Fluorescent Probes

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Multiplex Detection of Enzymatic Activity with Responsive Lanthanide-Based Luminescent Probes**

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Abstract: *Multiplex analyte detection in complex dynamic systems is desirable for the investigation of cellular communication networks as well as in medical diagnostics. A family of lanthanide-based responsive luminescent probes for multiplex detection is reported. The high modularity of the probe design enabled the rapid assembly of both green and red emitters for a large variety of analytes by the simple exchange of the lanthanide or an analyte-cleavable caging group, respectively. The real-time three-color detection of up to three analytes was demonstrated, thus setting the stage for the non-invasive investigation of interconnected biological processes.*

The reliable determination of enzymatic activity is crucial for fundamental cell biology and biochemistry, drug development, diagnosis, and therapeutic follow-up.[1] As enzymatic activity is dependent on the protein's expression level, localization, microenvironment, and substrate as well as cofactor availability, it is most accurately measured directly in situ.[2] The ability to monitor the activities of multiple enzymes in real-time, potentially in conjunction with the concentrations of regulators, would be immensely valuable. Information from such experiments would reduce the risk of false-positive or -negative diagnoses, facilitate the profiling of both healthy and diseased cells, and could establish connected nodes in cellular communication networks. We present a palette of lanthanide (Ln) based turn-on luminescent probes that enable the simultaneous, two-color detection of an enzyme and either a small molecule or a second enzyme. In combination with an organic-based fluorophore, the three-color detection of two enzymes and a regulatory small molecule was achieved. The well-separated Ln emissions make data interpretation and real-time analyte detection straightforward (Figure 1 a).

Ln emission in these probes is turned-on by the formation of a light-harvesting antenna from a caged precursor (Figure 1 b).[3] The design is highly modular, as a new analyte can be detected simply by altering the chemoselectively cleavable cage. Probes for β-glucosidase (β-Glu), β-galactosidase (β-Gal), α-mannosidase (α-Man), and phosphatase were built (Scheme 1). Analytes were chosen due to their importance as reporter enzymes,[4] in disease diagnosis,[5] in drug development,[5,6] and in biochemical assays.[7] A boronate-caged probe for reactive oxygen and nitrogen species (RONS; H_2O_2[8] and $ONOO^-$[9]) was also prepared. RONS are essential for cellular communication.[10] Protein tyrosine phosphatases (PTPs) can be regulated by RONS upon oxidation of their catalytically active Cys residue.[11] The co-detection of phosphatase activity and [RONS] has potential ramifications for drug discovery.[12] While Ln-based enzyme probes are known, these are either not able to detect enzymatic activity in real time,[13] require potentially damaging high-energy excitation,[14] or have low brightness.[3] Crucially, methods (luminescent or other) for the real-time measurement of enzymes and their regulators (e.g. PTPs and RONS[15]) are currently lacking.

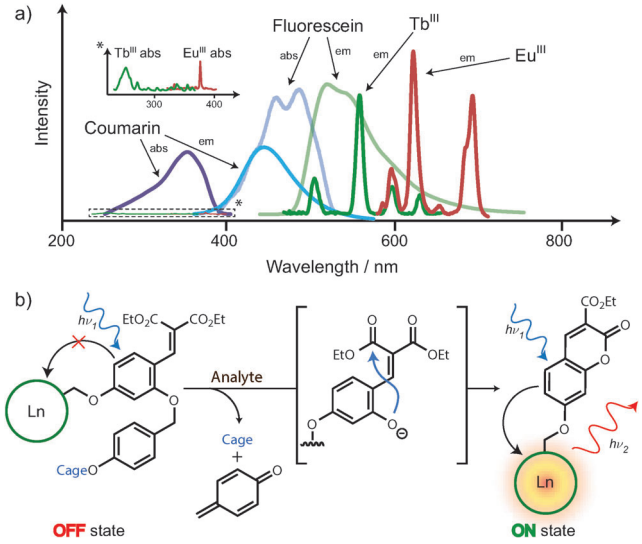

Figure 1. a) Comparision of the absorption and emission spectra of Ln- and organic-based emitters. b) Design of the turn-on probe.

The responsive probe **LnCou** ("on") and model compound **LnBn** ("off") were prepared in short, high-yielding syntheses (see the Supporting Information). Photophysical characterization was carried out on the model compounds (Table 1, see Figures S2–S5 in the Supporting Information). **LnBn** and **LnCou** had absorption maxima at 335 and 350 nm, respectively. The Ln excitation spectra were similar to their absorption spectra, thereby confirming Ln sensitization by the antennae. Excitation was possible with near-UV light,

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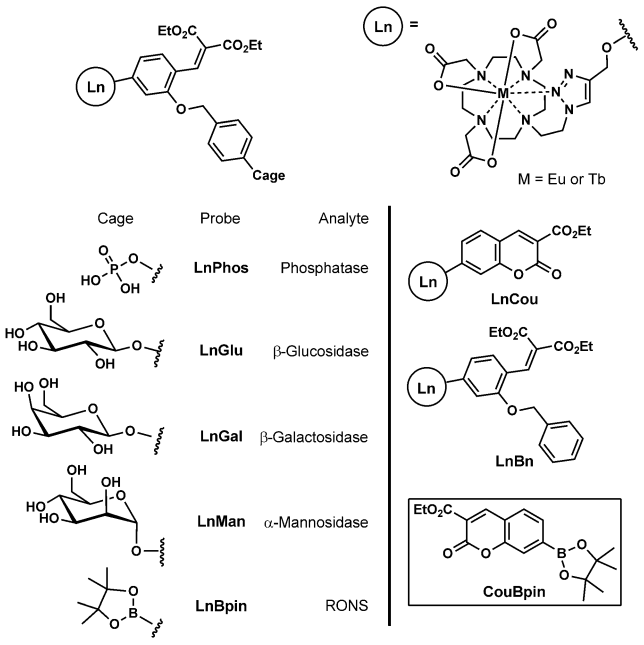

Scheme 1. Probes used in this work.

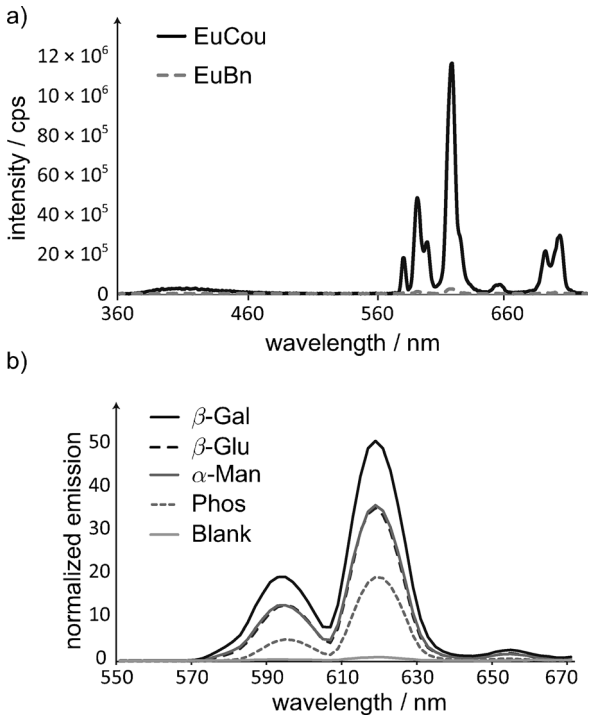

Figure 2. a) Time-resolved emission spectra of **EuBn** and **EuCou** in H_2O. b) Turn-on response of Eu probes recorded on a plate reader. The emission is normalized to those of the caged complexes. [Complex] = 50 μM; [enzyme] = 5 U β-Glu, 1 U α-Man, 0.25 U β-Gal, 0.1 U phosphatase; 25 °C; λ_{ex} = 356 nm; 250 μs delay; 1050 μs sample window; 100 mM HEPES, pH 7.0 or pH 8.0 (**EuPhos**). HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid.

Table 1: Photophysical characterization.

Entry[a]	λ_{max}[a] [nm]	ε[a] [$M^{-1}cm^{-1}$]	Φ_{Ln}[a,b] Φ_L [%]	τ_{H_2O} [ms][a]	τ_{D_2O} [ms][c]	q[d]
EuCou	350	13240	1.08 (37)	1.233	3.500	0.33
TbCou	350	13016	1.63 (32)	0.724	0.774	0.15
GdCou	350	15280	– (33)	–	–	–
EuBn	335	7580	0.12 (0.9)	1.165	3.375	0.37
TbBn	335	4540	0.41 (0.7)	0.971	0.689	−2.41
GdBn	335	4920	– (0.6)	–	–	–

[a] In H_2O. [b] With coumarin 2[18] or $Cs_3[Ln(dpa)_3]$[19] as reference. dpa = dipicolinic acid. [c] In D_2O. [d] Calculated as described in Ref. [20].

with a cut-off at approximately 380 nm. Such low-energy excitation is desirable for biological applications. The **LnBn** complexes showed weak residual fluorescence of the antenna (λ_{em} = 409 nm), which was intensified and red-shifted (λ_{em} = 457 nm) in **LnCou**. The lowest-energy triplet of **GdBn** was at 23500 cm^{-1}, with a lifetime at 77 K of (194 ± 8) ms, while that of **GdCou** was found at 21000 cm^{-1}, and had a long lifetime at 77 K of (1028 ± 74) ms. The low-temperature lifetime of the triplet state shortened in **EuCou** (299 ms) and **TbCou** (616 ms). These preliminary data do not enable the assignment of either a triplet[16] or a singlet-mediated[17] Ln-sensitization pathway. The **TbCou** emission was slightly sensitive to oxygen, probably because of energy back transfer from the Tb excited state to the coumarin triplet. An increase in the Tb lifetime to 2.62 ms at 77 K, and a 1.8-fold increase in the Tb emission upon deoxygenation support this proposal. However, the presence of oxygen did not interfere appreciably with analyte detection. The hydration numbers of $q < 1$ are typical of octadentate complexes. The negative q value of **TbBn** could be explained by multiple deactivation pathways.

To evaluate the turn-on response afforded by the probes, the integrated emission increases were determined (Figure 2b, see also Figures S6–S12 in the Supporting Information). These responses were 81- and 90-fold higher for **EuCou**/**EuBn** and **TbCou**/**TbBn**, respectively, which are among the highest observed for Ln-based turn-on probes.[21] An over 50-fold increase in the Ln emission was recorded for the β-Gal probe **EuGal**, and a 20–36-fold increase for **EuPhos**, **EuMan**, and **EuGlu** with alkaline phosphatase, α-Man, and β-Glu, respectively. Progress curves of different enzyme concentrations plotted versus time × [enzyme] were non-superimposable,[22] thus suggesting eventual deactivation of the enzyme, presumably caused by the quinone methide by-product.[23] The **EuBpin** response was linear for 0–100 μM [H_2O_2] (see Figure S11 in the Supporting Information).

One of the most attractive features of Ln-based probes is the interchangeability of metals, which results in probes with different emission colors: yellow (Dy), green (Tb), orange (Sm), red (Eu), and near-infrared (Yb, Nd). This streamlines the development of probes for multicolor and ratiometric detection.[24] The latter is advantageous due to its independence from probe concentration and bleaching. Ln-complex mixtures can afford a ratiometric readout if the ratio of the antenna's sensitization ability for the Ln changes during the experiment.[25] The addition of analyte to Eu- and Tb-probe mixtures resulted in an increase in the intensity ratio of the 618 nm/545 nm emission (see Figures S13 and S14 in the Supporting Information). The rate of this increase provides a measure of the in vitro enzyme activity. This method is

complementary to other Ln-based ratiometric detection strategies.[26]

Nonresponsive Ln complexes are well established for multicolor labeling.[27] However, multicolor analyte detection by the use of responsive Ln emitters has only recently become possible.[3] Tools for the real-time, simultaneous monitoring of enzymes with their small-molecule or enzyme regulators could greatly facilitate the mapping of cellular information flow. Our probes were created with such applications in mind, and in the following experiments their utility was explored. First, we established that we could detect two enzymes simultaneously by using probes with different emission wavelengths (see Figures S15 and S16 in the Supporting Information). It was also possible to co-detect a regulatory small molecule/enzyme pair,[11,15] specifically the RONS-dependent deactivation of human PTP1B, while measuring [H_2O_2] (see Figure S17 in the Supporting Information). Finally, the possibility of three-color detection was explored. Two Ln emitters were used in combination with an organic fluorophore, a RONS-responsive 7-pinacolborane coumarin (**CouBpin**, Scheme 1). H_2O_2-mediated oxidation of the boronate to afford the phenol yields blue fluorescence, which does not overlap with the Ln emissions. **CouBpin** is excitable at 410 nm, where the Ln complexes do not absorb appreciably, thus eliminating interfering fluorescence from the antenna. Three non-interacting analytes (β-Gal, alkaline phosphatase, H_2O_2) could be monitored independently of each other at 450, 545, and 655 nm, respectively (Figure 3a). Two interacting analytes (PTP1B, H_2O_2) were then studied together with a non-interacting analyte (β-Gal). The **EuGal** emission depended only on β-Gal. When H_2O_2 was present, the intensity of the **TbPhos** signal diminished compared to that in the absence of H_2O_2, thus reflecting the inactivation of PTP1B by H_2O_2. The **CouBpin** emission is essentially unaffected by PTP1B, which enables the accurate determination of [H_2O_2] (Figure 3b). Several chromophores are competent antennae for 5[28]–7[29] different lanthanides, which suggests that this three-color detection could be further extended with alternative antennae.

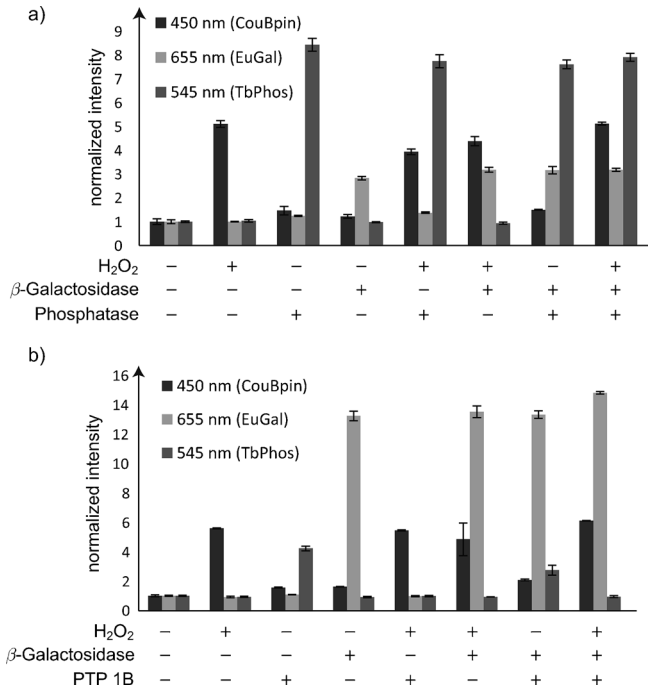

Figure 3. a) **EuGal** (6.25 μM), **TbPhos** (12.5 μM), and **CouBpin** (1 μM) were incubated separately with β-Gal (1 U), phosphatase (0.2 U), and H_2O_2 (100 μM) for 1 h at 37 °C (HEPES 100 mM, pH 8.0). b) **CouBpin** (2 μM) was incubated separately with β-Gal (0 or 0.25 U), PTP1B (0 or ca. 0.4 mU), and H_2O_2 (0 or 100 μM) for 30 min at 37 °C (100 mM HEPES, pH 7.0). **EuGal** (12.5 μM) and **TbPhos** (50 μM) were added, and incubated at RT for 30 min. Steady-state scan: $\lambda_{ex}=410$ nm $\lambda_{em}=450$ nm, time-resolved scan: $\lambda_{ex}=356$ nm, $\lambda_{em}=545$ and 655 nm, 250 μs delay, 1050 μs sample window. Error bars show the standard error based on three independent experiments.

In conclusion, a suite of responsive luminescent Ln complexes was prepared by exploiting analyte-triggered formation of an antenna. The probes have high stabilities in the absence of their analytes and excellent turn-on characteristics. By virtue of their modularity and ease of synthesis, they can be readily adapted to the detection of various analytes. The simultaneous detection of two analytes was straightforward without spectral cross-talk between the emitters. Furthermore, the Ln-based probes could be combined with an organic-based fluorophore with absorption and emission wavelengths outside of the probes' range to achieve three-color analyte detection. It is worth noting that all the experiments could be performed on a bench-top fluorimeter or a plate reader, and data analysis was simple due to the well-separated signals. The methods reported herein represent a significant step forward in multiplex detection in complex biological systems. The expansion of the probe palette and the use of these complexes for the investigation of cellular communication networks are ongoing.

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