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Binuclear Terbium(III) Complex as a Probe for Tyrosine Phosphorylation

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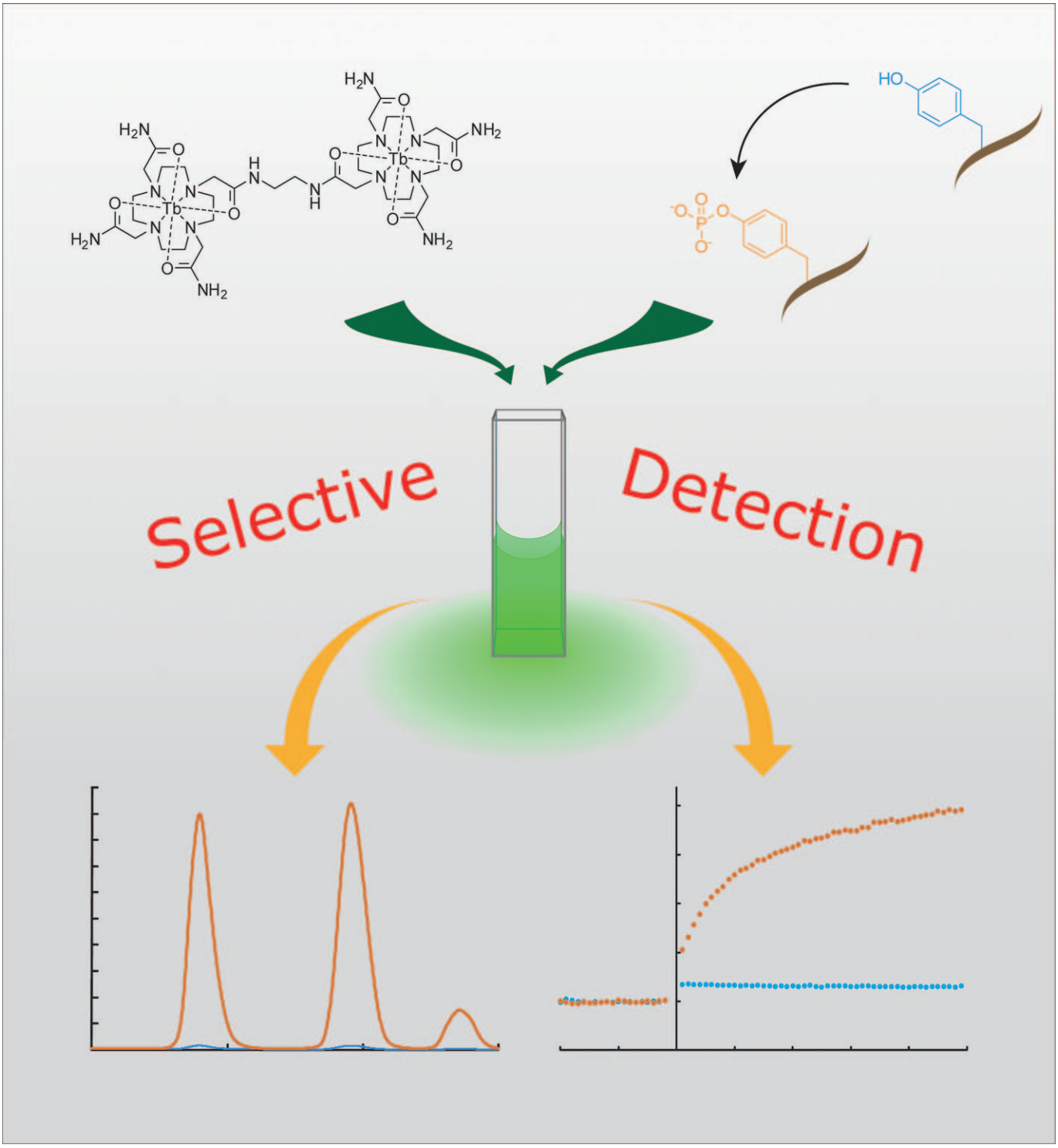


Abstract: By using the luminescence from binuclear complexes of Tb^{III} (**Tb_2-L^1** and **Tb_2-L^2**), phosphorylated Tyr residue in peptides was selectively detected in neutral aqueous solutions. Neither the non-phosphorylated Tyr, pSer, pThr, nor the other phosphate-containing biomolecules tested affected the luminescence intensity to any notable extent. Upon the binding of the pTyr to these Tb^{III} complexes, the luminescence from the metal ion was notably promoted, as the light energy absorbed by the benzene ring of pTyr is efficiently transferred to the Tb^{III} center. The binding activity of the binuclear Tb^{III} complexes towards pTyr is two orders of magnitude larger than that of the corresponding mononuclear complex. These binuclear complexes were successfully used for real-time monitoring of enzymatic phosphorylation of a peptide by a tyrosine kinase.

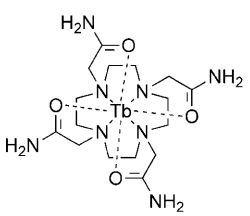
Keywords: kinases · lanthanides · luminescence · phosphorylation · tyrosine

Introduction

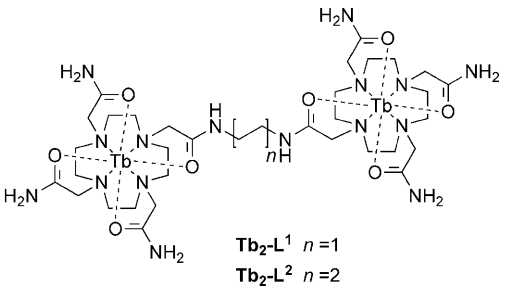
Phosphorylation of proteins controls many cellular events. In the course of signal transduction, Ser, Thr, and Tyr residues in proteins are reversibly phosphorylated and dephosphorylated.[1] Although Tyr phosphorylation accounts for only 0.05% of the total phosphorylation in cells (the majority occurs on Ser or Thr), it takes a crucial role in biological functions.[2] For example, the autophosphorylation of a Tyr in epidermal growth factor receptor (EGFR) triggers the signal-cascade inside the cell.[3] In the downstream of this kinase, there are Src family kinases, which are also controlled by Tyr phosphorylation and in turn phosphorylate Tyr residues in other proteins.[4] Excessive or insufficient Tyr phosphorylation causes serious problems.[1,2] Thus, selective detection of phosphorylated Tyr (pTyr) in proteins, with minimized signals from phosphoserine (pSer) or phosphothreonine (pThr), which exist more abundantly in biological systems, is quite important. Several metal complexes have been already reported to detect phosphorylation of proteins.[5,6] However, although these complexes recognize the phosphate groups of specimens, they barely discriminate pTyr from any other phosphate-bearing species, such as pSer and pThr.

Lanthanide ions and their complexes emit strong luminescence, when a chromophore ("antenna") is placed near them and transfers its excitation energy to the metal center.[7] In the absence of this antenna, however, the luminescence is very weak (the f–f transitions of the ions are Laporte-forbidden). By combining lanthanide complexes and antenna molecules, elegant systems to detect various anion guests have already been prepared.[7–12] A sophisticated example includes the analysis of benzoic acid derivatives using the antenna effect.[8] Phosphate-containing molecules were detected by attaching an antenna to lanthanide complexes. The binding of the phosphate group(s) of the guest molecule induces environmental changes around the lanthanide(III) ion and alters the luminescence from the ion.[10–13] However, selective detection of pTyr is difficult with this strategy, as coexisting molecules that also have phosphate groups (e.g., pSer, pThr, ATP, and DNA) could show similar effects as pTyr. These factors are more critical if a Tb^{III} ion (without any ligand) is used; nucleotides and nucleic acids show enormous antenna effects.[14,15] To obtain the signal from pTyr precisely, these background signals must be reduced.

Herein, we report binuclear Tb^{III} complexes (**Tb_2-L^1** and **Tb_2-L^2**), which selectively detect pTyr with respect to i) non-phosphorylated Tyr, ii) pSer and pThr, and iii) other coexisting phosphate-containing biomolecules. We have previously demonstrated by using **Tb-DOTAM** that the benzene ring of pTyr in the target peptides can be used as an antenna to enhance the emission from the Tb^{III} center (see Figure 1).[16] Of Tyr, Ser, Thr, and their phosphorylated products, only pTyr possesses both the notable antenna effect (benzene ring) and sufficient binding activity towards the Tb^{III} complex (phosphate group). Thus, the selectivities (i) and (ii) are fulfilled. Furthermore, the selectivity (iii) to other coexisting molecules is accomplished by using a bulky ligand



Tb-DOTAM



Tb_2-L^1 n =1
Tb_2-L^2 n =2

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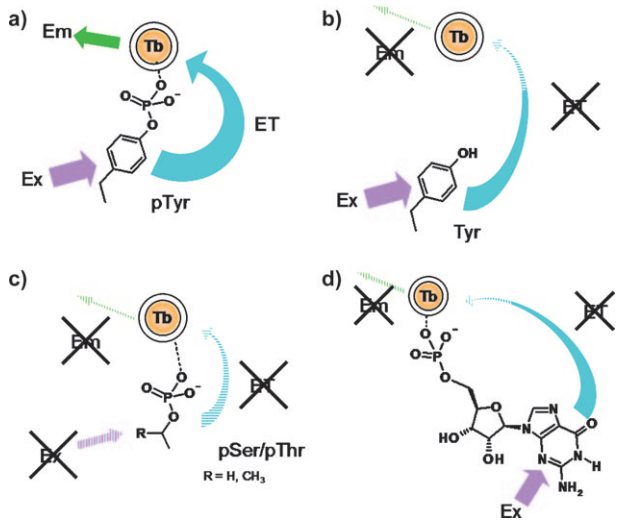


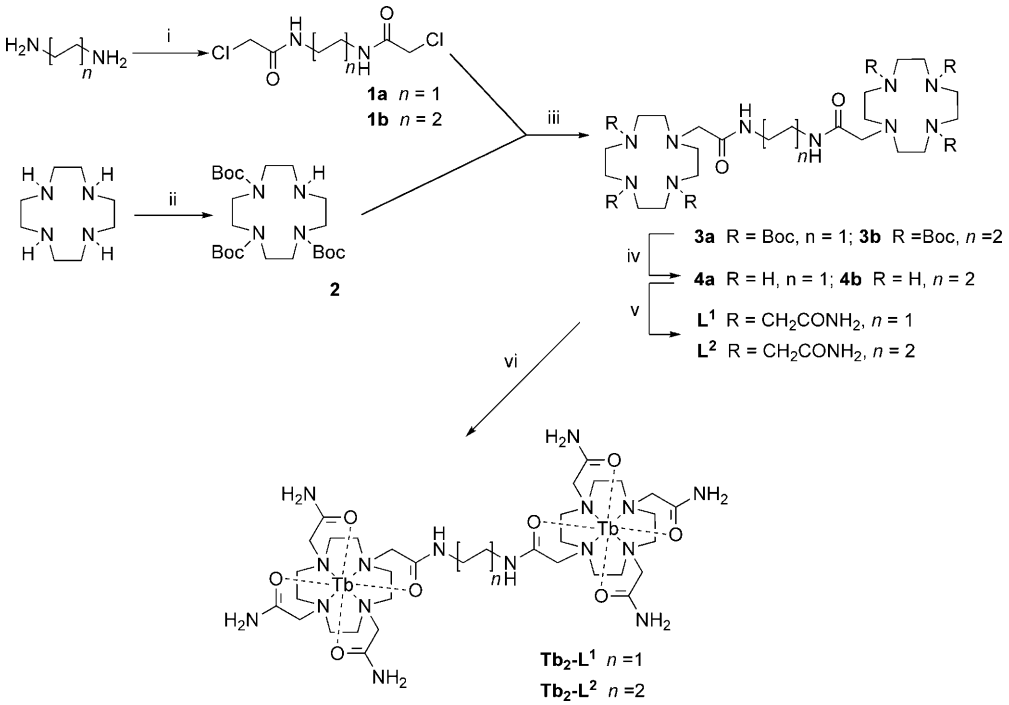
Figure 1. A phosphotyrosine-specific sensor based on the "antenna effect". a) The emission for the Tb^{III} complex is promoted by the transfer of excited energy of the benzene ring of pTyr to the Tb^{III} ion. b) Non-phosphorylated Tyr does not bind to the Tb^{III} complex so that no energy transfer occurs. c) pSer/pThr has no benzene ring to absorb the energy. d) The guanine group of GMP is located far away from the Tb^{III} complex even when its phosphate interacts with the metal center. Ex: excitation; ET: energy transfer; Em: emission.

(DOTAM), which extensively surrounds the metal ion and suppresses the coordination of various phosphate-bearing molecules. To further improve the detection ability, binuclear complexes (**Tb_2-L^1** and **Tb_2-L^2**) were developed.[17]

Under the situation described above, pTyr satisfactorily enhances the luminescence from the binuclear complexes **Tb_2-L^1** and **Tb_2-L^2** with a reasonable magnitude, because 1) the electrostatic interactions are primarily responsible for the binding of pTyr to the Tb^{III}, 2) the doubled positive charges of the binuclear Tb^{III} complex induce tighter interaction, and 3) the Tb^{III} center and the benzene ring of pTyr are in sufficient proximity for energy transfer. By using these binuclear Tb^{III} complexes, Tyr-phosphorylated peptides are clearly distinguished from non-phosphorylated peptides in neutral aqueous solutions. Furthermore, the enzymatic phosphorylation of Tyr in the peptides can be successfully monitored in real-time.

Results and Discussion

Design and synthesis of Tb^{III} complexes: The binuclear complexes (**Tb_2-L^1** and **Tb_2-L^2**) were synthesized according to Scheme 1, and characterized as described in the Supporting Information. These complexes, as well as their monomeric analog **Tb-DOTAM**, have no aromatic moiety, which could possibly work as an antenna, so that their intrinsic luminescence is minimized. In addition, both of these binuclear complexes carry a +6 net positive charge, which is able to electrostatically attract the phosphate residue of pTyr. As evidenced below, the **Tb_2-L^1** and **Tb_2-L^2** have one coordination water molecule on each of the Tb^{III} ions. This argument is also supported by the crystal structure analysis of **Tb-DOTAM** complex.[18,19]



Scheme 1. The synthetic procedures for **Tb_2-L^1** ($n=1$) and **Tb_2-L^2** ($n=2$). Reagents and conditions: i) chloroacetyl chloride, $CHCl_3$, reflux, 6 h; ii) $(Boc)_2O$, NEt_3, $CHCl_3$, 12 h; iii) $NaHCO_3$, KI, MeCN, reflux, 96 h; iv) 4 M HCl, 1,4-dioxane, 2 h; v) 2-bromoacetamide, $NaHCO_3$, KI, MeCN, reflux, 72 h; vi) $Tb(OTf)_3$, MeCN, reflux, 24 h.

Phosphotyrosine-selective promotion of luminescence from Tb_2-L^1, Tb_2-L^2, and Tb-DOTAM: Shown in Figure 2a are the emission spectra of Tb_2-L^1 in the presence or the absence of phenyl phosphate (PhOP), which is a model compound for the side chain of pTyr in proteins. The excitation wavelength was $\lambda = 262.5$ nm, which the benzene ring of PhOP absorbs. When PhOP was added to the solution, the luminescence assignments for the $^5D_4$–^7F_J ($J = 6, 5, 4$) transitions of the Tb^{III}. Similar enhancement was observed for mononuclear **Tb-DOTAM** and another binuclear complex Tb_2-L^2 (Figure 2b). It is noteworthy that these dimeric Tb^{III} complexes enhanced the luminescence much more remarkably than the monomeric complex **Tb-DOTAM**. There was not much difference in luminescence enhancement between Tb_2-L^1 and Tb_2-L^2. This is primarily because the interactions between Tb^{III} complexes and PhOP are electrostatic, as described below.

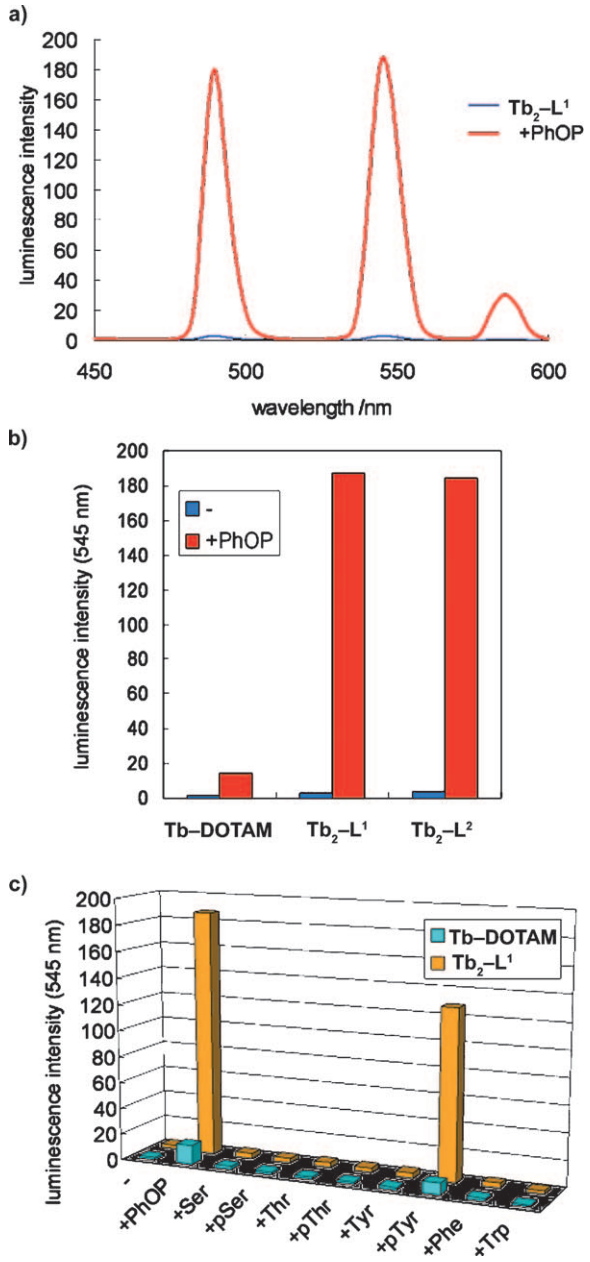


Figure 2. a) The luminescence spectra of Tb_2-L^1 in the presence and the absence of PhOP. b) The luminescence intensity at 545 nm of the solutions of Tb_2-L^1, Tb_2-L^2, and **Tb-DOTAM**. The luminescence was largely enhanced by PhOP. c) The luminescence intensity at 545 nm in the presence of PhOP or amino acids. The addition of PhOP or pTyr explicitly increased the emission. Conditions: [Tb^{III} complex] = [additive] = 100 μM, HEPES buffer (10 mM, pH 7.0), $\lambda_{ex} = 262.5$ nm.[20]

Similar enhancement of the luminescence was observed with the use of pTyr in place of PhOP (Figure 2c). In contrast, non-phosphorylated Tyr showed virtually no improvement, since not much of its excitation energy transfers to the Tb^{III} complexes. The promotion of luminescence was selective to pTyr, and all other amino acid derivatives investigated (pSer, pThr, Ser, Thr, Phe, and Trp) were inactive (see Figure 2c). Apparently, both the benzene ring (an antenna) of the pTyr residue and its phosphate (metal-binder) are essential to promote the luminescence, exactly as proposed in Figure 1. Although the Tb^{III} complexes also bind pSer and pThr, they do not affect the emission, owing to the absence of an antenna function.

These results strongly suggest that the present system is applicable to the detection of pTyr even if various phosphate-bearing species coexist in the same solution. Consistently, GMP (guanosine-5′-monophosphate), UMP, AMP, CMP, ADP, ATP, and α-D-glucose-1-phosphate hardly promoted the luminescence from Tb_2-L^1 (Table 1). These species and their analogues, such as DNA and RNA, are abundant in cells. In marked contrast, the luminescence from the Tb^{III} ion (without ligands) is notably enhanced by these species. The effect of GMP is especially remarkable. It has been proposed that GMP binds to a Tb^{III} ion through simultaneous coordination of the phosphate residue and the guanine ring (N7 and O6), using its guanine group as an antenna to promote the emission.[13] A similar coordination should occur for DNA and RNA. In the present Tb^{III} complexes, the coordination of these molecules to the Tb^{III} ion is prevented by the bulky ligands (Figure 1d). This deprives these species of proximity to the Tb^{III} center. On the other hand, pTyr has a benzene ring adjacent to the phosphate

Table 1. The luminescence intensity at 545 nm in the presence of various phosphate-bearing species.[a]

	Tb_2-L^1	Tb-DOTAM	$TbCl_3$
–[b]	3.03	1.60	1.03
PhOP	187	14.7	313
AMP	0.82	0.40	9.36
UMP	3.60	1.00	16.3
GMP	1.02	0.85	900
CMP	1.56	0.69	30.4
ADP	1.13	0.64	10.1
ATP	1.18	0.44	0.61
Glucose-P[c]	3.67	1.54	1.27

[a] Conditions: [Tb^{III} complex] = [additive] = 100 μM, HEPES buffer (10 mM, pH 7.0), $\lambda_{ex} = 262.5$ nm. [b] The value without any additive. [c] Glucose-P = α-D-glucose-1-phosphate.

group and sufficient proximity is maintained even if it is bound by the metal ions through electrostatic interactions (see below). The vital necessity of the ligands for selective detection of pTyr is evident.

Promoted phosphate-binding by binuclear complex Tb_2-L^1: The Job's plot between **Tb_2-L^1** and PhOP indicates the existence of an equimolar complex (see Figure S1 in the Supporting Information).[21] To compare the strength of interactions, an apparent dissociation constant (K_D) was estimated in terms of 1:1 complex formation. The change in luminescence intensity with increasing **Tb_2-L^1** concentration satisfactorily fits a curve based on this model (Figure 3). The K_D of

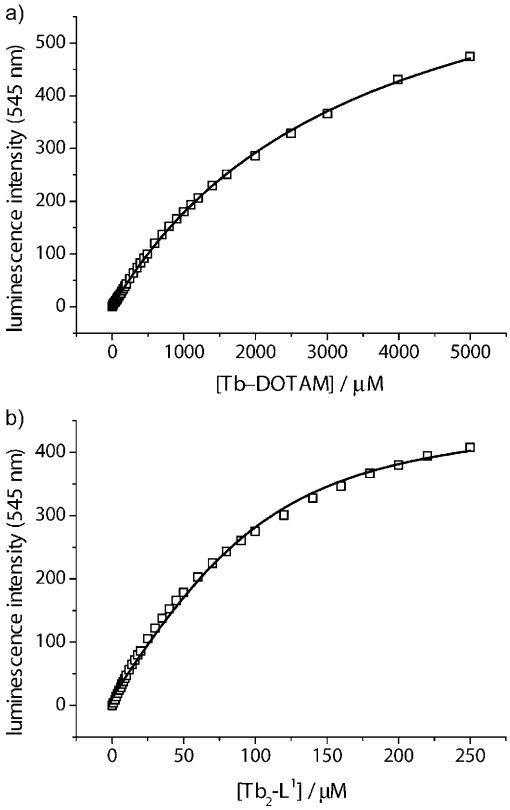


Figure 3. The titration of a) **Tb-DOTAM** and b) **Tb_2-L^1** to PhOP (100 μM) in HEPES buffer (10 mM, pH 7.0). λ_{ex} = 262.5 nm.

the **Tb_2-L^1**/PhOP complex was estimated to be 29 μM. This value is 110 times lower than corresponding value (3.3 mM) for **Tb-DOTAM**. Clearly, **Tb_2-L^1** binds pTyr more strongly than **Tb-DOTAM**, so that the pTyr-induced emission enhancement is larger for **Tb_2-L^1** (Figure 2b).[22]

To shed light on the manner of the interactions between the Tb^{III} complexes and PhOP, the number of the water molecules coordinated to the Tb^{III} ion was estimated. As the coordination of water molecules to Tb^{III} ion reduces the luminescence lifetime, owing to the energy transfer from the excited state of Tb^{III} to the higher O–H vibration overtones of the water, the lifetime would be increased by the replacement of the H_2O molecules with D_2O. Therefore the q value, which represents the number of coordinated water molecules, can be estimated by using the following Equation (1).[23] If the phosphate group of PhOP is directly coordinated with the Tb^{III} ion, the q value would be reduced, owing to its replacement with the corresponding coordinated water molecule.

$$q = 5.0(k_{H_2O} - k_{D_2O} - 0.06) \quad (1)$$

Here, k_{H_2O} and k_{D_2O} represent the rate constants of luminescence decay, measured in H_2O and D_2O, respectively (the decay curves are presented in Figures S2 and S3 in the Supporting Information). For both **Tb_2-L^1** and **Tb-DOTAM**, the q value was about 1, either before or after the addition of PhOP (Table 2).[24] Apparently, no ligand exchange with coordination water occurs in the binding of PhOP to the Tb^{III} complexes. Therefore, it is concluded that the interaction between the Tb^{III} complexes and PhOP is by means of ion-pairing, rather than a direct coordination.[25,26] Consistently, the luminescence from **Tb_2-L^1** in the presence of PhOP gradually decreased with increasing concentration of NaCl or KCl (see Figure S4 in the Supporting Information). **Tb_2-L^1** binds PhOP much more strongly (two orders of magnitude) than **Tb-DOTAM**, probably through simultaneous interactions of the phosphate with its two Tb^{III} ions. The electrostatic interactions of the **Tb_2-L^1** are also stronger, because the net positive charges of this binuclear complex (+6) are two times as large as that for **Tb-DOTAM** (+3).

Table 2. Half-lives of luminescence from the Tb^{III} complexes in H_2O (τ_{H_2O}) and in D_2O (τ_{D_2O}), and the q values calculated.[a]

	τ_{H_2O} [ms]	τ_{D_2O} [ms]	q
Tb-DOTAM	1.65	3.17	1.15
+PhOP	1.65	3.20	1.17
Tb_2-L^1	1.52	2.62	1.08
+PhOP	1.53	2.60	1.04

[a] See the Experimental Section for the experimental conditions.

Detection of phosphorylated Tyr in peptides by Tb_2-L^1: If the *Tyr*-phosphorylated P1 (Glu-Glu-Glu-Ile-*Tyr*-Glu-Glu-Phe-Asp) was added to a solution of **Tb_2-L^1**, the luminescence was considerably enhanced (Figure 4). The luminescence intensity was tenfold larger than that obtained if non-phosphorylated P1 was added. This peptide comes from a sequence that is heavily phosphorylated by the cancer-related protein *v*-Src.[27] The phosphorylated *Tyr* is surrounded by negatively charged amino acid residues, as is often observed in peptide sequences that can be tyrosine-phosphorylated by tyrosine kinases.[28] Assumedly, these negative charges promote the binding of the pTyr residue to **Tb_2-L^1**, resulting in the efficient detection of the Tyr phosphorylation of this peptide. Consistently, *Tyr*-phosphorylated P2 (Ser-Ala-Ala-Pro-*Tyr*-Leu-Lys-Thr-Lys), which is from another cancer-related protein STAT3 and has positive charges

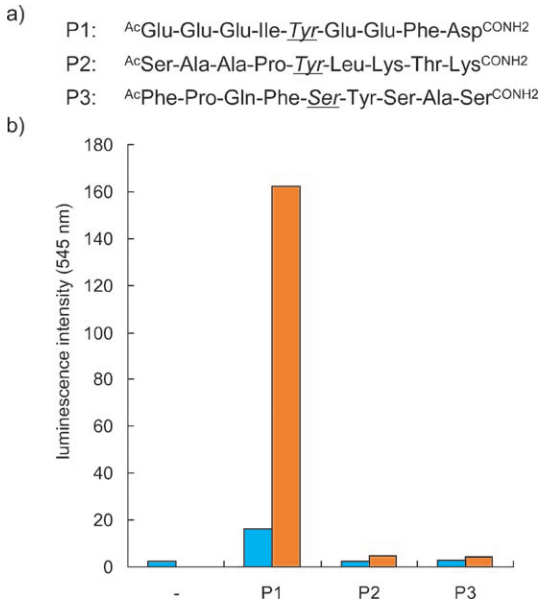


Figure 4. The luminescence intensity at 545 nm from $\mathbf{Tb_2\text{-}L^1}$ in the presence of phosphorylated (orange) and nonphosphorylated (light blue) peptides. The peptides used are shown in (a) with the phosphorylated residue underlined. Conditions: $[\mathbf{Tb_2\text{-}L^1}]=[\text{peptide}]=100\ \mu\text{M}$, HEPES buffer (10 mM, pH 7.0), $\lambda_{ex}=262.5$ nm.

near the *Tyr*,[29] exhibits only a 2 fold increase in the luminescence intensity of $\mathbf{Tb_2\text{-}L^1}$. The luminescence enhancement by *Ser*-phosphorylated P3 (Phe-Pro-Gln-Phe-*Ser*-Tyr-Ser-Ala-Ser) from c-Akt (PKB), which bears Tyr next to phosphorylated Ser,[30] was also small, as expected.

Real-time monitoring of enzymatic phosphorylation of Tyr in the P1 peptide: The process of phosphorylation of P1 peptide by Src kinase in aqueous solutions was directly monitored by fluorimetric analysis of $\mathbf{Tb_2\text{-}L^1}$ in real-time (Figure 5). To the solutions containing $\mathbf{Tb_2\text{-}L^1}$ and P1, as well as ATP and $MnCl_2$ (essential factors in this enzymatic reaction), the kinase was added and the luminescence at 545 nm was measured. The luminescence intensity increased time-dependently (red and green circles in Figure 5). The magnitude of increase in luminescence intensity exactly reflects the difference in the concentration of P1. To confirm that the increase of luminescence is owed to the increase in the amount of *Tyr*-phosphorylated P1, this enzymatic phosphorylation was independently analyzed under the same conditions by polyacrylamide gel-electrophoresis by using TAMRA-labeled P1. As presented in Figures S5–S7 in the Supporting information, the results of the two methods almost agree with each other, providing evidence for the argument. The $\mathbf{Tb_2\text{-}L^1}$ induced no notable change in the rate of Tyr-phosphorylation (compare the red squares with the blue ones in Figure S7 in the Supporting Information). In no experiment was the luminescence enhanced without the substrate peptide (blue squares in Figure 5). If *Tyr*-phosphorylated P1 was used as the substrate, the luminescence was strong from the beginning and not enhanced even after the

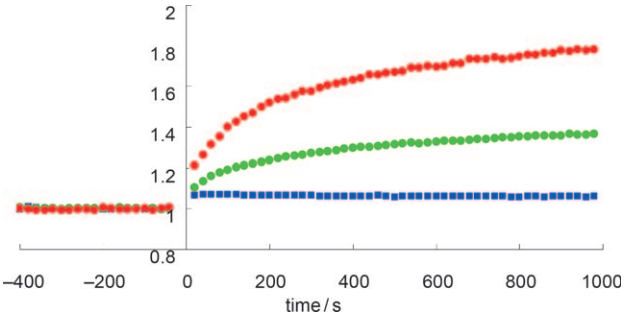


Figure 5. Change in luminescence intensity at 545 nm from $\mathbf{Tb_2\text{-}L^1}$ after the addition of Src kinase to the solution of P1 peptide. For red and green circles, the concentrations of P1 were 5 and 2.5 μM, respectively, and Src kinase (1.2 $\mu g\,mL^{-1}$) was added at time = 0. Note that the background signal satisfactorily remained constant before the addition of the enzyme (time <0). Conditions: $[\mathbf{Tb_2\text{-}L^1}]=100\ \mu\text{M}$, $[MnCl_2]=1$ mM, $[ATP]=10\ \mu\text{M}$, HEPES buffer (5 mM, pH 7.4), $\lambda_{ex}=262.5$ nm, $\lambda_{em}=545$ nm. The blue squares show the result without substrate peptide.

addition of kinase (data not shown). Apparently, the *Tyr*-phosphorylation of the substrate peptide was successfully monitored by $\mathbf{Tb_2\text{-}L^1}$ in the presence of ATP which competitively bound to the complex. The light absorption by the protein and ATP imposed no critical effects on this analysis. The present method is advantageous in that neither radiolabeling of the peptide nor its chemical labeling is required.[13,31,32]

Conclusion

With the use of pTyr-induced enhancement of luminescence from binuclear $\mathbf{Tb_2\text{-}L^1}$ and $\mathbf{Tb_2\text{-}L^2}$ complexes, pTyr in peptides has been selectively detected even in the presence of pSer, pThr, and other potential coexisting molecules (e.g., other amino acids, GTP, DNA, and RNA). The benzene ring of pTyr works as an antenna and enhances the luminescence from the Tb^{III} complex, which is otherwise very weak. The pTyr-induced enhancement of the luminescence from $\mathbf{Tb_2\text{-}L^1}$ was far larger than that observed for monomeric **Tb-DOTAM** complex. The process of enzymatic phosphorylation of Tyr in peptide was successfully monitored in real-time by $\mathbf{Tb_2\text{-}L^1}$. These results have opened a way to molecular design of pTyr-specific sensors.

The high pTyr selectivity of the present method, with respect to pSer/pThr and other phosphate-containing molecules, is certainly an advantage for various applications. The pTyr-detection limit is further improved by designing Tb^{III} complexes that can bind pTyr still more strongly, even under high salt conditions. Direct coordination of phosphate to the Tb^{III} center, in place of electrostatic interaction, could be effective there. Although the rather short wavelength of excitation light used ($\lambda=262.5$ nm) provides some limitations to the potential applications, use of multi-photon excitation systems could reduce some of these limitations. In vitro analyses, demonstrated here with a kinase, would be very effective in the biochemical analysis of Tyr-phosphorylation,

including the activation or inactivation of kinases and phosphatases.

Experimental Section

General: The reagents and compounds were purchased from Tokyo Chemical Industries, Wako Pure Chemicals, Sigma–Aldrich, or Nacalai Tesque at the highest grades available. Luminescence measurements were conducted in HEPES-NaOH buffer (pH 7.0, 10 mM) unless otherwise stated. The spectra were measured by using a FP-6500 fluorimeter (JASCO) with excitation at $\lambda = 262.5$ nm, soon after the additives were added. The procedures for the synthesis of the Tb^{III} complexes are included in the Supporting Information.

Lifetime measurements: Luminescence lifetime measurements of the Tb^{III} complexes were conducted in H_2O or D_2O solutions (10 mM HEPES buffer, pH 7 for H_2O solution). The D_2O solution was prepared by completely drying up this solution followed by the addition of the same volume of D_2O to the residue. The concentrations of Tb^{III} complexes were 100 and 500 μM, respectively, in the presence and the absence of PhOP (1 mM). Excitation was made at $\lambda = 355$ nm by pulse laser by means of a Nd:YAG laser (Spectra-Physics KK. INDI-40–10-HG-TRI-T). Luminescence at $\lambda = 545$ nm was measured by using a monochromator (JASCO CT-25TP) and a photomultiplier (Hamamatsu Photonics R446).

The preparation of the peptides: P1-TMR was TAMRA-labeled on its N-terminal and amidated on C-terminal (see the Supporting Information). All other peptides were acetylated on their N-terminal and amidated on C-terminal. The *Tyr*-phosphorylated P1, *Ser*-phosphorylated P3, and P1-TMR were synthesized by standard Fmoc-coupling chemistry followed by purification by using RP-HPLC; solid phase: ODS column; mobile phase: H_2O/MeCN (0.1% TFA). The other peptides were obtained from Life Technologies Japan Ltd. and used without further purification.

The conditions of the kinase reaction: The Src kinase, expressed as full-length human Src with a N-terminal GST-fusion protein using the baculovirus expression system, was obtained from Carna Biosciences, Inc, and stored in Tris-HCl (50 mM, pH 7.5) containing NaCl (150 mM), Brij 35 (0.05%), DTT (1 mM), and glycerol(10%). The reaction was assayed in HEPES-NaOH (5 mM, pH 7.4) containing **Tb_2-L[1]** (100 μM), $MnCl_2$ (1 mM), ATP (10 μM), P1 (2.5 or 5 μM), and Src kinase (1.2 $\mu g\,mL^{-1}$) as the final concentrations.

Acknowledgements

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