A new approach in protein phosphorylation for Nanofitin radiolabeling

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Introduction

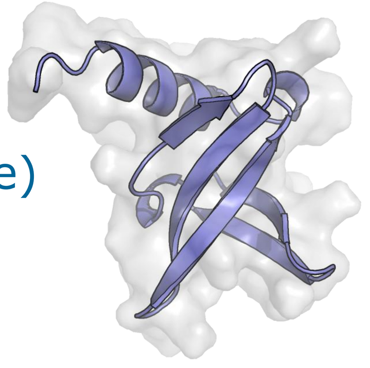
Recently, new strategies emerged in the field of monoclonal antibodies **radiolabeling** for PET imaging with the use of positron emitter such as **zirconium-89** or **gallium-68**[1]. Despite their important role in the therapeutic world, antibodies have many disadvantages related to their structure. Moreover, conjugation of chelating agent often occurs on lysines, which is non-regioselective and leads to a heterogeneous mixture of products. In addition, the slow clearance of antibodies can be a problem to obtain a good contrast when they are used in imaging.

To address these different limitations, we developed a **chemistry-free chelating system** consisting of a **highly phosphorylatable peptide tag**. A specific phosphorylation step, with the alpha subunit of the casein kinase II (CKIIα), can generate a nanocluster of four phosphates that **can interact strongly with metal ions** like zirconium[2].

Objectives:

1) Adapt the labeling tag to the stereoselective chelation of radionuclides for PET imaging.
2) Genetically fuse the tag to a Nanofitin, a protein scaffold developed as an alternative to antibodies, to ensure an efficient targeting of the radionuclide.

What are Nanofitins ?



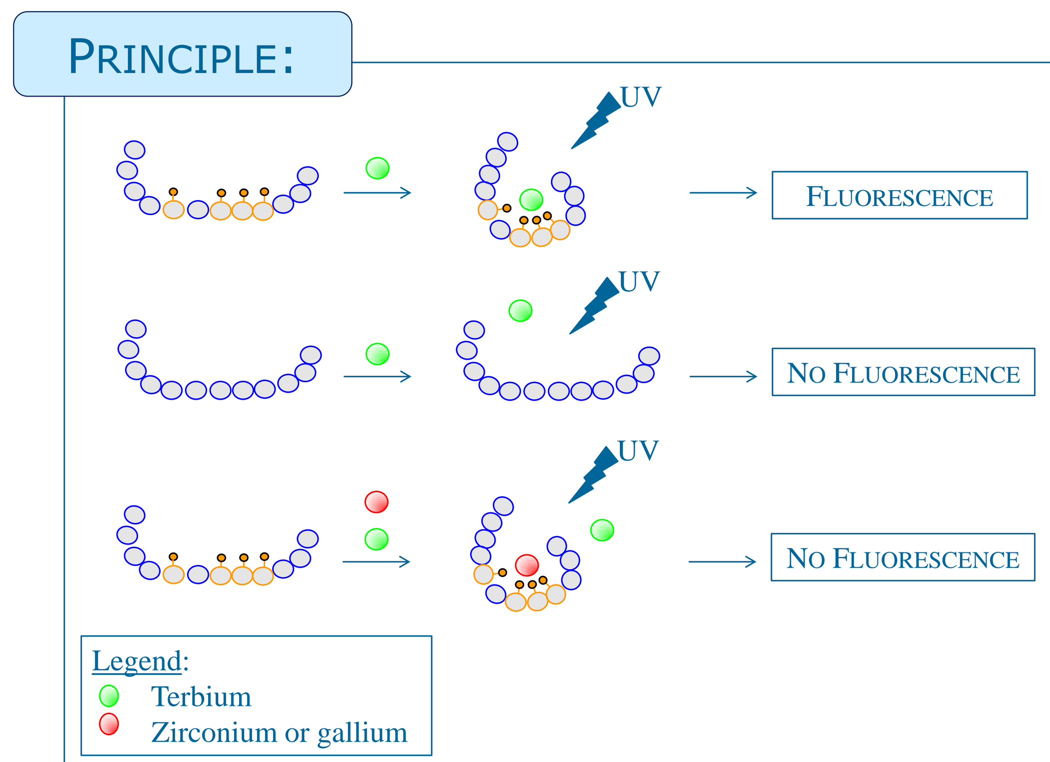
Small Protein:	10kDa (rapid clearance)
pH stability:	0-12
Temperature Stability:	Tm≈80°C
Production cost:	Low (Generated *in vitro* and produced in bacteria)
Affinity:	0,1nM

Results

Chelation with radionuclides

To optimize the sequence of the phosphorylatable tag, we studied the chelation of different mimetic peptides with a lanthanide (terbium).

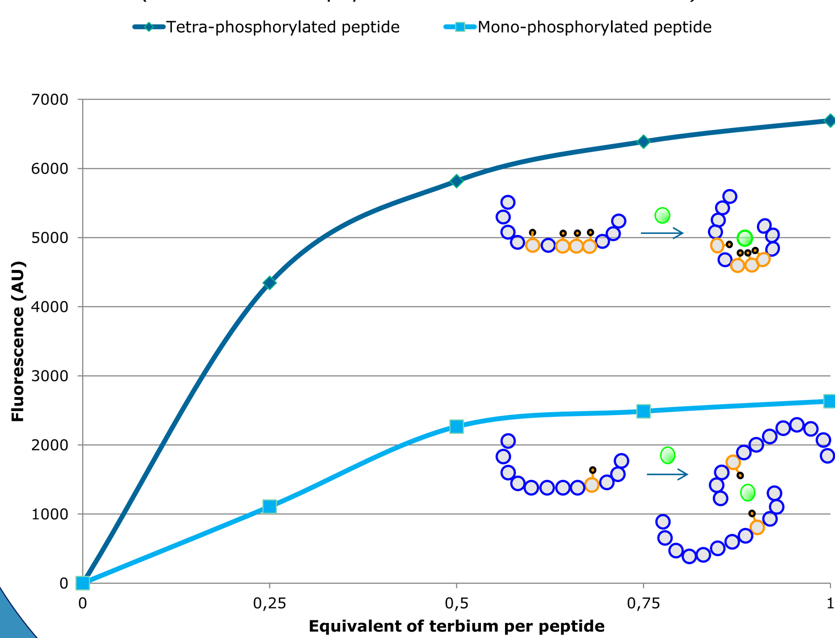
Principle:



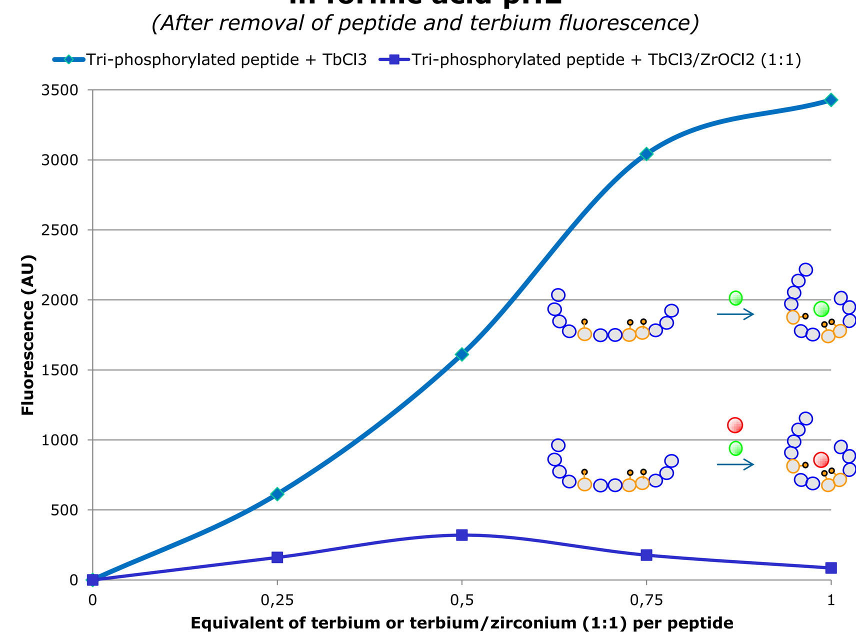
• Fluorescence increases as the peptide is phosphorylated.

• Chelation of the zirconium by the peptide tag was confirmed by a competition study.

Terbium(III) titration of mono- and tetra-phosphorylated peptides in formic acid pH2
(After removal of peptides and terbium fluorescence)

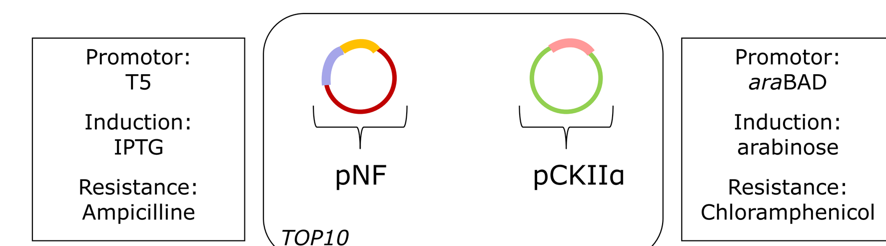


Terbium(III) titration of tri-phosphorylated peptide and competition with zirconium(IV) in formic acid pH2
(After removal of peptide and terbium fluorescence)



Development of an *in vivo* co-expression and phosphorylation system

In early development, the phosphorylation was realized *in vitro*. In order to save purification steps, we developed an ***in vivo* phosphorylation system** consisting in the co-expression of a Nanofitin® fused to the phosphorylatable tag (pNF) and the CKIIα.



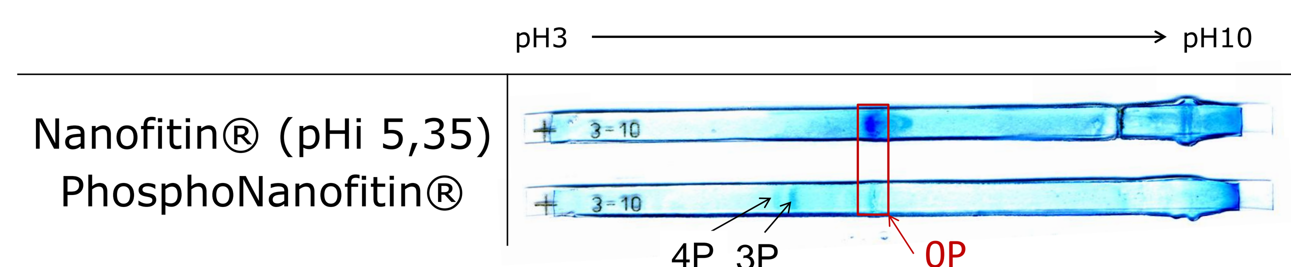
• Effectiveness of the *in vivo* system was demonstrated both by **electrophoretic mobility shift assay** and staining with a **specific phosphoprotein staining gel**: ProQDiamond®.

• Extra addition of ATP after lysis **improve significantly the rate of phosphorylation**.

Arabinose 0.2% / IPTG 0.5mM	- / +	+ / +	T-	T+
Coomassie blue staining				
ProQDiamond® staining				

Arabinose 0.2% / IPTG 0.5mM / ATP 5mM after lysis	+ / + / -	+ / + / +
Coomassie blue staining		
ProQDiamond® staining		

To evaluate the phosphorylation rate we developped an isoelectric focusing method based on the fact that the pHi of the Nanofitin® decreases as the protein is phosphorylated. Such a technique allowed us to isolate the **different phosphorylation states of the phospho-Nanofitin®** (from 0 to 4 phospho-serine).



Conclusion

In parallel to the development of the *in vivo* system, we are developing a phosphoprotein purification protocol by immobilized metal affinity chromatography, relying on the specific interaction of phosphate moeity with gallium(III) or iron(III). The use of a single tag for both the purification and the labeling will provide a much simpler and straightforward system. Finally this fusion system of phosphate nanocluster and a Nanofitin® will allows us to combine the advantages of an original single step regioselective labeling for both zirconium-89 and gallium-68, together with a highly stable targeting moiety. Concerning the chelation experiments, we have planned to realize a fluorescence studies with peptide-gallium complex and phospho-Nanofitin. Depending of those results, we will optimize the sequence of the tag to obtain an high affinity.

References : [1] Heuveling D. A. *et al.* (2011), *Oral Oncology*, 47, pp. 2–7 ; [2] Cinier M. *et al.* (2012), *Journal of Biological Inorganic Chemistry*, 17, pp. 399–407 ; [3] PhosphoProtein Handbook (2011), *QIAGEN®* ; [4] Machida M. *et al.* (2007), *FEBS Journal, 274, pp. 1576–1587*

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