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

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Magnetic resonance imaging (MRI) using redox-active, Eu^{II}-containing complexes is one of the most promising techniques for noninvasively imaging hypoxia *in vivo*. In this technique, positive (T_1 -weighted) contrast enhancement persists in areas of relatively low oxidizing ability, such as hypoxic tissue. Herein, we describe a fluorinated, Eu^{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 *in vivo*. Positive contrast is observed in ¹H-MRI with Eu in the +2 oxidation state, and chemical exchange saturation transfer and 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 fluorine interactions that cage a bound water molecule reduce relaxivity in a temperature-dependent fashion. Together, these advancements constitute the first report of *in vivo*, redox-responsive imaging using ¹⁹F-MRI.

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Introduction

Oxygen is critical to all forms of aerobic life, and imbalances of oxygen supply and consumption, such as hypoxia, are associated with disease.^{1–7} For example, hypoxic and nonhypoxic tumors have different responses to therapies, and regions of hypoxic tissue can be extremely heterogeneous,⁸ 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.⁹ MRI is capable of producing images of the inside of living organisms with excellent spatial resolution, and ¹⁹F has no detectable background signal *in vivo*. As a result of these properties, ¹⁹F-MRI has been used *in vivo* with perfluorocarbon

emulsions,¹⁰ perfluorocarbon nanoparticles,^{11,12} perfluorocarbon-labelled cells,^{13,14} and discrete lanthanide complexes.¹⁵ These examples of *in vivo* imaging with ¹⁹F-MRI make the technique one of the most promising for noninvasive imaging, and metal-based multimodal oxygen-responsive contrast agents for ¹⁹F-MRI would expand that promise to the imaging of hypoxia-related diseases. The design of contrast agents for 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 ¹⁹F nuclei lead to undetectable signal, but too many ¹⁹F nuclei are associated with low solubility.¹² Ideally, oxygen-responsive contrast agents for ¹⁹F-MRI would be soluble in water to enable distribution *in vivo* and be redox-responsive to yield molecular information regarding oxidation. Furthermore, because the ¹⁹F atoms in ligands are not intrinsically redox-active, redox-responsive probes for ¹⁹F-MRI need to include a redox-active element to impart a responsive nature to probes. Eu^{II/III}-based contrast agents react with oxygen to change MRI-relevant properties: Eu^{II} enhances contrast in T_1 -weighted MRI and Eu^{III} does not.^{16–22} We hypothesized that combining the favorable imaging properties of ¹⁹F-MRI with ¹H-MRI-active Eu^{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 ¹⁹F *via* strategic placement of 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, $2\text{Eu}^{\text{II/III}}$, 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 Eu^{II} , but not Eu^{III} that has a diamagnetic ground state, to dramatically influence the relaxation rates of nearby nuclei.¹⁷ Although Eu^{III} has low-lying excited paramagnetic electronic states that are thermally accessible, no measurable effect on relaxation rates have been observed at concentrations up to 6 mM.^{16,17,19} Eu^{II} is isoelectronic with Gd^{III} , which is commonly used in contrast agents for MRI. Fluorinated Gd^{III} -containing complexes influence the signal of ^{19}F in a distance-dependent manner;^{22–28} consequently, we hypothesized that introducing ^{19}F nuclei near 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 Gd^{III} that is not redox-active *in vivo*, oxidation of Eu^{II} to 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 ^1H -MRI in the +2 oxidation state and “turn-on” ^{19}F imaging probes in response to oxidation of Eu^{II} to Eu^{III} . Based on recent reports of Eu^{II} -based contrast agents,^{16,17,29} 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 ^1H -MRI, the $2\text{Eu}^{\text{II/III}}$ 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 EuCl_3 to produce 2Eu^{III} (59% yield), and 2Eu^{II} was synthesized in quantitative yield by reducing 2Eu^{III} with Zn^0 . Complex 2Eu^{III} can be recovered by oxidizing solutions of 2Eu^{II} with air as evidenced by UV-visible, ^{19}F -NMR, and luminescence spectroscopies (ESI Fig. S2†). Both 2Eu^{III} and 2Eu^{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.^{30–32} The structures of 2Eu^{III} and 2Eu^{II} suggest that the fluorophilic character of the CF_3 groups and π -stacking of the benzene rings contribute to the formation of a cage around the metal. Additionally, in both 2Eu^{III} and 2Eu^{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

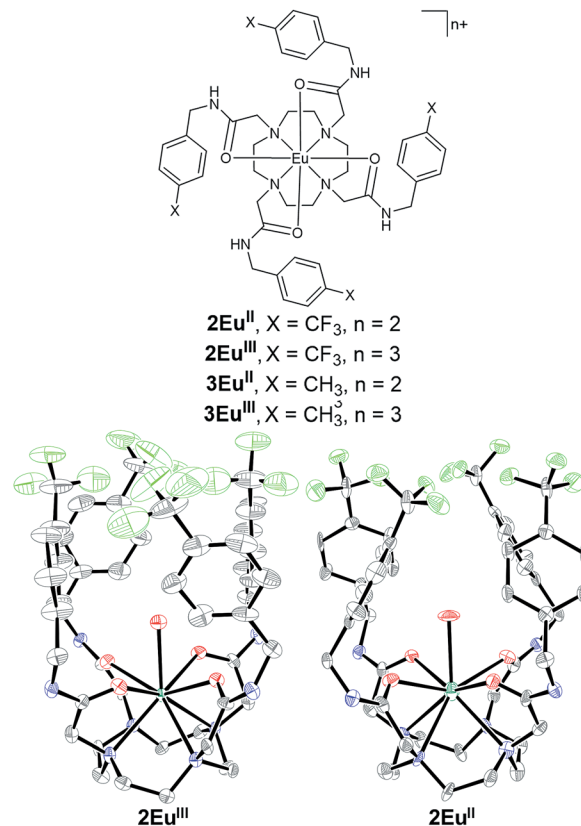


Fig. 1 (Left) Chemical structures of 2Eu^{II} , 2Eu^{III} , 3Eu^{II} , and 3Eu^{III} . Crystal structures of (center) 2Eu^{II} and (right) 2Eu^{III} grown from solutions in methanol and ethanol, respectively. Chloride counterions, outer-sphere water molecules, and hydrogen atoms are not shown for clarity. Thermal ellipsoids are drawn at 50% probability. Grey = C; blue = N; red = O; yellow-green = F; sea green = Eu. Crystallographic data for these structures have been deposited at the Cambridge Crystallographic Data Centre under deposition numbers CCDC 1562330 (2Eu^{II}) and CCDC 1562331 (2Eu^{III}).†

trapping of water has implications for T_1 -weighted MRI because impeded water exchange influences relaxivity,³³ the pseudo-axial 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 2Eu^{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 2Eu^{II} is similar to Eu^{II} -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),³⁴ and the relaxivity at 36.5 °C is within the range of reported Eu^{II} -containing complexes that have exchanging water.^{17,18,34–39} The



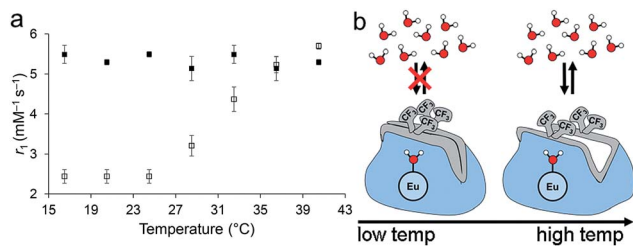


Fig. 2 (a) Relaxation rates (11.7 T) of 2Eu^{II} (□) (2–8 mM in aqueous 3-morpholinopropane-1-sulfonic acid buffer, pH 7.0) and 3Eu^{II} (■) (2–15 mM in aqueous 3-morpholinopropane-1-sulfonic acid buffer, pH 7.0). Error bars represent the standard error of mean of measurements from three independently prepared samples. (b) Cartoon explanation of mechanism of temperature-dependent change in relaxivity. The coin purse represents ligand 2 that is held shut by the fluororous interactions and π -stacking at low temperatures, preventing water exchange and, consequently, inner-sphere-based contrast enhancement. As temperature increases, thermal energy overcomes the interactions, opening the ligand to enable water exchange consistent with an increase in relaxivity.

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.⁴⁰ 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.^{41,42} Additionally, the change in relaxivity is consistent with Gd^{III}-based responsive contrast agents that modulate water coordination number.⁴³ A possible explanation for this temperature-dependent shift in relaxivity is that thermal energy overcomes the fluororous 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.^{42,44–47} Finally, to check for the possibility of aggregation, we performed dynamic light scattering measurements of 2Eu^{II} (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 at higher temperatures overcame intramolecular fluororous interactions to enable water exchange.

To explore the influence of the fluororous interactions between the trifluoromethyl moieties on the temperature-dependent behavior of 2Eu^{II}, we synthesized the nonfluorinated methyl analog, 3Eu^{II}, 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, 3Eu^{III} and 3Eu^{II}, were synthesized similarly to 2Eu^{III} and 2Eu^{II} (ESI Fig. S1†). The methylated complex, 3Eu^{II}, did not exhibit a temperature-dependent change in relaxivity like the fluorinated complex 2Eu^{II}. Between 16.5 and 36.5 °C, 3Eu^{II} displayed relaxivities from 5.14 to 5.49 mM⁻¹ s⁻¹, similar to the relaxivity of the trifluoromethyl complex, 2Eu^{II}, at temperatures ≥ 36.5 °C. The lack of temperature dependence of the methyl analog 3Eu^{II} suggests that intramolecular fluororous interactions are the cause of the temperature-dependent relaxivity of 2Eu^{II}. Therefore, the fluororous 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^{II} to Eu^{III}, we studied the spectroscopic properties of 2Eu^{III/II}. To verify that the ¹⁹F-NMR signal changes with oxidation state, we acquired ¹⁹F-NMR spectra of aqueous, buffered solutions of 2Eu^{III} and 2Eu^{II} (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 2Eu^{III}, and no detectable signal was observed for 2Eu^{II}. Upon oxidation of the solution of 2Eu^{II}, ¹⁹F-signal appeared consistent with the presence of 2Eu^{III} (ESI Fig. S2†). The ¹⁹F-NMR signal was too broad to be observed for 2Eu^{II} because the Eu^{II} 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 2Eu^{III}. Amides near Eu^{III} and Eu^{II} provide a means of imaging oxygen content *via* CEST MRI.¹⁷ Complex 2Eu^{III} has a saturation offset of 49 ppm (ESI Fig. S3†), which is similar to other Eu^{III}-containing tetraamide complexes.^{17,30,31,48,49} Therefore, based on spectroscopic studies, 2Eu^{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 2Eu^{III}, 2Eu^{II}, and 2Eu^{III} post-oxidation (Fig. 3). ¹⁹F- and CEST-MRI signals were observed for Eu^{III}-containing complex 2Eu^{III}, but no T_1 -weighted enhancement was observed relative to buffer. No detectable ¹⁹F- or CEST-MRI signals were observed for Eu^{II}-containing complex 2Eu^{II}, but a 2.0× increase in brightness in the T_1 -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^{II}-containing tetraamide complex without benzyl groups that does not influence CEST-MRI but does shorten T_1 .¹⁷ Therefore, the pair of complexes, 2Eu^{II} and 2Eu^{III}, provide oxidation-dependent signal: the Eu^{II}-containing complex acts as a T_1 -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 2Eu^{III/II} *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 2Eu^{II} (200 μ L, 5.4 mM in aqueous 3-morpholinopropane-1-sulfonic acid buffer, pH 7.0) and imaged with T_1 -weighted and ¹⁹F-MRI scans (Fig. 4a–e). All animal studies were done in accordance with protocols preapproved by



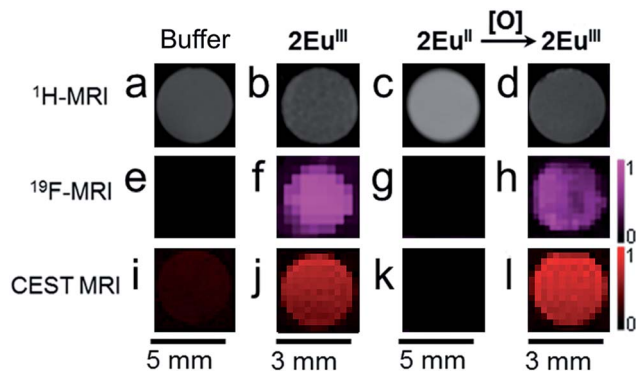


Fig. 3 MR images of solutions of samples in aqueous 3-morpholinopropane-1-sulfonic acid buffer (20 mM, pH 7.0) in glass (3 mm) or plastic tubes (5 mm). Scale bars correspond to the three images in the column above each bar. T_1 -weighted MRI images of solutions of (a) 3-morpholinopropane-1-sulfonic acid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

the Institutional Animal Care and Use Committee of Baylor College of Medicine. Intraperitoneal injections were selected to enable comparison with previous Eu^{II} studies.²⁰ 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 Eu^{II} -containing cryptate.²⁰ To detect 2Eu^{III} 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 2Eu^{III} 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*,^{50–53} 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 $[\text{M} + \text{Cl}]^{2+}$ adduct of 2Eu^{III} 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.⁵⁴ Furthermore, evidence of intact complex indicates that 2Eu^{III} remained soluble, diffused through the mouse, and was cleared intact.

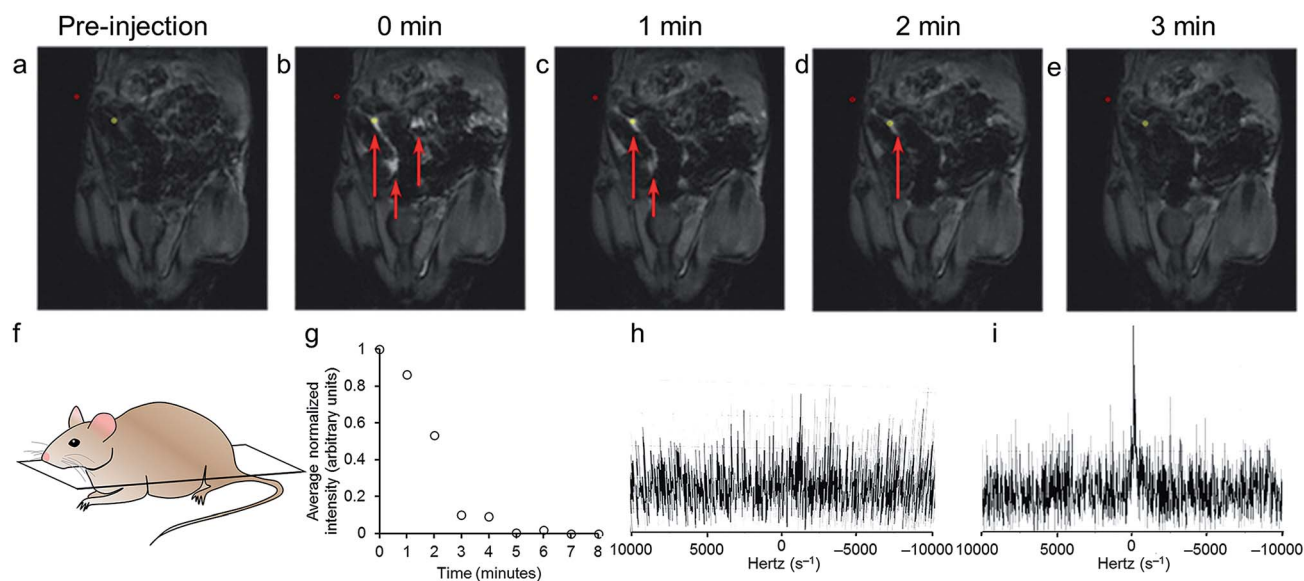


Fig. 4 Representative T_1 -weighted images demonstrating the response of 2Eu^{II} (5.4 mM in aqueous 3-morpholinopropane-1-sulfonic acid buffer, pH 7.0) after intraperitoneal injection (into a 20 g C57/Bl6 mouse). The images are (a) pre-injection, (b) immediately post-intraperitoneal injection, denoted 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 \times 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, 2Eu^{III} , that also displays temperature-dependent relaxivity, which was used as a tool for probing mechanism. The intramolecular fluorine interactions among *p*-trifluoromethylbenzylamides 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^{II} and water with intramolecular fluorine 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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