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Detection of phosphorylation states by intermolecular sensitization of lanthanide-peptide conjugates

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The luminescence of a designed peptide equipped with a coordinatively-unsaturated lanthanide complex is modulated by the phosphorylation state of a serine residue in the sequence. While the phosphorylated state is weakly emissive, even in the presence of an external antenna, removal of the phosphate allows coordination of the sensitizer to the metal, yielding a highly emissive supramolecular complex.

Phosphorylation is the fundamental signaling mechanism by which eukaryotic cells control all their basic processes, including cell cycle, motility, metabolism or genetic expression, to name just a few.[1] Therefore, there is a great interest in studying the role of kinases and phosphatases in natural processes and their connection with the etiology of diseases such as cancer.[2] In this context, the development of chemical sensors that allow monitoring of their activity is of great relevance.[3] Accordingly, a number of optical sensors based on environment-sensitive,[4] or chelation-enhanced fluorophores,[5,6] have been described. Lanthanides, as privileged emissive species,[7] have also been applied to the design of specific kinase sensors, often by taking advantage of the chelating effect of the phosphate anion to induce the formation of luminescent lanthanide complexes.[8]

In the context of our work in the development of fluorescent,[9] and lanthanide-based luminescent sensors,[10] we envisioned a new strategy for probing phosphorylation states, and thereby monitoring the enzymatic activity of kinases and phosphatases, based on the use of peptide conjugates containing coordinatively-deficient lanthanide complexes.[11] The designed lanthanide-chelating peptides include a C-terminal Protein Kinase C alpha (PKCα) recognition sequence,[12] a Pro-Gly β-turn inducing sequence that preorganizes the peptide chain to promote chelation,[13] and a sensing unit consisting on an N-terminal Trp residue acting as sensitizer and a terbium complex attached to the peptide chain through Asp (**1**, **1^P**) or Glu (**2**, **2^P**) side chains.[14] The heptacoordinating DO3A (1,4,7,10-tetraazacyclododecane-1,4,7-triacetatic acid), selected as macrocyclic ligand, does not satisfy all the available coordination positions of the lanthanide ion,[15] and therefore in the unphosphorylated state the unfilled positions should be occupied with water molecules, which are known to deactivate the luminescence from the metal ion. Phosphorylation of the serine residue should promote the intramolecular displacement of the water by the pSer side chain phosphate group, thus leading to an increased emission from the lanthanide ion (Fig. 1).[16,17]

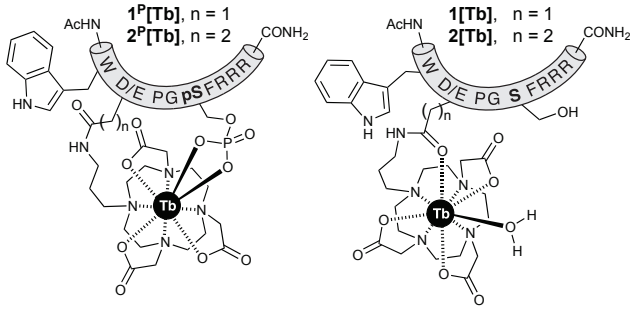

Fig. 1 Hypothetical coordination of the lanthanide ion in the alternative peptide phosphorylation states. The figure shows the sequences of the Asp, (**1[Tb]**, **1^P[Tb]**) and Glu-derived (**2[Tb]**, **2^P[Tb]**) metallopeptides.

Peptides were synthesized following standard Fmoc solid-phase synthesis procedures.[18] The Asp and Glu residues were inserted with their side chains orthogonally protected as allyl esters, and selectively deprotected by Pd catalysis once the peptide sequences were fully assembled in the solid phase.[19] The DO3A propylamino derivative was then conjugated to the resulting carboxylic acids.[20] The final peptides were deprotected/cleaved from the resin and purified by reverse-phase HPLC.

As expected, the peptides were able to coordinate Tb(III) ions, as shown by the appearance of the typical luminescence emission bands of the metal (488, 544 and 585 nm) upon irradiation at 280 nm of equimolar mixtures of the peptide chelates and $TbCl_3$. Peptides **2[Tb]** and **2^P[Tb]** displayed very similar spectra with comparable emission intensities, suggesting that the coordination environment of the metal ion was similar in both species; therefore these species were not considered for further studies (Fig. S2, supporting information). Surprisingly, we found that the luminescence of the unphosphorylated **1[Tb]** complex was approximately 2.5 times more intense than that of its phosphorylated counterpart, **1^P[Tb]** (Fig. S1 and S3, supporting information). In order to understand these unexpected relative emission intensities, we measured the luminescence lifetimes of both species in varying proportions of H_2O/D_2O. Fitting the resulting emission decays to single exponentials provided the metal emission lifetimes for **1^P[Tb]** and **1[Tb]** in H_2O and D_2O that were then used to calculate the number of inner-sphere water molecules bound to the metal center (q), which turned out to be $q \approx 1$ for **1[Tb]** and $q \approx 0$ for **1^P[Tb]** (Fig. S4-S5 and Table S1, supporting information).[21] Additionally, the coordination of the

pSer side chain to the lanthanide was confirmed by ^{31}P NMR; therefore, while **1^{P}**, lacking the metal ion, displayed a clear singlet at 3.63 ppm, complexation with Eu^{3+} in **1^{P}[Eu]** lead to a complete suppression of the phosphorus signal, and no resonance could be observed in the range of 250 to –250 ppm (Fig. S14, supporting information). In light of these results it can be concluded that the pSer phosphate is indeed displacing the inner-sphere water molecule and saturating the coordination of the lanthanide ion as we intended. The observed decrease in emission of **1^{P}[Tb]** could then be explained assuming that the coordination of the phosphoserine side chain in **1^{P}[Tb]** pulls the terbium macrocycle away from the Trp antenna, decreasing the energy transfer, and thus resulting in weaker emission, even in absence of the quenching effect of the displaced water molecule.[22]

Following on the above results, we reasoned that further increasing the emission of the unphosphorylated state might provide an effective phosphatase sensor. Phosphatases have been linked to perturbations in phosphorylation-mediated signaling pathways and the occurrence of illnesses, and therefore the detection of phosphatase activities is of major relevance .[23] We envisioned that the desired increase in luminiscence might be achieved by displacing the quenching water molecule with an external bidentate ligand working as lanthanide sensitizer. To test this approach, we selected europium as emissive ion, and 4,4,4-trifluoro-1-(2-naphtyl)-1,3-butanedione (**TNB**) as external antenna.[24] We expected that upon phosphorylation the intramolecular coordination of the phosphate in the pSer side chain to the europium ion could displace the antenna, thereby promoting a decrease in the luminescence emission (Fig. 2).[25]

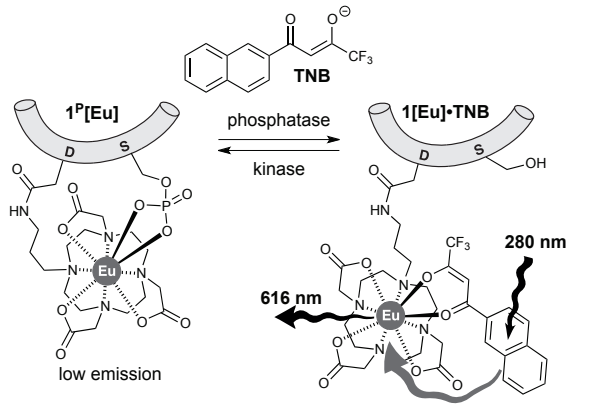

Fig. 2 Proposed sensing mechanism by selective coordination of **1[Eu]** with the external antenna and formation of a luminescent **1[Eu]•TNB** complex.

Irradiation at 280 nm of both peptide complexes in the presence of **TNB** resulted in the typical long-wavelength luminescence emission bands from Eu^{3+}. As expected, the emission intensity was significantly higher for the unphosphorylated peptide **1[Eu]** (Fig. 3). Titrations of both peptides **1^{P}[Eu]** and **1[Eu]** with **TNB** showed that the formation of the complex with **1[Eu]** was more favorable (K_{D1} ≈ 11 μM) than with **1^{P}[Eu]** (K_{D1} ≈ 99 μM). Therefore, at appropriate concentrations, **TNB** could selectively bind and sensitize the europium ion in **1[Eu]**, resulting in a significant enhancement of the difference in emission between both the unphosphorylated and the phosphorylated complexes, so that **1[Eu]** is about 20 times more luminescent than **1^{P}[Eu]** in the presence of the external **TNB** sensitizer, in the conditions described in the caption of Fig. 3.

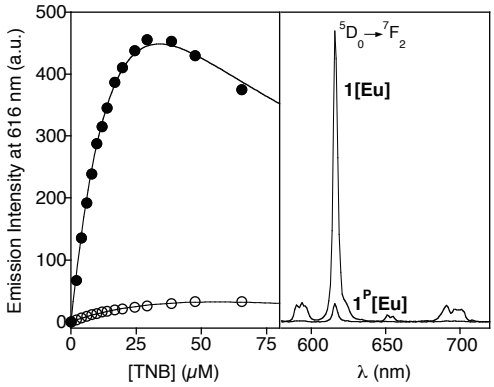

Fig. 3 Left: Luminescence titrations of 10 μM solutions of **1[Eu]** (●) and **1^{P}[Eu]** (○) monitoring the Eu(III) $^5D_0 \rightarrow {}^7F_2$ (ΔJ = 2) hypersensitive transition at 616 nm with increasing concentrations of **TNB**,[26] and best fit to 1:1 and 1:2 mixed binding model.[27] Right: Luminescence spectra of 10 μM **1[Eu]** and **1^{P}[Eu]** in the presence of 40 μM **TNB**. Excitation at 280 nm, 10 mM HEPES, 100 mM NaCl, pH 7.6. Same scale as the titration.

Having developed Eu(III) peptide complexes that display large luminescence changes between phosphorylation states, we decided to explore the use of this sensor for monitoring the course of enzymatic dephosphorylation (alkaline phosphatase, AP), and phosphorylation (PKCα) reactions. Thus, incubation of optimized concentrations of **1^{P}[Eu]** and **1[Eu]** in the presence of **TNB** with AP or PKCα, respectively, resulted in the typical response-time profiles shown in Fig 4.

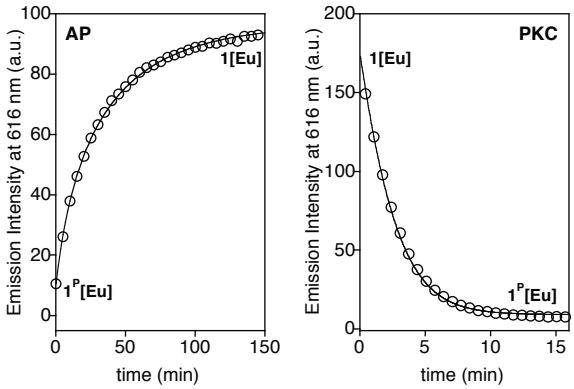

Fig. 4 Partial time courses monitoring the hypersensitive Eu(III) band at 616 nm upon excitation at 280 nm (AP) and 290 nm (PKCα).[28] Left: dephosphorylation of 25 μM **1^{P}[Eu]** with 20 units of AP in the presence of 77 μM **TNB**. 10 mM HEPES, 100 mM NaCl, pH 7.6 and 0.1X AP buffer. Right: phosphorylation of a 0.5 μM solution of **1[Eu]** with 10 μM **TNB** and 0.17 μg/mL of PKCα. 20 mM HEPES, 2.5 mM $MgCl_2$, 225 μM $CaCl_2$, 500 μM ATP, 1 mM DTT, 0.5 μg/mL phosphatidylserine, 0.1 μg/mL diacylglycerol, pH 7.4. Graphs include the best fit to the kinetic models described in the text and in the supporting information.

The differential equations governing each reaction step of the Michaelis-Menten mechanism for dephosphorylation of **1^{P}[Eu]** by AP, including the equilibrium accounting for product (inorganic phosphate) inhibition, were simultaneously fitted to the entire set of progress curves (Fig. 4 and S10). As a result, the Michaelis and inhibition constants were K_m = 14.5 ± 2.5 μM and

K_i = 4.4 ± 0.6 μM. Likewise, **1[Eu]** was readily phosphorylated by PKCα in the presence of 500 μM ATP, and the kinase catalysis could also be monitored by the decrease in the europium emission sensitized by the **TNB** antenna, following Michaelis-Menten kinetics with apparent submicro-molar Michaelis constant K_m = 0.23 ± 0.01 μM (Fig. 4 and S13).

Conclusions

We have demonstrated that phosphorylation of a serine residue of designed lanthanide metallopeptides can lead to a modification of the coordination sphere of the metal ion, leading to a significant luminescence change. Moreover, we have shown that addition of an external antenna that displays selective binding affinity for the non-phosphorylated species leads to a significant improvement in the ratio between the emission of the two states of the sensor, allowing the real time monitoring of enzymatic activity.

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† Electronic Supplementary Information (ESI) available: Peptide synthesis, luminescence titrations, inner-sphere water calculations, time-delayed luminescence experiments, kinetic studies and data analysis. See DOI: 10.1039/b000000x/

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