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# Transposing Molecular Fluorescent Switches into the Near-IR: Development of Luminogenic Reporter Substrates for Redox Metabolism

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Optical reporters enable the direct and minimally invasive probing of molecular events in complex environments such as living cells and tissues. While significant achievements have been made in the area of fluorescent sensors for metal ions and small organic compounds,[1] probes that report directly on enzyme activity are less developed by comparison, with the exception of reporters for hydrolases and kinases.[2]

Lanthanide complexes are attractive for the design of responsive probes owing to their sharp emission bands at long wavelengths and long luminescent lifetimes. While studies in this area have been dominated by the visible light emitting lanthanides $Tb^{3+}$ and $Eu^{3+}$,[3] the development of reporters based on the near-infrared (NIR) emitting metals is still in its infancy. The ions $Nd^{3+}$, $Yb^{3+}$, $Er^{3+}$, $Ho^{3+}$, and $Pr^{3+}$ have emission transitions spanning the region from 800 to 1600 nm and comprise the NIR lanthanide series.[4] NIR imaging agents have particular appeal for in vivo applications since the spectral region in which they operate allows greater tissue penetration.[5,6] Herein, we communicate the development of a NIR luminescent redox switch, based on a Nd(III) complex, that serves as a reporter substrate for the human aldo-ketoreductase 1C2 (AKR1C2).

As part of a program aimed at the development of fluorescent and luminescent enzyme activity reporters,[7] we have previously described a class of fluorogenic substrates for AKR1C2,[7a,e] an enzyme involved in steroid hormone metabolism and stress response pathways.[8] These reporters, based on an aminocoumarin fluorophore, emit a strong green fluorescence ($\lambda_{max} \approx 510$ nm) upon enzymatic reduction of the corresponding nonfluorescent ketone substrate. The importance of AKR1C2 as an emerging cellular stress marker motivated us to explore the possibility of adapting the fluorogenic substrate to a NIR emitting format. Since many organic chromophores are known to sensitize lanthanide luminescence via energy transfer (ET), we envisioned a strategy whereby our coumarin substrates could be directly converted to NIR reporters by tethering them to an appropriate lanthanide complex, thus obviating the need to evolve a new substrate based on an organic NIR fluorescent scaffold (e.g., Cy5.5)[6] (Figure 1).

Following this approach, we were faced with two key questions. The first one is whether the ketone/alcohol transformation would effect the significant change in ET efficiency necessary to afford a good luminescent switch.[9] Since the sensitization of lanthanide metals is assumed to occur via a triplet excited state of the organic chromophore,[3,4] it may be expected that groups promoting intersystem crossing (from singlet to triplet state), such as the ketone, would influence the energy transfer efficiency. However, the direction of this effect was not readily predictable on the basis of previous studies.[10,11]

The second question is how the size and polarity of the metal complex would affect the probe's ability to function as an enzyme substrate. We supposed that both ET efficiency and enzyme substrate capacity would be dependent on the length of the tether

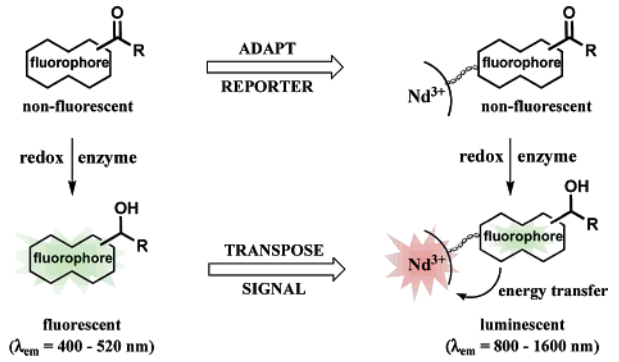


***Figure 1.*** The concept of adapting a fluorogenic redox reporter (left) to a NIR emitting format by the incorporation of lanthanide ions (right).

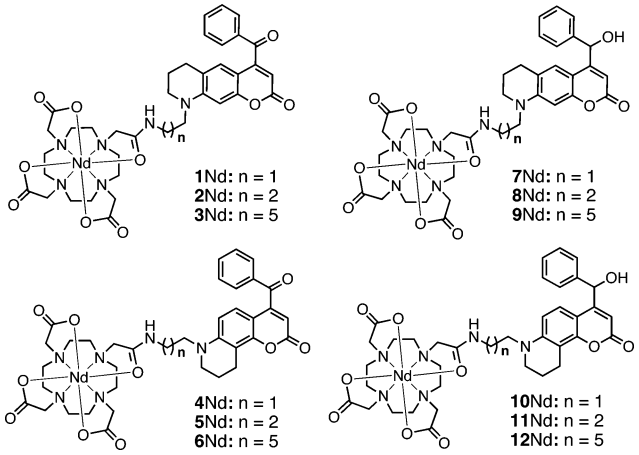


***Figure 2.*** Compounds **1**Nd−**12**Nd were synthesized to examine the effect of the linker length and the mode of attachment of the Nd:DOTA complex on luminescence switching and enzyme-substrate properties.

between the coumarin and the lanthanide complex, and we expected these parameters to be mutually antagonistic - a short tether would increase ET efficiency, while a longer tether would relieve the steric bulk from the site of enzymatic activity. We also anticipated the relative orientation of the coumarin and the complex to be a factor in enzyme recognition.

Guided by these design considerations, we synthesized a series of probe candidates comprised of macrocyclic Nd:DOTA chelates tethered via two-, three-, or six-carbon alkyl linkers to two regioisomeric aminocoumarin scaffolds (Figure 2, **1**Nd−**6**Nd). The corresponding alcohol products were also prepared (**7**Nd−**12**Nd).

Importantly, alcohols **7**Nd−**12**Nd all displayed characteristic $Nd^{3+}$ luminescence with the emission maximum at ~1060 nm (excitation at 395 nm). In contrast, ketones **1**Nd−**6**Nd had negligible emission in this region, making *the ketone/alcohol pair a robust luminogenic switch* in the reduction direction (Figure 3A). The relative luminescence intensity was calculated by integrating the


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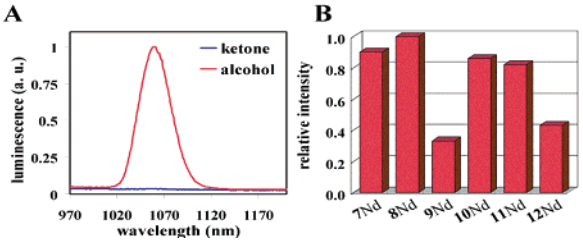
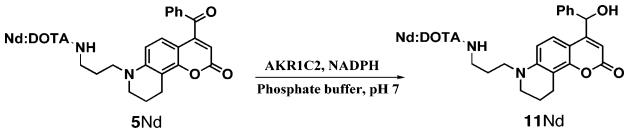



***Figure 3.*** The ketone/alcohol pairs constitute excellent redox switches (off/on switch in the reduction direction). (A) Luminescence spectra for ketone **2**Nd (blue) and alcohol **8**Nd (red); it is representative of the trend found for all examined $Nd^{3+}$ complexes. (B) Relative intensities of complexes **7**−**12**Nd (integrated intensity from 1020 to 1110 nm, normalized to **8**Nd). 5 $\mu$M solution in 100 mM phosphate buffer, pH 7, 25 °C, excitation at 395 nm.

***Table 1.*** Kinetic Parameters for AKR1C2

| | $K_m$ ($\mu$M) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ ($min^{-1}\mu M^{-1}$) |
|---|---|---|---|
| **1**Nd | 27.7 ± 2.0 | 4.1 | 0.15 |
| **2**Nd | 12.2 ± 1.1 | 2.3 | 0.19 |
| **3**Nd | 4.8 ± 0.6 | 8.4 | 1.75 |
| **4**Nd | 11.3 ± 1.4 | 2.5 | 0.22 |
| **5**Nd | 3.8 ± 0.5 | 5.7 | 1.50 |
| **6**Nd | 3.5 ± 0.4 | 3.0 | 0.86 |

***Scheme 1.*** Enzymatic Reduction of Reporter Substrates




emission intensity from 1020 to 1110 nm of 5 $\mu$M solutions at pH 7 and normalizing it to the value obtained for the most luminescent complex **8**Nd (Figure 3B). The two different coumarin scaffolds seemed to differ only slightly in their sensitizing ability. Of great interest to us was the finding that the fidelity of the energy transfer process is not significantly perturbed by extending the alkyl tether from two to three carbons (**7**Nd vs **8**Nd and **10**Nd vs **11**Nd) and the six-carbon tether supported a viable sensitized luminescent signal. This gave us confidence that some of the complexes would serve as suitable enzyme reporters since the optical switch was preserved even when the bulky lanthanide moiety was removed from the ketone substrate.

Probes **1**Nd−**6**Nd were subsequently tested against the enzyme AKR1C2 in the presence of NADPH. Gratifyingly, all of the probes were readily reduced by the enzyme, as observed by the increase in luminescence at 1060 nm. Quantitative measurement of enzymatic conversion afforded the kinetic parameters for each probe (Table 1). In accordance with our hypothesis, these parameters can be tuned by the length of the alkyl linker. For example, one carbon extension afforded nearly an order of magnitude increase in the catalytic efficiency ($k_{cat}/K_m$) between compounds **4**Nd and **5**Nd, which is a composite of 4-fold decrease of the $K_m$ value and 2-fold increase in the $k_{cat}$ (Table 1). Thus, complex **5**Nd is an excellent substrate for AKR1C2 with kinetic parameters comparable to those of the parent coumarin probe and the physiological steroid substrates (Scheme 1).[7e]

More detailed photophysical studies were carried out on alcohol products **8**Nd and **11**Nd (see Supporting Information). Time-resolved emission experiments revealed a luminescent lifetime of 81 ± 1 ns for both complexes, which is significantly longer than cellular autofluorescence. Relative to the free chelates **8** and **11**, coumarin fluorescence (510 nm) is attenuated in **8**Nd and **11**Nd by 85 and 90%, respectively. Phosphorescence measurement of the ketone/alcohol pair **5** and **11** furnished the emission maxima of 542 and 540 nm. This suggests that the luminescence switching phenomenon is not likely founded on differences in triplet energies between the ketone and alcohol chromophores, but rather on the availability of their emissive excited states.

In summary, this study describes the rational design of NIR luminescent lanthanide redox reporters. It also introduces a general platform for transposing a visible fluorescent switch to longer wavelengths by appending a luminescent lanthanide complex to a small fluorogenic substrate and demonstrated the viability of this concept in preserving enzyme substrate fidelity. Complex **5**Nd represents a promising NIR imaging probe for the cellular stress marker AKR1C2. Coupled with two-photon excitation,[12] this probe will operate in the 800/1060 nm regime, which is ideal for in vivo optical imaging.

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**Supporting Information Available:** Synthesis, characterization, and emission profiles for complexes **1**Nd−**12**Nd and enzymology assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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