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Prototypes of Lanthanide(III) Agents Responsive to Enzymatic Activities in Three Complementary Imaging Modalities: Visible/Near-Infrared Luminescence, PARACEST- and T_1 -MRI

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KEYWORDS: responsive probe, near-infrared, lanthanide complex, luminescence, PARACEST, MRI, optical imaging.

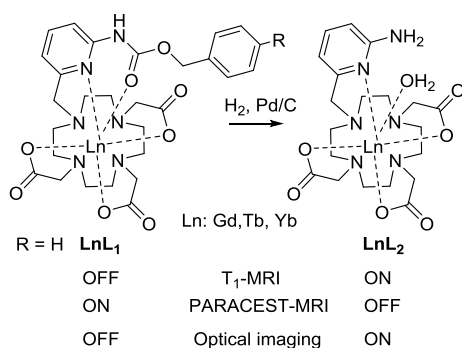
ABSTRACT: We report first prototypes of responsive lanthanide(III) complexes that can be monitored independently in three complementary imaging modalities. Through the appropriate choice of lanthanide(III) cations, the same reactive ligand can be used to form complexes providing detection either by (i) visible (Tb^{3+}) and near-infrared (Yb^{3+}) luminescence, (ii) PARACEST- (Tb^{3+} , Yb^{3+}) or (iii) T_1 -weighted (Gd^{3+}) MRI. The use of lanthanide(III) ions of different natures for these imaging modalities induces only a minor change in the structure of complexes that are therefore expected to have a single biodistribution and cytotoxicity.

Molecular imaging aims at visualizing biological objects or biochemical events at physiological, cellular and molecular levels within living cells, tissues or whole organisms for research, diagnostic or personalized medicine purposes.¹ It relies on selective and/or responsive imaging agents optimized for a specific imaging modality,² *i.e.* magnetic resonance imaging (MRI),³ optical imaging,⁴ ultrasonography,⁵ tomography⁶ etc. As each modality has its advantages and limitations, additional and less ambiguous information becomes accessible by sequentially or simultaneously combining several complementary techniques.⁷ The broad diversity of molecular events requires innovative solutions for their monitoring. Among them, enzymatic activities are of interest since their dysregulation is involved in many diseases. Smart imaging agents endowed with a responsive mechanism are required to detect these activities.⁸ Despite long-term efforts, progress in *in vivo* responsive imaging remains limited.⁹ An appealing strategy to accelerate the development of molecular imaging relies in the design of responsive probes suitable for different detection techniques using versatile molecular platforms, thus optimizing both time and efforts for synthetic work. Such platforms must combine a trigger, which can be adapted to a broad variety of molecular events, and a reporter moiety that can be detected by several imaging modalities.¹⁰ We previously designed a platform suitable for detection of enzymatic activities by PARACEST-MRI¹¹ in which an enzyme-specific trigger was coupled to a Ln^{3+} -based reporter using a self-immolative spacer. We have demonstrated that the

action of the selected enzyme resulted in elimination of the spacer associated with a change in MRI signal. In the present work, we set the basis of smart imaging probes which, in addition to PARACEST-MRI, allow the detection of enzymatic event by visible and near-infrared (NIR) luminescence and by T_1 -MRI. Bio-imaging in the NIR provides a major advantage of enhanced detection sensitivity due to the limited autofluorescence of biological tissues in this energy window.¹² Several NIR-emitting Ln^{3+} ions¹³ proved attractive for microscopy imaging of living cells.¹⁴ Moreover, Ln^{3+} -based imaging agents possess several complementary advantages over organic fluorophores, such as sharp emission bands (for spectral discrimination) and strong resistance to photobleaching.¹⁵

As Ln^{3+} cations possess versatile magnetic and optical properties while having similar reactivities,¹⁶ we have designed a molecular platform that can be made responsive and that is compatible with the complementary imaging modalities previously described (Scheme 1). Ligand H_3L_1 is a DO_3A derivative substituted by a pyridylmethyl moiety. The pyridine has multiple roles here: (i) to provide a donor atom for stable Ln^{3+} complexation,¹⁷ (ii) to act as a sensitizer for Ln^{3+} luminescence (antenna effect),¹⁸ and (iii) to introduce additional functionalities, in particular a self-immolative benzyl carbamate which will make this reporter responsive to molecular events such as enzymatic cleavage, as previously reported.¹¹ The enzymatic cleavage of such probe and the elimination of the self-immolative arm will induce the transformation of the carbamate into

an amine¹⁹ with subsequent changes in the coordination environment of the Ln³⁺ ion and modification of its hydration state. We hypothesized that such switching would be detectable by both visible and NIR luminescence as well as by T₁- and PARACEST-MRI depending on the nature of the Ln³⁺. We note that PARACEST and T₁ agents cannot be used simultaneously since the T₁ agent destroys the CEST signal, however, sequential imaging of the same animal in the two techniques can ascertain the findings and provide more accurate information. On the other hand, the luminescence capability provides higher sensitivity and resolution at the cellular level giving complementary information. LnL₁ are synthetically accessible model complexes of the responsive probes that will bear the self-immolative benzyl carbamate linker and an enzyme-specific substrate (R). For instance, β-D galactopyranoside can be used as a substrate for the detection of β-galactosidase. LnL₂ complexes are the end product of the enzymatic cleavage of LnL₁.



Scheme 1. Ln³⁺ complexes as prototypes of enzyme responsive probes for detection by visible (Tb³⁺) or near-infrared (Yb³⁺) luminescence, by T₁-weighted- (Gd³⁺) or by PARACEST- (Yb³⁺, Tb³⁺) MRI. The R in LnL₁ can be replaced by an appropriate substrate to afford enzyme sensitivity and specificity.

In order to test this concept, we synthesized and investigated Gd³⁺, Yb³⁺ and Tb³⁺ complexes of H₃L₁ and H₃L₂ (Scheme 1, Supporting Information). Synthesis of H₃L₁ was achieved in eight steps with a 13% overall yield starting from commercially available 2-bromo-6-amino pyridine. LnL₁ complexes were formed at room temperature in a pH-controlled aqueous solution upon addition of the corresponding LnCl₃ to the ligand. Their hydrolysis over palladium-charcoal in water led to the formation of the corresponding LnL₂ chelates.

Absorption spectra of the ligands H₃L₁ and H₃L₂ (Figure 1) display broad bands in the UV range centered at 232, 279 and 234, 303 nm, respectively, due to π→π* transitions. Upon formation of the Tb³⁺ or Yb³⁺ complexes, the low-energy absorption bands undergo a bathochromic shift of 5-10 nm. It is important to note a pronounced red-shift (25 nm, *i.e.* 320 vs. 345 nm) of the cutoff wavelengths of the absorption bands upon conversion of LnL₁ to LnL₂

which can be used to our advantage in the design of responsive probes.

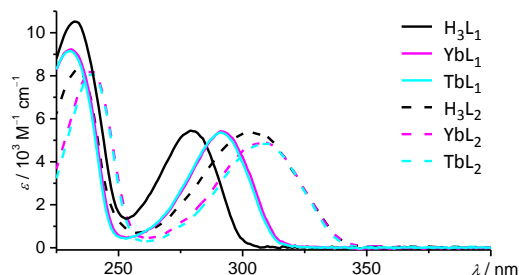


Figure 1. Absorption spectra of H₃L₁ and H₃L₂ and their corresponding Yb³⁺ and Tb³⁺ complexes (100 μM in HEPES buffer, pH 7.4, room temperature).

Excitation spectra recorded upon monitoring of the main transitions of Tb³⁺ and Yb³⁺ ions at 545 and 980 nm, respectively, match the shapes of the absorption ones, indicating that the sensitization of Ln³⁺ ions is occurring through the organic ligands (Figure 2, left). Upon excitation into ligand-centered absorption bands at 290-310 nm, TbL and YbL complexes exhibit characteristic emission in the visible or the NIR range due to ⁵D₄→⁷F_J (J = 6 - 0) or ²F_{5/2}→²F_{7/2} transitions, respectively (Figure 2, center and right). The differences in Stark splitting of the f-f transitions, that are more pronounced in the case of Yb³⁺ complexes, reflect changes in coordination environment around the Ln³⁺ ion.

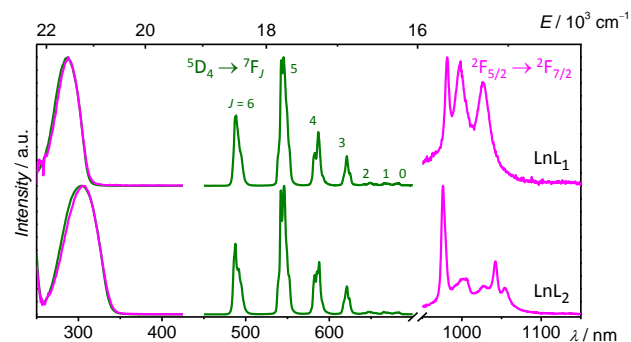


Figure 2. Left: excitation spectra of LnL₁ (top) and LnL₂ (bottom) (λ_{em} = 545 nm (Tb³⁺) and 980 nm (Yb³⁺)); center and right: emission spectra of LnL₁ (top, λ_{ex} = 290 nm) and LnL₂ (bottom, λ_{ex} = 310 nm). 100 μM in HEPES buffer, pH 7.4, room temperature. Ln = Tb³⁺ (green traces), Yb³⁺ (magenta traces).

Luminescence lifetimes and quantum yields have been determined in H₂O and D₂O solutions (Table S1). In H₂O, quantum yields and luminescence lifetime values of TbL₁ are larger than those observed for TbL₂ (37(1) vs. 22.2(2) % and 2.78(1) vs. 1.94(4) ms) but are similar when placed in D₂O (~36% and 3 ms), reflecting the role of O-H vibrations in the latter complex. Indeed, comparison of τ_{obs} in H₂O and D₂O using phenomenological equations²⁰ confirms that the Tb³⁺ ion is monohydrated in TbL₂ while no water molecule is coordinated to Tb³⁺ in TbL₁. For Yb³⁺ complexes, the situation is different due to the

intrinsically higher impact of vibrations on the non-radiative quenching of luminescence.^{13,21} Quantum yield and lifetime values are lower even in D₂O by 1.4-1.5 times for **YbL**₂ compared to **YbL**₁ (0.181(6) vs. 0.261(5) % and 5.02(2) vs. 7.7(2) μ s). This result can be explained by a stronger non-radiative quenching contribution of N-H vibrations in **YbL**₂. Moreover, in H₂O, **YbL**₂ exhibits a bi-exponential luminescence decay with lifetimes of 1.44 and 0.77 μ s. We can hypothesize the presence of an equilibrium between mono- and non-hydrated species in this case.

To analyze the abilities of the Tb³⁺ and Yb³⁺ complexes to operate as luminescence responsive probes upon elimination of the benzyl carbamate spacer, emission spectra of **LnL**₁ and **LnL**₂ were measured under ligand excitation at 340 nm (Figure 3). The choice of the excitation wavelength was dictated by the differences in absorption for the two series of complexes (Figure 1). The integrated emission intensities of **TbL**₂ and **YbL**₂ were found to be 20- and 6.5-times higher, respectively, than that of the corresponding **LnL**₁ complexes. Thus, conversion of visible (Tb³⁺) or NIR (Yb³⁺) emitting probes bearing the enzyme-specific substrate to **LnL**₂ complexes will lead to a turn-on luminescence response which will be the first example of a NIR-emitting Ln³⁺ complex for monitoring enzymatic reactions.

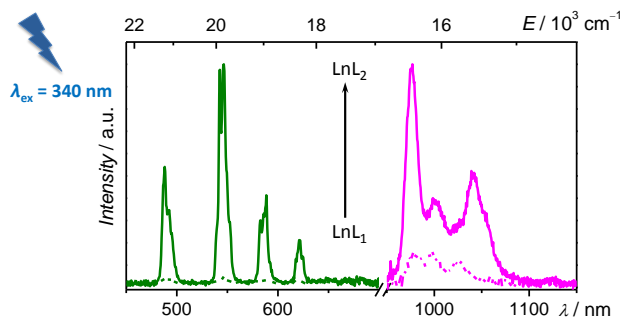


Figure 3. Demonstration of OFF-ON luminescence switch from **LnL**₁ (dotted line) to **LnL**₂ (plain line) under excitation at 340 nm (100 μ M in HEPES buffer, pH 7.4, room temperature). Ln= Tb³⁺ (green traces), Yb³⁺ (magenta traces).

To assess the potentiality of the Gd³⁺ analogues as enzyme responsive T_1 MRI agents, longitudinal water proton relaxivities, r_1 , were determined for **GdL**₁ and **GdL**₂ (0-80 MHz, 25 and 37 °C). At all fields and both temperatures, the r_1 values are considerably (90-100%) larger for **GdL**₂ than for **GdL**₁ (Supporting Information; $r_1 = 1.91 \text{ mM}^{-1}\text{s}^{-1}$ and $3.77 \text{ mM}^{-1}\text{s}^{-1}$ for **GdL**₁ and **GdL**₂, respectively; 60 MHz, 25°C). This relaxivity difference should lead to a remarkable turn-on response in T_1 -MRI upon enzymatic activation of the probe. The low relaxivity of **GdL**₁ points to a non-hydrated state, in accordance with the crystal structure (Figure S8) and the luminescence lifetimes (see above), while the higher relaxivity of **GdL**₂ is consistent with a monohydration of the Ln³⁺.

CEST spectra were recorded for **TbL**₁, **TbL**₂ and **YbL**₁, **YbL**₂ complexes in aqueous solution by applying a 3s selective saturation at 25 μ T in 1 ppm increments from -90 to +90 ppm (Yb³⁺) or -500 to +400 ppm (Tb³⁺) and

plotted as normalized water signal intensity (M_z/M_0 , %) against frequency offset (ppm). A single CEST effect (~25%) was observed at +44 ppm for **YbL**₁, while two CEST peaks appear at -105 and -115 ppm for **TbL**₁ (Figure S2), which were assigned to carbamate protons. While amide protons are a common source of PARACEST in Ln³⁺ complexes, this is the first example of carbamate protons that generate PARACEST effect. In contrast, no CEST is produced by **LnL**₂ analogues (Figures 4 and S2). Thus, the conversion of the carbamate complexes to the amine analogues upon enzymatic reaction will lead to a turn-off PARACEST response. For **TbL**₁, the two CEST peaks likely belong to two isomers, as it was reported for YbHPDO₃A.²² Indeed, Ln³⁺ complexes of cyclen-based ligands may exist as two diastereomeric pairs,²³ which are observable in the ¹H NMR spectrum of **TbL**₁ (Figure S23). The two CEST signals belonging to the same molecule can be later exploited for a ratiometric calibration that reports on enzyme activities irrespective of probe concentration.

The exchange rate of the mobile carbamate protons has been assessed by QUEST measurements for **YbL**₁ ($k_{ex}=6100\pm100 \text{ s}^{-1}$) and by the omega plot method for the two isomers of **TbL**₁ ($k_{ex}=4300\pm300 \text{ s}^{-1}$ at -115 ppm and $5000\pm400 \text{ s}^{-1}$ at -105 ppm; pH 7.4, 25°C). They are similar or slightly higher compared to values reported for Ln³⁺ tetraamide DOTA-derivatives with PARACEST effect.²⁴

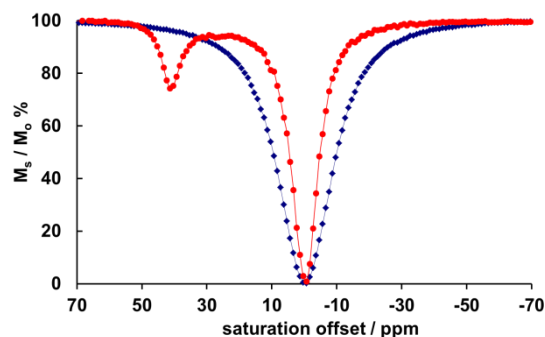


Figure 4. CEST spectra of **YbL**₁ (red) and **YbL**₂ (blue). 500 MHz, pH 7.5, 37 °C, irradiation power 25 μ T, saturation time 3 s, [**YbLi**]_{i=1,2} = 20 mM.

To further demonstrate the utility of our platform system, we have acquired T_1 -weighted and CEST MR images of the Gd³⁺ and Yb³⁺ complexes, respectively (Figure 5). These images are in accordance with the relaxivities of **GdL**₁ and **GdL**₂ and with the CEST effect observed for **YbL**₁. They evidence the potential of obtaining MRI detectable responses upon the transformation of the carbamate to the corresponding amine derivative complexes, representing off/on or on/off responses in T_1 or CEST MRI, respectively.

In conclusion, we have designed, synthesized and characterized the physico-chemical properties of complexes formed with a ligand system that can be tuned for specific detection of a wide variety of enzymatic activities using complementary detection modalities including visible and NIR optical imaging, T_1 or PARACEST MRI depending on the nature of the Ln³⁺ ion. A common triggering molecular mechanism is used for

the operation of different imaging modalities. LnL_1 and LnL_2 complexes described here model the states prior- and post-enzymatic cleavage occurring in these probes.

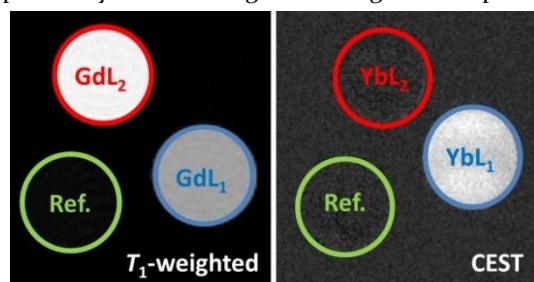


Figure 5. T_1 weighted and CEST MR images of phantoms containing complexes of H_3L_1 and H_3L_2 with Gd^{3+} (2.86 mM, pH 7.8) and Yb^{3+} (20.8 mM; pH 7.0). T_1 weighted images were acquired at 7 T using spin echo sequence with TE = 11.0 ms and TR = 200ms. CEST difference images were obtained by subtracting on-resonance (+44 ppm) from off-resonance (-44 ppm) images at 9.4 T (irradiating pulse of 25 μT , 4 s).

This is the first example where a single molecular system can be used as a responsive imaging agent in three different independent modalities. In these compounds, we have evidenced a remarkable variation in relaxivity (Gd^{3+}), in PARACEST as well as in luminescence properties (Tb^{3+} and Yb^{3+}) which open the way towards multimodal enzyme detection by using a single ligand.

ASSOCIATED CONTENT

Supporting Information. Synthetic procedures, compound characterization, ^1H NMR, NMRD, CEST spectra, photophysical measurements, ORTEP plot, crystallographic information, CIF files, HPLC analyses. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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