

Super para-magnetic micro particles with different surface modification and their use for the enrichment of peptide classes and their potential as Biomarker Discovery tool.

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Introduction

The identification of particular peptides or their PTMs from proteins involved in signal transduction has become one of the most challenging aspects of protein research. Phosphorylation, one of the most important PTMs, is estimated to occur on 30% of the mammalian proteome and perturbed regulation has been implicated in many pathologies. The rarity of phosphotyrosine compared to phosphoserine or phosphothreonine has prompted the development of more targeted and more sensitive approaches for its detection. Here we demonstrate the coupling of an antibody specific for tyrosine phosphorylation (4G10) to super para-magnetic beads with protein G covalently attached. These beads are used for the enrichment of tyrosine phosphopeptides from phosphoprotein digests and subsequently identifying the phosphorylation site by MALDI-TOF/TOF MS.

We also have used these super para-magnetic beads with an Immobilised Metal Affinity Chromatography (IMAC) function and immobilised Fe-(III) ions for the enrichment of Serine and Threonine phosphorylated peptides from phosphoprotein digests. The sites of phosphorylation have been subsequently identified by MALDI-TOF/TOF MS analysis. The super para-magnetic micro particles can also be a part of the ClinProt™ platform (Bruker Daltonics, Bremen, Germany) for Biomarker discovery. In this fully automated system, including integrated software for cluster analysis, the particles are available with different specificities to pull out sub fractions of proteins from plasma or other body fluids.

Results and Discussion

Super para-magnetic beads with protein G covalently attached were incubated with phosphotyrosine specific antibody 4G10 and then with a mixture of 10 pmol Bovine Serum Albumin (BSA) digest and three synthetic phosphotyrosine peptides at 1 pmol each. Phosphopeptides were then eluted with 3 µl 2,5-Dihydroxybenzoic acid (DHB) (5-10 mg/ml) in water with 0.1 or 1% Phosphoric acid for 7 minutes. 1 µl out of the 3 µl eluted was used and directly spotted on a 600 µm AnchorChip target. Once crystallised, MALDI TOF mass spectra were acquired using a Bruker ultraflex III MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Bremen,

Germany) operating in reflectron mode under the control of the flexControl software (Version 3.0, Bruker Daltonics, Bremen, Germany). External calibration was performed using peptide standards (Bruker Daltonics, Bremen, Germany). The MALDI-TOF MS spectrum before (Upper positive spectrum) and after enrichment (lower negative spectrum) is shown in Figure 1. Two of the three peptides could be enriched but there was also two BSA peptides enriched. These non-specifically bound peptides are showing two and three tyrosine residues in their sequence.

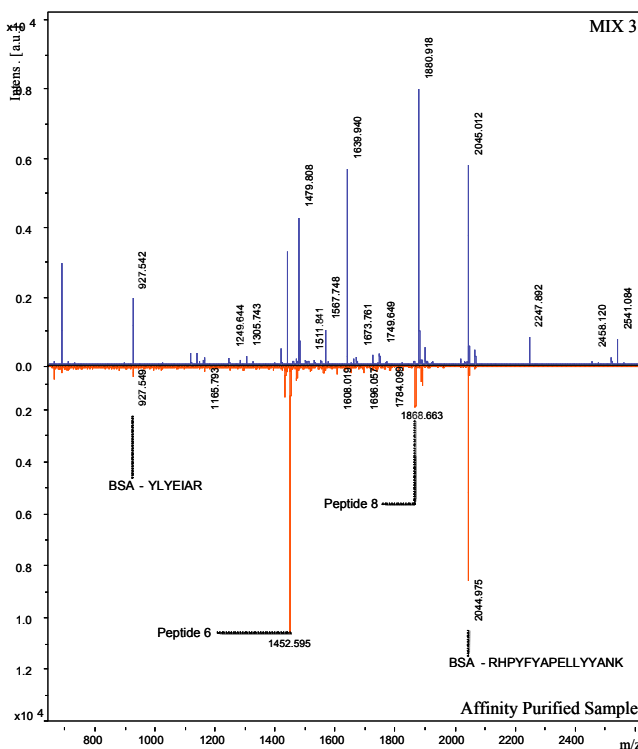


Fig. 1: MALDI-TOF MS spectrum of MIX 3 (table 3) (10pmol BSA digest +1pmol peptide 6 (CDFNGPpYLGPPH) +1 pmol peptide 8 (AEQAERpYDDMAACMK)+ 1pmol Y179 (Biotinyl-SGSGRASVFYpYEILNSK) ratio 10:1:1:1

Upper: prior to enrichment: Lower: after enrichment through 4G10 affinity-coupled MB-IAC Prot G protocol

Enriched phosphopeptide 6 [M+H]⁺: 1452.595 Da and 8 [M+H]⁺: 1868.663 as well as two unspecific enriched BSA-peptides are highlighted in the lower spectrum.

Super para-magnetic beads with IMAC function and immobilized Fe(III) ions were used for the enrichment of serine phosphorylated peptides from β -casein. The application of 1 pmol of a β -casein digest showed a good enrichment of the two phosphorylated peptides with these beads (data not shown). In order to elucidate the sensitivity of the beads a 100 fmol digest of β -casein was applied onto the beads and eluted with 3 μ L of 2,5 DHB in 50% Acetonitrile and 1% phosphoric acid. After elution 1 μ L was directly spotted on a 600 μ m AnchorChip target and used for MALDI-TOF MS analysis using a Bruker ultralex III MALDI-TOF/TOF mass spectrometer. The spectra of this analysis in positive and negative ion mode are shown in Figure 2.

We have used derivatised super para-magnetic beads to compare serum and plasma from genetically modified mice developing gastric cancer with those from normal mice in order to find changes specific to the disease. We have used beads with a hydrophobic interaction capacity and C 8 function to pull out sub fractions of peptides and could map differences in the gastric cancer samples. This work aims to establish cluster cohorts of diseased and non-diseased mice in order to allocate an unknown sample to the diseased or non-diseased state and identify potential biomarkers [1].

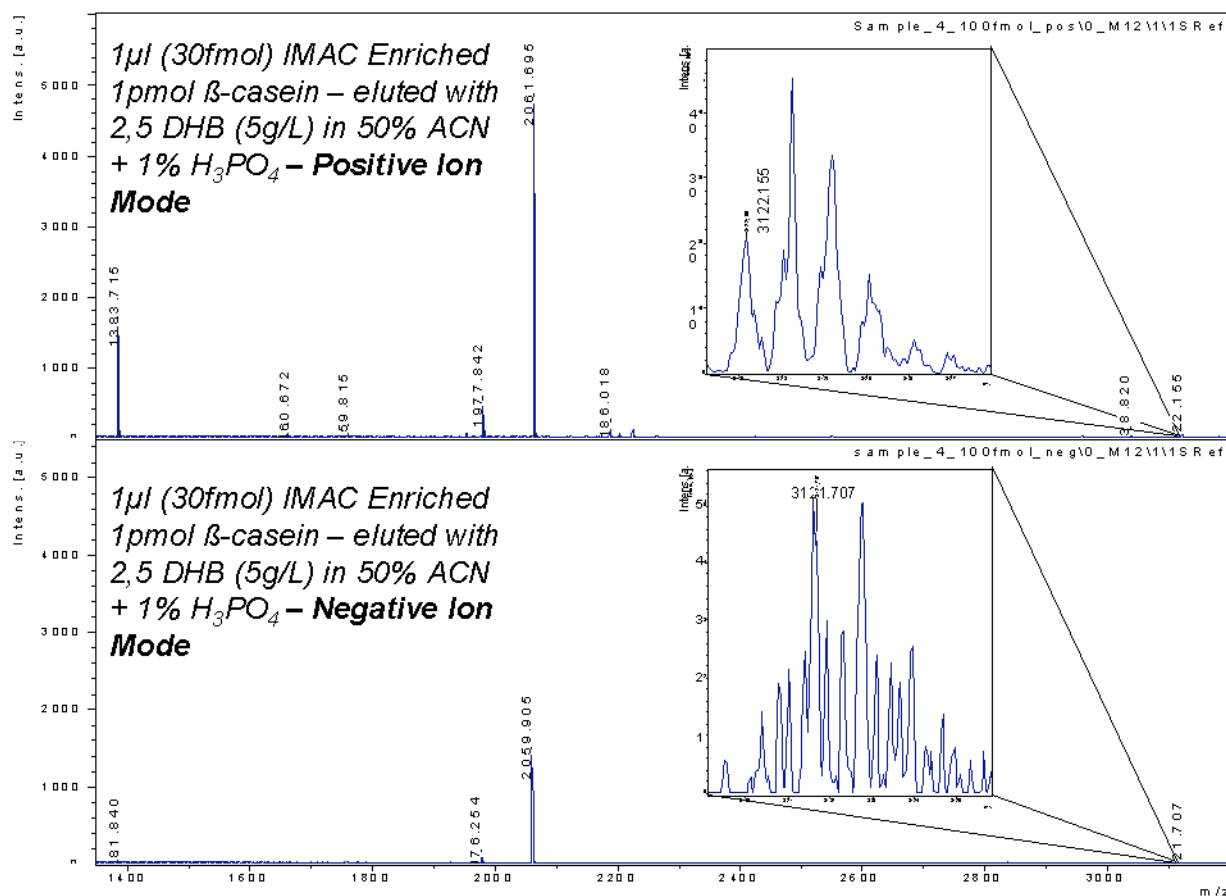


Fig. 2: MALDI-TOF MS spectra of 100fmol β -casein digest after enrichment with super para-magnetic IMAC beads measured in positive ion mode upper panel and negative ion mode lower panel showing the singly phosphorylated peptide at m/z of 2061.7 and 2059.9 and the quadruply phosphorylated peptide at m/z of 3122.1 and 3120.1.

The singly phosphorylated peptide is the base peak in the spectrum and the quadruply phosphorylated peptide is visible at a good signal to noise ratio at least in positive ion mode. The beads were also tested on in vitro phosphorylated protein digest and on in vivo phosphorylated proteins and we could successfully enrich serine and threonine phosphorylated peptides from these proteins.

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References

1. Xinyi Zhang, Sau-Mei Leung, Claudia R. Morris, and Mark K. Shigenaga (2004) *Journal of Biomolecular Techniques*, **V15**, **I3**, 167-175