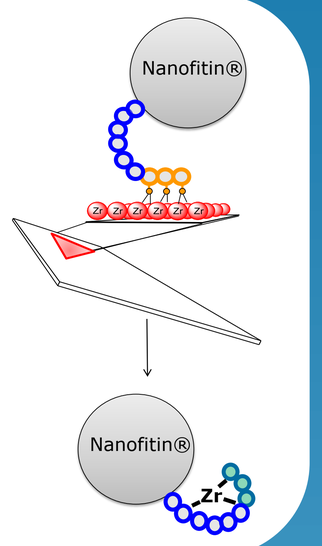
Development of an *in vivo* phosphorylation system in *Escherichia coli*

Goux M.[1,2], Defontaine A.[1], Fateh A.[1], Cinier M.[2] and Tellier C.[1]

[1]UFIP, University of Nantes, France and [2]Affilogic SAS, Nantes, France

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.

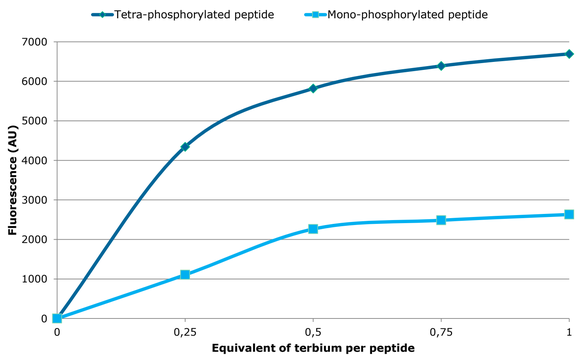
To address these different limitations, we developed a **chemistry-free chelating system** consisting of a **highly phosphorylatable peptide tag**. A specific phosphorylation step, with the alpha subunit of the casein kinase II (CKIIα), generates a nanocluster of four phosphates that **can interact strongly with metal ions** like zirconium. This strategy has already demonstrated its powerfulness for the stable and specific anchoring of protein on zirconium phosphonate-based microarray[2]. We are now adapting the use of this labeling tag to the stereoselective chelation of radionuclides for PET imaging. To ensure an efficient targeting of the radionuclide, the tag was genetically fused to a **Nanofitin®**, a protein scaffold developed as an alternative to antibodies.



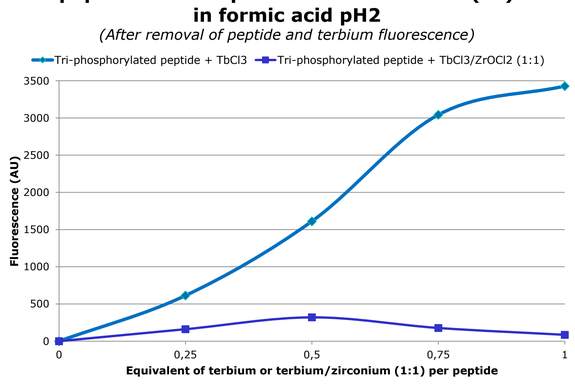
1. Chelation of the peptide tag with terbium(III) and zirconium(IV)

In order to optimize the sequence of the phosphorylatable tag, we studied the chelation of different mimetic peptides with terbium(III). The 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)



➡ Fluorescence increases as the peptide is phosphorylated.

➡ Fluorescence decreases in presence of zirconium.

2. Co-expression system TOP10/pNF-pCKIIα

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

Promotor : T5 Induction : IPTG Resistance : Ampicilline	pNF — pCKIIα *TOP10*	Promotor : *ara*BAD Induction : arabinose Resistance : Chloramphenicol

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

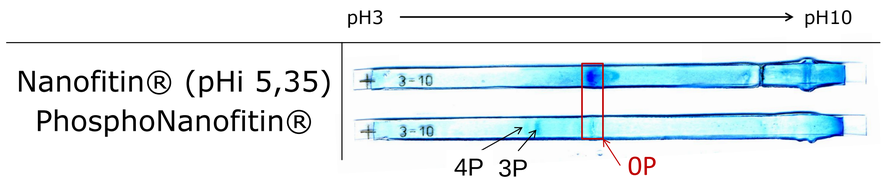
➡ Effectiveness of the *in vivo* system was demonstrated both by **electrophoretic mobility shift assay** and staining with a **specific phosphoprotein staining gel**: ProQDiamond®.

3. Optimization of the phosphorylation

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

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

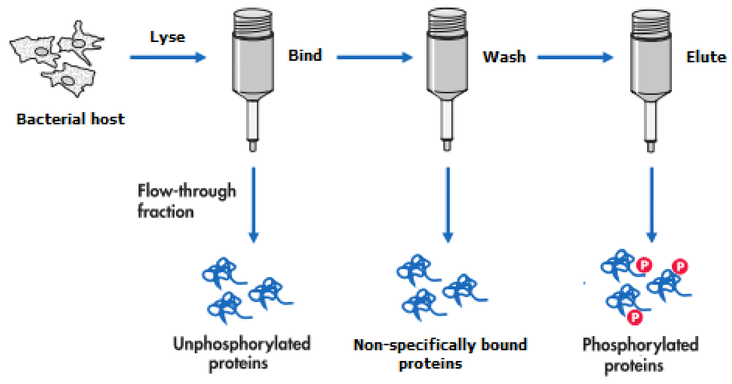
4. Evaluation of the phosphorylation rate by isoelectric focusing



The isoelectric focusing method is 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).

5. Purification of the phosphorylated Nanofitin® by Fe-NTA and Ga-NTA

Using the specific interaction of phosphate moeity with gallium(III) or iron(III), we are currently developing a phosphoprotein purification protocol by immobilized metal affinity chromatography[3,4].



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*

FRE CNRS 3478
Unité Fonctionnalité et Ingénierie des Protéines
2 rue de la Houssinière 44322 Nantes France

AFFILOGIC SAS
2 rue de la Houssinière 44322 Nantes France

Contact : *marine.goux@univ-nantes.fr*

2nd NanoFar Autumn School

October 21-25, 2013

University of Santiago de Compostella, Spain