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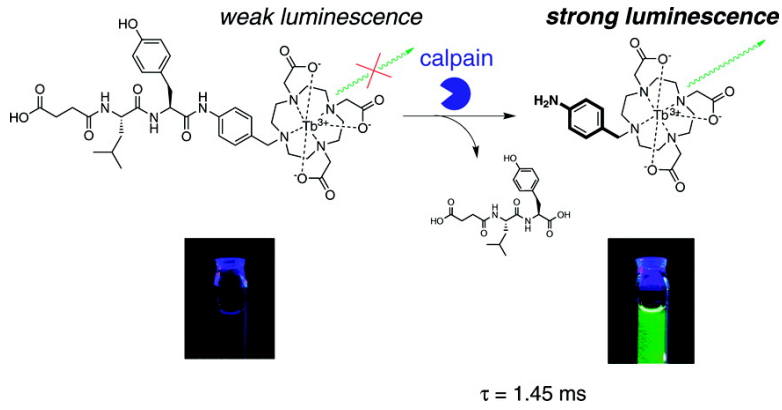

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Lanthanide-Based Protease Activity Sensors for Time-Resolved Fluorescence Measurements

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Time-resolved fluorescence measurements with long-lifetime luminescent lanthanide complexes[1] have attracted great attention from scientists. This is because the time-resolved luminescence signals are highly sensitive and are scarcely affected by other fluorescent compounds that coexist in the sample, which often interfere with steady-state fluorescence measurements. This method is quite useful, especially in high-throughput drug screening.[2] Protease inhibitors can be important drug targets for diverse diseases, including cancer, AIDS, inflammatory disorders, and so on. Therefore, lanthanide-based luminescent sensors that detect protease activities assume great significance.

Karvinen et al. reported a protease assay based on time-resolved fluorescence resonance energy transfer (TR-FRET).[3] However, the synthesis of TR-FRET probes is generally laborious because it involves the attachment of both the luminescent lanthanide complex and the appropriate quencher on the substrate peptides. Additionally, when the probe concentration is high, this system is associated with the risk of diffusion-enhanced intermolecular FRET.[4] Although another simpler assay based on quenching by photoinduced electron transfer is known, the luminescent substrate becomes nonluminescent after enzyme reaction in this case.[5] In principle, such quenching-type fluorescent probes are inferior to fluorogenic probes, which fluoresce after enzyme reaction, because fluorescence is generally quenched by several factors such as collisional quenching, energy transfer, electron transfer, and so on. Besides TR-FRET probes, there exist no other fluorogenic protease probes based on lanthanide luminescence. Therefore, fluorogenic probes such as substrate peptides attached with the short-lifetime fluorophore MCA (4-methylcoumarinyl-7-amide) are widely used in protease assays, despite the above-described limitations.[6] We here report simple-structure luminogenic lanthanide probes that detect protease activities.

The long-lifetime luminescence of lanthanide ions is due to the forbidden f−f transitions of metal electrons. Generally, energy transfer from an adjacent chromophore to a lanthanide ion is utilized for the efficient excitation of lanthanide ions. The structures of the antenna chromophores regulate the lanthanide luminescence intensity.[7] Thus, the antenna groups can be strategic targets for designing luminogenic lanthanide probes. By modifying the antenna structures, several kinds of lanthanide-based probes have been developed so far, for instance, for detecting pH,[8] metal ions,[9] and other molecules.[10] To develop luminogenic lanthanide probes for detecting protease activities, the candidate antenna groups should fulfill two requirements: (1) antenna groups with an amino group should yield strong lanthanide luminescence and (2) the lanthanide luminescence should be very weak when the amino group is protected with an acyl group. We thoroughly investigated the known antenna groups for the above requirements. We found that very few antenna groups that have an amino group could emit lanthanide luminescence in aqueous solution.[11] We considered this is because

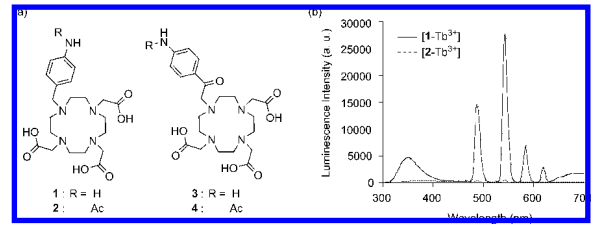
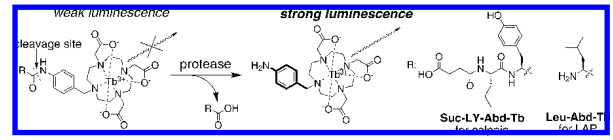


Figure 1. (a) Structures of synthesized compounds; (b) emission spectra of [**1**-Tb^{3+}] and [**2**-Tb^{3+}]; $\lambda_{ex} = 250$ nm.

Scheme 1. Structures and Schematic Representation of the Probes for Detecting Protease Activity



amino groups generally function as quenching groups for lanthanide luminescence.[5,12,13] Generally, amino substituents cause the red shift of the absorption spectra, and long-wavelength dyes are not adequate for the efficient excitation of Tb^{3+} or Eu^{3+}.[12] Thus, we hypothesized that only simple aromatic compounds with an amino group can act as efficient antenna groups for luminescent lanthanide ions such as Tb^{3+} and Eu^{3+}. Since tyrosine is known to sensitize Tb^{3+} and Eu^{3+},[14] we expected that even aniline derivatives could serve as good antenna groups.

For confirming the hypothesis, we designed and synthesized antenna-chelator conjugates **1** and **3**, which have simple aniline derivative groups as the antennas, and the corresponding acetylated compounds **2** and **4** (Figure 1a). We chose DO3A (1,4,7-tricarboxymethyl-1,4,7,10-tetraazacyclododecane) as the chelator for its strong binding ability to trivalent lanthanide ions.[15] The complex [**1**-Tb^{3+}] showed the characteristic emission spectrum of lanthanide complexes besides the antenna fluorescence at 350 nm (Figure 1b). Meanwhile, [**2**-Tb^{3+}]—the Tb^{3+} complex of the acetylated compound **2**—did not show lanthanide luminescence. On the other hand, although [**3**-Tb^{3+}] did not show lanthanide luminescence, [**4**-Tb^{3+}] showed strong lanthanide luminescence (see Supporting Information). Since the two acetylated compounds **2** and **4** are the model compounds of peptide conjugates, the above results indicated that the 4-aminobenzyl group can be a suitable antenna for luminogenic lanthanide probes detecting protease activities and that 4-aminobenzoylmethyl group can be an antenna for quenching-type protease probes. In addition, none of the Eu^{3+} complexes of compounds **1**−**4** showed lanthanide luminescence (data not shown).

Next, we designed Suc-LY-Abd-Tb to detect calpain activity, as shown in Scheme 1. Calpains are a family of intracellular cysteine proteases that are involved in many cellular processes.[16] Calpains

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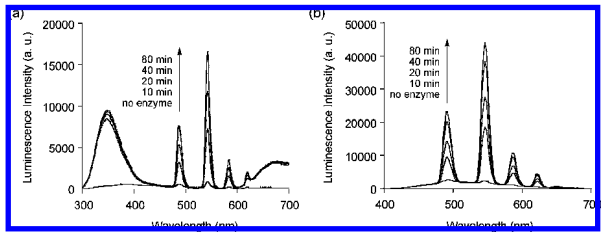

Figure 2. (a) Steady-state and (b) time-resolved (delay time, 10 μs; gate time, 3.0 ms) emission spectral change of Suc-LY-Abd-Tb with calpain I. $\lambda_{ex} = 250$ nm.

Table 1. Photophysical Properties of the Synthesized Compounds

	λ_{abs}/nm	ϵ/M^{-1} cm^{-1}	Φ	τ/ms
[**1**-Tb^{3+}]	240	12 000	0.051	1.47
[**2**-Tb^{3+}]	284	3 500	<0.001	1.49
Suc-LY-Abd-Tb	254	11 000	<0.001	1.46
Suc-LY-Abd-Tb + calpain I[a]	263	13 000	0.012	1.45

[a] It was confirmed by reversed-phase HPLC that enzyme reaction was completed.

are also related to several diseases such as muscular dystrophy, Alzheimer's disease, Parkinson's disease, and so on.[17] Thus, highly sensitive detection methods for calpain activities are crucial for developing drugs for such diseases.[18] The peptide sequence Suc-LY is known to be recognized efficiently by both calpains I and II.[17] The peptide-ligand conjugate Suc-LY-Abd was synthesized by a liquid-phase method, and the ligand was then complexed with Tb^{3+}. As we expected, the emission spectrum of Suc-LY-Abd-Tb was scarcely luminescent, similar to that of [**2**-Tb^{3+}]. Then, the addition of calpain I to the Suc-LY-Abd-Tb solution increased the emission intensity in a time-dependent manner (Figure 2a). The steady-state spectra in Figure 2a also include fluorescence around 350 nm derived from the antenna and the protein. In such cases, time-resolved measurement is very effective. The luminescence lifetimes of the synthesized compounds were approximately 1.5 ms (Table 1), much longer than those of general organic fluorescent compounds. Thus, the short-lifetime components in the emission spectra were completely excluded by performing measurements after a delay of 10 μs (Figure 2b).

We investigated the practical usefulness of Suc-LY-Abd-Tb. When fluorescent molecules are present in samples, they are very likely to significantly affect the results of fluorescence assays. For example, fluorescent drug candidates would give rise to false results for enzyme screening assays. We performed calpain assays with Suc-LY-Abd-Tb and the commercial probe Suc-LY-MCA in the presence of fluorescent compounds such as umbelliferone. The fluorescence intensity of Suc-LY-MCA was considerably increased in the presence of such compounds as compared to that in their absence. The time-resolved fluorescence intensity of Suc-LY-Abd-Tb was barely affected under the same conditions (see Supporting Information). This result indicates the superiority of our lanthanide-based probes over conventional fluorescent probes in practical applications.

Finally, to demonstrate the generality of our sensing system, we synthesized another lanthanide-based probe, Leu-Abd-Tb, for leucine aminopeptidase (LAP); this enzyme hydrolyzes the peptide bond of N-terminal hydrophobic amino acids such as leucine (Scheme 1). Leu-Abd-Tb showed an increase in the time-resolved luminescence when incubated with LAP (see Supporting Information), similar to the case of Suc-LY-Abd.

In conclusion, we developed novel luminogenic lanthanide probes for detecting protease activities. The probe design principle could be widely applicable to time-resolved assays for any proteases. These lanthanide-based probes could accelerate drug-screening processes and also contribute to the clarification of biological systems. Furthermore, achievement of longer wavelength excitation could enable a microscopic time-resolved fluorescence imaging of protease activities in living cells.[13]

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Supporting Information Available: Detailed synthetic procedures; supplementary spectra; physical properties; photostability experiment; enzyme reaction experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

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