mM $(GdL^a)^{2-}$] were obtained horizontally and are displayed. Both samples were dissolved in distilled water in small vials.

which have weak chelating ability. However, the carboxylate substituent, which L^a has, chelates Zn^{2+} strongly and has a smaller steric repulsion than the pyridyl substituent of L^c. Therefore, it can be considered that the $Zn^{2+}/(GdL^a)^{2-}$ 1:1 complex is more stable than the Zn^{2+}/GdL^c 1:1 complex, and $(GdL^a)^{2-}$ does not form a $Zn^{2+}/(GdL^a)^{2-}$ 2:1 complex, in contrast to GdL^c.

In MR images of $(GdL^a)^{2-}$ solution, the change in T_1 generated by the addition of Zn^{2+} [1 equivalent to $(GdL^a)^{2-}$] could be visualized (Figure 6). Complex $(GdL^a)^{2-}$ was dissolved in distilled water (0.25 mM). Zn^{2+} [1 equivalent to $(GdL^a)^{2-}$] was added to one sample but not to the other. The images displayed in Figure 6 reveal that the T_1-mediated contrast of $(GdL^a)^{2-}$ was altered by chelation of Zn^{2+} with $(GdL^a)^{2-}$.

For a Zn^{2+}-sensitive MRI contrast agent, high selectivity against Mg^{2+} and Ca^{2+} is crucial. Therefore, we examined the effects of Ca^{2+}, Mg^{2+}, Na^+, K^+, and H^+ on the R_1 relaxivity of $(GdL^a)^{2-}$ aqueous solution. There was no large effect of H^+ on the R_1 relaxivity between pH 6 and 8 in the absence of Zn^{2+} (data not shown). The R_1 curve exhibits no marked change and no pH dependence over the pH range of 6–8. No effect of 100 mM Na^+ or K^+ on the R_1 relaxivity of $(GdL^a)^{2-}$ solution was observed in the presence or absence of Zn^{2+}. The R_1 relaxivity of $(GdL^a)^{2-}$ solution upon the addition of 1 equivalent of Ca^{2+} or Mg^{2+} at pH 7.20 (25 mM KMOPS buffer) is illustrated in Figure 2, showing no change. Further, the R_1 relaxivity of $(GdL^a)^{2-}$ in 25 mM KMOPS buffer (pH 7.20) containing 5 mM Ca^{2+} or 5 mM Mg^{2+} showed no effects of Ca^{2+} or Mg^{2+} in the presence or absence of Zn^{2+} (data not shown). Compound $(GdL^a)^{2-}$ thus showed high selectivity for Zn^{2+} against Ca^{2+} and Mg^{2+}.

We also designed and synthesized the Gd^{3+} DTPA-bisamide complex $(GdL^b)^{2-}$ (Figure 1). Compound $(GdL^b)^{2-}$ was synthesized by using trimethylenediamine as a starting material, instead of ethylenediamine for $(GdL^a)^{2-}$. Compound $(GdL^b)^{2-}$ was synthesized according to the reaction scheme shown in Supplemental Data. The water proton R_1 relaxivity of $(GdL^b)^{2-}$ solution at pH 7.20 (25 mM KMOPS buffer) in the presence of various concentrations of Zn^{2+} did not change with Zn^{2+} concentration (Figure 4). We also measured the UV-visible spectra of $(GdL^b)^{2-}$ solution (300 μM) at pH 7.20 (25 mM KMOPS buffer) upon the addition of Zn^{2+} (data not shown). The absorbance spectrum of $(GdL^b)^{2-}$ solution changed similarly to that of $(GdL^a)^{2-}$ solution. The absorbance between 220 and 300 nm changed linearly with Zn^{2+} concentration up to 1.16:1 $Zn^{2+}/(GdL^b)^{2-}$ molar ratio and remained at a plateau with further increase of Zn^{2+} concentration. It can be considered from these results that $(GdL^b)^{2-}$ and Zn^{2+} form a complex, and the water accessibility of the chelated Gd^{3+} ion is not changed by this complexation. We presume that the difference between $(GdL^a)^{2-}$ and $(GdL^b)^{2-}$ is due to the linkage between amide and tertiary amine. The ethylene bonding is shorter than the trimethylene bonding and thus may result in exclusion of water molecules from Gd^{3+} upon Zn^{2+} coordination. We also examined the effects of Ca^{2+} and Mg^{2+} on the R_1 relaxivity of $(GdL^b)^{2-}$ aqueous solution. The R_1 relaxivity of $(GdL^b)^{2-}$ solution at pH 7.20 (25 mM KMOPS) showed no change, even when 1 equivalent of Ca^{2+} or Mg^{2+} was added.

These characteristics of the complexes are favorable for in vivo imaging of Zn^{2+} concentration changes, for example, by comparing signal intensity decay in a region of interest with and without various stimuli, given that the clearance of the agent itself should remain unchanged.

Significance

Previously, we designed and synthesized the Gd^{3+} DTPA-bisamide complex GdL^c as a Zn^{2+}-sensitive MRI contrast agent. For this agent, the decrease of the R_1 relaxivity upon addition of Zn^{2+} can be understood in terms of the Zn^{2+} coordination geometry, which is proposed to be as shown in Figure 3. This mechanism was supported by the UV-visible spectra, CSI mass spectra, and the experimental data of compound GdL^d. Here, we report the novel Gd^{3+} DTPA-bisamide complex $(GdL^a)^{2-}$, which was designed on the basis of our work on GdL^c. The R_1 relaxivity of $(GdL^a)^{2-}$ solution decreased in the presence of Zn^{2+} from 0:1 to 1:1 $Zn^{2+}/(GdL^a)^{2-}$ molar ratio and remained at a plateau upon further addition of Zn^{2+}. Moreover, compound $(GdL^a)^{2-}$ had high selectivity for Zn^{2+} against Ca^{2+} and Mg^{2+}. We also synthesized the Gd^{3+} DTPA-bisamide complex $(GdL^b)^{2-}$, which is an analog of $(GdL^a)^{2-}$. The R_1 relaxivity of $(GdL^b)^{2-}$ solution at pH 7.20 (25 mM KMOPS) in the presence of various concentrations of Zn^{2+} did not change, and it also showed no change upon addition of Ca^{2+} or Mg^{2+}. Thus, these novel compounds, $(GdL^a)^{2-}$ and GdL^c, are excellent candidates for incorporation into sensors designed for selective detection of Zn^{2+} in biological applications. The molecule $(GdL^a)^{2-}$ may also have the potential to form responsive luminescent lanthanide complexes (Eu, Tb) [24, 25].

Experimental Procedures

Materials

DTPA-bisanhydride was purchased from Aldrich Chemical Co. Inc., USA. All other reagents were purchased from either Tokyo Kasei Kogyo Co., Ltd., Japan, or Wako Pure Chemical Industries, Ltd., Japan. All solvents were used after distillation.

Instruments

^1H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-LA300. Mass spectra were measured with a JEOL JMS-DX 300 mass spec-

trometer (EI^+) or a JEOL JMS-700T mass spectrometer (FAB^+). UV-visible spectra were obtained on a Shimadzu UV-1600. HPLC purification was performed on a reverse-phase column (GL Sciences, Inertsil Prep-ODS 30 mm × 250 mm) fitted on a Jasco PU-1587 System. MR images were obtained on a Varian INOVA200 (4.7 T): Spin Echo Tr/Te = 300/9.4 ms, Matrix 256*128, NEX = 4, FOV = 6*6 cm, slice thickness = 2 mm.

Preparation of Solutions

The gadolinium complexes $K_2(GdL^a)$ and $K_2(GdL^b)$ were prepared by a modification of the literature procedures [26, 27]. Ligand H_5L^a or H_5L^b was dissolved in distilled water. To this solution, 1 N KOH was added until the pH reached 7.0. An equal molar amount of an aqueous solution of $GdCl_3$-$6H_2O$ was then added slowly, while a pH of 7 was maintained by further addition of 1 N KOH. The solution was kept at room temperature under vigorous stirring for 1 hr until the pH was stabilized. The compound was then purified by precipitation by the addition of acetone. $K_2(GdL^a)$: a colorless solid; HRMS (FAB^+) Calcd for ($M\text{-}K^+ + 2H^+$) m/z 969.2150, found 969.2177. HPLC analysis: retention time, 10.5 min (purity, 98.4% integrated intensity); Inertsil ODS-3 4.6 mm × 250 mm (GI Sciences); eluent, a 20 min linear gradient, from 0% to 80% solvent B (solvent A, 0.1 M Et_3N-AcOH; solvent B, acetonitrile-H_2O [80:20]); flow rate, 1.0 ml; UV, 254 nm. $K_2(GdL^b)$: a colorless solid; HRMS (FAB^+) Calcd for ($M\text{-}2K^+ + 3H^+$) m/z 959.2907, Found 959.2847. HPLC analysis: retention time, 10.7 min (purity, 98.3% integrated intensity); Inertsil ODS-3 4.6 mm × 250 mm (GI Sciences); eluent, a 20 min linear gradient, from 0% to 80% solvent B (solvent A, 0.1 M Et_3N-AcOH; solvent B, acetonitrile-H_2O [80:20]); flow rate, 1.0 ml; UV, 254 nm. All samples were assessed for the absence of free gadolinium ions using xylenol orange as the indicator. The solutions of the gadolinium complex for the relaxation time measurement were prepared by dissolving the above solid compound in a buffer solution.

Relaxation Time Measurement

The relaxation time T_1 of aqueous solutions of the Gd^{3+} complex $(GdL^a)^{2-}$ or $(GdL^b)^{2-}$ was measured in KMOPS buffer (25 mM; pH 7.20) by using the standard inversion-recovery procedure (JEOL JNM-LA300, 25°C). The R_1 relaxivity of these compounds was determined from the slope of the plot of $1/T_1$ versus $[(GdL^a)^{2-}]$ or $[(GdL^b)^{2-}]$ (0.4, 0.6, 0.8, 1.0 mM). The buffered Gd^{3+} complex $[(GdL^a)^{2-}$ or $(GdL^b)^{2-}]$ solution was allowed to equilibrate for at least 10 min after the addition of $ZnCl_2$, $CaCl_2$, or $MgCl_2$ aqueous stock solution.

CSI Mass Measurement

CSI mass spectra were measured with a JEOL JMS-700 mass spectrometer. The desolvating plate temperature was 23°C. In CSI mass measurement, $Zn(ClO_4)_2$ aqueous solution was used as Zn^{2+} stock solution. In the mass spectrum measurement, Zn^{2+} and Gd^{3+} complexes were mixed in H_2O:2% DMF (1 mM), H_2O:2% DMF (4 mM), and H_2O/MeOH = 1/1 (2.5 mM), corresponding to solutions of $ZnGdL^c$, Zn_2GdL^c, and $ZnGdL^a$, respectively.

Supplemental Data

Experimental details for the synthesis of $\mathbf{H_5L^a}$ and $\mathbf{H_5L^b}$. CSI mass spectra for 1:1 complex; $ZnGdL^c$ and 2:1 complex; Zn_2GdL^c. Please write to chembiol@cell.com for a PDF.

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