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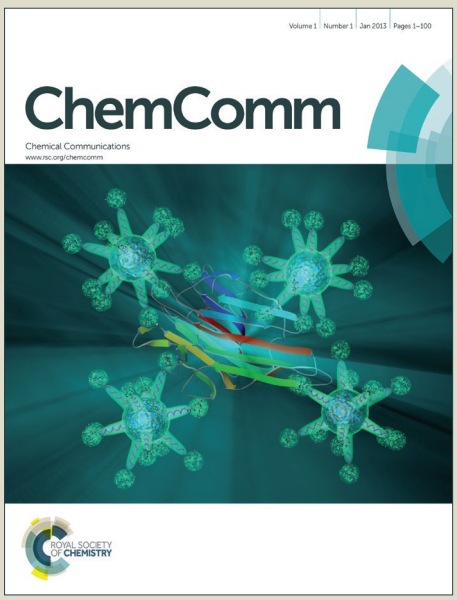

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Selective detection of tyrosine-containing proximally phosphorylated motifs using an antenna-free Tb^{3+} luminescent sensor

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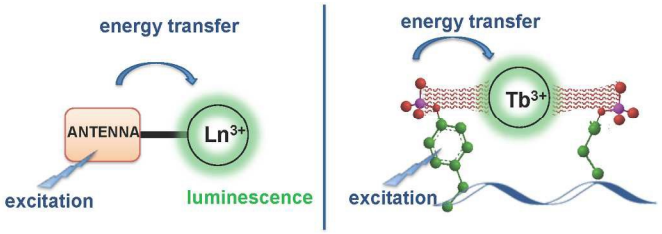
We herein report the first application of Tb^{3+} for the selective detection of an important subset of the phosphoproteome, namely, proximally di-phosphorylated peptide motifs where at least one phosphorylated residue is tyrosine

As a post-translational modification, phosphorylation often controls a proteins' activity status.[1] While some proteins become activated through single residue phosphorylation, others require di- or poly- phosphorylation on neighbouring or proximal residues. While phosphorylation on a tyrosine (Y) residue is less common, a variety of perturbations associated with this specific phospho-site are commonly implicated in the onset and progression of many diseases.[2] For example, proximally di-phosphorylated pY-containing motifs can be found in the activation loops of JAK2 (pYpY)[3] and MAPK protein family members (pTXpY, T = threonine, X = any amino acid),[4] which are known contributors to cancer progression.[2] Similar to JAK2, Syk kinase, whose dysregulated activity is implicated in autoimmune diseases, requires phosphorylation on two neighbouring tyrosine residues for its activation.[5] Analysis of a post-translational modifications database (PhosphoSitePlus®) suggested that over a thousand proximally di-phosphorylated pY-containing motifs are present in the human phospho-proteome (Table S1).[6] Thus, it appears that these motifs have broad impact in intra-cellular signalling, and their detection could be of importance.

Luminescent chemo-sensors incorporating transition metals with vacant coordination sites have been successfully employed for the detection of phosphorylated motifs found on peptides and proteins.[7-9] Using this design, academic and industrial groups (i.e. Hamachi et. al. and Molecular Probes Inc.) have achieved significant progress in the detection of mono-phosphorylated peptide and protein sequences.[10-13] Additionally, sensors have been developed that allow for the selective detection of proximally di-phosphorylated peptide and protein motifs over mono-phosphorylated motifs.[14-17] Although transition metal-based luminescent phospho-protein sensors were demonstrated to be effective and versatile in their applications, they cannot be readily optimized to recognize among the residues that are phosphorylated, i.e. pY versus pS or pT. On the other hand, while the use of lanthanide-based sensors for the detection of phosphopeptides is less explored, it has been shown that Tb^{3+}-based sensors are capable of the selective detection of pY-containing mono-phosphorylated peptides.[18-23]

The signal selectivity of Tb^{3+} for pY arises from the luminescence sensitization of the Tb^{3+} ion (Scheme 1). As a Lewis acid, Tb^{3+} can bind to negatively charged phosphate groups found on pY, pS and pT residues of phosphopeptides. Upon binding to pS- and pT-containing peptides, no significant luminescence enhancement occurs due to the low absorption coefficient of Tb^{3+}, a consequence of orbitally forbidden intra-configurational 4f-4f transitions.[24] On the other hand, the phenyl ring of the bound pY can efficiently transfer its excitation energy to the Tb^{3+} centre inducing "luminescence sensitization" of Tb^{3+}. This leads to an enhanced, long-lived, luminescence at well-defined wavelengths.[20]

Building upon this work and considering the importance of pY signalling pathways, we sought to determine if Tb^{3+} luminescence could be applied for the detection of proximally di-phosphorylated peptides containing at least one pY residue. This could be achieved through simultaneous association of a single Tb^{3+} ion with phosphate groups found on neighbouring residues, which, we rationalized, could lead to a more stable complex as compared to that of a mono-phosphorylated site (Scheme 1). An analogous, neighbouring group-assisted Tb^{3+} binding effect was reported by Sames *et. al.* in application to a synthetic peptide-sensor.[25]



Scheme 1. A schematic representation of the sensor's proposed mode of action

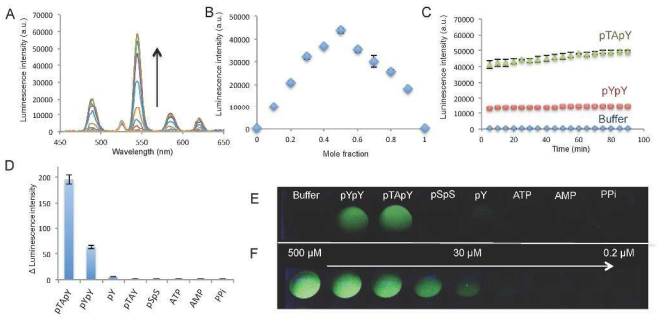


Fig. 1. (A) luminescence intensity spectra of 50 µM $TbCl_3$ titrated with 500-0.244 µM pTApY peptide; (B) Job plot of $TbCl_3$ with pTApY peptide (C) luminescence intensity of $TbCl_3$ (50 µM) in the presence of 50 µM peptides as a function of time (D) change in luminescence intensity of 100 µM $TbCl_3$ in the presence of various analytes (100 µM); (E) luminescence image of 500 µM $TbCl_3$ in the presence of various analytes (500 µM); (F) luminescence image of 500 µM $TbCl_3$ titrated with 500-0.2 µM of pTApY peptide. All experiments were performed in 50 mM HEPES, 50mM NaCl, pH 7.5. All luminescence spectra were acquired using time-resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN M1000

To determine whether Tb^{3+} is capable of detecting pY-containing proximally di-phosphorylated peptides, we measured the change in Tb^{3+} luminescence in response to target peptides (Ac-ApYpYAA-NH_2 or Ac-ApTApYA-NH_2) using time-resolved settings. Increasing concentrations of peptides (500-0.244 µM) were incubated with 50 µM of $TbCl_3$ (50 mM HEPES, 50 mM NaCl, pH 7.5) and the resultant luminescence intensity (450-650 nm) was measured upon irradiation at 263 nm. As expected, enhancement in luminescence at Tb^{3+}-specific wavelengths[21] was directly correlated with the concentration of target peptides in solution (Fig. 1A, Fig. S1). The binding of Tb^{3+} to pTApY peptide was shown to be 1:1 as demonstrated by Job Plot analysis (Fig. 1B), suggesting that the observed signal enhancement might require association of Tb^{3+} with both phosphate groups simultaneously. This sensing system was shown to be stable to photobleaching, a known advantage of lanthanide luminescence (Fig 1C).

To confirm that the observed Tb^{3+} signal was selective for the target pY-containing proximally di-phosphorylated motifs, a screen against variably phosphorylated short peptides was performed under time resolved settings (Fig. 1D). As hypothesized, Tb^{3+} displayed significant signal selectivity for a peptide proximally di-phosphorylated on Y residues over its mono-phosphorylated counterpart of identical primary amino acid sequence (Ac-AYYAA-NH_2).

Strong signal enhancement in response to pTApY suggested that of the two neighbouring phosphorylated residues, only one is required to bear an antenna. However, as the pTAY peptide did not induce significant signal enhancement, it suggested that direct phosphorylation on the Y antenna may be required. Additionally, a peptide proximally di-phosphorylated on serine residues (pSpS; lacking an antenna) did not induce significant Tb^{3+} luminescence enhancement. Although the signal was not observed, Tb^{3+} was expected to bind the pSpS phosphorylated site. Thus, one would expect that the signal from the target peptide, pYpY/pTApY, would be reduced in the presence of pSpS due to the decrease in the amount of Tb^{3+} available for binding the target. This was confirmed by performing a competition experiment (Fig. S4). However, the signal for the target peptide was readily recovered by the addition of excess Tb^{3+} (Fig. S4). Successful detection of the target motif in the presence of an off-target species competing for Tb^{3+} binding was encouraging, given the higher relative abundance of phosphorylation on S and T residues.[6] Consistent with previous studies[,18,19,21] we observed no Tb^{3+} luminescence enhancement in response to some biologically relevant phospho-anions including ATP, AMP, and PPi.

To assess if other detection techniques are compatible with this sensory approach, we repeated the screens described above (Fig. 1A and D) using a short-wave UV-lamp. As expected, the selectivity trend among the analytes tested was comparable for both detection techniques (Fig. 1E). Although the visible detection limit for target peptides obtained using a UV-lamp was inferior (30 µM, Fig. 1F) to that of a microplate reader (4 µM, Fig. S3), the compatibility of this system with a conventional UV-lamp makes this approach accessible to a majority of laboratories.

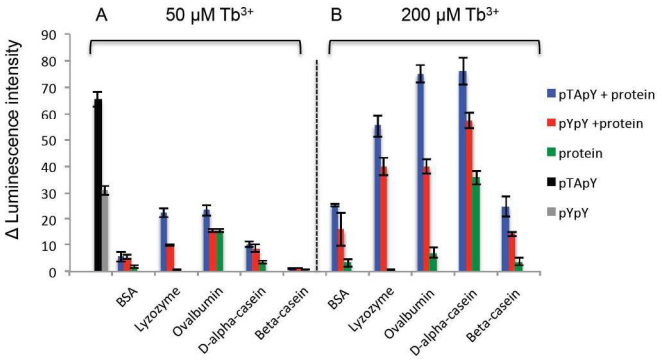


Fig. 2 (A) Change in luminescence intensity of $TbCl_3$ (50 µM) in the presence and absence of different analyte mixtures (40 µM peptide and 40 µM protein); (B) Change in luminescence intensity of $TbCl_3$ (200 µM) in the presence and absence of different analyte mixtures (40 µM peptide and 40 µM protein). All luminescence spectra were acquired using time-resolved settings (60 µs delay time and 1.5 ms integration time) on a TECAN M1000

The results of the peptide-based competition experiment described above, demonstrated that sensing of the target motifs could be retained in the presence of off-target peptides, which compete for binding to Tb^{3+} (Fig. S4). Next, to assess if the sensing of target motifs could be retained in the presence of full size proteins, Tb^{3+} (50 µM) was added to a mixture of 40 µM of target peptides (pTApY and pYpY) incubated with or without 40 µM of a full size

protein (BSA, lysozyme, ovalbumin, D-α-casein, or β-casein). Analogous to the peptide competition experiment, the detection signal decreased in the presence of a competing protein (Fig. 2A). This was expected as Tb^{3+} likely interacts with the proteins' surface, particularly with acidic residues. Increasing the concentration of Tb^{3+} to 200 μM was shown to compensate for the decrease in the effective concentration (Fig. 2B). As can be seen from Fig. 2B, the increase in Tb^{3+} concentration, in most cases, led to a significant recovery in the detection signal. Interestingly, D-α-casein induced significant Tb^{3+} luminescence in the absence of target peptides. This could be a result of Tb^{3+} binding to a site on the protein proximal to an antenna, inducing its luminescence sensitization. The observed off-target signal is not likely a result of Tb^{3+} binding to either of the two phosphorylated serine residues on D-α-casein,[33] since the signal induced by the ovalbumin protein, which also bears two phosphorylated serine residues,[6] was minimal. Notably, the recovery of signal towards target peptide was not as efficient in the presence of β-casein and BSA as compared to other proteins tested (Fig. 2B). This is likely due to the presence of five phosphorylated serine residues on β-casein[33] and a metal binding site potentially present on BSA.[26] On the other hand, incubating Tb^{3+} with the highly positively charged non-phosphorylated protein lysozyme alone, which was not expected to significantly interact with Tb^{3+}, did not induce luminescence enhancement. Consistent with this observation and among the proteins tested, lysozyme interfered the least with the Tb^{3+} luminescence originating from interaction with the target peptides (Fig. 2A and B). Therefore we have shown that at appropriate concentrations of Tb^{3+}, target peptides can be detected in the presence of full size proteins.

Conclusions

In conclusion, we have demonstrated significant enhancement in Tb^{3+} luminescence in the presence of proximally di-phosphorylated peptide motifs, where at least one of the phosphorylated residues is tyrosine. This facile approach towards the detection of an important subset of phosphorylated motifs warrants further exploration and could be of potential value to the phosphopeptide/phosphoprotein detection field.

Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x

1. T. Hunter, Cell, 1995, 80, 225–236.
2. P. Cohen, Nat Rev Drug Discov, 2002, 1, 309–315.
3. I. S. Lucet, E. Fantino, M. Styles, R. Bamert, O. Patel, S. E. Broughton, M. Walter, C. J. Burns, H. Treutlein, A. F. Wilks, and J. Rossjohn, Blood, 2006, 107, 176–183.
4. N. Dephoure, C. Zhou, J. Villén, S. A. Beausoleil, C. E. Bakalarski, S. J. Elledge, and S. P. Gygi, Proc. Natl. Acad. Sci. U.S.A., 2008, 105, 10762–10767.
5. J. Zhang, M. L. Billingsley, R. L. Kincaid, and R. P. Siraganian, J. Biol. Chem., 2000, 275, 35442–35447.
6. P. V. Hornbeck, J. M. Kornhauser, S. Tkachev, B. Zhang, E. Skrzypek, B. Murray, V. Latham, and M. Sullivan, Nucleic Acids Res., 2012, 40, D261–70.
7. T. Sakamoto, A. Ojida, and I. Hamachi, Chem. Commun., 2009, 141–152.
8. A. Ojida and I. Hamachi, Bull. Chem. Soc. Jpn., 2006, 79, 35–46.
9. H. T. Ngo, X. Liu, and K. A. Jolliffe, Chem Soc Rev, 2012, 41, 4928–4965.
10. A. Ojida, Y. Mito-oka, and M. Inoue, J. Am. Chem. Soc., 2002, 124, 6256–6258.
11. A. Ojida, Y. Mito-Oka, K. Sada, and I. Hamachi, J. Am. Chem. Soc., 2004, 126, 2454–2463.
12. A. Ojida, T. Kohira, and I. Hamachi, Chem. Lett., 2004, 33, 1024–1025.
13. T. H. Steinberg, B. J. Agnew, K. R. Gee, W.-Y. Leung, T. Goodman, B. Schulenberg, J. Hendrickson, J. M. Beechem, R. P. Haugland, and W. F. Patton, Proteomics, 2003, 3, 1128–1144.
14. A. Ojida, M.-A. Inoue, Y. Mito-Oka, and I. Hamachi, J. Am. Chem. Soc., 2003, 125, 10184–10185.
15. A. Ojida, T. Sakamoto, M.-A. Inoue, S.-H. Fujishima, G. Lippens, and I. Hamachi, J. Am. Chem. Soc., 2009, 131, 6543–6548.
16. Y. Ishida, M.-A. Inoue, T. Inoue, A. Ojida, and I. Hamachi, Chem. Commun., 2009, 0, 2848–2850.
17. D. Kraskouskaya, M. Bancerz, H. S. Soor, J. E. Gardiner, and P. T. Gunning, J. Am. Chem. Soc., 2014, 136, 1234–1237.
18. H. Akiba, J. Sumaoka, and M. Komiyama, ChemBioChem, 2009, 10, 1773–1776.
19. H. Akiba, J. Sumaoka, and M. Komiyama, Chem. Eur. J., 2010, 16, 5018–5025.
20. S. C. Zondlo, F. Gao, and N. J. Zondlo, J. Am. Chem. Soc., 2010, 132, 5619–5621.
21. D. P. Ringer, J. L. Etheredge, B. L. Dalrymple, and J. S. Niedbalski, Biochemical and Biophysical Research Communications, 1990, 168, 267–273.
22. A. M. Lipchik and L. L. Parker, Analytical chemistry, 2013, 85, 2582–2588.
23. E. Pazos and M. E. Vázquez, Biotechnology Journal, 2014, 9, 241–252.
24. J.-C. G. Bünzli, Chem. Rev., 2010, 110, 2729–2755.
25. M. S. Tremblay, Q. Zhu, A. A. Martí, J. Dyer, M. Halim, S. Jockusch, N. J. Turro, and D. Sames, *Org. Lett.*, 2006, **8**, 2723–2726.
26. UniProt Consortium, Nucleic Acids Res., 2014, 42, D191–8.