Review

Advances in Lanthanide-based luminescent peptide probes of kinase and phosphatase activity

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Abbreviations: DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; ET, energy transfer; ISC, intersystem crossing; OSSu, *N*-hydroxysulfosuccinimide; *t*-Bu, *tert*-butyl.

Abstract

Signaling pathways based in protein phosphorylation and dephosphorylation play critical roles in the orchestration of complex biochemical events and are at the core of most signaling pathways in the cells (i.e. cell cycle regulation, cell motility, apoptosis, etc.). The understanding of such complex networks is based to a great extent in the biochemical study of their components, kinases and phosphatases, and therefore the development of luminescent sensors for monitoring their activity is an active field of research. Examples in the literature usually rely on the modulation of the fluorescence emission of organic fluorophores, but given the exceptional photophysical properties of lanthanide ions, there is an increased interest in their application as emissive species for monitoring kinase and phosphatase activity. This review summarizes the advances in the development of lanthanide-based luminescent peptide sensors as tools for the study of these fundamental enzymes and provides a critical description of current examples and synthetic approaches to these types of sensors.

Keywords

kinase sensing, phosphatase sensing, luminescence, sensors, fluorescent assays

1. Introduction

Luminescence techniques, allowing the visualization of molecular events with extraordinary sensitivity and selectivity, have transformed the study of biological systems [1, 2, 3]. Part of the increasing success of the luminescent techniques derive from the development of fluorophores with improved photophysical properties with extreme sensitivity to chemical variations in their surroundings that lead to measurable changes in their emissive properties. These changes can thus be exploited for monitoring many physico-chemical variables [4, 5, 6,7, 8], including pH [9], viscosity [10], polarity [11], or redox state [12]. Moreover, these fluorophores can be integrated in receptor systems for the development of smart chemical sensors capable of monitoring selected molecular targets and events, such as proteins,[13, 14] DNA and RNA [15, 16], metal ions [17], or enzymatic activity [18, 19, 20, 21]. In addition to the development of new organic fluorophores, there is an increasing interest in the application of lanthanide ions as emissive species in luminescent sensors, owing to their narrow bands and long wavelength emission, and the possibility of avoiding background fluorescence using time-resolved experiments.

Lanthanides comprise the fourteen elements from $_{58}$Ce to $_{71}$Lu. Lanthanides display marked chemical similarities, their coordination chemistry dominated by the +3 oxidation state, a clear predilection for hard donor ligands (preferentially oxygen), and high coordination numbers (usually higher than six) with little stereochemical preferences. With the exceptions of La(III) ($4f^0$) and Lu(III) ($4f^{14}$), all of the trivalent lanthanide cations are paramagnetic species, for which the electronic configuration in the inner 4f orbitals progressively changes from $4f^1$ in Ce(III) to $4f^{14}$ in Lu(III); these ions are characterized in most cases to low-lying excited states with multiple J sublevels due to significant spin-orbit coupling interactions. The spectroscopic properties of the lanthanide complexes arise from transitions between these states in the 4f sub-shell, which is shielded from the influence of the environment by the higher energy $6s^2$ and $5p^6$ orbitals, and therefore lanthanide complexes display similar spectroscopic properties—characteristic of each metal ion—regardless of their chemical environment [22].

Many of the lanthanides excited states can relax to the ground state with emission of light (Figure 1a). As a result of the relative isolation of the 4f orbitals from their environment, lanthanide ions exhibit very sharp absorption and emission bands, usually in the orders of tens of nm (in comparison with the wide emission bands of transition metal complexes or organic fluorophores that often extend over 100 nm) [23]; this feature is potentially useful in multiplex experiments that require simultaneous monitoring of more than one spectroscopic signal [24]. Moreover, transitions between different configuration states of the f sub-shell are forbidden by parity selection rules, and therefore lanthanides display long-lived excited states that in the case of Eu^{3+} and Tb^{3+} can be in the order of milliseconds, and their decay is mainly governed by non-radiative transitions, i.e. interaction of the excited electronic levels of the lanthanide ions with the vibrational modes of solvent molecules in their vicinity. The resulting long lifetimes open the

possibility for time-resolved experiments in which the emission is measured after a short delay—typically tens of μs—after the excitation pulse, thus eliminating interferences from scattered light and autofluorescence, and greatly enhancing the specificity and signal-to-noise ratio for detection (Figure 1b) [23, 25, 26, 27]. A second consequence of the forbidden nature of the f-f transitions is the weak absorption of the trivalent lanthanide ions (with extinction coefficients in the order of 1-5 M^{-1} cm^{-1}), which make the direct excitation of these species impractical. This drawback is bypassed by taking advantage of energy transfer processes between nearby organic fluorophores and the lanthanide ion. This indirect excitation mechanism, called "*sensitization*" or "*antenna effect*", is the basis of most applications of lanthanide luminescence in sensing strategies, as it is extremely sensitive to changes in the immediate surroundings of the metal ion or the antenna. The sensitization process is extremely complex, and involves a number of energy levels from both the donor fluorophore (usually the lowest triplet state is reached by intersystem crossing (ISC) after the initial population of the S_1 state upon excitation of the antenna) and the accepting metal ion; the coupling between the donor and the acceptor involves various mechanisms, including through-bond energy transfer (Dexter mechanism) [28], as well as through-space couplings between the dipole moment of the T_1 state of the antenna and the dipole moment of the 4f orbitals (Förster mechanism) in the acceptor lanthanides (Figure 1c) [29, 30]. As far as sensor development, Tb^{3+} is particularly relevant among the lanthanide ions, because it can be efficiently sensitized by the indole and phenol side chains of the tryptophan and tyrosine residues, thus allowing its use in the context of natural peptide and protein sequences with these natural antennas; on the other hand, although europium (III) is not effectively sensitized by these native residues it can be excited by the 7-azatryptophan analog, which can also be incorporated into proteins in place of natural tryptophan residues [31]. Additionally, terbium and europium (III) ions are the most strongly emitting members of the lanthanide series, which also contributes to their widespread application as emissive centers in luminescent lanthanide sensors [32].

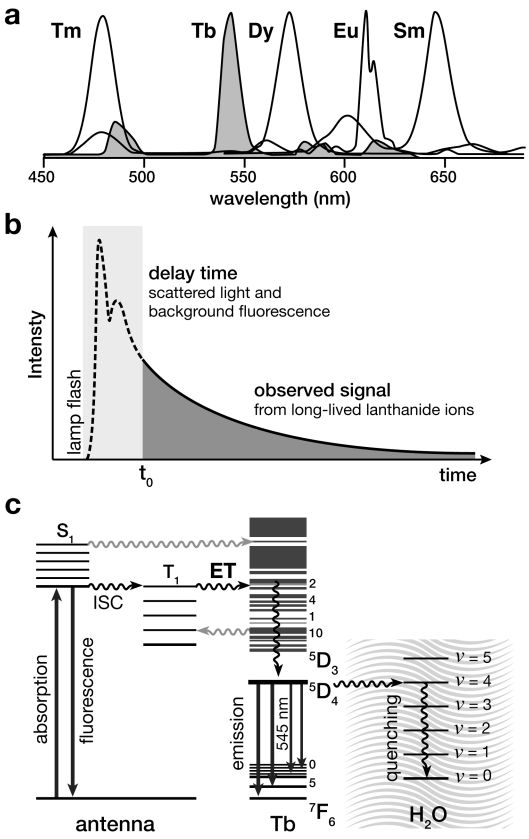

Figure 1. a) Luminescence spectra of UV/visible-emitting lanthanide ions highlighting the Tb(III) emission spectrum; b) Outline of a time-gated experiment in which the luminescence from the lanthanide ion is recorded after a delay time and relaxation of the autofluorescence of organic fluorophores; c) Simplified Jablownsky diagram of the lanthanide sensitization process. Excitation of the organic antenna by absorption of light populates the lowest singlet state (S_1). Fluorescence emission from S_1 recovers the ground state of the organic fluorophore whereas intersystem crossing (ISC) yields electrons in the lowest triplet state of the antenna (T_1), from which the energy can be transferred to a Terbium (III) ion. Finally, the Tb(III) ion can reach its ground state ($^7F_6$) through light emission from the lowest excited state ($^5D_4$), or vibrational quenching by solvent water molecules. Non-radiative transitions are depicted with wavy arrows; black arrows represent more probable processes than grey ones.

Kinase-mediated protein phosphorylation together with dephosphorylation catalyzed by phosphatases represents the basic metabolic switch by which cells control the flow of information [33], as well as most metabolic processes, particularly those that involve the coordination of complex functions, including for example gene expression, cell cycle, apoptosis, differentiation or motility, to name just a few [34]. Therefore, given the relevance of phosphorylation/dephosphorylation processes in cellular homeostasis and disease, there is a great interest in studying the role of kinases and phosphatases and their connection with

the etiology of diseases such as cancer [35], for which chemical sensors that allow the straightforward and continuous monitoring of their activity are of great relevance [6, 36, 37]. Therefore, many fluorescent sensors of kinase activity, based on a variety of mechanisms, such as conformational changes [38], environment- [39], or chelation-enhanced fluorescence [40, 41, 42] have been reported in the design of kinase sensors, and are even commercially available [43]. Although some of these assays perform well as *in vitro* tests, the unique properties of lanthanide ions (long excite state lifetimes, long emission wavelengths, sharp emission bands, etc.) would open new interesting possibilities in kinase/phosphatase sensing. In this review we will discuss the use of lanthanide complexes as privileged emissive species applied to the design of specific kinase sensors, particularly in the context of peptide-based probes that provide an ideal platform for interrogating specific enzymes with known sequence selectivity.

2. Strategies in the development of lanthanide-based sensors

The complexity of the sensitization mechanism, in which several electronic states and species are involved, provides multiple opportunities for the modulation of the metal luminescence and their application for the design of luminescent biosensors. Thus, the modification of the hydration state has been exploited in the development of lanthanide-based bicarbonate [44], or pH sensors [45], which rely on the displacement of metal-bound water molecules to eliminate a competing non-radiative quenching mechanism and enhance the emission of the metal ion (Figure 2a). Alternatively, non-luminescent coordinatively-unsaturated lanthanide complexes can be turned into highly emissive species upon complexation with appropriate fluorophores that act as antennas, as demonstrated by the use of a lanthanide-chelating cyclodextrin as chemosensor for aromatic hydrocarbons (Figure 2b) [46]. Likewise, the supramolecular (non-covalent) interaction between a non-fluorescent ligand bearing a lanthanide chelate to an analyte that can function as an antenna can also result in a large luminescence increase; this was demonstrated by the application of lanthanide metallopeptides as cyclin A sensors in which a Tb(III) ion in the peptide is sensitized upon binding to the protein by a tryptophan residue located in the vicinity of the cyclin A recognition site [47]. As an alternative demonstration that noncovalent interactions can be used to sensitize lanthanide ions, it has been shown that the luminescence of a terbium ion bound to the head group of a synthetic amphiphyle incorporated into a micelle can be effectively sensitized by aromatic residues of integral membrane proteins [48]. Likewise, it is also possible to combine the displacement of inner-sphere bound water molecules and the coordination of an external antenna (strategies a and b, Figure 2), to trigger luminescence emission, as shown with the development of ternary lanthanide luminescent complexes and their application in displacement assays to detect anions [49, 50, 51]. Another common strategy relies in the chemical or conformational modification of the lanthanide-binding ligand to modulate the affinity for the metal ion, so that the coordination takes place in only one of the states of the sensor (Figure 2c).

This approach has been used for example in the development of simpler protease sensors that generate an appropriate chelating ligand upon proteolysis [52], but this strategy has proven particularly powerful for the development of kinase sensors, because the phosphorylation event creates a good chelating group that significantly improves the metal-binding properties of the phosphorylated species. A final sensing strategy relies on the modulation of the antenna effect through changes in the distance between the metal center and the sensitizing fluorophore, so that reducing the distance between the sensitizing antenna and the metal ion in the "on" state results in increased luminescence from the lanthanide (Figure 2d). This effect is particularly effective when large conformational changes take place, such as in the folding of peptide chains involved in protein-protein [53], or protein-oligonucleotide interactions [54]. Each strategy has advantages and shortcomings, and sometimes the same chemical event (i.e. tyrosine phosphorylation) can be approached through more than one of these schemes, as we will review in the next section.

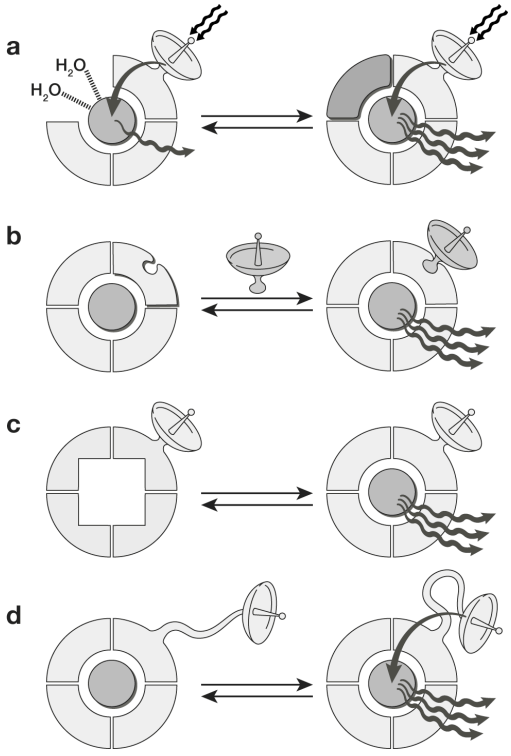

Figure 2. Strategies used to modulate lanthanide sensitization. a) binding of an analyte displaces quenching water molecules increasing the emission; b) binding to the antenna yields an emissive complex; c) conformational or chemical changes in the probe increase the binding affinity for the lanthanide ion generating the emissive state of the probe; d) conformational changes reduce the distance between the antenna and the ion increasing the luminescence intensity.

Before considering specific examples of lanthanide-based luminescent sensors for kinases and phosphatases, we will briefly discuss the incorporation of the lanthanide emissive ions into organic receptors/transduction units. Lanthanide ions cannot be covalently attached to designed ligand or receptors, and therefore they are incorporated into the organic scaffolds in the form of coordination complexes with organic chelators. Lanthanides usually display high coordination numbers and a marked preference for oxygen ligands. The most commonly documented ligands for the complexation of lanthanide ions include linear polycarboxylates, such as DTPA (diethylenetriaminepentaacetic acid) or BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid), and azacrown macrocycles—particularly *cyclen* (1,4,7,10-tetraazacyclododecane) and *cyclam* (1,4,8,11- tetraazacyclotetradecane) derivatives, which feature cavity sizes appropriate for lanthanide ions—containing acetate pendant arms that provide strong binding donor groups. Prominent examples of these family of compounds are DO3A (1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid), DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and TETA (1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid) chelators. Additionally, derivatives of the smaller azamacrocyle *tacn* (1,4,7-triazacyclononane), such as NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) are being increasingly used [55, 56, 57].

The conjugation of these chelators to the rest of the molecular sensing device is usually made through one of the carboxylate side arms, which is modified as an amide. This strategy has the advantage of maintaining the chelating properties of that arm—through the lone pair of the oxygen atom in the resulting amide—while providing a stable and synthetically amenable connection. This basic approach can be implemented in various ways. The simplest approach involves reaction of amines in the ligand with the commercially available DTPA anhydride or other electrophilic species reported in the literature [58, 59, 60, 61]. Another approach requires the derivatization of a chelating macrocycle (i.e. DOTA) as an electrophilic *N*-hydroxysulfosuccinimide ester, which then can be used to modify any reactive amine in a biomolecule or ligand of interest [62, 63] (Figure 3a). Alternatively, the versatile DOTA tris-*t*-Bu ester derivative (DOTA(t-Bu)$_3$, Figure 3b, 3c) can be synthesized in two steps from the cyclen tetraazamacrocyle [64, 65] which, having only one reactive carboxylate, can be easily incorporated into most synthetic schemes. This approach has been extensively applied for the modification of peptides in solid phase peptide synthesis schemes [47]. Multiple variations of this strategy, including the synthesis of numerous structural analogs of the macrocyclic chelates—particularly cyclen derivatives—have been reported in the literature, and reviewed elsewhere [66, 67, 68]. Once the macrocycle is incorporated into the organic ligand, the incorporation of the lanthanide ion is straightforward, as incubation in aqueous solution with the appropriate chloride salt will quantitatively yield the desired product. Finally, in addition to high thermodynamic stability, DOTA-lanthanide complexes are kinetically inert. This property is particularly important for *in vivo* applications because free lanthanide ions are toxic and can disrupt calcium signaling

and irreversibly associate with proteins and tissues replacing calcium; this has been extensively associated in the context of Gd(III) complexes extensively used as MRI contrast agents [69].

In addition to the use of synthetic chelating ligands to bind the lanthanide ions, peptides provide a unique opportunity for binding metal ions, as their sequence can be tailored to create appropriate coordination environments to bind metal ions with high affinity and selectivity [70, 71, 72]. Thus, it is known that natural calcium-binding loops of EF-hand proteins, can also bind lanthanide ions with good affinity [73]. Optimization of the consensus sequence of these chelating loops through combinatorial peptide synthesis afforded lanthanide-binding oligopeptides with low nanomolar binding affinity towards lanthanide ions. Moreover, the resulting lanthanide binding peptides were highly luminescent owing to the presence of a sensitizing tryptophan residue in the sequence, and could be used as highly versatile tags for the introduction of lanthanide ions (and luminescent properties) into proteins [74, 75, 76].

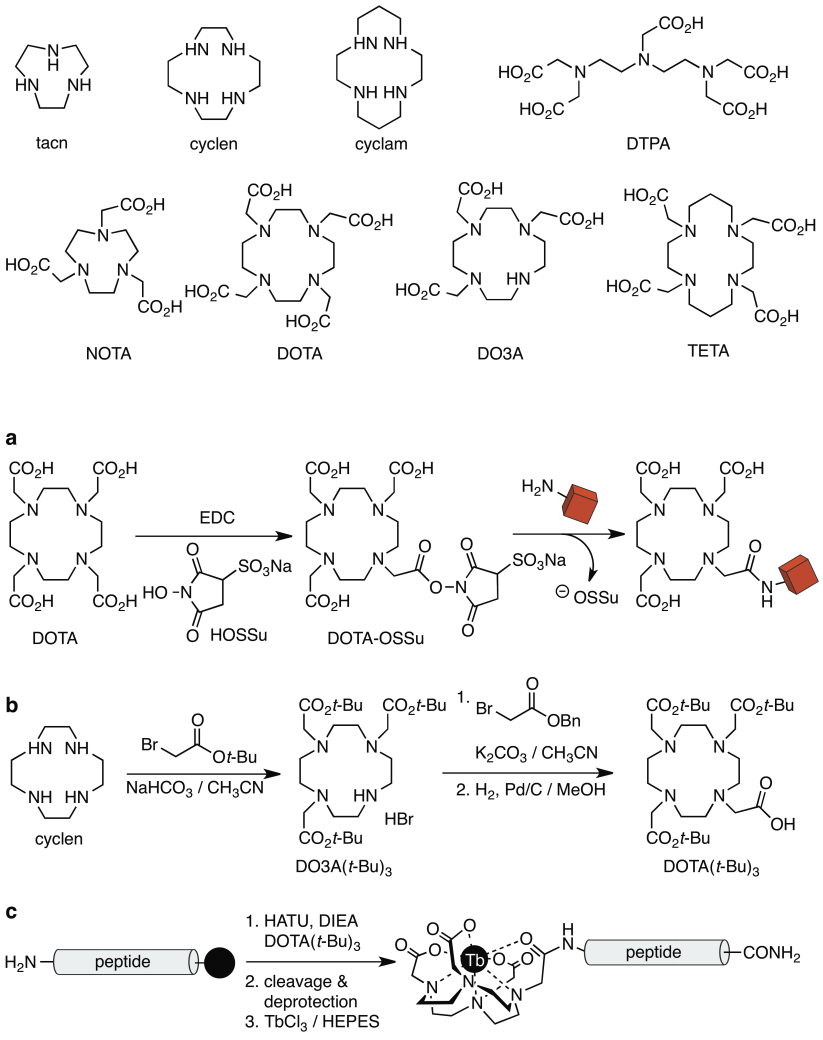

Figure 3. Top, Structures of common lanthanide chelators; Bottom, synthetic strategies to access peptide-DOTA conjugates: a) Preparation of the DOTA *N*-hydroxysulfosuccinimide ester by incubation of DOTA with EDC and HOSSu in solution; the resulting DOTA-OSSu is conjugated through an amine. b) Synthesis of protected DOTA tris-*t*-Bu ester. c) solid-phase modification of a selectively deprotected amine in a peptide chain with DOTA tris-*t*-Bu ester. Cleavage and deprotection, followed by incubation with $TbCl_3$ yields the desired DOTA[Tb(III)]-labeled peptide.

3. Lanthanide-based kinase and phosphatase sensors

Lanthanides have been used as probes for the determination of a broad array of enzyme activities, mostly through the detection of small molecules implicated in the catalytic reaction, either as reactants or as products, such as H_2O_2, ATP, pyrophosphate or phosphate [25, 77, 78, 79, 80, 81]. We will focus our review in the development of smart peptidic sensors incorporating lanthanide ions as emissive species, particularly those that monitor kinase or phosphatase activity.

One of the earliest reports on the use of lanthanide ions to (indirectly) monitor the phosphorylation state of a peptide describes the effect of tyrosine phosphorylation on metal binding affinity [82]. The study by K. J. Franz *et. al.* was focused on the metal-binding properties of C-terminal α-synuclein peptide fragments, which are believed to be involved in calcium binding, and in modulating the aggregation pathways of the protein *in vivo* [83]. Terbium luminescence was used as a straightforward and simple spectroscopic handle that could signal whether the phosphorylation state, residue mutations, or sequence truncations had any effect on the metal-binding properties of the α-synuclein peptides, and through competitive displacement titrations, it also allowed to monitor the binding of other spectroscopically silent metals, such as Al^{3+}, Fe^{3+}, Ca^{2+}, Mg^{2+}, Mn^{2+} or Co^{2+}, to the peptide fragments. This approach was made possible by exploiting the natural tyrosine residue—particularly in its phosphorylated form—as effective sensitizer of the terbium ion luminescence [84, 85, 86]. Moreover, the authors found that the unphosphorylated α-synuclein peptide fragment, containing an arrangement of carboxylate groups similar to those found in Ca binding loops, did not show any significant binding affinity for the Tb(III) ion and was only weakly emissive, whereas the analogous peptide containing the phosphorylated tyrosine residue displayed excellent binding properties ($K_D \approx 300$ nM) and strong luminescence, therefore constituting a novel metal-binding site in which the pTyr was playing an active role in the coordination of the Tb(III) ion. Further studies showed that metal coordination is also supported by carboxylic acid groups at positions 119, 121 and 126 that together created a structured multidentate chelating environment [87].

The generation of an effective coordination environment upon phosphorylation has proven to be a particularly powerful strategy for the development of lanthanide-based kinase sensors. The first example of a designed sensor based on this approach makes use of a phosphoserine residue as an isosteric mimic of a

natural glutamic residue (Glu is indeed considered as a pSer mimetic) [88]. The probe design is based on the mutation of a key glutamic acid residue, which is required for tight metal binding in the calcium-binding loop of EF hands, for a serine residue. This replacement would result in a significant loss of metal binding affinity, which could be recovered upon phosphorylation by specific kinases and generation of a chelating phosphoserine residue that reconstitutes the metal binding site [89] (Figure 4a). The design was completed with the replacement of a natural tyrosine at position 7 by a tryptophan sensitizer, and the introduction of various kinase recognition sequences at the C-terminal section of the peptide, thus yielding sensors for PKA, PKC and Erk MAP kinase. As expected, the unphosphorylated peptides were poor Tb^{3+} binders, and displayed only very weak luminescence. In contrast the phosphorylated peptides displayed in all cases strong emission from the terbium (III) ion. Metal binding was also confirmed with competitive titrations with the non-luminescent Ho^{3+} ion that resulted in the loss of Tb^{3+} luminescence, consistent with the formation of a competitive peptide-Ho^{3+} complex. Moreover, NMR experiments in the presence of the diamagnetic La^{3+} demonstrated that the binding to the metal induced significant conformational changes in the peptide chain.

The initial concept of phosphorylation-dependent generation of a lanthanide-coordinating side chain was later applied in the design of peptidic sensors of tyrosine kinase activity. The elegant approach described by the group of Neal J. Zondlo involved, as in the example discussed before, the replacement of the invariant Glu^{12} residue present in EF hands (DKNADGYIDAA**E**K). However in this case, considering the increased length of the phosphotyrosine side chain relative to the Glu, it was thought that the tyrosine residue could be introduced in the peptide sequence not in place of the Glu^{12}, but actually one residue before it. Thus, it was expected that the introduction of a pTyr residue at position 11 should replicate the electrostatic and coordinating properties of the Glu residue at position 12, which would be replaced by a simple Ala residue in the final sensor (Figure 4b); as in the initial report, the Tyr at position 7 was replaced by a Trp residue acting as efficient Tb^{3+} sensitizer, and the C-terminal sequence included the Abl recognition sequence (DKDADGWEAI-**Y**-AAPFAK) [90].

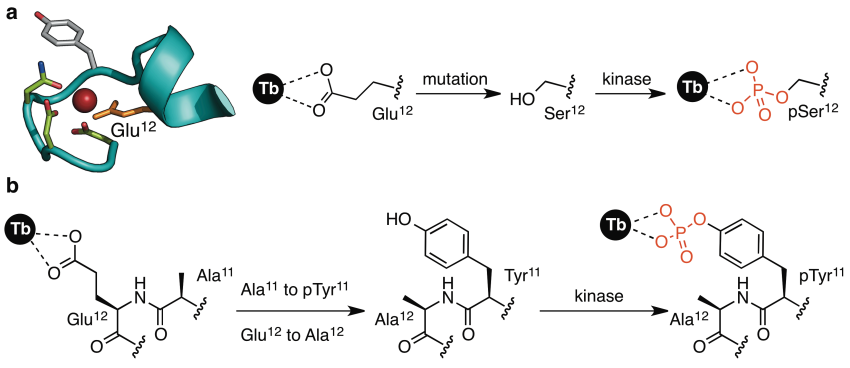

Figure 4. a) Structure of the EF hand calcium-binding loop indicating the natural Tyr residue that is replaced by a Trp in the final sensor, and the metal-chelating side chains, including the key Glu^{12} side chain. The replacement strategy is outlined, showing the regeneration of the chelating ligand upon phosphorylation of the Ser^{12} residue; b) Design of a tyrosine kinase peptide sensor through double mutation: the chelating Glu^{12} residue is replaced by an alanine, while simultaneously the Ala^{11} is replaced by a tyrosine. Phosphorylation of the tyrosine generates a chelating side chain approximately at the same position as the original Glu^{12} carboxylate.

As expected, the control peptide containing the double mutation (Glu12 to Ala and Ala11 to Tyr) did not show significant luminescence emission in the presence of Tb(III). In contrast, the phosphorylated peptide exhibiting strong terbium luminescence and tighter binding (K_D(pTyr) ≈ 54 μM while K_D(Tyr) ≈ 1.9 mM), indicating that $pTyr^{11}$ effectively mimics Glu^{12} (K_D(Glu) ≈ 88 μM). NMR experiments with La^{3+} demonstrated significant conformational changes in the peptide chain upon incubation with the metal ion, consistent with the adoption of a ordered structure and the organization of the EF hand upon coordination. The peptide including the Abl recognition motif also displayed phosphorylation-dependent terbium binding and luminescence, consistent with the results discussed with the model peptides. Moreover, incubation of the unphosphorylated peptide sensor with Abl kinase resulted in a large increase in terbium Tb^{3+} luminescence, showing that the peptide could be used as a sensor of Abl kinase activity; likewise, incubation of the phosphorylated peptide with YOP tyrosine phosphatase resulted in a rapid reduction in Tb^{3+} luminescence, consistent with dephosphorylation and dissociation of the metal ion from the peptide.

The generation of an appropriate coordination environment upon phosphorylation as a viable sensing strategy is not restricted to peptide-based lanthanide ligands. The group of D. Sames demonstrated that it is also possible to integrate the phosphorylation event in the generation of a hybrid metal-binding ligand, comprising a synthetic diethylenetriaminepentaacetic acid (DTPA) and a phosphoserine side chain in a short tripeptide (DTPA-Gly-Trp-Ser/pSer). Unfortunately, this simple design displayed multiple equilibria involving both monomeric and dimeric species and thus, while the unphosphorylated peptide preferentially bound Tb^{3+} as a highly luminescent dimeric complex, the equilibrium was shifted towards the weakly luminescent monomeric species in the case of the phosphorylated peptide, resulting in a higher emission in the unphosphorylated form (approximately two-fold increase in the luminescence intensity). This was used in an enzymatic assay to monitor the dephosphorylation of the pSer residue with the nonspecific alkaline phosphatase [91]. The observation of multiple association states is a relatively common phenomenon observed with coordinatively-unsaturated lanthanide complexes, and requires careful fine tuning of the assays or the redesign of the sensors to avoid these undesired association states.

The same strategy was used a couple of years later for the development of a luminescent sensor for tyrosine phosphorylation. The new design was greatly improved over their initial DTPA/pSer approach, and was based on the use of phosphotyrosine as an iminodiacetate surrogate mimicking ethylene glycol tetraacetate (EGTA) cooperative chelation of a trivalent lanthanide cation [92]. In addition to the chelating ligand, the sensor contained a carbostyril 124 chromophore as antenna [93], which can efficiently sensitize Tb^{3+} and Eu^{3+} ions. The sensor also included the recognition elements common to both Src and Abl tyrosine kinases C-terminal to the sensing components (NTA/carbostyril 124). As expected for a coordinatively deficient ligand, the unphosphorylated probe displayed almost negligible emission in the presence of Tb^{3+} or Eu^{3+}. In contrast, the phosphorylated counterpart displayed intense luminescence emission in presence of both metal ions, which could be correlated to an apparent dissociation constant K_D of ≈160 μM. Although the titration profiles indicate that the equilibrium in solution involves more than a simple 1:1 complex, the system is well behaved and the distortion introduced by higher order associations is not significant; indeed, the variation in the luminescence intensity could also be used to monitor the activity of Src and Abl tyrosine kinases, which resulted in similar activities to those measured with the optimized peptide, thus demonstrating that the sensing components were well tolerated by the enzymes.

A flexible option for introducing chelating units into peptide sequences is described by Appelblom *et. al*., who developed an amine-reactive terbium complex (2,2',2",2"'-{{6,6'-{4"-[2-(4-isothiocyanato-phenyl)-ethyl]-pyrazole-1",3"-diyl)bis(pyridine)-2,2'-diyl}bis(methylenenitrilo)}tetrakis(acetato) Tb^{3+}) that can be attached to the side chain of a Lys residue to label a nonemissive peptide sequence (EAI-Y-AAPFA**K**, reactive Lys in bold) and turn it into a luminescent sensor. Within the appropriate sequence context, a phosphotyrosine residue might act as a complementary chelating ligand, thus modifying the emissive properties of the terbium ion in response to the phosphorylation state of the key residue (Figure 5a) [94]. While the phosphorylation reaction with Abl kinase was shown to be very slow, and the maximum change in the terbium luminescence was significant (≈ 3.2-fold increase in emission intensity) the approach proved to be flexible and relatively simple to implement. Additionally, the phosphorylated versions were tested as substrates of the T-cell tyrosine phosphatase (TC PTP), for which they were excellent substrates. Interestingly, the authors studied several peptides with increasing separation between the reactive Lys (and hence the terbium chelate) and the phosphotyrosine residue (EAI-pY-AP**K**, EAI-pY-AAP**K**, EAI-pY-AAAP**K**, EAI-pY-AAPFA**K**), but the distance had only a modest effect on the performance of the sensors (this might suggest that intramolecular coordination of the phosphotyrosine is not relevant for the sensing mechanism but, unfortunately, the report does not include lifetime measurements in D_2O/H_2O mixtures that, together with NMR experiments, would have been helpful in identifying the structural changes and mechanism responsible of the observed luminescence enhancement).

An alternative approach to sensing kinase and phosphatase activity is based on the coordination of the peptide sensor to an external antenna, which is selectively bound to one of the phosphorylation states.

Following this strategy our group has recently reported a peptide conjugate containing a heptacoordinating DO3A chelate (1,4,7,10- tetraazacyclododecane-1,4,7-triacetic acid, Figure 3), which does not satisfy all the available coordination positions of the lanthanide ion [95]; in addition to the chelate, the peptide chain includes a C-terminal Protein Kinase C alpha (PKCα) recognition sequence [96], and a Pro-Gly β-turn inducing sequence that preorganizes the peptide chain to promote the coordination of the phosphoserine residue to the lanthanide ion [97]. The sensitization of the lanthanide ion in the sensor relies on the coordination to an external antenna, in this case 4,4,4-trifluoro-1-(2-naphtyl)-1,3-butanedione (**TNB**), which is known to effectively sensitize Eu^{3+} [49]. The sensor is designed so 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 upon phosphorylation and conversely, dephosphorylation should open coordination positions in the europium chelate that could be satisfied by coordination to the external antenna (Figure 5b) [98]. As expected, the luminescence of the Eu^{3+} chelate is modulated by the phosphorylation state of a serine residue in the sequence, so that the unphosphorylated peptide **[Eu]** displays significantly higher emission intensity from the metal ion upon irradiaton of the TNB antenna than the phosphorylated counterpart ^{P}**[Eu]**. Titrations of both ^{P}**[Eu]** and the unphosphorylated Eu^{3+} peptide **[Eu]** with increasing concentrations of TNB showed that the formation of the TBN-Eu^{3+} complex was much more favorable with the unphosphorylated peptide than with the phosphorylated analog (the apparent binding constants were, $K_D \approx 11$ μM, and $K \approx 99$ μM, respectively). The supramolecular sensor was used to monitoring the course of enzymatic dephosphorylation (alkaline phosphatase, AP), and phosphorylation (PKCα) reactions, allowing the measurement of kinase and phosphatase time courses.

A related approach makes use of a dinuclear DOTAM terbium complex for the selective detection of phosphorylated tyrosine residues in the presence of non-phosphorylated tyrosines, phosphoserine, phosphothreonine, or other coexisting phosphate-containing molecules. The selectivity in the sensor relies on the unique combination of effective chelation (through the phosphate) and sensitization properties (thanks to the phenol ring) found in phosphotyrosine residues [99]. Thus, upon binding to phosphotyrosine, the terbium ion is sensitized and its luminescence is enhanced, this enhancement could be used to monitor the enzymatic phosphorylation of Tyr residues in model peptides (Figure 5c). The design has the clear advantage that it does not require the modification of the peptide substrate, and therefore it should be easily applicable to studying the phosphorylation of full proteins; on the downside, it is not possible to distinguish the phosphorylation of different Tyr residues, and therefore would be hard to implement in complex biological settings, where multiple phosphorylated tyrosine species would be simultaneously present. Moreover, the bis-DOTAM dinuclear complex almost fully coordinates the lanthanide ions, therefore relying mainly on electrostatic attraction for the formation of the pTyr-sensor complex, which results in a low binding constant ($K_D \approx 29$ μM) and a strong dependency on the ionic strength of the medium.

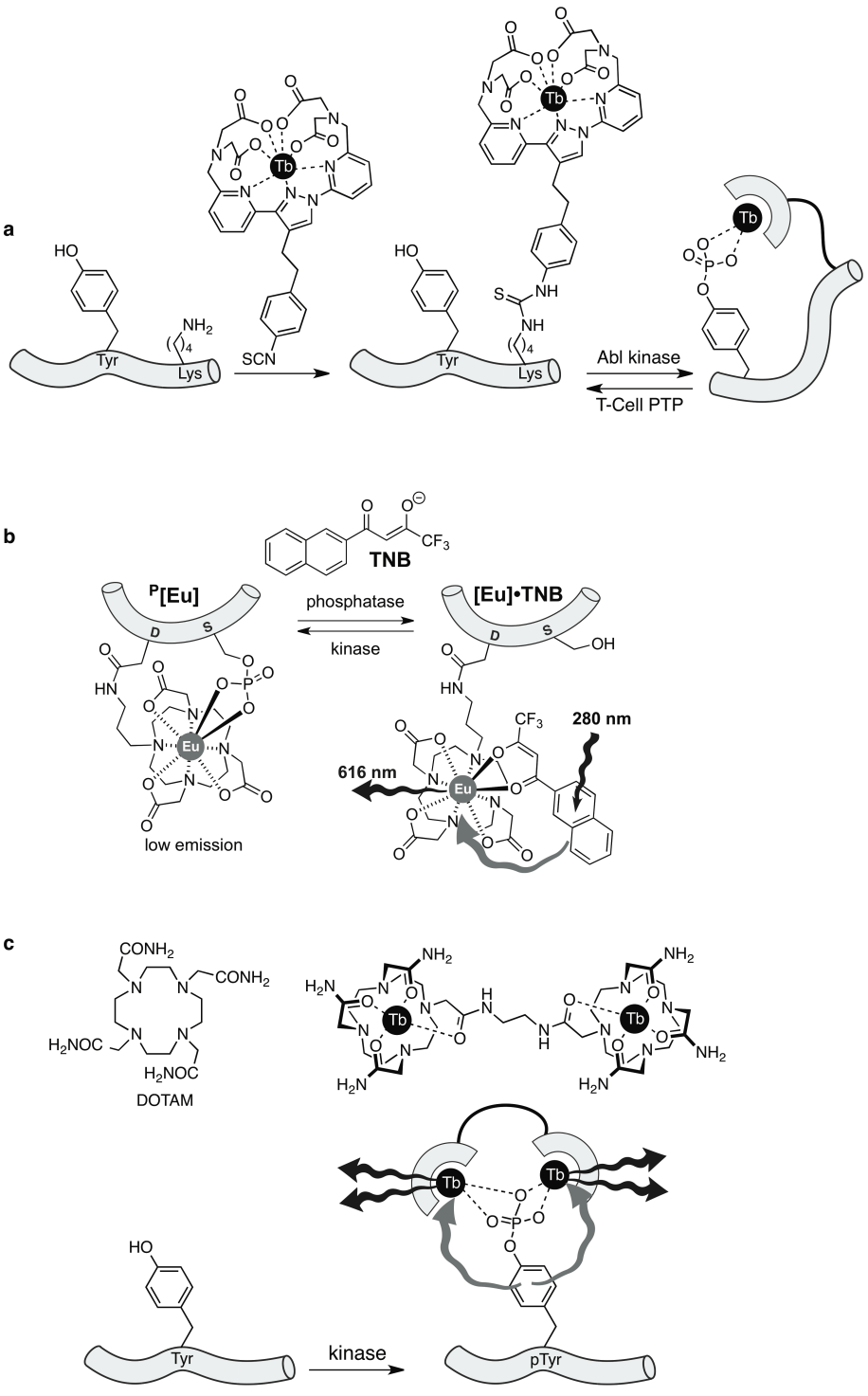

Figure 5. a) Tyr peptide containing a nucleophilic Lys in its sequence is reacted with the isothiocyanate terbium chelate, and the phosphorylated peptide sensor responds to Abl tyrosine kinase activity by increasing its emission. Dephosphorylation with TC PTP recovers the unphosphorylated (and less emissive)

peptide; b) Proposed sensing mechanism by selective coordination of **[Eu]** with the external antenna and formation of a luminescent **[Eu]•TNB** complex; c) structure of the macrocyclic DOTAM (amide derivative of DOTA) and structure of the dinuclear Tb(III) complex used as sensor and sensing mechanism by interaction of the terbium complex with the phosphotyrosine by electrostatic interactions.

The most recent report so far describing the use of peptide probes to monitor phosphorylation takes advantage of the long luminescence lifetimes of the terbium ions [100]. In this report, the authors study the binding of Tb^{3+} to phosphorylated and unphosphorylated peptides of a Spleen tyrosine kinase (Syk)-specific artificial substrate peptide (SAStide: GGDEED-Y/pY-EEPDEPGGKGG). The peptide displays differential terbium binding so that the phosphorylated peptide pSAStide formed a 1:1 complex with Tb3+ with a K_D of approximately 1.5 μM; in comparison, the K_D for the formation of the complex with the unphosphorylated SAStide was of 7.6 μM. Interestingly, the steady-state luminescence of the unphosphorylated SAStide in the presence of Tb^{3+} was lower than that displayed by the phosphorylated peptide. This difference is likely the consequence of the presence of one H_2O molecule in the coordination sphere of the SAStide peptide, which seems to be displaced in the pSAStide complex, as shown by lifetime measurements. Moreover, the authors were able to enhance the sensitivity and dynamic range of the probe by optimizing the integration times in time-resolved luminescence assays, and while the luminescence ratio between the phosphorylated and unphosphorylated peptides (pSAStide/SAStide) in steady state experiments is about 2:1, it can be enhanced up to approximately 32:1, thus demonstrating an additional optimization strategy in lanthanide-based sensors of phosphorylation.

4. Concluding Remarks

Although the first reported kinase peptide sensor based on lanthanide luminescence dates from 2006, the advantages afforded by lanthanide luminescence have fueled the development of a number of different sensors. The rich photophysics of the lanthanide sensitization process provides many opportunities for the development of sensors exploiting a variety of mechanisms, very often based on the intrinsic chelating properties of the phosphorylated side chains. Future developments will probably show alternative mechanistic approaches and will exploit new bioconjugation reactions to translate these findings into full-protein sensors, or into biologically relevant contexts.

Acknowledgments. We thank the support given by the Spanish grants SAF2010-20822-C02, CTQ2012-31341, CTQ2009-14431, SAF2007-61015, and the Xunta de Galicia INCITE09 209 084PR,

GRC2010/12, PGIDIT08CSA-047209PR. E.P. thanks the *Fundación Barrié de la Maza* for her postdoctoral fellowship.

The authors declare no commercial or financial conflict of interest

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Figure 1. a) Luminescence spectra of UV/visible-emitting lanthanide ions highlighting the Tb(III) emission spectrum; b) Outline of a time-gated experiment in which the luminescence from the lanthanide ion is recorded after a delay time and relaxation of the autofluorescence of organic fluorophores; c) Simplified Jablownsky diagram of the lanthanide sensitization process. Excitation of the organic antenna by absorption of light populates the lowest singlet state (S_1). Fluorescence emission from S_1 recovers the ground state of the organic fluorophore whereas intersystem crossing (ISC) yields electrons in the lowest

triplet state of the antenna (T_1), from which the energy can be transferred to a Terbium (III) ion. Finally, the Tb(III) ion can reach its ground state ($^7F_6$) through light emission from the lowest excited state ($^5D_4$), or vibrational quenching by solvent water molecules. Non-radiative transitions are depicted with wavy arrows; black arrows represent more probable processes than grey ones.

Figure 2. Strategies used to modulate lanthanide sensitization. a) binding of an analyte displaces quenching water molecules increasing the emission; b) binding to the antenna yields an emissive complex; c) conformational or chemical changes in the probe increase the binding affinity for the lanthanide ion generating the emissive state of the probe; d) conformational changes reduce the distance between the antenna and the ion increasing the luminescence intensity.

Figure 3. Top, Structures of common lanthanide chelators; Bottom, synthetic strategies to access peptide-DOTA conjugates: a) Preparation of the DOTA *N*-hydroxysulfosuccinimide ester by incubation of DOTA with EDC and HOSSu in solution; the resulting DOTA-OSSu is conjugated through an amine. b) Synthesis of protected DOTA tris-*t*-Bu ester. c) solid-phase modification of a selectively deprotected amine in a peptide chain with DOTA tris-*t*-Bu ester. Cleavage and deprotection, followed by incubation with $TbCl_3$ yields the desired DOTA[Tb(III)]-labeled peptide.

Figure 4. a) Structure of the EF hand calcium-binding loop indicating the natural Tyr residue that is replaced by a Trp in the final sensor, and the metal-chelating side chains, including the key Glu^{12} side chain. The replacement strategy is outlined, showing the regeneration of the chelating ligand upon phosphorylation of the Ser^{12} residue; b) Design of a tyrosine kinase peptide sensor through double mutation: the chelating Glu^{12} residue is replaced by an alanine, while simultaneously the Ala^{11} is replaced by a tyrosine. Phosphorylation of the tyrosine generates a chelating side chain approximately at the same position as the original Glu^{12} carboxylate.

Figure 5. a) Tyr peptide containing a nucleophilic Lys in its sequence is reacted with the isothiocyanate terbium chelate, and the phosphorylated peptide sensor responds to Abl tyrosine kinase activity by increasing its emission. Dephosphorylation with TC PTP recovers the unphosphorylated (and less emissive) peptide; b) Proposed sensing mechanism by selective coordination of **[Eu]** with the external antenna and formation of a luminescent **[Eu]•TNB** complex; c) structure of the macrocyclic DOTAM (amide deriva-

tive of DOTA) and structure of the dinuclear Tb(III) complex used as sensor and sensing mechanism by interaction of the terbium complex with the phosphotyrosine by electrostatic interactions.

Author's Short Biographies

Elena Pazos studied Chemistry at the University of Santiago de Compostela, graduating in 2006. She obtained her PhD in February 2012 under the supervision of Prof. José Luis Mascareñas and Prof. M. Eugenio Vázquez, and stayed as a visiting PhD student at the School of Chemistry of the Trinity College Dublin, with Prof. Thorfinnur Gunnlaugsson (2008), and in 2010 with Prof. Scott Silverman at the University of Illinois at Urbana-Champaign; she is currently working as a *Barrié de la Maza* fellow with Prof. Samuel Stupp at Northwestern University where she is focused on the development of supramolecular peptide nanostructures.

M. Eugenio Vázquez (1973) graduated in Chemistry from the University of Santiago de Compostela in 1996, and obtained his PhD under the supervision of Prof. José Luis Mascareñas, working on synthetic DNA-binding peptides. In 2001 received the "*Human Frontier Science Program*" long-term fellowship and joined Prof. Barbara Imperiali at MIT, where he worked for three years on the development of caged compounds and fluorescent probes. He returned to Santiago as a "*Ramón y Cajal*" researcher in 2004 and obtained his permanent position in 2010. In 2008 he received the RSEQ *Sigma-Aldrich* Young Researchers Award and in 2012 the Lilly Young Researchers Award.