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Screening of ligands for redox-active europium using magnetic resonance imaging

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Abstract

We report a screening procedure to predict ligand coordination to Eu^{II} and Eu^{III} using magnetic resonance imaging in which bright images indicate complexation and dark images indicate no complexation. Here, paramagnetic Gd^{III} is used as a surrogate for Eu^{III} in the screening procedure to enable detection with magnetic resonance imaging. The screening procedure was tested using a set of eight ligands with known coordination to Eu^{II} and Eu^{III}, and results were found to be consistent with expected binding. Validation of the screening procedure with known coordination chemistry enables use with new ligands in the future.

Graphical abstract

Keywords

coordination chemistry; europium; magnetic resonance imaging; screening

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1. Introduction

The presence of hypoxia in tumors has been correlated to the propagation and persistence of many cancers,^{1–6} making hypoxic tissue an important therapeutic and diagnostic target. In pursuit of monitoring hypoxia for clinical and preclinical applications, contrast-enhanced magnetic resonance imaging (MRI) is one technique that has been studied.^{7–10} Contrast agents for MRI commonly contain Gd^{III} because this ion accelerates the T_1 relaxation of water protons, thus inducing positive contrast enhancement in T_1 -weighted MRI. However, Gd^{III} is not redox-active under physiological conditions, necessitating that Gd^{III}-containing contrast agents contain redox-sensitive ligands to report hypoxia.^{11–15} Consequently, some level of contrast enhancement from Gd^{III} is observable at all times, potentially confounding experiments focused on imaging hypoxia. Contrast agents based on redox-active Eu^{II} are of interest for the study of hypoxia because the Eu^{II} ion is isoelectronic to the Gd^{III} ion and exhibits similar T_1 -shortening behavior to Gd^{III};^{16–22} however, unlike Gd^{III}, Eu^{II} is redox-active under physiological conditions and ceases to enhance contrast at imaging-relevant concentrations upon oxidation to the +3 oxidation state. The redox-activity and contrast-enhancing properties of Eu^{II} and Eu^{III} have recently been used to differentiate oxygen-rich and oxygen-deficient regions *in vivo*,^{23–25} opening the door for research into other ligands that are compatible with redox-active europium. Oxygen-sensitive Eu-containing complexes for MRI involve the coordination chemistry of both Eu^{II} and Eu^{III}, and these ions often require different coordination environments. The rational design of new Eu^{II}-based contrast agents requires information regarding the ability of ligands to bind both Eu^{II} and Eu^{III} to enable sequestration of europium in either oxidation state. To rapidly detect ligands that bind both the relatively soft, large Eu^{II} ion and the harder, smaller Eu^{III} ion, we sought to develop screening procedure that uses MRI to indicate binding. We chose MRI for our screening protocol over other analytical techniques because not all complexes produce observable contrast enhancement. For example, complexes with no inner-sphere water might not enhance contrast. Because ligand environments directly affect parameters relating to positive contrast enhancement in T_1 -weighted MRI, the use of MRI enables selection of ligands that both coordinate europium ions and show promise for use in MRI. Here, we report a proof-of-concept study that uses a set of eight reported ligands to test our new MRI-based ligand screening procedure.

To design a screening procedure with MRI, we sought to display contrast enhancement (bright images) in T_1 -weighted images only upon the interactions of metals and ligands, and no contrast enhancement (dark images) in the absence of interactions between metals and ligands. This selective contrast enhancement is possible due to the precipitation of uncomplexed metal with phosphate-buffered saline (PBS) prior to imaging. Although this design is potentially useful for the study of Eu^{II}, it precludes use with the Eu^{III} ion. Because Eu^{III} does not shorten the T_1 of water protons, solutions containing Eu^{III} display the same dark contrast in T_1 -weighted images as solutions containing no metal ions.²⁵ To use MRI to study the coordination of Eu^{III}, we suspected that the T_1 -shortening Gd^{III} ion would be a viable surrogate due to the similar size and coordination chemistry of the two ions.^{26,27} Studying Gd^{III}, which produces MRI contrast enhancement, to predict the binding of Eu^{III} reverses the common practice of using luminescence spectroscopy of Eu^{III} to estimate the

binding properties of Gd^{III}-containing complexes.^{28–30} Therefore, in our screening procedure (Figure 1), we assume that a bright signal in a T_1 -weighted MR image of a solution of ligand and Gd^{III} predicts the binding of that ligand to Eu^{III}. Furthermore, to eliminate the need for large-scale syntheses of potential ligands, we sought to design our screening procedure to require small quantities of ligand (<1 mg). Therefore, to simultaneously screen multiple ligands for binding to both Eu^{II} and Eu^{III} with MRI, we chose to use multiwell plates, mirroring other MRI-based screening procedures.^{31–33} Additionally, multiwell plates are central to screens throughout chemical biology that involve small amounts of substrates or ligands, as exemplified by techniques developed by Laura Kiessling and coworkers.^{34–36}

2. Materials and Methods

2.1. General Methods

Commercially available chemicals were of reagent-grade purity and were used without purification unless otherwise noted. Water was purified using a PURELAB Ultra Mk2 water purification system (ELGA) and degassed under reduced pressure before use. PBS (10×) was purchased from Fisher Bioreagents. Ligand **1** (4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane) was purchased from TCI chemicals. Ligand **2** (5,6-benzo-4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacos-5-ene) was purchased from Sigma-Aldrich as a 50 wt % solution in toluene, and the toluene was removed under reduced pressure before use. Ligands **3** (4,7,13,16,21-pentaoxa-1,10-diazabicyclo[8.8.5]tricosane) and **5** (diethylenetriaminepentaacetic acid) were purchased from Alfa Aesar. Ligand **7** (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) was purchased from Adamas-Beta. Ligands **4** (1,4,7,10,13,16,21,24-octaazabicyclo[8.8.8]hexacosane),^{37,38} and **8** (2,2', 2'', 2'''-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetrakis(*N*-(4(trifluoromethyl)benzyl)acetamide))²⁵ were synthesized following reported procedures. ¹H and ¹³C-NMR spectra were acquired at 65 °C (to improve peak resolution relative to lower temperatures) using a Varian VNMRs-500 spectrometer (499.42 MHz for ¹H and 125.59 MHz for ¹³C). Chemical shifts are reported relative to residual solvent signal (D₂O: ¹H δ 4.75 ppm) or an internal standard (acetonitrile in D₂O: ¹³C δ 1.47 ppm). NMR spectra are assumed to be first order and the multiplicity is reported as “brs” = broad singlet, and “m” = multiplet. High-resolution electrospray ionization mass spectrometry (HRMS) was performed using a Waters LCT Premier XE time-of-flight high-resolution mass spectrometer.

2.2. Synthesis and Characterization of ligand **6** (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis-(acetamidoacetate))

t-Butyl-protected ligand **9** (tetra-*tert*-butyl-2,2', 2'', 2'''-((2,2', 2'', 2'''-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetrakis(acetyl))tetrakis-(azanediyl))tetraacetate) was synthesized following reported procedures (See Supplementary Material).^{39,40} A mixture of *t*-butyl-protected ligand **9** (602.7 mg, 0.7036 mmol) and aqueous HCl (3 M, 20 mL) was stirred for 4.5 hours. Solvent was evaporated, and the resulting residue was dissolved in water and freeze-dried to yield **6** as a white solid (440.4 mg, 99%). ¹H NMR (499.42 MHz, D₂O): δ 3.67 (brs, 16H), 4.00–4.50 (m, 16H); ¹³C NMR (125.59 MHz, D₂O): δ 41.8, 50.8,

55.8, 169.6, 173.3. HRMS (m/z): $[M + H]^+$ calcd. for $C_{24}H_{41}N_8O_{12}$, 633.3767; found, 633.2821.

2.3. Screening Procedure and MRI

All samples for the screening procedure were prepared under N_2 in a wet glovebox (water allowed but not O_2) by stirring $EuCl_2$ or $GdCl_3$ (4.0 μ mol, 1.3 equiv) with ligand (3.0 μ mol, 1.0 equiv) in degassed water (2 mL) for 20 h at 25 or 60 °C. All samples were sealed in glass vials with Teflon caps to prevent water evaporation throughout the stirring step. Samples prepared at 60 °C were allowed to cool to ambient temperature for 2 h after the 20 h heating step. PBS was added to each sample resulting in solutions (3 mL each) that contained 1.6 mM phosphate (pH 7.4 ± 0.2). Phosphate was added to precipitate unreacted metal by forming insoluble salts with phosphate anions. After stirring for 20 h, samples were filtered through 0.20 μ m Millex-LG low protein binding hydrophilic polytetrafluoroethylene filters to remove insoluble phosphate salts before transfer of aliquots (0.5000 mL) of the filtrates into a specially engineered polyetherimide (ULTEM[®]) 47-well plate. Use of the polyetherimide plate aids in shimming and thus prevents artifacts in gradient echo images (See Supplementary Information for details of the design).⁴¹ Due to the limited gradient linearity and B_1 homogeneity of the MRI scanner, only data from the center wells (48 mm $\times$ 60 mm) of the plate were used in this study. The filled plate was capped with standard NMR-tube caps and sealed with wax to maintain inert atmosphere and thus prevent oxidation from molecular oxygen during MRI.⁴² MRI was performed using a Bruker ClinScan small animal scanner (300.43 MHz, 7.06 T) equipped with a 30 cm bore magnet. T_1 -weighted images were acquired at 21 °C using a three-dimensional fast low angle shot (FLASH) pulse sequence with an echo time of 3.26 ms; a repetition time of 21 ms; flip angles of 5, 10, 20, 30, 40, 50, and 60°; a pixel bandwidth of 260 Hz; two averages; a field of view of 48 mm $\times$ 110 mm $\times$ 32 mm; and a matrix size of 112 $\times$ 256 $\times$ 16; resulting in a resolution of 0.43 mm $\times$ 0.43 mm $\times$ 2 mm. The determination of T_1 values from the data followed a reported procedure.⁴³ T_1 maps were obtained based on pixel-by-pixel fitting to a two-parameter (T_1 and proton density) function. Perfect B_1 homogeneity and flip angle calibration were assumed. One slice covering all wells was processed. SPIN software was used to measure T_1 values. T_1 maps were extrapolated (2 $\times$) with the nearest-neighbor zoom type; therefore, the pixel number was increased by a factor of four, and the resolution was reduced to 0.215 mm $\times$ 0.215 mm $\times$ 2 mm. After zooming, regions of interest were manually drawn inside each well on the T_1 maps to obtain the mean and standard deviation of T_1 for pixels in that well. Near-edge pixels were avoided in the regions of interest to minimize partial volume effects.

2.4. Elemental Concentration Measurements

Energy-dispersive X-ray fluorescence measurements of Eu and Gd concentration were performed with a Shimadzu EDX-7000 spectrometer in the Lumigen Instrument Center at Wayne State University. A serial dilution was used to prepare calibration solutions from commercially available standards (Fluka ICP standard solutions for Eu, 1002 mg/L, and Gd, 1000 mg/L). Calibration curves consisting of four points were created using the fluorescence intensity at 5.845 keV for Eu and 6.056 keV for Gd of standards in a 31–500 ppm

concentration range. Concentrations in Tables 1 and 2 are reported at the 90% confidence interval from analysis of the calibration curves.

2.5. Minimum Detectable Concentrations of Eu and Gd

The minimum detectable concentrations of Eu and Gd for the energy-dispersive X-ray fluorescence experiments were determined using a reported procedure.⁴⁴ Calibration curves consisting of four points between 63 and 500 ppm Eu or Gd and seven independently prepared samples each of 60 ppm Eu and 60 ppm Gd were prepared from commercially available standard solutions (Fluka ICP standard solution for Eu, 1002 mg/L; Fluka ICP standard solution for Gd, 1000 mg/L). The corresponding fluorescence intensities of each sample and calibration point were plotted as a function of concentration. To determine the limit of detection, eq. 1 was employed,⁴⁴ where m is the slope of the emission intensity versus concentration (0.0189 units mg^{-1} L for Eu; 0.0229 units mg^{-1} L for Gd), and σ is the standard deviation of the emission intensities of the seven independently prepared samples of the same concentration (0.096 units for Eu; 0.066 units for Gd). From these calculations, the minimum detectable concentrations (90% confidence interval) of Eu and Gd were 100 and 60 μM , respectively.

$$\text{minimum detectable concentration} = 3\sigma/m \quad (1)$$

3. Results and Discussion

To use MRI to predict ligand coordination, our screening procedure displays bright T_1 -weighted images when paramagnetic metal ions, like Eu^{II} or Gd^{III} , interact with water. To ensure that bright signals correspond to the interaction of a ligand of interest with Eu^{II} or Gd^{III} and not uncomplexed metal ions, uncomplexed metal ions were removed prior to imaging. The removal involves introducing PBS after mixing of the metal salt and ligand solutions. Uncomplexed Eu^{II} and Gd^{III} ions form insoluble salts with the phosphate anions in PBS,^{45,46} and the solids can be removed by filtration. Therefore, when the filtrate is imaged using T_1 -weighted MRI, a bright signal is only observed if interactions between Eu^{II} or Gd^{III} ions and ligand prevent precipitation with phosphate and result in the formation of water-soluble complexes (Figure 1).

To demonstrate that uncomplexed Gd^{III} and Eu^{II} ions precipitate and are removed below detectable levels prior to MRI, control experiments were performed. The control experiments involved stirring EuCl_2 or GdCl_3 (1.3 mM) in water without added ligand. Upon the addition of PBS and subsequent filtration, energy-dispersive X-ray fluorescence measurements indicated that no metal was present in the filtrate ($<100 \mu\text{M}$ Eu; $<60 \mu\text{M}$ Gd). These results are in line with the solubility products for GdPO_4 ($10^{-25.39}$)⁴⁷ and $\text{Sr}_3(\text{PO}_4)_2$ (10^{-31}).⁴⁸ The solubility product of Sr^{II} phosphate was used as an approximation for Eu^{II} phosphate because Eu^{II} and Sr^{II} have been reported to form isostructural complexes.¹⁷ Therefore, any MRI-detectable metal remaining in ligand-containing samples after filtration must be due to ligand association.

To establish the utility of the screening procedure, a proof-of-concept experiment was performed involving a series of ligands that were selected based on their ability to bind Eu^{II} and Gd^{III} (Figure 2). Ligands **1–4** were selected because they bind Eu^{II} ,^{26,49,50} and ligands **5–8** were selected because they bind Gd^{III} or Eu^{III} .^{25,51,52} Ligands **1**, **3**, and **5–8** have been reported as complexes with Eu^{II} and Eu^{III} or Gd^{III} .^{25,26,51–55} Additionally, ligands **4** and **8** enable exploration of the constraints of usefulness of our screening procedure: Because **4** binds Eu^{II} only at pH values >8 , it was selected to demonstrate a constraint of our procedure with respect to pH-dependent complexation. Because ligand **8** is insoluble in water, but Eu^{II} - and Eu^{III} -containing complexes of **8** are soluble in water after synthesis in a mixture of methanol and acetonitrile,²⁵ this ligand was selected to study a constraint of our screening procedure with respect to ligand solubility.

To test our screening procedure, solutions of ligands **1–8** were combined with EuCl_2 or GdCl_3 . We also prepared samples of EuCl_2 or GdCl_3 without ligand to corroborate our energy-dispersive X-ray fluorescence data. After subsequent addition of PBS, stirring (20 h), and filtration, MRI was performed (Figure 3). For the solutions of EuCl_2 or GdCl_3 without ligand, no contrast enhancement was observed, further supporting the premise that uncomplexed metal is removed prior to imaging. For solutions initially containing Eu^{II} , a bright T_1 -weighted signal was observed with ligands **1** and **2**, as expected.^{26,53} Although the relative intensities of contrast enhancement are not equivalent for ligands **1** and **2** with Eu^{II} , all visible contrast enhancement is indicative of the interaction of metal ions with ligands. Specifically, the absolute intensity depends on both the concentration of complex in solution and the relaxivity of the complex, which depends on the structure of the ligand. For ligand **3** with Eu^{II} , no contrast enhancement was observed despite reports of complexes containing the Eu^{II} ion.²⁶ The lack of observable complexation with **3** is supported by reports of the complex of Eu^{II} with **3** being more labile than Eu^{II} -containing complexes of ligands **1** and **2**.^{26,53} Additionally, no T_1 -weighted contrast enhancement was observed for ligand **4** after stirring in the presence of Eu^{II} . The lack of complexed Eu^{II} ion with **4** arises due to the neutral pH imposed by the addition of PBS; prior to the addition of buffer, solutions of Eu^{II} have a pH of 10.0, but dissociation of Eu^{II} is expected at pH values <8 .⁵⁰ Dissociation of Eu^{II} from **4** after the addition of PBS is supported by the observed color change from yellow (indicative of complexation of **4** with Eu^{II}) to colorless (indicative of no complexation) upon addition of PBS. Additionally, T_1 -weighted MRI indicated interaction between Eu^{II} and ligand **6**, consistent with reports of the Eu^{II} -containing complex of this ligand.^{52,56} The interaction between Eu^{II} and ligand **6** is further supported by the observed change in color from colorless (indicative of no complexation) to yellow (indicative of complexation) upon the addition of PBS. Although the change in color from colorless to yellow upon complexation of Eu^{II} to ligands **4** and **6** is useful in indicating complexation, many complexes of Eu^{II} are colorless, including the other complexes of Eu^{II} examined in this study. The lack of color limits the use of optical assays to detect complexation of Eu^{II} . As expected, no interactions of Eu^{II} with ligands **5**, **7**, or **8** were observed. The lack of observed contrast enhancement in solutions of Eu^{II} with ligands **5** and **7** is likely because the Eu^{II} -containing complexes of ligands **5** and **7** possess relatively negative electrochemical potentials,^{42,54,55} and therefore, we do not expect Eu^{II} in these complexes to remain in the +2 oxidation state throughout the timeframe of our screening procedure. For ligand **8**, the

low aqueous solubility of the ligand prevented Eu^{II} from interacting with the ligand. This conclusion is supported by the observation of white solid following 20 hours of stirring in water containing Eu^{II} .

As a secondary method to corroborate our imaging data, X-ray fluorescence measurements were performed (Table 1). The results were consistent with the imaging data with two outliers. Although the samples containing ligands **5** and **7** with Eu^{II} displayed no contrast enhancement in T_1 -weighted images, energy-dispersive X-ray fluorescence measurements indicated the presence of europium in these samples. This phenomenon is likely due to redox events resulting in the formation of Eu^{III} -containing complexes. Eu^{II} -containing complexes of **5** and **7** are known to have relatively negative electrochemical potentials that could enable reduction of advantageous O_2 , the ligand, or solvent.^{54,55} This phenomenon is consistent with the observation that low concentrations (~ 1 mM) of Eu^{II} **5** exhibit an oxidative half-life of ~ 1 h.⁵⁴ Therefore, despite the presence of europium in the samples containing ligands **5** and **7**, the negative electrochemical potentials and consequent redox events preclude contrast enhancement. This observation indicates that an electrochemical boundary of selectivity for our screening method lies between -1.135 and -0.903 V vs Ag/AgCl. This boundary is based on the most positive $E_{1/2}$ value of a complex that did not display contrast enhancement (Eu^{II} **5**, $E_{1/2} = -1.135$ V vs Ag/AgCl),⁴² and the most negative $E_{1/2}$ value associated with a complex that did exhibit enhanced contrast (Eu^{II} **6**, $E_{1/2} = -0.903$ V vs Ag/AgCl).⁴²

In the same multiwell experiment used to examine binding to Eu^{II} at ambient temperature, ligands **1–8** were screened for binding to Gd^{III} (Figure 3). For the screening of ligands with Gd^{III} , T_1 -weighted contrast enhancement was only observed with ligands **5** and **7**. Contrast enhancement with these two ligands was expected because Gd^{III} -containing complexes of these ligands are used clinically.⁵⁷ The lack of observed contrast enhancement for ligands **1–3** was also expected for aqueous complexation,⁵⁸ due to the ethereal donors of the cryptands being insufficient to compete with protic solvents for coordination to Gd^{III} .⁵⁹ Ligand **4** is likely unable to form inert complexes with tripositive lanthanide ions at neutral pH, as has been suggested in the literature.⁶⁰ Additionally, Gd^{III} -containing solutions with ligand **6** showed no visible contrast enhancement in T_1 -weighted images. We suspect the lack of observed binding to be due to the slow, pH-dependent formation of Gd^{III} **6**.⁶¹ Like Eu^{II} , Gd^{III} did not form a detectable complex with ligand **8** because of the low aqueous solubility of **8**.

To gain quantitative information regarding ligand interactions with Eu^{II} or Gd^{III} from the MR images, we calculated average T_1 values for each solution from a T_1 map of the entire multiwell plate (Table 1).⁴³ By comparing the water proton T_1 of each solution to that of PBS in the same multiwell experiment, we were able to detect the presence of paramagnetic metal complexes, some of which were not visible in the T_1 -weighted images. For samples containing Eu^{II} , the only samples with shortened values of T_1 were also the samples that displayed visible contrast enhancement in the T_1 -weighted image. For samples containing Gd^{III} , we detected one inconsistency between the visible contrast enhancement in the T_1 -weighted image and the T_1 values: a shortening of T_1 for ligand **6** and Gd^{III} compared to PBS alone (Table 1). This observation was corroborated by energy-dispersive X-ray fluorescence measurements that revealed the presence of gadolinium in the solution with

ligand **6** and indicates that Gd^{III} interacts with ligand **6** after stirring at ambient temperature, but not as strongly as with ligands **5** and **7**. This observation is consistent with the slow formation of Gd^{III} with **6**,⁶¹ and it demonstrates the utility of T_1 measurements for detecting relatively weak metal-ligand interactions that result in concentrations of complexes that are not visible in T_1 -weighted images.

In analyzing the results of a screening protocol, it is important to examine ligand associations that could lead to false-positive or false-negative results. A possible issue with the screening procedure, specifically relevant to 1,4,7,10-tetraazacyclododecane (cyclen)-based ligands with carboxylate arms, is the possibility of metal ions associating with the arms of the ligand without fully binding to the ring.^{61–64} For example, in the synthesis of $\text{Gd}^{\text{III}}\text{7}$, a Gd^{III} ion binds to the carboxylate arms of a ligand first, forming a diprotonated intermediate.^{61–64} For Gd^{III} to settle into the cyclen ring of the ligand, the protons must be removed, either by heating or by pH-adjustment to more basic pH from the acidic pH imposed by proton liberation from the ligand interacting with metal.^{61–64} Therefore, it is reasonable to conclude that the MRI signal observed for $\text{Gd}^{\text{III}}\text{7}$ after stirring at ambient temperature might be due, at least in part, to ligand association and not full complexation. The stepwise formation of $\text{Gd}^{\text{III}}\text{7}$ potentially leads to a false positive result in our screening protocol after stirring only at ambient temperature, despite $\text{Gd}^{\text{III}}\text{7}$ being a clinically approved contrast agent that is inert at neutral pH. Further, evidence of only a small amount of interaction of ligand **6** with Gd^{III} based on the T_1 map is likely because of incomplete complexation. In this case, the T_1 map detected what would have been a false-negative based on the T_1 -weighted image. However, these points do not diminish the usefulness of the screening procedure for selecting potential candidates for detailed studies. After complex formation at ambient temperature, phosphate precipitation, and MRI screening, the ligands were observed to interact with Eu^{II} and Gd^{III} as expected based on previous reports, with the exception of ligand **3** with Eu^{II} and ligand **6** with Gd^{III} . With the consideration of incomplete binding in mind, we expanded the stirring conditions to include heat.

To use our screening procedure to extract additional information regarding complexation of Eu^{II} and Gd^{III} (and therefore Eu^{III}), the procedure was repeated with heating at 60 °C during the stirring of metal ions with ligands (Figure 4). With the addition of heat, Eu^{II} and Gd^{III} displayed binding to the ligands similar to that at ambient temperature with four important exceptions: EuCl_2 with ligands **3**, **4**, and **6** and GdCl_3 with ligand **6**. Although Eu^{II} displayed no binding activity at ambient temperature with **3**, shortening of T_1 was observed after stirring with heat. Energy-dispersive X-ray fluorescence measurements confirmed the presence of europium and, therefore, of complexed europium in the PBS solutions of Eu^{II} with ligand **3** (Table 2). Because Eu^{II} only displays interaction with ligand **3** after heating, we can conclude that heat either overcomes a thermal barrier to metallation or increases the reaction rate of the complexation with ligand **3**. However, this is a small change that was not detectable in the T_1 -weighted image. Although the sample containing EuCl_2 and ligand **4** displayed a slightly shorter T_1 than that of PBS, we were unable to detect europium using energy-dispersive X-ray fluorescence, suggesting that this is not a real difference. Interestingly, the addition of heat to the stirring step decreased the observed interaction between Eu^{II} and ligand **6**, as shown by a longer T_1 compared to that measured in the

ambient stirring experiment. Although the relatively negative electrochemical potential of Eu^{II} **6** is not as negative as that of Eu^{II} -containing complexes of ligands **5** and **7**,⁴² the addition of heat promotes redox chemistry, and therefore could mask a positive result. This premise is further supported by X-ray fluorescence measurements indicating similar europium content in samples prepared at ambient temperature and at 60 °C. Therefore, we conclude that the sample prepared by mixing Eu^{II} with **6** with heating contains a mixture of Eu^{III} - and Eu^{II} -containing complexes. Notably, Gd^{III} only displayed a slight interaction with ligand **6** after mixing at ambient temperature, as indicated by T_1 measurement. However, with heating during the stirring step, this mixture yields contrast enhancement visible in the T_1 -weighted image, signaling that heat increased the amount of binding of ligand **6** to Gd^{III} . Therefore, the addition of heat leads to positive results for subtler ligand-metal interactions for both Eu^{II} and Gd^{III} . Furthermore, the addition of heat to the stirring step possibly eliminates false-positive results with Gd^{III} samples and cyclen-based ligands that contain carboxylates. However, because Eu^{II} is readily oxidized, adding heat, and thereby promoting redox chemistry, limits the application of our screening technique to ligands with electrochemical potentials more positive than can be evaluated with stirring at ambient temperature.

4. Conclusions

To aid in the search for ligands that bind Eu^{II} and Eu^{III} , we developed an MRI-based screening procedure to evaluate multiple ligands simultaneously. In the resulting MR images, bright contrast corresponds to the presence of metal-ligand interactions, and dark contrast indicates a lack of interactions with paramagnetic ions. Although Eu^{III} does not detectably shorten T_1 , we were able to estimate the binding of Eu^{III} by studying the binding of Gd^{III} . Using a library of eight ligands with known binding interactions to Eu^{II} and Eu^{III} , we detected complexation from T_1 -weighted images and T_1 values. Although several parameters—including temperature, time, and pH—can be adjusted to optimize complex formation, those parameters are often ligand-specific. The screening protocol reported here includes two common sets of conditions (ambient-temperature and heated stirring) for metallation. These conditions provide a baseline for studying the interactions of a collection of ligands. Furthermore, we envision modifications with respect to the size of the wells and the number of plates to make the reported method into a high-throughput screening procedure. We expect that this screening protocol will provide a powerful technique for the pursuit of redox-responsive Eu-based contrast agents because it opens the door to testing large arrays of new ligands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A. Supplementary data

Supplementary material associated with this article can be found, in the online version at.

Figure 1.

Flow chart describing the experimental procedures and two possible outcomes for the screening protocol. (Top) After stirring ligand L_1 with Eu^{II} or Gd^{III} in water, a complex forms. Uncomplexed metal ions precipitate ($\downarrow$) with the addition of PBS, leaving the complex in solution. Imaging of the filtrate results in a bright T_1 -weighted image. (Bottom) After stirring ligand L_2 with Eu^{II} or Gd^{III} in water, a complex does not form. Metal ions precipitate ($\downarrow$) with the addition of PBS, leaving no lanthanide complex in solution. Imaging of the filtrate results in a dark T_1 -weighted image that is not different from images of buffer. Spectator ions are not shown for clarity.

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Figure 3.

(Left) T_1 -weighted MR image (flip angle of 50°) of PBS solutions of GdCl_3 or EuCl_2 with ligands **1–8** after stirring at ambient temperature. Dashed-line circles are a guide to denote the location of wells. (Right) Key for the image on the left where (a) denotes PBS; (b) indicates a control sample prepared by stirring GdCl_3 in water without ligand, adding PBS, and filtering; and (c) indicates the analogous control prepared from EuCl_2 without ligand. The other wells contain samples of GdCl_3 or EuCl_2 with ligands **1–8** in PBS after filtration, with the numbers in the circles corresponding to the identities of the ligands. The solid circle denotes an empty well. Each well has a diameter of 6.5 mm and a volume of 500 μL .

Figure 4.

(Left) T_1 -weighted MR image (flip angle of 50°) of PBS solutions of GdCl_3 or EuCl_2 with ligands **1–8** after stirring at 60°C . Dashed-line circles are a guide to denote the location of wells. (Right) Key for the image on the left where (a–c) denote PBS. The other wells contain samples of GdCl_3 or EuCl_2 with ligands **1–8** in PBS after filtration, with the numbers in the circles corresponding to the identities of the ligands. The solid circle denotes an empty well. Each well has a diameter of 6.5 mm and a volume of 500 μL .

Table 1

T_1 values and metal content after stirring at ambient temperature and phosphate precipitation.

Sample ^a	T_1^b (ms)	Metal Content (mM)
Eu ^{II} 1	520 ± 20	0.6
Eu ^{II} 2	1100 ± 20	0.4
Eu ^{II} 3	6300 ± 600	<0.1
Eu ^{II} 4	6500 ± 500	<0.1
Eu ^{II} 5	4800 ± 400	0.8
Eu ^{II} 6	1030 ± 20	0.6
Eu ^{II} 7	5900 ± 500	0.6
Eu ^{II} 8	6200 ± 300	<0.1
Gd ^{III} 1	4800 ± 800	<0.06
Gd ^{III} 2	5100 ± 300	<0.06
Gd ^{III} 3	6000 ± 1000	<0.06
Gd ^{III} 4	5800 ± 300	<0.06
Gd ^{III} 5	169 ± 5	1
Gd ^{III} 6	3000 ± 200	>0.06 and <1
Gd ^{III} 7	374 ± 7	1
Gd ^{III} 8	5900 ± 700	<0.06
Eu ^{II} control ^c	6000 ± 800	<0.1
Gd ^{III} control ^c	6000 ± 400	<0.06
PBS	4500 ± 400	not applicable

^aCounter ions are not listed for clarity.

^bValues are the mean ± standard error of 372 pixels.

^cwithout ligand.

Table 2

T_1 values and metal content after stirring at 60 °C and phosphate precipitation.

Sample ^a	T_1^b (ms)	Metal Content (mM)
Eu ^{II} 1	680 ± 10	0.5
Eu ^{II} 2	1830 ± 80	0.2
Eu ^{II} 3	3900 ± 300	0.1
Eu ^{II} 4	4000 ± 400	<0.1
Eu ^{II} 5	5800 ± 900	0.8
Eu ^{II} 6	3600 ± 300	0.5
Eu ^{II} 7	5200 ± 700	0.7
Eu ^{II} 8	4400 ± 400	<0.1
Gd ^{III} 1	7300 ± 900	<0.06
Gd ^{III} 2	6100 ± 600	<0.06
Gd ^{III} 3	6000 ± 900	<0.06
Gd ^{III} 4	4900 ± 600	<0.06
Gd ^{III} 5	334 ± 4	1
Gd ^{III} 6	1150 ± 20	>0.06 and <1
Gd ^{III} 7	341 ± 5	1
Gd ^{III} 8	4800 ± 600	<0.06
PBS	5300 ± 400	not applicable

^aCounter ions are not listed for clarity.

^bValues are the mean ± standard error for 289 pixels (for PBS, values are the mean ± standard error of 867 voxels).