



Lanthanide-based peptide biosensor to monitor CDK4/cyclin D kinase activity[†]

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We describe a lanthanide biosensor that responds to CDK4 kinase activity in melanoma cell extracts through a significant and dose dependent increase in luminescence, thanks to sensitization of a DOTA[Tb³⁺] complex incorporated into a CDK4 substrate peptide by a unique tryptophan residue in an adjacent phosphoaminoacid binding moiety.

Phosphorylation triggered by extracellular signals and carried out by protein kinases is a fundamental signal transduction mechanism involved in the regulation of most basic processes in eukaryotic cells.¹ Consequently, chemical tools that perturb the function of protein kinases and fluorescent probes that enable imaging of their spatio-temporal dynamics offer means of dissecting their behaviour and unravelling the complexity of signalling cascades in which they are involved. Fluorescent reporters of protein kinases constitute potent tools for probing changes in the subcellular localization and activation of these enzymes, thereby allowing to re-examine and address questions relative to their biochemical function and regulation in complex biological samples and in living cells in a sensitive and non-invasive fashion.² Accordingly, a number of fluorescent peptide biosensors for monitoring kinase activity, based on environment-sensitive³⁻⁶ or chelation-enhanced⁷⁻⁹ fluorophores, have been described. More recently, lanthanides have also been applied to the design of kinase biosensors, often taking advantage of the chelating effect of the phosphate anion to induce the formation of luminescent lanthanide complexes.¹⁰⁻¹⁶ Indeed lanthanide-based probes are particularly attractive as their luminescence is significantly longer than the nanosecond lifetimes of organic fluorophores. Moreover their

use in biological applications allows to circumvent issues associated with organic dyes relative to background fluorescence of living cells.¹⁷

CDK4/cyclin D is a heterodimeric member of the cyclin-dependent kinase family involved in regulation of cell cycle progression at the G1/S transition through phosphorylation of the Retinoblastoma tumor suppressor protein (Rb).¹⁸ This kinase is frequently hyperactivated in several human cancers, and in particular in melanoma, due to overexpression or mutation of CDK4, genetic amplification of cyclin D, methylation or loss of function of p16^{INK4a}.¹⁹⁻²² Recently, our group developed an environmentally-sensitive fluorescent peptide biosensor to probe CDK4/Cyclin D activity, which was further implemented to quantify kinase activity in skin biopsies and melanoma xenografts.²³ Herein we describe a luminescent, terbium-based peptide biosensor that reports on CDK4 activity in melanoma cell extracts with much greater sensitivity than the previously reported TAMRA-labelled biosensor. Tb-CDKACT4-OH biosensor is a bipartite peptide derived from the backbone of the first CDKACT4 biosensor, comprising a CDK4-specific substrate sequence derived from Rb and a phosphoamino acid binding domain (PAABD) derived from the interface of the WW domain of Pin1 with a phosphopeptide.²³ To generate the Tb-based biosensor, we took advantage of the unique Trp residue in the PAABD and further incorporated a terbium complex in the substrate moiety, proximal to the CDK4-specific phosphorylation site, thereby generating a luminescent transduction unit which would emit luminescence when brought into spatial proximity with the Trp antenna, following phosphorylation of the substrate moiety and its consequent recognition by the PAABD (Figure 1A).

Peptide biosensor variants harbouring either the native CDK4-specific substrate sequence, or the corresponding synthetic phosphopeptide were prepared by Fmoc-based solid-phase peptide synthesis. The Tb³⁺ complex was introduced into the peptide sequence as DOTA-Tb³⁺ maleimide (chelating macrocycle DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) through a Cys residue. Peptide identities and

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purities were confirmed by electrospray mass spectrometry and high-performance liquid chromatography (HPLC) analysis. Detailed synthetic procedures are described in ESI[†].

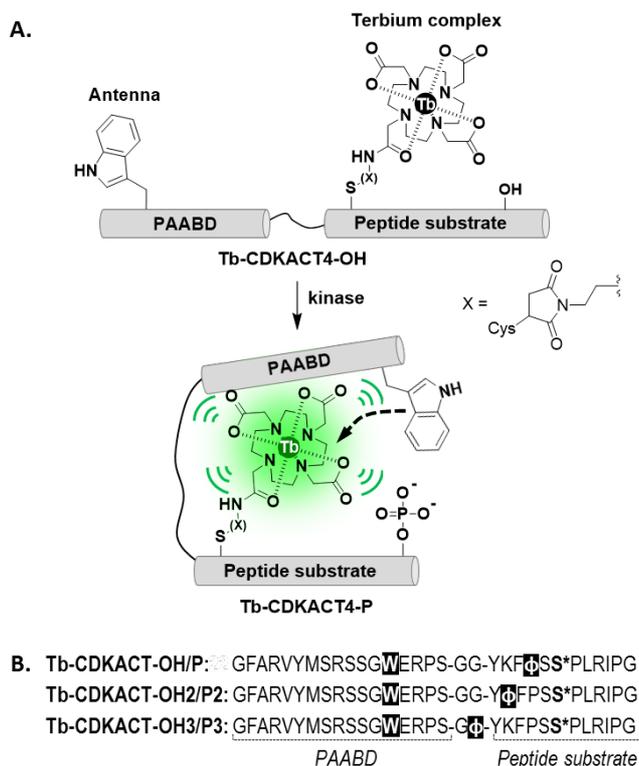


Fig. 1 A) Schematic representation of the biosensor. Upon phosphorylation, the phosphopeptide should bind to the recognition domain (PAABD), leading to a conformational change that should bring together the sensitizing antenna and the terbium complex, resulting in an increase in the luminescence emission. B) The peptide sequence of the designed biosensors, where the phosphorylatable residue is bold and ϕ = Cys(DOTA-Tb³⁺).

To determine the optimal position of the lanthanide complex within the substrate sequence relative to the Trp antenna in the PAABD moiety, a small peptide library was prepared, in which the chelating DOTA unit was positioned at various sites (-2, -4, and -6 positions) relative to the phosphorylatable serine residue (Figure 1B). In order to validate the functionality of these biosensor peptides and to assess their dynamic range of response, we first determined the increase in luminescence intensity between the phosphorylated and the corresponding unphosphorylated variants. We found that the greatest response was observed when the lanthanide complex was incorporated at position -2 (peptides Tb-CDKACT-OH and Tb-CDKACT-P; Figure S1). Indeed, upon excitation at 280 nm, the synthetic phosphate-containing biosensor Tb-CDKACT-P, showed a significant increase (3.2-fold) in the typical luminescence emission bands of the terbium ion, at 488, 544, 585, and 620 nm (Figure 2A). However, when the unique Trp residue in the PAABD moiety was replaced by Ala [peptides Tb-CDKACT-OH(-W) and Tb-CDKACT-P(-W)], no increase in luminescence was observed, demonstrating that the tryptophan antenna is indeed required for sensitization (Figure 2B). Furthermore, when an independent PAABD peptide was

incubated with the DOTA-Tb³⁺ labelled Rb phosphopeptide (Tb-Rb-P), no terbium luminescence was observed, indicating that an intramolecular link was required for proper spatial positioning between the tryptophan residue and the terbium complex (Figure S2). Moreover, we found that the luminescent response of Tb-CDKACT4-P relative to Tb-CDKACT4-OH increased with the temperature of the reaction (between 25 °C and 37 °C), indicating that the response involving recognition of the phospho-substrate by the PAABD is a dynamic process that relies on kinetic energy (Figure S3).

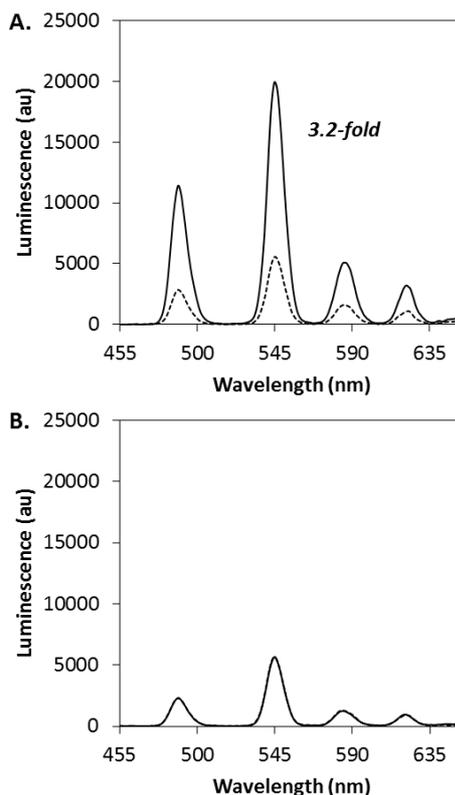


Fig. 2 A) Time-resolved luminescence emission ($\lambda_{\text{exc}} = 280$ nm, 0.4 ms delay time) of a 5 μM solution of the phosphopeptide Tb-CDKACT4-P (solid line) and the corresponding unphosphorylated biosensor Tb-CDKACT4-OH (dashed line) at 30 °C, in 50 mM HEPES buffer, pH 7.5, 100 mM NaCl. B) Time-resolved luminescence emission ($\lambda_{\text{exc}} = 280$ nm, 0.4 ms delay time) of a 5 μM solution of the phosphopeptide Tb-CDKACT4-P(-W) (solid line) and the corresponding unphosphorylated biosensor Tb-CDKACT4-OH(-W) (dashed line) at 30 °C, in 50 mM HEPES buffer, pH 7.5, 100 mM NaCl.

When further assessing the best working conditions for biosensor application, we observed that overall luminescent signal of Tb-CDKACT-OH and Tb-CDKACT-P increased with the concentration of the peptide. However, the dynamic range of the reporter (ratio between Tb-CDKACT4-P and Tb-CDKACT4-OH) remained within the same average value of 3.2-3.7-fold, indicating that the sensitivity of response was independent of biosensor concentration (Figure 3A). Furthermore, when the same peptides (Tb-CDKACT-OH and CDKACT-P) were labelled with a variety of thiol reactive derivative fluorophores (4-DMN, TP-2Rho, Bodipy, coumarine, oxazine, Cy3, Cy5, TAMRA, FITC and merocyanine 53), none of them responded to the same extent as the peptides labelled with the terbium complex,

emphasizing the much greater sensitivity of the lanthanide probe in this biosensor design (Figure 3B).

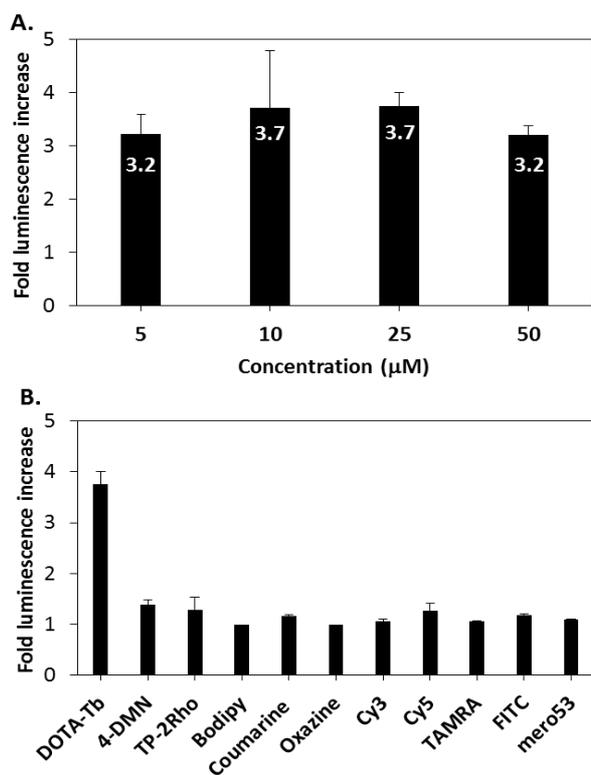


Fig. 3 A) Luminescence difference at 545 nm between Tb-CDKACT4-P and Tb-CDKACT4-OH, at increasing concentrations (5, 10, 25 and 50 μM) of a solution of the corresponding peptide in 50 mM HEPES buffer, pH 7.5, 100 mM NaCl ($\lambda_{\text{ex}} = 280$ nm). B) Comparison of the luminescence difference between CDKACT4-P and CDKACT4-OH, labelled with either diverse fluorophores or the DOTA-Tb³⁺ complex.

We therefore further implemented the Tb-CDKACT4-OH peptide biosensor to probe CDK4/Cyclin D kinase activity in A375 melanoma cell extracts. Incubation of 25 μM biosensor with 40 μg of cell extracts resulted in a robust 3-fold increase in luminescence (Figure 4A), corresponding to the maximal increase in luminescence observed between Tb-CDKACT4-OH and its synthetic phospho-counterpart (Figure 3A), highlighting the robustness of the biosensor in a complex environment, i.e. cell extracts. Moreover, Tb-CDKACT4-OH responded to A375 melanoma cell extracts in a dose-dependent fashion, between 5 and 100 μg, thereby indicating its utility and potential for quantification of kinase activities within this range (Figure 4B). In contrast, addition of BSA (40–200 μg), did not have any significant impact on the luminescence of the biosensor, indicating that Tb-CDKACT4 does not simply respond to the presence of unrelated proteins (Figure 4B). The specificity of response to CDK4 activity was further confirmed by incubation with melanoma cell extracts (40 μg) pretreated with different concentrations of the CDK4-specific inhibitor Abemaciclib (LY2835219), or the pan-CDK inhibitor Roscovitine, which inhibits several CDKs (CDK2, CDK1, CDK5, CDK7, CDK9), but not CDK4/CDK6. Whilst the luminescent signal emitted by the biosensor upon incubation with A375 cell extracts remained

unperturbed when incubated with extracts from Roscovitine-treated cells, it was reduced in a dose-dependent fashion upon incubation with extracts from Abemaciclib-treated cells (Figure 4C). Moreover, the concentration-dependent luminescent response reported by Tb-CDKACT biosensor mirrored the inhibition profile of A375 cell proliferation (Figure 4D), revealing that 50% inhibition of both cell proliferation and CDK4 activity were achieved at a concentration of 1 μM Abemaciclib in this cell line. In contrast, neither Abemaciclib, nor Roscovitine had any significant effect on the luminescence of Tb-CDKACT4-OH peptide biosensor (Figure S4).

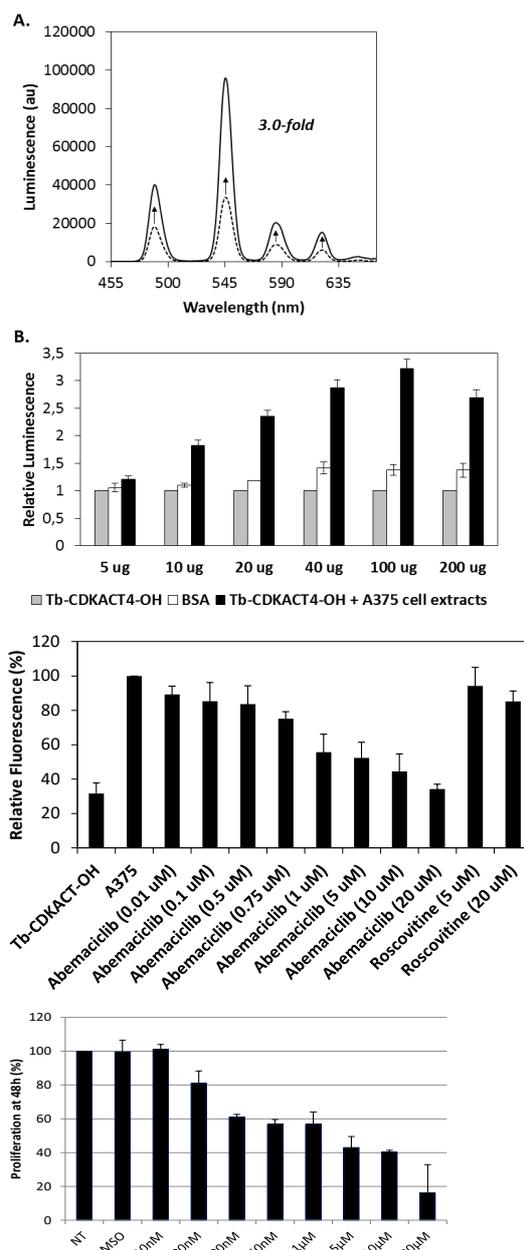


Fig. 4 A) Time-resolved luminescence emission (0.4 ms delay time) of a 25 μM solution of the Tb-CDKACT4-OH biosensor at 30 °C, in 50 mM HEPES buffer, pH 7.5, 100 mM NaCl (dashed line), and the same peptide in the presence of 40 μg of A375 melanoma cell extracts (solid line). B) Tb-CDKACT4-OH peptide biosensor responds to A375 melanoma cell extracts in a dose-dependent fashion, but not upon addition of BSA. C) Tb-CDKACT4-OH biosensor responds to inhibition of CDK4/Cyclin D activity in A375 melanoma cell extracts by Abemaciclib and Roscovitine. D) the corresponding inhibition of A375 cell proliferation by Abemaciclib

In summary, we have engineered the first lanthanide-based peptide biosensor of CDK4 and have demonstrated that its luminescence intensity is significantly and specifically enhanced upon phosphorylation by this kinase in melanoma cell extracts. Our biosensor displays remarkable sensitivity owing to its original design, which involves the intramolecular sensitization of a luminescent DOTA[Tb³⁺] macrocyclic complex by a sensitizing tryptophan antenna within the PAABD upon phosphorylation of the substrate moiety by CDK4. The luminescent properties of this biosensor make it a performant tool for probing kinase activities in cell extracts, thereby providing major improvement over assays which report on the kinase activity of purified recombinant enzymes, since cell extracts constitute a complex yet more physiological environment. We have further demonstrated that this biosensor mirrors the response of living cells to inhibition by kinase-specific inhibitor, thereby highlighting the attractiveness of this technology for drug discovery purposes.

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Notes and references

- 1 T. Hunter, *Cell*, 2000, **100**, 113-127.
- 2 J. A. González-Vera, *Chem. Soc. Rev.*, 2012, **41**, 1652-1664.
- 3 R. Agnes, F. Jernigan, J. Shell, V. Sharma and D. S. Lawrence, *J. Am. Chem. Soc.*, 2010, **132**, 6075-6080.
- 4 Q. Wang and D. S. Lawrence, *J. Am. Chem. Soc.*, 2005, **127**, 7684-7685.
- 5 Q. Wang, S. M. Cahill, M. Blumenstein and D. S. Lawrence, *J. Am. Chem. Soc.*, 2006, **128**, 1808-1809.
- 6 V. Sharma, R. Agnes and D. S. Lawrence, *J. Am. Chem. Soc.*, 2007, **129**, 2742-2743.
- 7 M. Shults and B. Imperiali, *J. Am. Chem. Soc.*, 2003, **125**, 14248-14249.
- 8 M. Shults, K. Janes, D. Lauffenburger and B. Imperiali, *Nat. Methods*, 2005, **2**, 277-284.
- 9 E. Luković, J. A. González-Vera and B. Imperiali, *J. Am. Chem. Soc.*, 2008, **130**, 12821-12827.
- 10 M. Tremblay, M. Lee and D. Sames, *Org. Lett.*, 2008, **10**, 5-8.
- 11 M. Tremblay, Q. Zhu, A. Martí, J. Dyer, M. Halim, S. Jockusch, N. Turro and D. Sames, *Org. Lett.*, 2006, **8**, 2723-2726.
- 12 A. M. Lipchik, M. Perez, S. Bolton, V. Dumrongprechachan, S. B. Ouellette, W. Cui and L. L. Parker, *J. Am. Chem. Soc.*, 2015, **137**, 2484-2494.
- 13 W. Cui and L. L. Parker, *Sci. Rep.*, 2016, **6**, 28971.
- 14 W. Cui and L. L. Parker, *Chem. Commun.*, 2014, **51**, 362-365.
- 15 N. P. Damayanti, L. L. Parker and J. M. K. Irudayaraj, *Angew. Chem. Int. Ed.*, 2013, **52**, 3931-3934.
- 16 E. Pazos and M. E. Vázquez, *Biotechnol. J.*, 2014, **9**, 241-252.
- 17 M. C. Heffern, L. M. Matosziuk and T. J. Meade, *Chem. Rev.*, 2014, **114**, 4496-4539.
- 18 R. H. Grafstrom, W. Pan and R. H. Hoess, *Carcinogenesis*, 1999, **20**, 193-198.
- 19 K. E. Sheppard and G. A. McArthur, *Clin. Cancer Res.*, 2013, **19**, 5320-5328.
- 20 D. M. Miller and K. T. Flaherty, *Pigment Cell Melanoma Res.*, 2014, **27**, 351-365.
- 21 T. Mahgoub, A. J. Eustace, D. M. Collins, N. Walsh, N. O'Donovan and J. Crown, *Int. J. Oncol.*, 2015, **47**, 900-908.
- 22 R. Sotillo, J. F. García, S. Ortega, J. Martín, P. Dubus, M. Barbacid and M. Malumbres, *Proc. Natl. Acad. Sci.*, 2001, **98**, 13312-13317.
- 23 C. Prével, M. Pellerano, J. A. González-Vera, P. Henri, L. Meunier, J. Voltaire, V. Jossierand and M. C. Morris, *Biosens. Bioelectron.*, 2016, **85**, 371-380.