Luminescent Saccharide Biosensor by Using Lanthanide-Bound Lectin Labeled with Fluorescein

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In biosensing, it is considered that metal-based luminescence has advantages over fluorescence on several points, such as the unique longer lifetime of emission, a lower scattering of excited light, the lack of disturbance with fluorescent impurities, and the absence of analogues properties in the biological sample.[1] Thanks to these benefits, a luminescence mode sensing system is anticipated to be promising particularly for application to complicated mixtures of biological samples. Unfortunately, the high sensitivity of luminescence to dissolved oxygen frequently prevents precise and reproductive luminescent analysis.[2] Although ratiometric sensing might overcome such a problem,[3] it requires two distinct luminophores within one chemosensor molecule, which is generally difficult from a synthetic point of view.

Here we describe a new luminescent biosensor for complicated glycoconjugates basis on a lanthanide-complexed sugar-binding protein (lectin) modified with a fluorophore. By using luminescence resonance energy transfer (LRET)[4] within the engineered protein, ratiometric luminescent sensing can be carried out. Furthermore, it can be successfully applied to a luminescent assay for enzymatic trimming of a glycoprotein.

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Site-specific introduction of Fluorescein (Fl) to Concanavalin A (Con A), a mannose-binding protein,[5] was conducted by post-photoaffinity labeling modification (P-PALM), as we reported recently (Scheme 1).[6] That is, the benzylthiol group newly incorporated into the Tyr100 site of the Con A surface was labeled with fluorescein-5-maleimide to yield fluorescein-modified Con A (Fl-Con A).[7] It is reported that there is a binding site for metal ions such as Mn^{II} or Tb^{III} in proximity to the sugar-binding pocket of Con A.[8] In fact, when Tb^{III} chloride ($TbCl_3$) was added to native Con A, three main luminescence peaks (490, 544 and 583 nm) appeared under the phosphorescence mode of experimental conditions (excitation 280 nm and delay time 0.1 ms, Figure 1 A). These peaks, due to the Tb^{III} emission sensitized by fluorescent amino acids (Trp, Tyr, etc) of Con A, are direct evidence of the Tb^{III} complexation with Con A (Con A/Tb), because of the close distance (within 3 Å) required for the sensitization.[9] Interestingly, as shown in Figure 1 A, an additional peak at 513 nm was observed in the case of $TbCl_3$ titration of Fl-Con A. Figure 1 B shows that the Fl-Con A fluorescence-mode excitation spectrum overlaps significantly with one of the Tb^{III} emission peaks (490 nm) and that the emission fluorescent peak is coincident with the new peak (513 nm). In addition, the excitation maximum of the phosphorescence of Fl-Con A monitored at the Fl emission peak (513 nm) appeared at 280 nm in the presence of Tb^{III}; this value is identical to the excitation spectrum monitored at the Tb^{III} emission peak (544 nm, see Figure S3 in the Supporting Information). Thus, it is clear that a Tb^{III} complex was formed with Fl-Con A (Fl-Con A/Tb) and that the Fl emission appeared in the rather long time range by a LRET from Tb^{III}. Such a long-lived luminescence of Fl-Con A/Tb is advantageous over short-lived fluorescence. For example, fluorescent impurities—such as another contaminated fluorophore (DMACA[10]), a protein emission, and the scattered light due to the excitation, all of which were observed in the fluorescence spectra—can be cancelled in luminescence spectra so as to obtain a simplified spectrum (see Figure S4 in the Supporting Information).

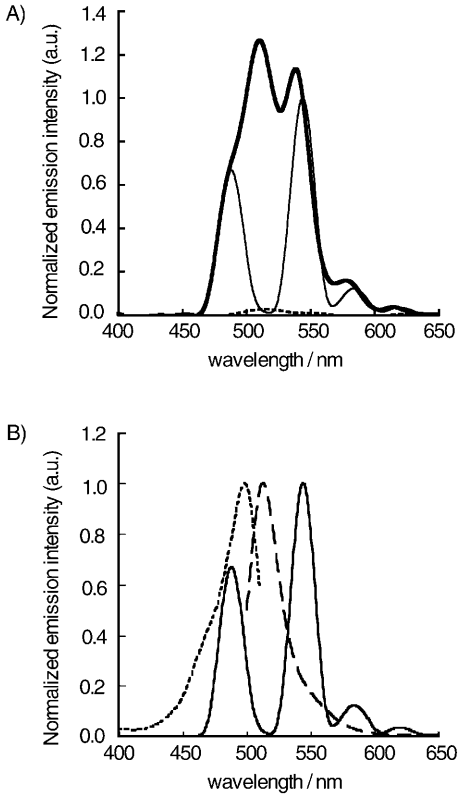
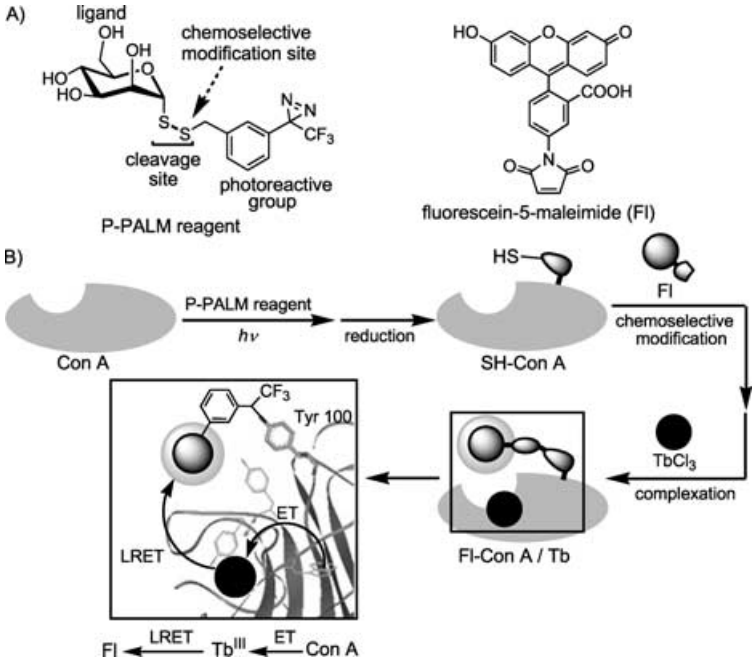


Figure 1. A) Emission spectra of 3 μM native Con A with 50 μM $TbCl_3$ (light line) and 1 μM Fl-Con A in the absence (dashed line) and presence (heavy line) of 50 μM $TbCl_3$. B) Excitation (dotted line) and emission (dashed line) spectra of 1 μM Fl-Con A in the fluorescence mode, and the emission spectrum of 3 μM native Con A with 50 μM $TbCl_3$ (solid line) in the phosphorescence mode. All experiments were conducted under the conditions of 10 mM HEPES buffer (pH 7.5), 5 mM $CaCl_2$, 0.1 M NaCl, $T = 20 \pm 1$ °C.



Scheme 1. A) Molecular structure of a P-PALM reagent and fluorescein-maleimide. B) P-PALM scheme for semisynthesis of the luminescent biosensor and the mechanism of LRET on Con A.

Thanks to the LRET emission of Fl, two distinct luminescent fluorophores (i.e., Tb^{III} and Fl), essential for ratiometric luminescence analysis, are found within one protein scaffold (Fl-Con A/Tb). Thus, we conducted luminescent titration experiments of Fl-Con A/Tb with several saccharide derivatives, such as monosaccharides, oligosaccharides, and glycoproteins. Figure 2 A shows a typical luminescent spectral change of Fl-Con A/Tb upon addition of a branched mannopentaose (Man-5), an essential fragment of glycoprotein surfaces. Clearly, the LRET peak at 513 nm decreases relative to the Tb luminescence at 544 nm with increasing Man-5 concentration.[11] This implies that a ratiometric value of the intensity of the LRET peak over the Tb^{III} peak is successfully utilized, whereas a varied content of the dissolved oxygen seriously disturbs the reproductive analysis when using a single luminescent peak during the ti-

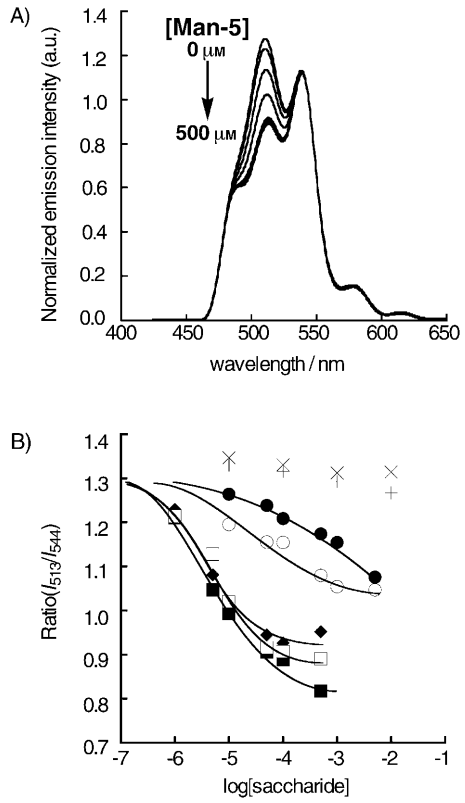


Figure 2. A) Luminescent spectral changes of Fl-Con A/Tb (1 μM Fl-Con A and 50 μM $TbCl_3$) upon addition of Man-5 (0→500 μM). B) Luminescent titration plots of the ratiometric intensity (I_{513}/I_{544}) versus saccharide concentration (log[saccharide]): Man-1 (○), Man-2 (●), Man-3 (◆), Man-4 (■), Man-5 (□), Gal (×), Cel (+). Conditions: 10 mM HEPES buffer (pH 7.5), 5 mM $CaCl_2$, 0.1 M NaCl, $T = 20 \pm 1$ °C, $\lambda_{ex} = 280$ nm.

tration measurements. The titration curve for Man-5 obeys a typical saturation, as shown in Figure 2, and gives us a binding constant of $1.0 \times 10^5\ M^{-1}$. The value is comparable to that of native Con A. Figure 2B also shows other titration curves with different saccharide derivatives monitored by ratiometric luminescence. The ratio change occurs for all mannose derivatives with corresponding sensitivity, whereas Me-α-galactose (Gal) and a β-linked glucose derivative, cellobiose (Cel), are not sensed luminescently.[12] The selectivity and binding affinity thus evaluated—Man-5 ≈ Man-4 ≈ Man-3 > Man-2 ≈ Man-1 > Cel ⩾ Gal (Table 1)—are almost identical to literature values of native Con A determined by isothermal titration calorimetry (ITC).[5b] This indicates that Fl-Con A/Tb is a luminescent biosensor that retains a natural selectivity in spite of the double modification. We also noticed that the saturated values of the LRET intensity roughly parallel the bulkiness of the oligomannoside family, that is, in the order of Man-5 = Man-4 = Man-3 > Man-2 and Man 1, similar to the case of Fl-Con A. In addition, no response is observed either for a randomly modified Fl-Con A/Tb or for native Con A/Tb.[13] Therefore, it is assumed that the attached Fl senses a microenvironmental change, such as micropolarity or micro-pH, caused by saccharide binding. The bulkier saccharide pushed Fl further out of the binding cavity of Con A, thus inducing the luminescence change.[14]

Table 1. Comparison of the association constants of Fl-Con A/Tb and native Con A to various saccharide and glycoproteins.

Saccharide	K [M^{-1}]	
	Fl-Con A/Tb	native Con A[b]
Man-1	1.6×10^4	1.1×10^4
Man-2	4.3×10^3	1.3×10^4
Man-3	2.4×10^5	2.5×10^5
Man-4	2.2×10^5	2.0×10^5
Man-5	1.0×10^5	2.0×10^5
Gal	_[a]	_[a]
Cel	_[a]	_[a]
Ribo B (Man-5 derivative)	1.8×10^5	_[c]
Ribo A (Man-5 nonderivative)	_[a]	_[c]

[a] Precise values cannot be determined because of the low affinity. [b] Determined by ITC.[5b] [c] No data were reported in the previous literature.

In addition to sugars, glycoprotein sensing can be carried out by LRET of Fl-Con A/Tb with a microM sensitivity ($K = 1.8 \times 10^5\ M^{-1}$ for ribonuclease B (Ribo B),[15] an enzyme modified with a Man-5 derivative), while mannose and the nonglycosylated protein (Ribo A) were less sensitively detected. By using this selectivity, a luminescent assay for an enzymatic trimming reaction on a glycoprotein surface can be designed. In a proof-of-principle experiment, α-mannosidase, an exoglycosidase,[16] and Ribo B were employed as a model enzyme and substrate, respectively. Figure 3A shows a typical luminescence change of Fl-Con A/Tb depending on the reaction time. The time course monitored by the ratiometric intensity is plotted in Fig-

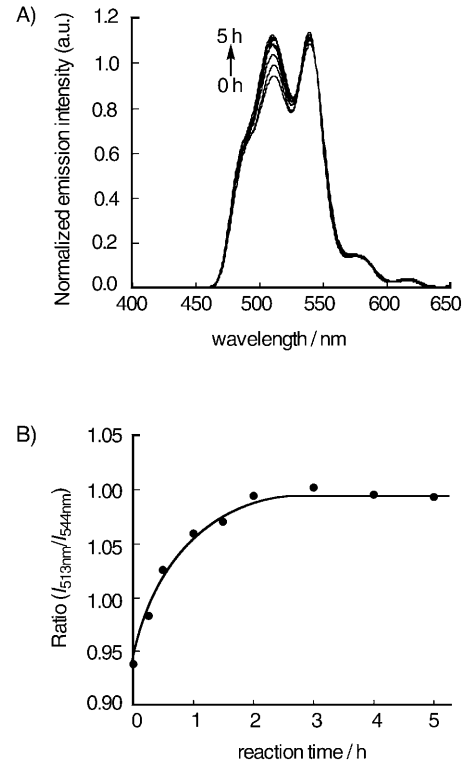
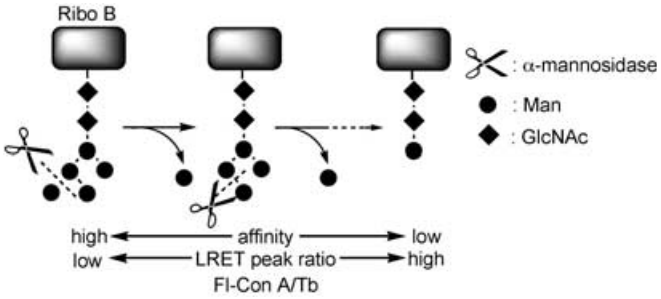


Figure 3. A) Time dependent spectral change of the emission of Fl-Con A/Tb (1 μM Fl-Con A and 50 μM $TbCl_3$) in the presence of Ribo B by addition of α-mannosidase (0→5 h). B) Time profile of the ratiometric intensity (I_{513}/I_{544}).

ure 3B. The LRET peak increases upon α-mannosidase injection, then the change gradually levels off. The observed change is reasonably explained as follows: initially, the LRET peak intensity was suppressed due to the complexation of Ribo B with Fl-Con A/Tb, it recovered following the decrease in the concentration of the complexed species due to the hydrolytic cleavage of the Man-5 moiety of Ribo B (Scheme 2),[17] because the mannose produced has a much lower affinity to Fl-Con A/Tb. It should be noted that this simple method needs neither a tedious labeling process of saccharides nor fully equipped mass spectrometers.



Scheme 2. Luminescent sensing scheme of the branched mannoside on Ribo B by α-mannosidase.

In conclusion, we have successfully demonstrated that ratiometric sensing of complicated saccharide derivatives and luminescent monitoring of the glycoprotein trimming process are possible using LRET of the engineered lectin biosensor. This result is expected to extend the validity of luminescent sensing in biological systems. The sensing method might be applicable to other enzymatic processes of glycoconjugates; investigation of which is now under way in our laboratory.

Experimental Section

Con A was labeled by our previous method and separated.[6c] The second fraction was collected and mainly used in the following study. Preparation of Fl-Con A can be found in the Supporting Information. In luminescence titration of saccharides, saccharide solution was added dropwise to Fl-Con A (1 μM) and $TbCl_3$ (50 μM) in HEPES buffer (10 mM, pH 7.5; 5 mM $CaCl_2$, 0.1 M NaCl) at 20 °C, and the luminescence spectrum was measured. Titration curves thus obtained were analyzed by the nonlinear least-squares curve-fitting method or the Benesi–Hildebrandt plot to give the association constants for various saccharides. In the α-mannosidase assay, Ribo B (0.05 μmol) in MES buffer (pH 6.5, 0.5 mL, 50 mM) was mixed with 1 unit of α-mannosidase in distilled water. The reaction mixture was incubated at 20 °C for a reaction time of 0, 0.25, 0.5, 1, 1.5, 2, 3, 4 or 5 h, then aliquots (25 μL) were added dropwise to Fl-Con A (1 μM) and $TbCl_3$ (50 μM) in HEPES buffer (10 mM, pH 7.5; 5 mM $CaCl_2$, 0.1 M NaCl) at 20 °C. The luminescence spectrum was measured to determined the luminescence ratio (513/544 nm). The hydrolytic process of the branched mannoside attached to Ribo B was also monitored by MALDI-TOF MS.

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Keywords: biosensors · lanthanides · lectins · luminescence · protein engineering

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[7] The fluorescence quantum yield (QY) of fluorescein is not greatly lessened by attachment to a Con A surface, that is, QY of fluorescein = 0.95, QY of fluorescein-5-maleimide = 0.47, QY of Fl-Con A = 0.52 (see Figure S2 in the Supporting Information).

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[10] 7-Dimetylaminocomarin-4-acetic acid (see Figure S5a in the Supporting Information).

[11] As a standard method, the wavelength at 490 nm (energy donor) was used for quantitative analysis. However, the emission peak at 490 nm overlaps partially with the Fl emission to make the precise analysis difficult. Thus, we normalized the spectra using the peak at 544 nm instead of 490 nm in this study.

[12] It is known that UV excitation is sometimes problematic because of the UV-induced strong photobleaching. However, we confirmed that the photobleaching of the emission of Fl-Con A by 280 nm excitation scarcely occurred under our measurement conditions (see Figure S6 in the Supporting Information). Thus, it is considered that the luminescent ratio change in the titration experiments was not affected by this factor.

[13] It is clear that the added saccharides scarcely interact with Tb^{III} in these titrations, because the luminescence spectra of native Con A/Tb were never changed by the addition of Man-1 (see Figure S8 in the Supporting Information).

[14] When Fl-Con A was titrated against Man type saccharides, the fluorescence intensity was gradually lessened and saturated (e.g. Man-5, see Figure S9 in the Supporting Information). This spectral change is the same as that of IAEDANS–Con A,[6c] and thus the mechanism for the fluorescence change, that is, the fluorophore being pushed out by the sugar, as with IAEDANS–Con A, is suggested in this case.

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[17] The reaction was also monitored by MALDI-TOF MS. The time course was in agreement with that monitored by LRET.

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