Selective isolation at the femt mole level of phosphopeptides from proteolytic digests using 2D-nanoLC-ESI-MS/MS and titanium oxide pre-columns

Martijn W. H. Pinkse1, Pauliina M. Uitto1, Martijn J. Hilhorst2, Bert Ooms2 and Albert J. R. Heck1

1 Utrecht University, Utrecht, The Netherlands. 2 Spark Holland BV, Emmen, The Netherlands.

Introduction
Selective detection of phosphopeptides from proteolytic digests is a challenging and highly relevant task in many proteomics applications. Often phosphopeptides are present in small amounts and need selective isolation and/or enrichment before identification. This poster describes a novel automated method for the enrichment of phosphopeptides from complex mixtures using Titanosphere. This relatively new base material for HPLC columns consists of porous titanium oxide (TiO2) microspheres with a smooth and alkaline surface that displays amphoteric ion-exchange properties. A unique characteristic of TiO2 is to effectively absorb organic phosphates in acidic conditions and desorb in alkaline conditions [1]. We have used this new base material in an automated two-dimensional nano-LC-MS3 setup (Figures 1-2), with Titanosphere as the first dimension and reversed-phase material as the second dimension. A unique advantage that lesser column handling steps are required, and therefore it seems to be a more robust enrichment strategy.

Results
Figure 3A shows the BPI chromatograms of a normal reversed phase run of a peptide mixture at 125 fmol RKIpSASEF and around 2 minutes both peptides elute with similar mass spectrometric responses. Figure 3B shows the first reversed phase run, using the double TiO2/C18 pre-column. This time only one peak is observed, which is the non-phosphorylated peptide. Elution of the TiO2 pre-column at high pH and a second gradient successfully recovered the phosphopeptide (Figure 3C).

PKG phosphorylation.
PKG autophosphorylates in the presence of Mg2+-ATP and cAMP or cGMP [3]. In order to identify autophosphorylation sites proteolytic digest of non-, partially, and highly autophosphorylated PKG were analyzed using the developed method (Figure 4). In total 8 sites were identified, including two previously uncharacterized (Table 1). Figure 5 shows MS/MS spectra of three phosphopeptides.

Experimental
2D-LC-MS3 was performed on a modified LC-packings Ultimate nano-HPLC and a Micromass QqTOF. Titanosphere was a gift from GL-Sciences Inc., Tokyo, Japan. Porous ceramic frits, trapping columns and analytical columns were prepared as essentially described by Meirng et al [2].

Conclusions
Figure 6 shows that methylation [4] reduces this affinity as demonstrated for [Glu+] tri- and tetrabromophenol II (EVQNDEEGFSSAR).

Non specific absorptions.
Although less pronounced, relative acidic peptides also showed certain affinity for TiO2. Figure 7 shows that methylation [4] reduced this affinity as demonstrated for [Glu+] tri- and tetrabromophenol II (EVQNDEEGFSSAR).

Acknowledgements
We acknowledge GL sciences Inc., Tsuchiura, Japan for making Titanosphere available to us. We kindly thank Jennifer L. Bush, Jackie D. Corbin and Sharon H. Francois for the preparation of autophosphorylated PKG. We thank Klara Rumpel and Frank Pullen of PFIair Research UK for financial support.

References