Fluorinated Eu\textsuperscript{II}-based multimodal contrast agent for temperature- and redox-responsive magnetic resonance imaging\textsuperscript{†}

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Magnetic resonance imaging (MRI) using redox-active, Eu\textsuperscript{II}-containing complexes is one of the most promising techniques for noninvasively imaging hypoxia \textit{in vivo}. In this technique, positive (T\textsubscript{1}-weighted) contrast enhancement persists in areas of relatively low oxidizing ability, such as hypoxic tissue. Herein, we describe a fluorinated, Eu\textsuperscript{II}-containing complex in which the redox-active metal is caged by intramolecular interactions. The position of the fluorine atoms enables temperature-responsive contrast enhancement in the reduced form of the contrast agent and detection of the oxidized contrast agent via MRI \textit{in vivo}. Positive contrast is observed in \textit{1H}-MRI with Eu in the +2 oxidation state, and chemical exchange saturation transfer and \textit{19F}-MRI signal are observed with Eu in the +3 oxidation state. Contrast enhancement is controlled by the redox state of Eu, and modulated by the fluororous interactions that cage a bound water molecule reduce relaxivity in a temperature-dependent fashion. Together, these advancements constitute the first report of \textit{in vivo}, redox-responsive imaging using \textit{19F}-MRI.

Introduction

Oxygen is critical to all forms of aerobic life, and imbalances of oxygen supply and consumption, such as hypoxia, are associated with disease.\textsuperscript{1–7} For example, hypoxic and nonhypoxic tumors have different responses to therapies, and regions of hypoxic tissue can be extremely heterogeneous,\textsuperscript{*} complicating the differentiation of different types of tissues. Consequently, many visualization techniques, including optical imaging, positron emission tomography, photoacoustic imaging, and magnetic resonance imaging (MRI), are being investigated for the imaging of oxygen levels to study the progress and treatment of diseases.\textsuperscript{9} MRI is capable of producing images of the inside of living organisms with excellent spatial resolution, and \textit{19F} has no detectable background signal \textit{in vivo}. As a result of these properties, \textit{19F}-MRI has been used \textit{in vivo} with perfluorocarbon emulsions,\textsuperscript{10} perfluorocarbon nanoparticles,\textsuperscript{11,12} perfluorocarbon-labelled cells,\textsuperscript{13,14} and discrete lanthanide complexes.\textsuperscript{15} These examples of \textit{in vivo} imaging with \textit{19F}-MRI make the technique one of the most promising for noninvasive imaging, and metal-based multimodal oxygen-responsive contrast agents for \textit{19F}-MRI would expand that promise to the imaging of hypoxia-related diseases. The design of contrast agents for \textit{19F}-MRI is complicated by the conflict between the need for a large number of chemically equivalent fluorine atoms and the hydrophobicity of fluorine. Too few \textit{19F} nuclei lead to undetectable signal, but too many \textit{19F} nuclei are associated with low solubility.\textsuperscript{12} Ideally, oxygen-responsive contrast agents for \textit{19F}-MRI would be soluble in water to enable distribution \textit{in vivo} and be redox-responsive to yield molecular information regarding oxidation. Furthermore, because the \textit{19F} atoms in ligands are not intrinsically redox-active, redox-responsive probes for \textit{19F}-MRI need to include a redox-active element to impart a responsive nature to probes. Eu\textsuperscript{III}-based contrast agents react with oxygen to change MRI-relevant properties: Eu\textsuperscript{II} enhances contrast in T\textsubscript{1}-weighted MRI and Eu\textsuperscript{III} does not.\textsuperscript{16–22} We hypothesized that combining the favorable imaging properties of \textit{19F}-MRI with \textit{1H}-MRI-active Eu\textsuperscript{II} based complexes would produce oxidation-state-dependent imaging and enable detection of both oxidation states of Eu. We overcame the solubility challenges associated with \textit{19F} \textit{via} strategic placement of \textit{19F} atoms in ligands for europium that sequestered the fluorine atoms via intramolecular interactions. An important consequence of these intramolecular interactions was the formation of a cage that trapped a molecule of water bound to europium, enabling control over contrast enhancement.

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in a temperature-responsive manner. Here, we report a new redox-active contrast system, $2\text{Eu}^{III}$, that has twelve chemically equivalent $^{19}$F nuclei, is water-soluble, and is detectable in vivo before and after oxidation using different imaging protocols with a single MRI scanner.

Our strategy was based upon the ability of $\text{Eu}^{II}$, but not $\text{Eu}^{III}$ that has a diamagnetic ground state, to dramatically influence the relaxivity for $\text{Eu}^{III}$ near multiple chemically equivalent $^{19}$F nuclei. Consequently, we hypothesized that introducing $^{19}$F nuclei near $\text{Eu}^{II}$ would cause severe line-broadening (via shortening of the transverse relaxation time of $^{19}$F) to the point of no observable signal. However, unlike $\text{Gd}^{III}$ that is not redox-active in vivo, oxidation of $\text{Eu}^{II}$ to $\text{Eu}^{III}$ would be expected to remove quenching of $^{19}$F signal. Therefore, Eu-containing complexes would act as $T_1$-shortening contrast agents for $^1\text{H}$-MRI in the $+2$ oxidation state and "turn-on" $^{19}$F imaging probes in response to oxidation of $\text{Eu}^{II}$ to $\text{Eu}^{III}$. Based on recent reports of $\text{Eu}^{II}$-based contrast agents, we thought that a cyclen scaffold would provide a synthetically viable opportunity to coordinate both oxidation states of Eu near multiple chemically equivalent $^{19}$F nuclei while maintaining solubility in water. Our design incorporates twelve equivalent $^{19}$F nuclei that enable detection in vivo but also provides solubility for clearance via the excretory system. Because a single MRI scanner can be used for $^{19}$F- and $^1\text{H}$-MRI, the $2\text{Eu}^{III}$ system would be detectable before and after oxidation using the same instrument.

**Results and discussion**

Fluorinated ligand 2 was synthesized in two steps from commercially available starting materials (see the ESI† for detailed procedures, yields, and characterization). Ligand 2 was metalated with $\text{EuCl}_3$ to produce $2\text{Eu}^{III}$ (59% yield), and $2\text{Eu}^{II}$ was synthesized in quantitative yield by reducing $2\text{Eu}^{III}$ with $\text{Zn}$. Complex $2\text{Eu}^{III}$ can be recovered by oxidizing solutions of $2\text{Eu}^{II}$ with air as evidenced by UV-visible, $^{19}$F-NMR, and luminescence spectroscopies (ESI Fig. S2†). Both $2\text{Eu}^{III}$ and $2\text{Eu}^{II}$ are soluble in neutral, buffered water suitable for in vivo injections. Furthermore, both complexes share similar conformations in the solid state (Fig. 1). All four amide arms are pseudo-axial, resulting in caged structures around the Eu ions. This conformation is unusual for tetraamide complexes with lanthanides.

In all reported examples, amide arms favor pseudo-equatorial positions unless forced to be pseudo-axial by intramolecular steric interactions. The structures of $2\text{Eu}^{III}$ and $2\text{Eu}^{II}$ suggest that the fluorophilic character of the CF$_3$ groups and $\pi$-stacking of the benzene rings contribute to the formation of a cage around the metal. Additionally, in both $2\text{Eu}^{III}$ and $2\text{Eu}^{II}$, one coordinated water molecule is held within the pocket formed by the p-trifluoromethylbenzyl groups, effectively caging the coordinated water molecule from the surroundings. Although trapping of water has implications for $T_1$-weighted MRI because impeded water exchange influences relaxivity, the pseudoaxial amides could be useful for temperature control of relaxivity. However, crystal structures describe solid-state conformations and do not account for dynamics in solution; therefore, we turned to solution-phase characterization.

To probe the stability of the pseudo-axial conformation in solution, relaxivity was measured as a function of temperature at 11.7 T between 16.5 and 40.5 °C (Fig. 2). This temperature range was selected because it spans from slightly below room temperature to slightly above body temperature. The plot of relaxivity versus temperature of $2\text{Eu}^{II}$ shows two regimes: there is no change in relaxivity between 16.5 and 24.5 °C, and there is a linear increase in relaxation rate between 24.5 and 40.5 °C. The relaxivity at 36.5 °C is 2.0 × higher than the relaxivity at 16.5 °C. At the colder temperature, the relaxivity of $2\text{Eu}^{II}$ is similar to $\text{Eu}^{III}$-containing complexes with purely outer-sphere contrast enhancement (a coordinated water molecule does not measurably influence relaxivity if it is not exchanging with bulk water), and the relaxivity at 36.5 °C is within the range of reported $\text{Eu}^{III}$-containing complexes that have exchanging water.
observed dependence on temperature is the opposite of what would be expected for a system that does not change the number of coordinated water molecules: increased temperatures lead to more rapid molecular rotations that are associated with lower relaxivity values. A similar, but smaller, effect in relaxivity caused by hydrophobic groups around a caged water molecule has been previously observed with a structurally similar Gd(III) complex that has four N-(1-phenylethyl)propionamide arms and exhibits a similar increase in relaxivity over the same temperature range. Additionally, the change in relaxivity is consistent with Gd(III)-based responsive contrast agents that mediate water coordination number. A possible explanation for this temperature-dependent shift in relaxivity is that thermal energy overcomes the fluoruous and π-stacking interactions above 24.5 °C, resulting in accessibility of bulk water to participate in exchange with the coordinated water inside of the cage formed by the p-trifluoromethylbenzyl arms. Conformational changes of similar Eu(III) and Gd(III)-containing, cyclen-based complexes have been reported to influence water-exchange rates. Finally, to check for the possibility of aggregation, we performed dynamic light scattering measurements of Eu(III) (3.1 mM in aqueous 3-morpholinopropane-1-sulfonic acid buffer) at 5 and 37 °C. No aggregates were detected at either temperature, supporting a mechanism of relaxivity modulation based on water exchange instead of molecular reorientation. The observed changes in relaxivity are consistent with what would be expected if pseudo-axial amides in the solid-state structure were similar to solution-phase at lower temperatures, and thermal energy overcomes the interactions, opening the ligand to enable water exchange consistent with an increase in relaxivity.

To explore the influence of the fluoruous interactions between the trifluoromethyl moieties on the temperature-dependent behavior of Eu(III), we synthesized the nonfluorinated methyl analog, Eu(III), and measured relaxivity over the same range of temperatures. Ligand 3 was synthesized in two steps from commercially available starting materials, and the corresponding Eu-containing complexes, Eu(III) and Eu(III), were synthesized similarly to Eu(III) and Eu(III) (ESI Fig. S1†). The methylated complex, Eu(III), did not exhibit a temperature-dependent change in relaxivity like the fluorinated complex Eu(III). Between 16.5 and 36.5 °C, Eu(III) displayed relaxivities from 5.14 to 5.49 mM⁻¹ s⁻¹, similar to the relaxivity of the trifluoromethyl complex, Eu(III), at temperatures ≥36.5 °C. The lack of temperature dependence of the methyl analog Eu(III) suggests that intramolecular fluoruous interactions are the cause of the temperature-dependent relaxivity of Eu(III). Therefore, the fluoruous interactions impart an element of kinetic control that has implications for long-term storage and could be used to measure temperature using relaxivity.

To test if the p-trifluoromethylbenzylamide arms would enable detection after oxidation of Eu(III) to Eu(III), we studied the spectroscopic properties of Eu(III). To verify that the 19F NMR signal changes with oxidation state, we acquired 19F-NMR spectra of aqueous, buffered solutions of Eu(III) and Eu(III) (5.4 mM in aqueous 3-morpholinopropane-1-sulfonic acid buffer, pH 7.0). A singlet at –62.1 ppm relative to sodium triflate was observed for Eu(III), and no detectable signal was observed for Eu(III). Upon oxidation of the solution of Eu(III), 19F-signal appeared consistent with the presence of Eu(III) (ESI Fig. S2†). The 19F-NMR signal was too broad to be observed for Eu(III) because the Eu(III) ion shortened the transverse relaxation times of 19F to the point of no observable signal. Upon oxidation to Eu(III), 19F signal appeared. Furthermore, because not all MRI scanners are capable of imaging 19F, we also acquired the chemical exchange saturation transfer (CEST) spectrum of Eu(III). Amides near Eu(III) and Eu(III) provide a means of imaging oxygen content via CEST MRI. Complex Eu(III) has a saturation offset of 49 ppm (ESI Fig. S3†), which is similar to other Eu(III)-containing tetramide complexes. Therefore, based on spectroscopic studies, Eu(III) has detectable 19F- and CEST MRI after oxidation.

To verify that the oxidation-state-dependent signal was observable with MRI at imaging-relevant concentrations, we imaged solutions of complexes Eu(III), Eu(III), and Eu(III) post-oxidation (Fig. 3). 19F- and CEST-MRI signals were observed for Eu(III)-containing complex Eu(III), but no T₁-weighted enhancement was observed relative to buffer. No detectable 19F- or CEST-MRI signals were observed for Eu(III)-containing complex Eu(III), but a 2.0× increase in brightness in the T₁-weighted scan was observed relative to buffer (see ESI Fig. S8 and Table S4† for signal-to-noise ratios). This observation is consistent with another Eu(III) containing tetramide complex without benzyl groups that does not influence CEST-MRI but does shorten T₁. Therefore, the pair of complexes, Eu(III) and Eu(III), provide oxidation-dependent signal: the Eu(III)-containing complex acts as a T₁-enhancing contrast agent, and the Eu(III)-containing complex acts as an imaging probe for 19F- and CEST-MRI. Due to the well-behaved redox- and temperature-responsive contrast system Eu(III), in vitro, we investigated the behavior of the system in mice.

To verify that oxidation-state-dependent signals were observable with MRI in vivo, mice were injected in the peritoneal cavities with solutions of Eu(III) (200 µL, 5.4 mM in aqueous 3-morpholinopropane-1-sulfonic acid buffer, pH 7.0) and imaged with T₁-weighted and 19F-MRI scans (Fig. 4a–e). All animal studies were done in accordance with protocols preapproved by...
the Institutional Animal Care and Use Committee of Baylor College of Medicine. Intraperitoneal injections were selected to enable comparison with previous EuII studies.28 Because intraperitoneal injections in a normal mouse are in non-hypoxic conditions, it is expected that the contrast agent would lose $T_1$ contrast and develop $^{19}$F signal intensity upon oxidation. $T_1$-weighted MRI images of the coronal planes of mice showed contrast that appeared immediately upon injection and faded over the course of three minutes. The duration of $T_1$ enhancement is consistent with intraperitoneal injection of a EuII-containing cryptate.29 To detect 2EuIII post-oxidation in vivo, we collected $^{19}$F spectra of mice between $T_1$-weighted scans. $^{19}$F signal intensity appeared nine minutes post injection (Fig. 4i). The in vivo $^{19}$F spectra demonstrated that 2EuIII could be detected post-oxidation in vivo using a $^{19}$F coil of an MRI scanner. Other $^{19}$F-MRI, redox-responsive contrast agents have been demonstrated to display oxidation-dependent signal in vitro or ex vivo,30–31 but to the best of our knowledge, this is the first demonstration of detection of $^{19}$F-MR signal in vivo in a redox-responsive manner.

Encouraged by the $T_1$ and $^{19}$F oxidation-response in vivo, we investigated the in vivo solubility and clearance by collecting a sample of urine after imaging and within one hour of injection. The urine was analyzed using coupled high-performance liquid chromatography mass spectrometry. The [M + Cl]$^{2+}$ adduct of 2EuIII was observed with the correct mass-to-charge ratio and expected isotope pattern (ESI Fig. S4†). Although this technique is not quantitative, evidence of intact complex post-injection is promising for future in vivo studies with respect to minimization of toxicity from decomplexation or transmetallation.34 Furthermore, evidence of intact complex indicates that 2EuIII remained soluble, diffused through the mouse, and was cleared intact.

![Fig. 3](image-url) MR images of solutions of samples in aqueous 3-morpholinopropane-1-sulfonic acid buffer (20 mM, pH 7.0) in glass (3 mm) or plastic tubes (5 mm). Scale bars correspond to the three images in the column above each bar. $T_1$-weighted MRI images of solutions of (a) 3-morpholinopropane-1-sulfonic acid (20 mM, pH 7.0) (b) 2EuIII (3.5 mM); (c) 2EuII (3.5 mM); and (d) 2EuIII after oxidation of 2EuII (3.5 mM). $^{19}$F-MRI images of (e) 3-morpholinopropane-1-sulfonic acid (20 mM, pH 7.0); (f) 2EuIII (3.5 mM); (g) 2EuII (3.5 mM); and (h) 2EuIII after oxidation of 2EuII (3.5 mM). The scale bar on the right of (h) represents signal intensity for all $^{19}$F-MRI images in Fig. 3. CEST MRI images of (i) 3-morpholinopropane-1-sulfonic acid (20 mM, pH 7.0); (j) 2EuIII (3.5 mM); (k) 2EuII (3.5 mM); and (l) 2EuIII after oxidation of 2EuII (3.5 mM). The scale bar on the right of (l) represents signal intensity for all CEST MRI images in Fig. 3.

![Fig. 4](image-url) Representative $T_1$-weighted images demonstrating the response of 2EuII (5.4 mM in aqueous 3-morpholinopropane-1-sulfonic acid buffer, pH 7.0) after intraperitoneal injection (into a 20 g C57/B16 mouse). The images are (a) pre-injection, (b) immediately post-intraperitoneal injection, denoted as 0 min, (c) 1 min post-intraperitoneal injection, (d) 2 min post-intraperitoneal injection, and (e) 3 min post-intraperitoneal injection. Red arrows denote areas of positive contrast enhancement. The area represented by each image is 26 mm × 40 mm. (f) Diagram showing plane of the mouse in images a–e. (g) Average normalized pixel brightness, which were subtracted with the pre-injection image intensities, plotted over time post-intraperitoneal injection from panels (a)–(e), the area from the red circles (background) was subtracted from the area in the yellow circles to calculate average pixel brightness. Single pulse $^{19}$F signal intensity of a whole mouse (h) pre-injection and (i) nine minutes post-injection.
Conclusions

In conclusion, we have synthesized a new redox-active contrast agent, $2\text{Eu}^{2+}$, that also displays temperature-dependent relaxivity, which was used as a tool for probing mechanism. The intramolecular fluoruous interactions among p-trifluoromethylbenzamides lead to the formation of a cage-like structure that modulates the interactions of water with Eu as a function of temperature, evidenced by the change in relaxivity. Because of the presence of fluorine atoms and amide protons, the system can be tracked in vivo after oxidation with a variety of instruments that are equipped with either CEST or $^{19}$F-MR imaging capabilities. We also observed intact complex in mouse urine post-injection, which is promising for future toxicity studies and in vivo imaging. Together, our results allude to a new molecular strategy for rationally designing ligand architectures by modulating interactions between Eu$^3+$ and water with intramolecular fluoruous attractions. These results could pave the way to using existing imaging techniques with new chemical agents to access previously unattainable oxidative information noninvasively in vivo.

Conflicts of interest

There are no conflicts to declare.

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