

## Lanthanide Contrast Agents

International Edition: DOI: 10.1002/anie.201511649  
German Edition: DOI: 10.1002/ange.201511649Oxidative Conversion of a Europium(II)-Based  $T_1$  Agent into a Europium(III)-Based paraCEST Agent that can be Detected In Vivo by Magnetic Resonance Imaging

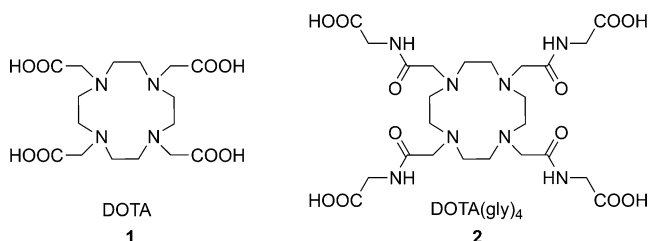
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**Abstract:** The  $\text{Eu}^{\text{II}}$  complex of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) tetra(glycinate) has a higher reduction potential than most  $\text{Eu}^{\text{II}}$  chelates reported to date. The reduced  $\text{Eu}^{\text{II}}$  form acts as an efficient water proton  $T_1$  relaxation reagent, while the  $\text{Eu}^{\text{III}}$  form acts as a water-based chemical exchange saturation transfer (CEST) agent. The complex has extremely fast water exchange rate. Oxidation to the corresponding  $\text{Eu}^{\text{III}}$  complex yields a well-defined signal from the paraCEST agent. The time course of oxidation was studied in vitro and in vivo by  $T_1$ -weighted and CEST imaging.

Gadolinium complexes are commonly used as contrast agents in magnetic resonance imaging (MRI). They generate image contrast by shortening the longitudinal ( $T_1$ ) relaxation time of bulk water protons. The efficiency of a  $T_1$  agent is defined by  $r_1$  relaxivity, which is dependent on several parameters, including the metal bound water exchange rate, rotational correlation time of the complex, and the electronic relaxation time of the metal ion.<sup>[1]</sup> An alternative to the  $\text{Gd}^{3+}$ -based contrast agents is the isoelectronic  $\text{Eu}^{2+}$  ion.<sup>[2]</sup> Both ions have a  $4f^7$  electron configuration and a symmetric  $^8S_{7/2}$  ground state but  $\text{Eu}^{2+}$  complexes in general display much faster water exchange rates and faster electronic relaxation times.<sup>[2]</sup> Analogous complexes of  $\text{Eu}^{2+}$  and  $\text{Gd}^{3+}$  can produce similar relaxivity values at lower fields while at higher fields the  $\text{Eu}^{2+}$  complexes tend to be more efficient.<sup>[3]</sup>

The  $\text{Eu}^{2+}$  aqua ion is extremely sensitive to oxidation as demonstrated by its strongly negative reduction potential ( $-585$  mV vs.  $\text{Ag}^+/\text{AgCl}$ ).  $\text{Eu}^{2+}$  poly(amino carboxylate) chelates usually have lower reduction potential, although some  $\text{Eu}^{2+}$  cryptates have been reported to be more stable towards oxidation.<sup>[4,5]</sup>  $\text{Eu}^{2+}$  complexes have been proposed as redox sensitive  $T_1$  agents,<sup>[3,5,6]</sup> because oxidation of  $\text{Eu}^{2+}$  leads to the formation of weakly paramagnetic  $\text{Eu}^{3+}$ , which has little impact on water proton  $T_1$ . The  $\text{Eu}^{3+}$  ion however

generates a moderately strong magnetic dipolar field that produces large hyperfine shifts of NMR signals of nearby ligand protons. While  $\text{Eu}^{3+}$  complexes are very poor  $T_1$ -shortening agents,  $\text{Eu}^{3+}$  DOTA tetra(amides) (Figure 1)



**Figure 1.** Structure of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid **1** and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid tetra(glycine amide) **2**.

belong to a conceptually different class of MRI contrast agents, known as paraCEST agents that alter image contrast by transferring selectively saturated spins from a highly shifted small pool of proton spins (metal-bound water) to the bulk water pool.<sup>[7]</sup> Chemical exchange saturation transfer (CEST) occurs when the proton exchange rate between the two pools ( $k_{\text{ex}}$ ) is in the slow-to-intermediate exchange regime ( $k_{\text{ex}} \leq \Delta\omega$ , where  $\Delta\omega$  is the chemical shift difference between the two pools). A redox responsive liposomal  $\text{Eu}^{2+}/\text{Eu}^{3+}$  system was recently reported that showed  $T_1$  shortening effect and lipoCEST effect ( $\Delta\omega \approx 1$  ppm) originating from the exchange between the water protons inside the liposomes and the bulk water protons not associated with the liposomes. Upon oxidation, the  $T_1$  enhancement disappeared while the lipoCEST remained unaffected. The CEST effect in this system is not due to the formation of a  $\text{Eu}^{3+}$  complex.<sup>[8]</sup>

$\text{Eu}^{2+}$  complexes have several orders of magnitude faster water exchange rates in comparison to the corresponding  $\text{Eu}^{3+}$  complexes and with a suitable ligand system, may offer a unique opportunity in the design of redox responsive MR agent that shortens  $T_1$  in the reduced state and produces a CEST signal in the oxidized state. In the present work we show that  $\text{Eu}^{2+}\mathbf{2}$  is an efficient  $T_1$  shortening agent because of the rapid water exchange of the  $\text{Eu}^{2+}$  bound water, but upon oxidation it turns into the well-known paraCEST agent,  $\text{Eu}^{3+}\mathbf{2}$ , which has slow water exchange kinetics. Merbach suggested in 2003 that the redox stability of  $\text{Eu}^{2+}\mathbf{1}$  could be increased by substituting nitrogen containing donor groups for the carboxylate side-arms.<sup>[3,6]</sup> Here, we also show that

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$\text{Eu}^{2+}\mathbf{2}$  indeed has significantly improved redox stability compared to  $\text{Eu}^{2+}\mathbf{1}$ .

The CEST effect can be expressed as a decrease in total bulk water signal intensity, and assuming complete and instantaneous saturation of the bound water peak, the net magnetization of water protons at steady-state is given by the following equation:

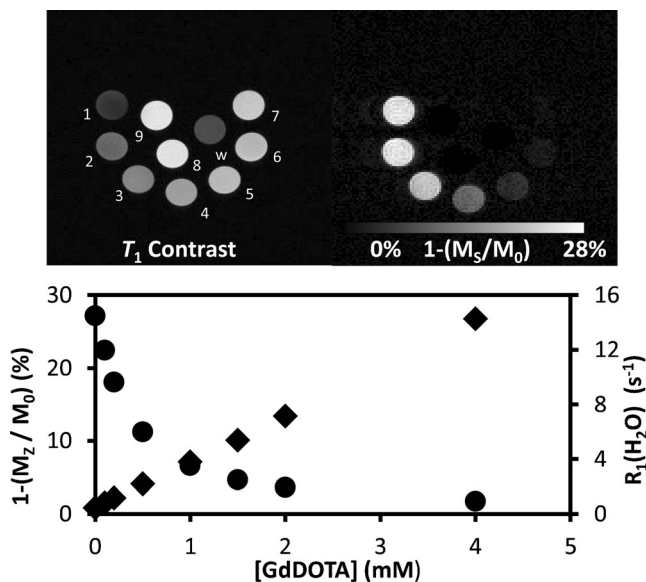
$$\text{CEST effect (\%)} = 1 - \left( \frac{M_s}{M_0} \right) = 100 \left( 1 + \frac{cqT_1}{111\tau_M} \right)^{-1} \quad (1)$$

where  $c$  is the concentration of the agent,  $q$  is the number of protons per agent, 111 represents the molar concentration of bulk water protons,  $T_1$  is the longitudinal (spin-lattice) relaxation time of bulk water, and  $\tau_M$  is the lifetime of the exchanging proton ( $\tau_M = 1/k_{\text{ex}}$ ).<sup>[7]</sup> Thus, the magnitude of the CEST effect is dependent on both the agent concentration and the bulk water  $T_1$ . Obviously, as  $\text{Eu}^{2+}\mathbf{2}$  are oxidized, the  $T_1$  shortening effect of  $\text{Eu}^{2+}$  will diminish while the paraCEST agent concentration will increase over the course of the reaction. To study the dependence of CEST on  $T_1$ , we designed a model experiment in which the paraCEST agent  $\text{Eu}^{3+}\mathbf{2}$  were mixed with varying concentration of the  $T_1$  shortening agent  $\text{Gd}^{3+}\mathbf{1}$  (Supporting Information, Table S1). Figure 2 shows that the proton relaxation rate ( $R_1 = 1/T_1$ ) of bulk water protons increases with increasing  $[\text{Gd}^{3+}\mathbf{1}]$  while the CEST signal from the paraCEST agent diminishes. At the two extremes of  $[\text{Gd}^{3+}\mathbf{1}]$ , the images are dominated by either CEST (when  $[\text{Gd}^{3+}\mathbf{1}] = 0$ ) or  $T_1$  (when  $[\text{Gd}^{3+}\mathbf{1}] = 4$  mM) but

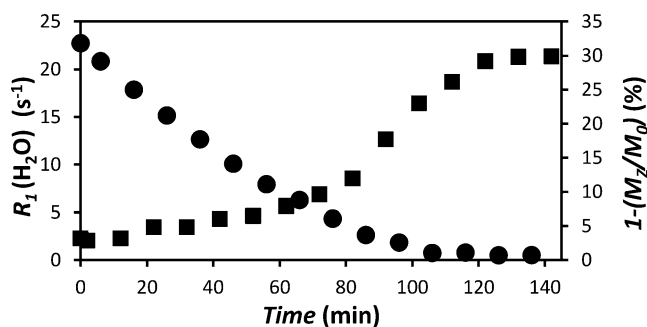
there is a range of  $\text{Gd}^{3+}$  concentrations (samples 4, 5, and 6) where both CEST and  $T_1$  enhancement contribute to the signal. The CEST signal was  $< 10\%$  when  $R_1 = 5 \text{ s}^{-1}$ . From the fitting of the  $T_1$  and CEST intensities to Equation (1), a bound water residence lifetime ( $\tau_M$ ) of 410 ms was obtained for  $\text{Eu}^{3+}\mathbf{2}$  at  $19^\circ\text{C}$ , in agreement with the  $\tau_M$  value determined by other methods.<sup>[9]</sup> This same phenomenon, the sensitivity of CEST to water proton  $T_1$ , formed the basis of a recently reported redox-sensitive paraCEST agent.

$\text{Eu}^{2+}$  complexes of **1** and **2** were conveniently prepared by directly reacting the ligands with commercially available  $\text{EuCl}_2$  under oxygen free conditions.<sup>[10]</sup> The relaxivity of  $\text{Eu}^{2+}\mathbf{2}$  was measured as  $3.2 \text{ mM}^{-1} \text{ s}^{-1}$  at 9.4 T and 1 T (Supporting Information, Figures S1 and S2). The  $\text{Eu}^{2+}$  bound water exchange rate as estimated by fits of variable temperature  $^{17}\text{O}$  NMR water linewidth data to theory was  $k_{\text{ex}} = 0.21 \times 10^9 \text{ s}^{-1}$  for  $\text{Eu}^{2+}\mathbf{2}$  and  $k_{\text{ex}} = 0.63 \times 10^9 \text{ s}^{-1}$  for  $\text{Eu}^{2+}\mathbf{1}$  in 20 % dioxane – 80 % water solutions (Supporting Information, Figures S3, S4 and Tables S2–S4), which are in the range of previously reported values.<sup>[3,6,11,12]</sup> It is worth noting that the water exchange for  $\text{Eu}^{2+}\mathbf{2}$  is nearly the same as that of  $\text{Eu}^{2+}\mathbf{1}$ . This indicates that the glycinate amide side-chains in  $\text{Eu}^{2+}\mathbf{2}$  do not affect the water exchange rate in comparison to the corresponding  $\text{Eu}^{3+}$  complexes where the difference between carboxylate and amide donor ligands is typically three orders of magnitude.<sup>[1,2]</sup> Unlike  $\text{Gd}^{3+}$  complexes, the  $r_1$  value of  $\text{Eu}^{2+}\mathbf{2}$  did not decrease significantly at high field, in agreement with previously published data for other  $\text{Eu}^{2+}$  complexes. The redox stability of  $\text{Eu}^{2+}\mathbf{2}$  was studied by cyclic voltammetry. The reduction potential measured for the  $\text{Eu}^{2+}\mathbf{2}/\text{Eu}^{3+}\mathbf{2}$  redox couple was found to be  $-226 \text{ mV}$  vs.  $\text{Ag}^+/\text{AgCl}$  electrode (Supporting Information, Figure S5), which is far less negative than that of  $\text{Eu}^{2+}\mathbf{1}$  ( $-1135 \text{ mV}$ ), or than the  $\text{Eu}^{2+}$  aqua ion ( $-585 \text{ mV}$  against  $\text{Ag}^+/\text{AgCl}$  electrode).<sup>[3]</sup> The rates of conversion of  $\text{Eu}^{2+}\mathbf{2}$  and  $\text{Eu}^{2+}\mathbf{1}$  to their respective  $\text{Eu}^{3+}$  complexes were also investigated by NMR by measuring the decay of the relaxation rate of the bulk water in a sealed NMR tube under  $\text{N}_2$  atmosphere at 9.4 T (Supporting Information, Figures S6 and S7). The measured half-lives ( $t_{1/2}$ ), 19 and 7 days, respectively, also show that  $\text{Eu}^{2+}\mathbf{2}$  is the most stable cyclen-based  $\text{Eu}^{2+}$  complex reported so far. Similar stabilizing effect of the charge neutral amide group was reported for  $\text{Eu}^{2+}$  complexes of 1,10-diaza-18-crown-6 derivatives in which picolinamide pendant arms were substituted for picolinate groups.<sup>[13]</sup>

Next, a fresh solution of  $\text{Eu}^{2+}\mathbf{2}$  was prepared in a 6 mm tall vessel and exposed to air while both  $T_1$  and CEST were measured as a function of time by imaging a slice 4 mm below the surface of the sample (Figure 3). As shown, the bulk water  $R_1$  decreased steadily for about 100 minutes in this slice reflecting oxidation of  $\text{Eu}^{2+}$  to  $\text{Eu}^{3+}$ . The CEST signal increased following a sigmoid curve with an inflection point at around 100 min reaching 10% at around  $6 \text{ s}^{-1}$  bulk water relaxation rate in agreement with the results shown in Figure 2. These data reveal that relaxation of bulk water protons limits the intensity of the CEST signal and at about 80 min, the  $T_1$  relaxation is slow enough for CEST to become efficient. As expected, the rate of oxidation depends heavily on the slice selected. For example, the  $T_1$  enhancement in



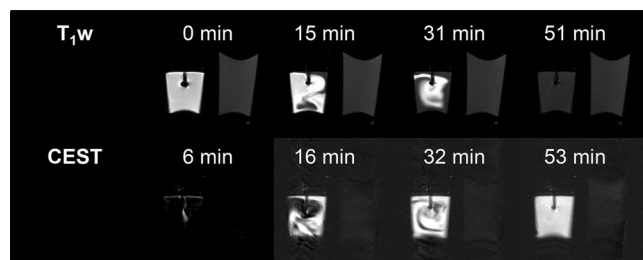
**Figure 2.** Top: Correlation between relaxation rates (♦) and the CEST effect (●) in a mixture of  $\text{Gd}^{3+}\mathbf{1}$  and  $\text{Eu}^{3+}\mathbf{2}$ . Each circle in the image represents a separate sample.  $[\text{Eu}^{3+}\mathbf{2}]$  in each sample was 10 mM while  $[\text{Gd}^{3+}\mathbf{1}]$  ranged from 0 to 4 mM. Vial 9 contained 8 mM  $\text{Gd}^{3+}\mathbf{1}$  alone while vial w contained only water. Bottom: Plots of CEST ( $1-(M_s/M_0)$ ) and  $R_1$  versus  $[\text{Gd}^{3+}\mathbf{1}]$ . Imaging parameters:  $B_0 = 9.4 \text{ T}$ ,  $20^\circ\text{C}$ ;  $T_1$ w: GEMS sequence,  $\text{TR} = 9.9 \text{ ms}$ ,  $\text{TE} = 5.0 \text{ ms}$ ; CEST: FSEMS sequence,  $\text{sat time} = 3 \text{ s}$ ,  $\text{sat power} = 10 \text{ }\mu\text{T}$ ,  $\text{sat freq} = 54 \text{ (on)}$ ,  $-54 \text{ ppm}$  (off-resonance).



**Figure 3.** Plots of relaxation rate ( $R_1$ ) (●) and CEST (■) for a solution initially containing 10 mM  $\text{Eu}^{2+}2$  versus time. The data were collected from images in a single slice 4 mm away from the surface. Imaging parameters:  $B_0 = 9.4$  T,  $20^\circ\text{C}$ ,  $T_1\text{w}$ : FSEMS sequence,  $\text{TR} = 2$  s,  $\text{TE} = 3.0$  ms; CEST: FSEMS sequence, sat time = 3 s, sat power =  $10\text{ }\mu\text{T}$ , sat frq = 54 (on-resonance),  $-54$  ppm (off-resonance).

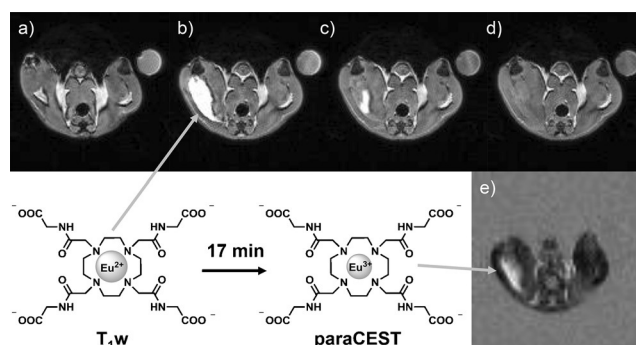
a slice just below the surface disappeared just after 10 minutes (Supporting Information, Figure S8).

To demonstrate the feasibility of using  $\text{Eu}^{2+}2$  as redox sensitive probe we studied its reaction with  $\text{H}_2\text{O}_2$ . A closed phantom containing  $\text{Eu}^{2+}2$  solution (1 mL, 10 mM) was constructed and a small volume (20  $\mu\text{L}$ ) of hydrogen peroxide solution (3 %) was injected into the container.  $T_1\text{w}$  and CEST images were recorded consecutively over a period of 1 h. As anticipated, the complex reacted rapidly with  $\text{H}_2\text{O}_2$  and the mixing and diffusion of  $\text{H}_2\text{O}_2$  in the solution could sequentially be observed by both  $T_1\text{w}$  and CEST imaging. These images also demonstrate that the complex is stable in both oxidation states and throughout the process of oxidation (Figure 4; Supporting Information, Figures S9, S10).



**Figure 4.** Sequential  $T_1\text{w}$  (top) and CEST (bottom) images of a phantom containing  $\text{Eu}^{2+}2$  (1 mL, 10 mM, left) and  $\text{H}_2\text{O}$  (right) after the injection of  $\text{H}_2\text{O}_2$  (3 %, 20  $\mu\text{L}$ ). Imaging parameters:  $B_0 = 9.4$  T,  $20^\circ\text{C}$   $T_1\text{w}$ : FSEMS sequence,  $\text{TR} = 2$  s,  $\text{TE} = 5$  ms; PARACEST: FSEMS sequence, sat time = 3 s, sat power =  $10\text{ }\mu\text{T}$ , sat frq = 54 (on-resonance),  $-54$  ppm (off-resonance).

Encouraged by the enhanced stability of  $\text{Eu}^{2+}2$  we tested the agent in vivo as well. The complex was injected into the thigh muscle of healthy male mice at a dose of  $0.05\text{ mmol kg}^{-1}$ . Oxidation of the agent at the injection site could be observed by both  $T_1\text{w}$  and CEST imaging (Figure 5). Given that diffusion of small molecules away from the injection site and into the vascular bed occurs relatively slowly, the relatively rapid decrease in water proton enhancement seen in  $T_1\text{w}$  images at the injection site over  $\approx 15$  min presumably reflects oxidation of  $\text{Eu}^{2+}$  to  $\text{Eu}^{3+}$ . To validate this assump-



**Figure 5.**  $T_1\text{w}$  and CEST imaging of an intramuscular injection of  $\text{Eu}^{2+}2$  (10 mM, 100  $\mu\text{L}$ ) into the thigh muscle of a healthy male C57BL/6J mouse at  $B_0 = 9.4$  T.  $T_1\text{w}$  images at a) pre-injection, b) 5 min, c) 12 min, d) 16 min and e) the CEST image at 17 min. Selected imaging parameters:  $T_1\text{w}$ : ge3D sequence,  $\text{TR} = 3.6$  ms,  $\text{TE} = 1.8$  ms.  $T_1\text{w}$  images have been normalized to muscle tissue; PARACEST: FSEMS sequence, sat time = 3 s, sat power =  $10\text{ }\mu\text{T}$ , sat frq = 42 (on-resonance),  $-42$  (off-resonance) ppm.

tion, after  $T_1\text{w}$  enhancement of the signal vanished, CEST images were collected to verify that  $\text{Eu}^{3+}2$  was indeed present. The CEST signal detected near the injection site (Figure 5e) remained evident over a period of about 20 min before disappearing (Supporting Information, Figure S11).

Since free  $\text{Eu}^{3+}$  does not produce a CEST effect, the detection of the agent by CEST imaging after the  $T_1\text{w}$  enhancement indicates that the complex remained intact in vivo throughout the oxidation process. All of the mice recovered after imaging and no evidence of toxicity was apparent after injection of  $\text{Eu}^{2+}2$ .

In conclusion, we have shown that ligand **2** forms a complex with  $\text{Eu}^{2+}$  that is surprisingly stable to oxidation. The  $\text{Eu}^{2+}$ -bound water exchange rate for this complex ( $k_{\text{ex}} = 0.21 \times 10^9\text{ s}^{-1}$ ) was found to be extremely fast, indicating that the amide side-arms do not have a significant decelerating effect on the water exchange. The agent has different contrast enhancing properties depending on the oxidation state of the metal. In its divalent form it is an efficient  $T_1$  shortening agent with an  $r_1$  relaxivity comparable to Gd-based contrast agents. Oxidation converts it into  $\text{Eu}^{3+}2$ , which is a commonly used paramagnetic chemical exchange saturation (paraCEST) agent. The oxidation of  $\text{Eu}^{2+}2$  by air or  $\text{H}_2\text{O}_2$  could be followed by both  $T_1\text{w}$  and CEST imaging. The improved oxidative stability of  $\text{Eu}^{2+}2$  When injected intramuscularly into healthy mice the complex generated strong  $T_1$  enhancement that gradually diminished over several minutes after which strong CEST effect could be observed at the injection site. This complex could serve as a design platform for a novel class of redox sensitive bimodal MR contrast agents in which the redox potential of the  $\text{Eu}^{2+}$  may be fine-tuned by the nature of the peripheral amide groups.

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**Keywords:** contrast agents · divalent lanthanides · europium · paraCEST ·  $T_1$  weighting

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