Magnetic Resonance Imaging

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Selective Sensing of Zinc Ions with a PARACEST Contrast Agent**

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Divalent zinc, the second most abundant transition-metal ion in seawater and in humans, is an essential integral component of numerous functional proteins involved in a wide range of physiological systems.[1–4] Zinc ions also bind to many membrane receptors, transporters, and channels, and modulate their activity.[5] Therefore, it is not surprising that zinc deficiency affects many organs, including the digestive, immune, and neuronal systems.[4,6,7] Free Zn^{II} ions are released from loosely bound metal–protein complexes found in selected cell types and cell organelles in brain and other tissues.[8] Various indirect evidence indicates that the concentration of free Zn^{II} ions ranges from 10^{-12} to 10^{-5} M in undifferentiated mammalian cells to approximately 0.3 mM in hippocampal-nerve synaptic vesicles.[9,10] Understanding the importance and control of the release of Zn^{II} ions in living cells will require suitable detection and imaging reagents.[11–13]

In the past two decades, magnetic resonance imaging (MRI) has grown into one of the most powerful techniques in diagnostic medicine and biomedical research. One of the first designs for sensing Zn^{II} ions by MRI was based on a Gd^{III} complex that showed an approximately 30% decrease in water relaxivity in the presence of Zn^{II} ions.[14] The ligand in this complex was prepared by treating diethylenetriaminepentaacetic acid (dtpa) bisanhydride with *N*,*N*-bis(2-pyridylmethyl)ethylenediamine to form a ligand having both a Gd^{III} site and a Zn^{II} site, which is presumably formed by the four pyridine donors situated above the water molecule bound to the Gd^{III} ion. On the basis of recent success at creating imaging agents that respond to changes in water-exchange

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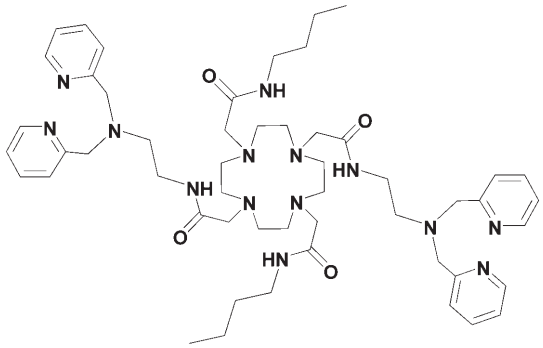
PARACEST = paramagnetic chemical exchange saturation transfer.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

rates rather than relaxation rates, we decided to test the hypothesis that a chemical exchange saturation transfer (CEST) agent may be advantageous for measuring the levels of free Zn^{II} ions in tissues.[15–18] Here, we report a first-generation paramagnetic CEST agent, [Eu(dotampy)] (dotampy = 1,7-bis(*N*,*N*-bis(2-pyridylmethyl)aminoethylcarbamoylmethyl)-4,10-bis(butylcarbamoylmethyl)-1,4,7,10-tetraazacyclododecane, **1**), as a novel sensor for Zn^{II} ions.



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The CEST profile of [Eu(dotampy)] is typical of a system exhibiting intermediate to slow water exchange between a Eu^{III}-bound water species with a resonance at $\delta = 50$ ppm and that of bulk water at $\delta = 0$ ppm (Figure 1). Somewhat surprisingly, addition of Zn^{II} ions to this complex in buffered solution at pH 7.1 resulted in broadening of the resonances of both the Eu^{III}-bound water molecule ($\delta = 50$ ppm) and the bulk water ($\delta = 0$ ppm), suggestive of more rapid water exchange. As a result, the most dramatic changes that are observed in the CEST spectrum upon addition of Zn^{II} ions occur in the region between these two exchanging water signals (15–30 ppm). This effect was even more dramatic when Zn^{II} ions were added to [Eu(dotampy)] at pH 8.0 (Figure 1). In this case, water (or proton) exchange is so fast that the entire CEST signal at $\delta = 50$ ppm disappeared upon addition of Zn^{II} ions. The sensitivity of CEST to base catalysis was not anticipated for this system nor was the origin of this effect immediately apparent. One possibility is that if a Zn^{II} ion forms a mononuclear complex by coordinating the four pyridine donors, such binding could introduce "strain" around the Eu^{III} coordination complex and perhaps alter the geometry of [Eu(dotampy)] from a square-antiprism (SAP) structure with intermediate-to-slow water exchange to a twisted-square-antiprism (TSAP) structure with faster water exchange. A high-resolution ^{1}H NMR spectrum of [Eu(dotampy)] shows a single, highly shifted resonance near $\delta = 25$ ppm that is characteristic of the four axial macrocyclic protons in a SAP coordination geometry (Figure 2) and no indication of even a small population of a TSAP coordination isomer.[19–22] Addition of Zn^{II} ions to [Eu(dotampy)] resulted in a slight shift of this resonance to low field and a broadening into three or more nearly resolved signals. This result indicates that [Eu(dotampy)] is less symmetric in the presence of Zn^{II} ions but, again, no new resonances were present in the region between $\delta = 12$ and 15 ppm, as would be anticipated if

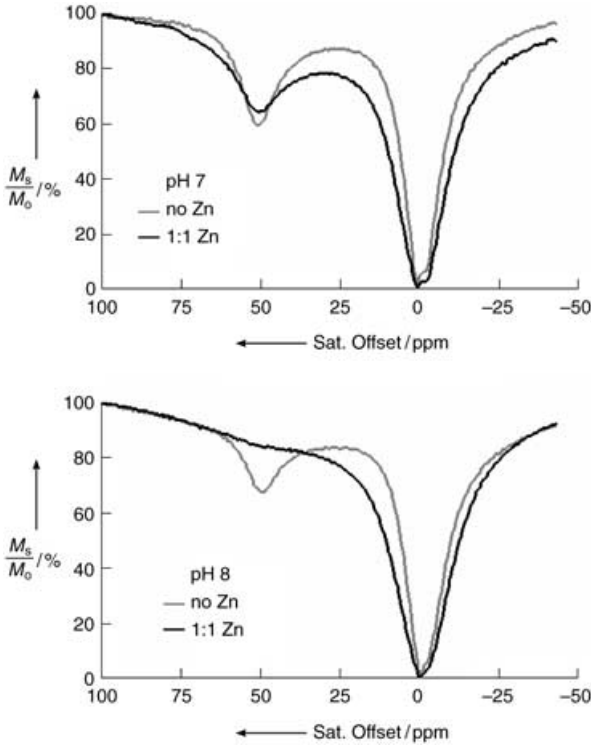


Figure 1. Top: Z-spectra of 20 mM [Eu(dotampy)] in the absence (gray line) and in the presence (black line) of 20 mM Zn^{II} ions in 1,4-piperazinebis(ethanesulfonic acid) (PIPES) buffer (100 mM; pH 7.1) at 25 °C. Bottom: Z-spectra of 20 mM [Eu(dotampy)] in the absence (gray line) and in the presence (black line) of 20 mM Zn^{II} ions in 2,4,6-tris[(dimethylamino)methyl]phenol (DMP) buffer (100 mM; pH 8.0) at 25 °C. The CEST profiles were collected by using a 2-s frequency-selective hard pulse ($B_1 = 1000$ Hz) followed by a non-frequency-selective 60° read-out hard pulse. M_s = bulk-water magnetization when a presaturation pulse is applied; M_0 = equilibrium magnetization without presaturation.

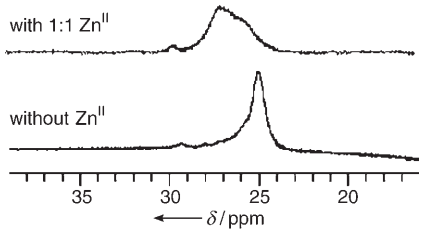
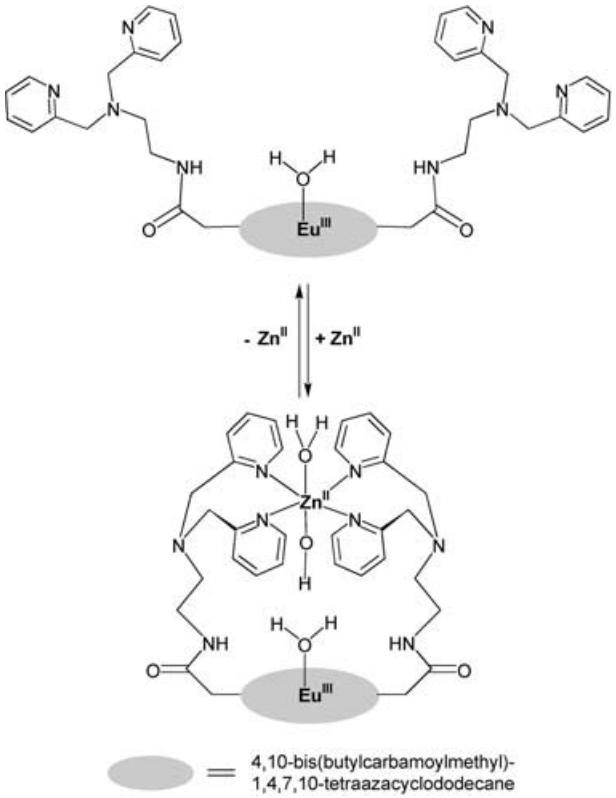


Figure 2. Low-field portion of a ^{1}H NMR spectrum (400 MHz, 25 °C) of 100 mM [Eu(dotampy)] in the absence (upper) and in the presence (lower) of 100 mM of Zn^{II} ions at pH 6.95.

there had been a change in structure from SAP to TSAP.[23] Thus, the enhanced water-exchange characteristics of the [Eu(dotampy)]–Zn^{II} adduct cannot be traced to a switch in the coordination geometry of the complex.

A second possible reason why water (or proton) exchange is faster in the [Eu(dotampy)]–Zn^{II} adduct is that the Zn^{II} ion may itself have a coordinated water molecule that is partially deprotonated at these pH values, thereby positioning a $Zn^{II}OH$ species near the Eu^{III}-bound water molecule (a hypothetical model is illustrated in Scheme 1). Such a species



Scheme 1. Hypothetical structures of [Eu(dotampy)] in the presence and absence of Zn^{II} ions.

could act to catalyze prototropic exchange between the Eu^{III}-bound water molecule and the bulk solvent. Other ligand systems containing two *N*,*N*-bis(2-pyridylmethyl)amine (dpa) units frequently form dinuclear complexes with Zn^{II} ions, in which each dpa arm provides three nitrogen donors and the fourth coordination site is occupied by a bridging hydroxide ion.[24–27] However, all of our data on this system (potentiometry, spectroscopy, and CEST titrations, see below) show that a mononuclear species is formed between [Eu(dotampy)] and Zn^{II} ions under all experimental conditions examined. Molecular modeling studies of the [Eu(dotampy)]–Zn^{II} adduct suggest that the positions of the tertiary amines on each side-arm are constrained by the size of bridging {Eu(dotam)} moiety and are therefore not likely to be coordinated to the Zn^{II} ion. This would leave only the four pyridyl nitrogen atoms and perhaps one or two water molecules available to form a complex with the Zn^{II} ions. Attempts to isolate crystals of [Eu(dotampy)]–Zn^{II} suitable for X-ray crystallographic analysis to confirm the structure illustrated in Scheme 1 have been unsuccessful.

Potentiometric titrations of [Eu(dotampy)] were performed in the absence and presence of Zn^{II} ions to examine whether either a Eu^{III}- or Zn^{II}-coordinated water molecule can be titrated over the pH range employed in the CEST studies (see Supporting Information). In the absence of Zn^{II} ions, [Eu(dotampy)] displays three stepwise protonation constants ($\log K_a = 3.60 \pm 0.03$, 5.75 ± 0.03, 11.29 ± 0.02) over the usual pH range covered by potentiometry. The $\log K_a$ values of 3.60 and 5.75 are consistent with protonations occurring at the two tertiary nitrogen atoms on the side-arms whereas the $\log K_a$ value of 11.29 likely corresponds to deprotonation of the Eu^{III}-bound water molecule.[28] A fit of the titration data collected in the presence of a stoichiometric amount of Zn^{II} ions gave a stability constant of $\log K_{st} = 7.59 \pm 0.02$ for the [Eu(dotampy)]–Zn^{II} adduct. The potentiometric data also provided evidence for formation of a $Zn^{II}OH$ species above approximately pH 6 which could act as a catalyst for the exchange of protons between the Eu^{III}-bound water molecule and the bulk solvent. A similar mechanism has been proposed for proton catalysis by the appended phosphonate groups in [Gd(dota-4amp)] (dota-4amp = 1,4,7,10-tetrakis(*N*-methylphosphate)-1,4,7,10-tetraazacyclododecane).[29] Spectrophotometric titrations of [Eu(dotampy)] at pH 7.1 confirmed this potentiometric binding model. The absorbance between 225 and 285 nm changed linearly with an increase in the Zn^{II} concentration up to a [Eu(dotampy)]/Zn^{II} mole ratio of 1:1, with isosbestic points at 248, 267, and 272 nm, but then remained unchanged with further increases in Zn^{II} concentration. A Job plot of these binding data provided further evidence for the formation of a 1:1 complex (see Supporting Information). Finally, additional support is given by the Z-spectra collected after incremental additions of Zn^{II} ions at pH 7 (Figure 3); the CEST effect was

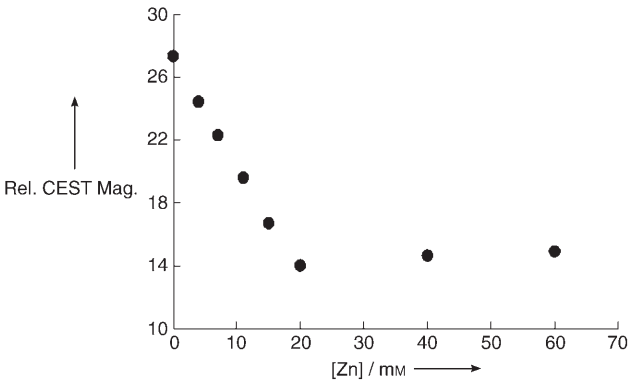


Figure 3. A plot of relative CEST magnitude versus Zn^{II} concentration for 20 mM [Eu(dotampy)] in PIPES buffer (100 mM; pH 7).

quenched linearly with addition of up to one equivalent of Zn^{II} ions, but then relatively small changes were observed upon further increases in the Zn^{II} concentration. This observation demonstrates that a 1:1 complex is formed both at 20 mM (the concentrations used to collect the CEST spectra) and 0.1–2.0 mM (the concentrations used in the potentiometric and UV/Vis experiments).

To demonstrate that Zn^{II} binding can be sensed in a CEST imaging experiment, a phantom consisting of four plastic tubes (i.d. 4 mm), each containing 20 mM [Eu(dotampy)] and different amounts of Zn^{II} ions, was prepared. Images were acquired after applying a selective Gaussian-shaped presaturation pulse at $\delta = 50$ ppm followed by a spin-echo gradient imaging sequence. A second image was collected after presaturation at $\delta = 25$ ppm and CEST images were generated by pixel-by-pixel subtraction of the two SE images. The image intensities (Figure 4) show a clear gradation that

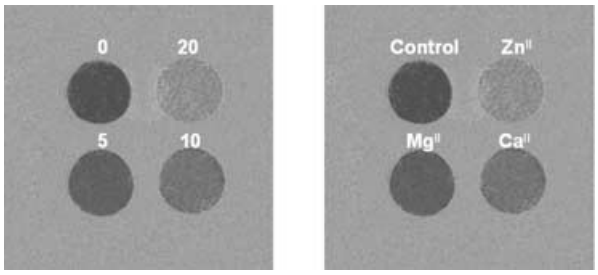


Figure 4. Left: CEST images of phantoms containing 20 mM [Eu-(dotampy)] and either 0, 5, 10, 20 mM Zn^{II} ions. Right: CEST images of phantoms containing 20 mM [Eu(dotampy)] plus stochiometric amounts of Zn^{II}, Mg^{II} or Ca^{II} ions. PIPES (100 mM) was used to buffer the solution at pH 7.1. A 1 s (left image) or 0.5 s (right image) frequency-selective Gaussian shaped pulse ($B_1 = 1000$ Hz) was used to presaturate the sample prior to applying spin-echo (SE) imaging pulses. The CEST images represent the intensity difference between the SE images for saturation at $\delta = 50$ ppm and $\delta = 25$ ppm from the bulk water. Other imaging parameters included: TR/TE 1300 ms/13 ms, thickness 2 mm, FOV 30×30 mm, data matrix 128×128, 4 dummy scans, each image was the average of 2 scans. TR = repetition time; TE = echo time; FOV = field of view.

parallels the Zn^{II} concentrations in each sample. To test for selectivity, CEST images were also collected by replacement of Zn^{II} ions with either Mg^{II} or Ca^{II} ions. Those ions could not be detected by CEST imaging.

Finally, one needs to consider the practical use of this system for imaging of Zn^{II} ions in a real biological system. From the CEST results illustrated above, one can estimate that a minimum of approximately 2 mM agent would be needed to satisfy the sensitivity limits for detection of Zn^{II} ions by MRI. Given that the CEST MRI signal would only detect changes in total Zn^{II} concentration covering the range from 0.4 to 1.6 mM (as set by the sensitivity limits of MRI) and that $\log K_{st} = 7.59 \pm 0.02$ for the [Eu(dotampy)]–Zn^{II} system, one can then estimate that the effective sensitivity range for this sensor in detecting changes in concentration of "free" Zn^{II} ions varies from 5 nM to 0.12 μM—a reasonable concentration range for levels of free Zn^{II} ions in tissues. Of course, the practicality of this detection method remains to be proven, but the idea—detection of the catalysis of proton exchange by a nearby coordinated hydroxide group, as demonstrated in the current Zn^{II} system—serves as an attractive model for the design other responsive PARACEST imaging agents.

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