Evaluation of EuII-based positive contrast enhancement after intravenous, intraperitoneal, and subcutaneous injections

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EuII-based contrast agents offer physiologically relevant, metal-based redox sensing that is unachievable with GdIII-based contrast agents. To evaluate the in vivo contrast enhancement of EuII as a function of injection type, we performed intravenous, intraperitoneal, and subcutaneous injections in mice. Our data reveal a correlation between reported oxygen content and expected rates of diffusion with the persistence of EuII-based contrast enhancement. Biodistribution studies revealed europium clearance through the liver and kidneys for intravenous and intraperitoneal injections, but no contrast enhancement was observed in organs associated with clearance. These data represent a step toward understanding the behavior of EuII-based complexes in vivo. Copyright © 2016 John Wiley & Sons, Ltd.

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1. INTRODUCTION

Redox balance is critical to the homeostasis of living tissues, and redox stress is associated with some cancers (1–4) and ailments such as cardiovascular (5,6), Alzheimer’s (7–10), liver (11,12), and chronic kidney disease (13). The ability to noninvasively detect changes in redox environments in real time would be invaluable to diagnosing diseases and monitoring responses to therapies. Magnetic resonance imaging (MRI) offers a noninvasive platform to image opaque objects, but often requires responsive contrast agents to relay chemical information regarding the local redox environment. The GdIII ion has dominated the clinical landscape and preclinical research in MRI because it provides excellent T1-shortening (positive) contrast enhancement (14–16), but GdIII is restricted to the +3 oxidation state under physiological conditions preventing metal-based redox responses (17). The EuII ion is isoelectronic with GdIII (4f7), and both ions provide positive contrast enhancement in MRI (18–27). Additionally, EuII can be oxidized by one electron to produce the EuIII ion that does not enhance positive contrast (19). The ability of EuII-222 to impart oxidative stabilization of the +2 oxidation state of europium has been studied (28,29), and recent reports have characterized the aqueous magnetic and electrochemical properties of EuII-222 and other EuII-containing complexes (18–27,30–33). Despite increased oxidative stability, the EuII ion of EuII-222 is prone to rapid oxidation by oxygen in solution (18). Oxidation of EuII in elevated oxygen partial pressures coincides with the loss of positive contrast enhancement (19), and we suspected this change in contrast enhancement would be observable in vivo. The +2 oxidation state of europium has been demonstrated to persist for hours within relatively oxygen-deficient necrotic tissue (pO2 < 10 mmHg) (18); therefore, we turned our attention to regions containing relatively higher levels of dissolved oxygen such as the subcutaneous space, fluids of the peritoneal cavity, and blood (Fig. 2).

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2. RESULTS AND DISCUSSION

To test our hypothesis regarding the in vivo response of EuII-222, we acquired T₁-weighted images of mice after administering EuII-222 (0.1 mL, 4 mM, europium dose of 3 mg/kg) through intravenous, intraperitoneal, and subcutaneous injections (Fig. 3). Mice were imaged prior to injection and at 3 and 8 min to compare responses with the three injection types. Based on these images, the intravenous injection resulted in no positive contrast enhancement; the intraperitoneal injection led to positive contrast enhancement in the peritoneal cavity that disappeared by 8 min; and the subcutaneous injection produced positive contrast enhancement both 3 and 8 min post-injection. The absence of positive contrast enhancement after the intravenous injection suggests that EuII-222 was oxidized within the first 3 min in the blood. Although this observation is inconsistent with the low oxygen content of venous blood (relative to the peritoneal cavity), the circulation time of blood in a mouse is approximately 8 s (38). This rapid circulation suggests that venous and arterial blood exchanged ~24 times over the course of the scan, allowing for blood solutes (including EuII-222) to be exposed to a relatively high level of oxygen. Therefore, the exchange between venous and arterial blood during circulation can explain our observations. It is unlikely that dilution alone could account for the complete loss of observable positive contrast enhancement because no positive contrast enhancement was observed in organs associated with clearance (liver, kidneys, or bladder; Fig. 3), whereas positive contrast enhancement was observed in the kidneys within 3 min after intravenous injection of an

Figure 1. Structure of EuII-222. Counterions have been omitted for clarity.

Figure 2. $pO_2$ ranges in necrotic and non-necrotic tumor (converted from percent hemoglobin saturation using a hemoglobin saturation curve) (34,35), subcutaneous space (36), venous blood (36), the peritoneal cavity (37), and arterial blood (36).

Figure 3. Representative T₁-weighted images demonstrating the response of EuII-222 after different injection types. The images are (A) pre-intravenous injection; (B) 3 min post-intravenous injection; (C) 8 min post-intravenous injection; (D) pre-intraperitoneal injection; (E) 3 min post-intraperitoneal injection; (F) 8 min post-intraperitoneal injection; (G) pre-subcutaneous injection; (H) 3 min post-subcutaneous injection; and (I) 8 min post-subcutaneous injection. Red arrows denote areas of positive contrast enhancement. The area represented by each image is 31 mm x 90 mm.
equivalent dose of Gd\textsuperscript{III}-diethylenetriaminepentaacetate. To ensure that Eu\textsuperscript{III}-222 had not been oxidized prior to the injection, we acquired T\textsubscript{1}-weighted images of the syringe before and after the injection and observed positive contrast enhancement for both, indicating that oxidation occurred in vivo.

An intraperitoneal injection placed Eu\textsuperscript{II}-222 into an intermediate pO\textsubscript{2} range (relative to intravenous and subcutaneous injections) and allowed positive contrast enhancement to be observed in the 3 min scan. However, the loss of positive contrast enhancement by 8 min suggests that Eu\textsuperscript{III}-222 diffused to regions of high oxygen level (vasculature), oxygen diffused into the peritoneal cavity, or both types of diffusion occurred. Relative to the peritoneal cavity, subcutaneous space has a lower rate of diffusion and a lower pO\textsubscript{2} (36,37,39). Consistent with these properties, positive contrast enhancement was observed both 3 and 8 min post-subcutaneous injection. Results of the intravenous, intraperitoneal, and subcutaneous imaging experiments suggest that both pO\textsubscript{2} and diffusion play a role in the persistence of Eu\textsuperscript{II}-based contrast enhancement in vivo. Furthermore, despite oxidation occurring in the mice, no adverse effects were observed during any of the in vivo studies reported here.

The imaging data presented here demonstrate that Eu\textsuperscript{II}-222 is oxidized faster than the MRI timescale used in our experiments for intravenous injections, that intraperitoneal injections offer transitory contrast enhancement, and that subcutaneous injections exhibit positive contrast enhancement for at least 8 min. Our observed trends correlate with reported values of pO\textsubscript{2} (34–37), where lower pO\textsubscript{2} values correspond to prolonged contrast enhancement. The lack and loss of positive contrast enhancement observed in the intravenous and intraperitoneal injections, respectively, led us to measure the biodistribution of europium, which we suspected would be informative regarding the route of clearance.

To understand the biodistribution of europium for the intravenous and intraperitoneal injections, we used inductively coupled plasma mass spectrometry (ICP–MS) to quantify europium in the blood, liver, kidneys, spleen, heart, bone (femur), muscle (thigh), brain, upper and lower gastrointestinal tract (GI), stomach, lungs, and brain (Fig. 4). The majority of detected europium was found in the liver and kidneys for both types of injections, with the relative quantities being higher for intravenous injections. ICP–MS data does not provide insight into speciation during clearance, a complicated topic that we are investigating using knowledge of the kinetic stability of Eu\textsuperscript{VIII}-containing cryptates (29); however, ICP–MS data provide valuable insight into the route of clearance. For intraperitoneal injections, the smaller amount of europium detected in the liver, kidneys, and blood might indicate relatively slow diffusion from the peritoneal cavity. Evidence of slow diffusion of Eu\textsuperscript{II}-222 from the peritoneal cavity supports a response dependent on the diffusion of oxygen into the peritoneal cavity. Furthermore, the presence of europium in detectable quantities after intravenous injections (there is no endogenous europium in mice), together with the images in Fig. 3, suggest that oxidation of Eu\textsuperscript{II}-222 occurs within 3 min of intravenous injection.

3. CONCLUSIONS

Our results demonstrate that Eu\textsuperscript{II}-based contrast enhancement is sensitive to the route of administration, with positive contrast enhancement expected for regions containing relatively low levels of oxygen and slow rates of diffusion. These results help define the boundaries of Eu\textsuperscript{II}-based positive contrast enhancement with Eu\textsuperscript{II}-222 in vivo, and might be helpful in the preclinical application of other Eu\textsuperscript{II}-based complexes. Furthermore, the in vivo use of lanthanide-based redox-response is a relatively unexplored realm. Although other redox-active molecules might contribute to the oxidation of Eu\textsuperscript{II}, we expect that the oxidation of Eu\textsuperscript{II} by oxygen is responsible for the correlation between oxygen content and the persistence of positive contrast enhancement in vivo, and efforts in our laboratory to understand aqueous Eu\textsuperscript{II} oxidation chemistry are currently underway. Additionally, our biodistribution studies revealed clearance of europium through the liver and kidneys, but no positive contrast enhancement was observed in these organs. These results are an important step towards understanding the scope of Eu\textsuperscript{II}-based positive contrast enhancement for a new class redox-active contrast agents based on lanthanide redox chemistry.

4. EXPERIMENTAL

4.1. General Procedures

Commercially available chemicals were of reagent-grade purity or better and were used without further purification unless otherwise noted. Water was purified using a PURELAB Ultra Mk2 water purification system (ELGA) and degassed prior to use.

4.2. Preparation of Contrast Agent Solutions

Contrast agent solutions for intravenous, intraperitoneal, and subcutaneous injections were prepared by adding aqueous EuCl\textsubscript{3} and aqueous 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (222) in a 1:1 ratio to a 4 mL glass vial equipped with a magnetic stir bar under an atmosphere of N\textsubscript{2}. The resulting clear, colorless solution was stirred for 1 h before addition of the 10x phosphate buffered saline (PBS, Fisher BioReagents) and water to achieve a final solution of Eu\textsuperscript{II}-222 (4 mM) in PBS (11.9 mM phosphates, 137 mM NaCl, and 2.7 mM KCl). The clear, colorless solution was stirred for 30 min then filtered through a 0.2 μm hydrophilic filter. The concentration of europium in the clear, colorless filtrate was determined by ICP–MS and was used directly for imaging studies.

![Figure 4](image-url)
4.3. ICP–MS

ICP–MS measurements were acquired on an Agilent Technologies 7700 series spectrometer in the Lumigen Instrument Center at Wayne State University. All dilutions were performed with 2% HNO₃ that was also used for blank samples during calibration. The calibration curve was created using the $^{153}$Eu isotope ion count for a 10–100 ppb concentration range (diluted from Fluka ICP standard solution, $\text{Eu}_2\text{O}_3$ in aqueous 2% HNO₃, 1000 mg Eu/L), and samples (with the exception of tissue digestion) were diluted to fall within this range.

4.4. Magnetic Resonance Imaging

Studies in animals were carried out with the assistance of the Animal Model and Therapeutics Evaluation Core of the Barbara Ann Karmanos Cancer Institute after approval from the Wayne State University Institutional Animal Care and Use Committee. MRI scans were performed in the Elliman Clinical Research Building at Wayne State University with a 7 T Bruker Clinscan small animal MRI scanner equipped with a 30 cm bore. $T_1$-weighted images (3D FLASH) were acquired with a body coil while using a water circulating set to 37 °C. The whole body coronal plane images were acquired using an echo time of 1.5 ms, repetition time of 11 ms, flip angle of 40 degrees, 44 image slices at 0.5 mm thickness, and a 31 mm × 90 mm field of view, and an in plane resolution of 0.352 mm × 0.352 mm.

For intravenous injections, mice were catheterized before being anesthetized with isoflurane. A micro-volume extension set was used to inject the solution of EuCl₂ into the tail vein with removing the mouse from the magnet. A correction volume (0.08 mL) was added to the calculated dose volumes for intravenous injections to account for the volume of the phosphate-buffered saline within the catheter. For intraperitoneal and subcutaneous injections, mice were first anesthetized with isoflurane, imaged prior to injection, and then the cradle with the mouse was removed from the magnet to perform the injection while still anesthetized. After injections, mice were imaged immediately to acquire the first time points post-injection. Intravenous injections were triplicated, intraperitoneal injections were duplicated, and the subcutaneous injection was performed once.

4.5. Biodistribution Studies

For biodistribution studies, mice were not catheterized or anesthetized. Mice were injected with the same europium dioxide used for imaging (3 mg/kg) before being sacrificed 1 h post-injection at which point the blood, liver, kidneys, spleen, heart, bone (femur), muscle (thigh), brain, upper and lower GI tract, stomach, and lungs were harvested. The samples were weighed, freeze dried for 72 h, and digested in 25 mL volumetric flasks using 6 mL of 3 M nitric acid at 75 °C with constant stirring for 16 h. The entirety of each sample was used for digestion with the exception of the liver, which was homogenized with mortar and pestle prior to addition to a volumetric flask and a fraction (~130 mg) of the homogenate was added to a volumetric flask. After 16 h, the digests were allowed to cool to ambient temperature before the addition of water to achieve a total volume of 25 mL. The digests were transferred to conical tubes (50 mL) and insoluble oils were removed by centrifugation. The clear, yellow supernatants were immediately transferred to conical tubes (15 mL) for analysis of europium concentration with ICP–MS.

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