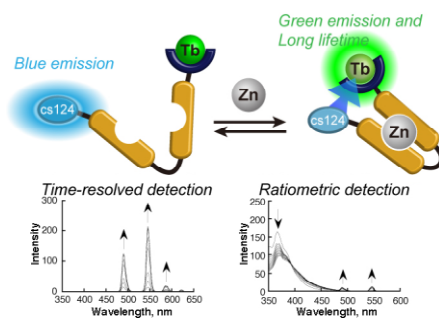


Graphical Abstract

Development of a dual functional luminescent sensor for zinc ion based on a peptidic architecture

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Development of a dual functional luminescent sensor for zinc ion based on a peptidic architecture

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ABSTRACT

A synthetic peptide bearing a lanthanide complex, TbOTZ exhibits a decrease of chromophore fluorescence and a concomitant luminescence enhancement due to sensitized Tb³⁺ upon Zn²⁺ binding. Thus, TbOTZ can be a valuable tool for ratiometric sensing of Zn²⁺ as well as for time-resolved fluorescence detection with a single molecule.

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Zinc (Zn²⁺) is the second most abundant transition metal in biological systems and is an essential component of many enzymes for its catalytic or structural roles.¹ While Zn²⁺ is tightly held in these proteins,^{2,3} labile zinc pools (weakly bound or free) also exist over various concentrations ranging from nanomolar to millimolar and are present in especially high concentrations in the brain,⁴ pancreas,⁵ and spermatozoa.⁶ Changes in such Zn²⁺ concentrations may affect a fundamental function of cells; therefore, these Zn²⁺ pools are homeostatically regulated in response to the cellular Zn²⁺ status.

To understand the biological roles of the labile Zn²⁺ in cells and tissues, numerous Zn²⁺-selective fluorescent sensors have been developed.⁷ Although, small molecules and protein-based fluorescent sensors⁸ have afforded many important insights into the physiological functions of Zn²⁺,^{7a,7c,9} most of these sensors require multi-step synthesis (usually exceeding five steps), including complicated synthetic reactions or labor-intensive purification procedures. Therefore, in these cases, essential redesigning of the molecules is needed to alter the chemical properties of these sensors such as binding affinity, metal ion selectivity, and water solubility. Regarding these points, a metal ion sensor based on a peptide scaffold offers several advantages. For example, structural optimization of peptides with appropriate binding affinity for Zn²⁺ can be achieved by alterations of amino acid sequences. Unnatural amino acids or synthetic organic

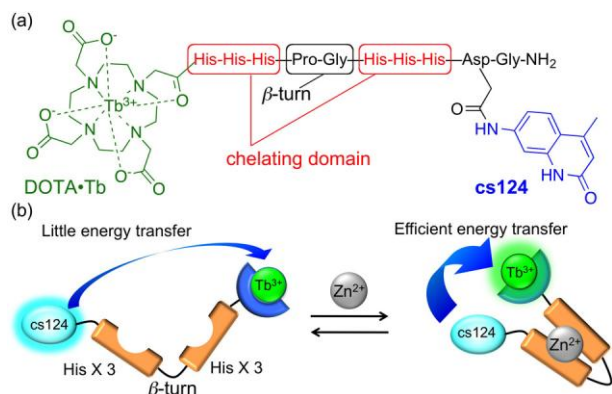
molecules can also be easily introduced into the terminal sequences and/or the amino acid side chains, which is hard to achieve with proteins, by using standard solid-phase synthetic techniques. Although a few peptide-based fluorescent sensors for Zn²⁺ have been reported,¹⁰ these sensors have some limitations for optical imaging applications. Particularly, they require ultraviolet excitation that can damage biological samples and induce auto-fluorescence from endogenous cellular molecules such as the reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD).¹¹

Lanthanide-based luminescence sensors (especially Eu³⁺ or Tb³⁺ complexes), which exhibit sensitized-lanthanide emission upon Zn²⁺ binding, are capable of overcoming such the limitations observed in UV-excitable fluorescent sensors. Because of long luminescence excited-state lifetimes of sub-milliseconds of these metals, auto-fluorescence and scattered light decay can be effectively eliminated by taking a delay time in the emission collection.¹² Furthermore, a shorter exposure time of UV irradiation in pulsed excitation can reduce photodamage by the excitation light during live-cell imaging. However, few examples of lanthanide-based luminescent sensor for Zn²⁺ have been reported.¹³ Herein, we present the design and photophysical properties of a short peptide sensor consisting of less than 10 amino acids (TbOTZ, Scheme 1) for detecting Zn²⁺ using a time-resolved luminescence (TRL) spectroscopy. Furthermore, we

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describe the potential use of TbOTZ as a sensor for ratiometric detection of Zn^{2+} .



Scheme 1. (a) Structure of the peptidic Zn^{2+} sensor TbOTZ. (b) Schematic representation of energy transfer induced by Zn^{2+} binding.

The present sensor, TbOTZ, where the sequence is DOTA(Tb)-HHHPGHHH-D(cs124)G, essentially consists of three domains: carbostyryl 124 (cs124) at the C-terminus as the energy donor to the Tb^{3+} core, two histidine trimers linking with the β -turn sequence of proline-glycine (PG), which pre-organizes the metal-binding cavity, and a Tb^{3+} complex supported by a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (DOTA) ligand at the N-terminus. In general, lanthanides are sensitized via energy transfer from an appropriate excited chromophore (antenna effect) rather than by direct excitation of spin-forbidden f-f transitions.¹² The efficiency of energy transfer from the chromophore to the metal ion strongly depends on the distance separating the two components. When the metal-binding motif recognizes Zn^{2+} and forms the complex, the cs124 chromophore becomes spatially close to the Tb^{3+} core, resulting in the increase in sensitized emission intensity.

We first synthesized a Fmoc-protected amino acid with cs124, Fmoc-Asp(cs124)-OH, according to a reported procedure.¹⁴ Prior to starting the synthesis of the designed peptide by Fmoc solid-phase peptide synthesis, the resins were pre-treated with Fmoc-glycine in order to mount Fmoc-Asp(cs124)-OH on the resin efficiently. At the end of the solid-phase synthesis, the N-terminus of the peptide on the resin was modified with tris(*tert*-butyl)ester of DOTA ligand having a free carboxyl group. After cleavage from the resin, the pure peptide of apo form was obtained by HPLC equipped with an ODS column (XBridge, Waters). Chelation of the peptide with Tb^{3+} followed by purification with gel permeation chromatography with Sephadex G10 gel (GE-Healthcare) gave the pure sensor peptide, TbOTZ, which was confirmed by ESI-TOF mass spectroscopy (Fig. S1 and S2).

As TbOTZ is highly water soluble, we measured the photophysical properties of the sensor peptide under physiological conditions (50 mM HEPES, pH 7.4, 0.1 M KNO_3) without any co-solvent. TbOTZ exhibited a strong emission band at 365 nm corresponding to cs124 fluorescence upon excitation at 330 nm, which is the absorption maximum of TbOTZ (Fig. S3). Addition of Zn^{2+} triggers the gradual decrease of the intensity at 365 nm and concomitant increase of the intense peaks at 490, 545, and 583 nm, those are attributed to the Tb^{3+} transition of $^5\text{D}_4 \rightarrow ^7\text{F}_J$ ($J = 6, 5$, and 4 , respectively), with a distinct isoemissive point at 392 nm (Fig. 1). The quantum yields of cs124 fluorescence and Tb^{3+} luminescence were decreased and increased, respectively, upon binding with Zn^{2+} (Table 1). These spectral features of TbOTZ indicate that this peptide sensor makes ratiometric detection of Zn^{2+} possible, which, in principle, offers the accurate determination of $[\text{Zn}^{2+}]$ by monitoring the

emission intensities of cs124 and Tb^{3+} . The formation of the peptide- Zn^{2+} complex with a 1:1 stoichiometry was confirmed by the Hill plot, where a Hill coefficient is calculated to be one by the slope of the line (Fig. S4). The plots of fluorescence intensities at 365 and 545 nm as a function of $[\text{Zn}^{2+}]$ can be well fitted by a pseudo-first-order equation (inset in Fig. 1), from which the apparent dissociation constant was calculated to be $K_d = 160 \pm 10 \mu\text{M}$, indicating that TbOTZ is suitable for detecting Zn^{2+} concentrations ranging from 50 to 750 μM . The lower affinity sensors with K_d values in the mid- to high-micromolar range has been required to understand the roles played by zinc in the physiological and pathological functioning of the brain, where near-millimolar concentrations of Zn^{2+} are presumed to exist in synaptic clefts.⁴ The peptide sensor TbOTZ would have a potential as a practical tool in these research fields.

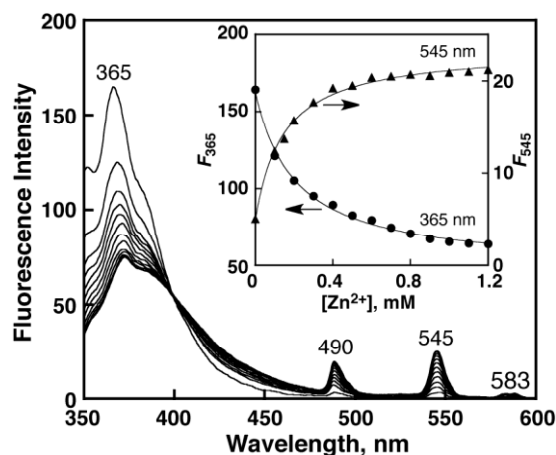


Figure 1. Emission spectral change (excitation at 330 nm) of TbOTZ (10 μM) upon addition of various concentrations of Zn^{2+} (0–1.2 mM). These spectra were acquired in 50 mM HEPES buffer (pH 7.4, 0.1 M KNO_3). Inset: plots of fluorescence intensities at 365 nm (circle) and 545 nm (triangle) with best-fit curves for the K_d of 160 μM .

We next performed TRL measurements with TbOTZ for detecting Zn^{2+} . When taking a delay time of 0.05 ms, a 15-fold enhancement of Tb^{3+} luminescence was observed upon addition of Zn^{2+} ions, whereas the fluorescence from cs124 as well as scattering light from pulses of the excitation lamp was completely eliminated (Fig. 2). This result allowed the detection of Zn^{2+} with TbOTZ by the TRL measurements. To the best of

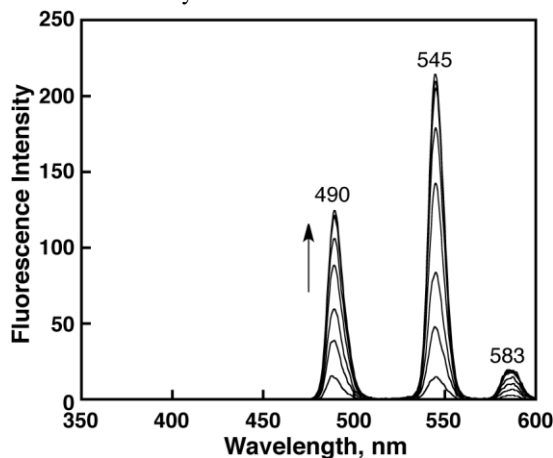


Figure 2. Time-resolved emission spectra (excitation at 330 nm) of TbOTZ (10 μM) upon addition of Zn^{2+} (0–1.0 mM, 0.1 mM each). These spectra were measured in 50 mM HEPES buffer (pH 7.4, 0.1 M KNO_3) using a delay time of 0.05 ms and a gate time of 1.00 ms.

our knowledge, TbOTZ is the first example that can monitor the changes of $[Zn^{2+}]$ not only by ratiometric analysis of emission peaks but also by TRL spectroscopy.

Table 1. Luminescence and Chemical Properties of TbOTZ

	Zn ²⁺ free		Zn ²⁺ complex	
	cs124	Tb ³⁺	cs124	Tb ³⁺
Φ (%) ^a	23.4	0.18	18.0	1.68
τ (ms) ^b	in H ₂ O	in D ₂ O	in H ₂ O	in D ₂ O
	1.62	2.71	1.25	1.85
q ^c	0.95		0.99	

^a Quantum yields were calculated using quinine ($\Phi = 0.53$ in 0.1 N H₂SO₄) for cs124 fluorophore and [Ru(bipy)₃]Cl₂ ($\Phi = 0.028$ in water) for Tb³⁺ complex as standards, respectively.

^b In 50 mM HEPES buffer solutions at pH 7.4 and pD 7.4.

^c q values were estimated using the equation $q^{Tb} = 5(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1} - 0.06)$.

The selectivity profiles of TbOTZ were examined by titrations with various biologically important metal ions (Fig. 3). High concentrations (10 mM) of alkali and alkali earth metal ions, which are highly abundant species in living cells, induced little emission spectral change of TbOTZ. More importantly, this exerted a negligible effect on the fluorescence response for Zn²⁺, indicating the potential use of this peptide sensor for biological samples. While paramagnetic transition metals including Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, and Cu²⁺ induced fluorescence quenching and interfere with Zn²⁺ binding (Fig S5), these free cations would have little influence in biological samples, because these paramagnetic metals exist at very low concentrations as compared with Zn²⁺.¹⁵ It should be noted that the fluorescence of TbOTZ was not affected by Cd²⁺ (Fig S5), which often exhibits the same or similar fluorescence responses to Zn²⁺ ions as well as

competitive inhibition of the response because of their chemical similarities. This may suggest the metal-binding site consisting of six histidines in TbOTZ creates a highly restricted binding pocket for first-row transition metals rather than for Cd²⁺ with large ionic radius.

In order to confirm whether the Zn²⁺-induced luminescence enhancement occurred with conformational change of the peptide platform, lifetimes of Tb³⁺ luminescence (τ) were measured in the absence and presence of Zn²⁺ (Table 1 and Fig. S6). Both before and after the Zn²⁺ binding, the numbers of coordinated water molecules (q values) on the Tb³⁺ center were unchanged to be ~ 1 . Because the emission intensity of lanthanide ions is directly dependent on the q value, some lanthanide-based luminescent sensors have utilized the displacement of metal-bound water by an analyte-binding as a luminescent switch of the sensor.¹⁶ In case of TbOTZ, however, the luminescence OFF-ON mechanism involving water displacement can be excluded. We then compared the phosphorescence spectra of TbOTZ before and after binding with Zn²⁺. In the absence of Zn²⁺, the phosphorescence emission (22,500 cm⁻¹) from the triplet state of the cs124 chromophore¹⁷ and intense sharp peaks of Tb³⁺ were observed in MeOH/EtOH (4/1, v/v) glasses at 77 K (Fig. S7). On the other hand, the triplet state completely disappeared upon the addition of Zn²⁺. These indicate that Zn²⁺ binding triggers enhancement of the energy transfer rate from the triplet excited-state of chromophore to Tb³⁺, which strongly support the conclusion that the terminals of TbOTZ become spatially close as illustrated in Scheme 1.

Finally, we tested the application of TbOTZ to TRL detection of Zn²⁺ in a biological buffer containing 10% fetal bovine serum (FBS). TRL measurement provided distinct turn-on response to Zn²⁺ without being affected by biological contaminants in the serum (Fig. S8).

In conclusion, we have successfully developed a new peptidic dual functional sensor TbOTZ, which enables both the ratiometric and TRL measurements for the detection of Zn²⁺ with a single molecule. Furthermore, TbOTz is able to correctly detect Zn²⁺ in the presence of FBS by TRL measurement. The main advantage of peptidic sensors is the high versatility of the sensor design. For instance, the binding affinity for Zn²⁺ would be increased by tuning the β -turn sequence of the peptide in a way that enables peptide to adopt a more stable β -hairpin conformation.^{10d} Moreover, the metal-binding sequence in TbOTZ can be altered with other chelatable amino acids such as methionine and aspartic acid in order to change the metal ion selectivity. Such the peptide-based fluorescence probes would be useful chemical sensors for monitoring extracellular and environmental metal ion concentrations.

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Supplementary Material

Supplementary data (synthesis and characterization of TbOTZ, absorption spectra, Hill plot, luminescence lifetimes, phosphorescence spectra, metal competitive assay, and TRL measurements with FBS) associated with this article can be found, in the online version.

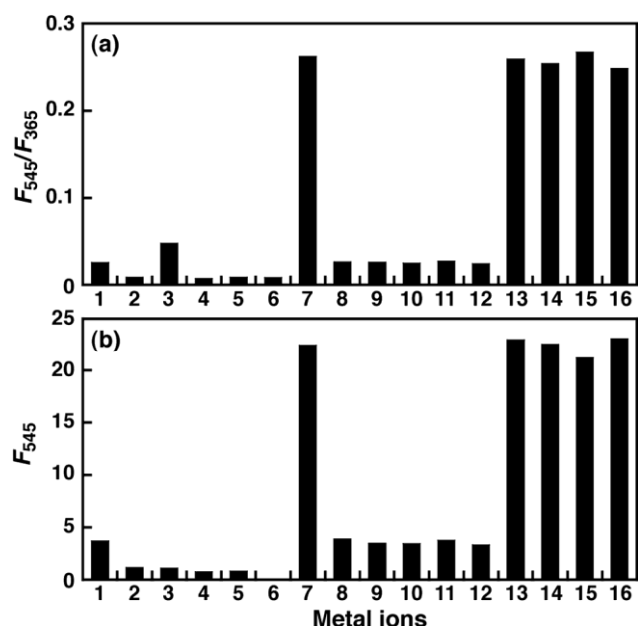


Figure 3. Metal ion selectivity profiles of TbOTZ (10 μ M) in 50 mM HEPES (pH 7.4, 0.1 M KNO₃). Bars represent (a) emission intensity ratio (F_{545}/F_{365}) and (b) luminescence intensity at 545 nm to various metal cations (10 mM for Na⁺, K⁺, Ca²⁺, and Mg²⁺, 500 μ M for all other metals); 1: no metal; 2: Mn²⁺; 3: Fe²⁺; 4: Co²⁺; 5: Ni²⁺; 6: Cu²⁺; 7: Zn²⁺; 8: Cd²⁺; 9: Na⁺; 10: K⁺; 11: Mg²⁺; 12: Ca²⁺; 13: Na⁺+Zn²⁺; 14: K⁺+Zn²⁺; 15: Mg²⁺+Zn²⁺; 16: Ca²⁺+Zn²⁺.

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