Evaluation of Novel Supports for Selective and Efficient Enrichment of Phosphorylated Peptides

Dalila Bensadek[1], Wenzhang Chen[1],Justin Jordaan[2]; Isak Gerber[2] , Douglas Lamont[1]

[1] College of Life Sciences, University of Dundee, Scotland, UK [2] CSIR Biosciences, Pretoria, South Africa

Overview

A series of novel emulsion based supports MagReSyn™ TiO_2 (A-D) were evaluated using ^{32}P labelled phosphopeptides and nano LC-MS/MS.

The performance of MagReSyn™ TiO_2 (D) was found to be significantly better in terms of selectivity and specificity for phosphopeptides when compared to A-C and GL Sciences Titansphere.

Introduction

Protein phosphorylation plays a key role in various cellular processes and is therefore the most widely studied post-translational modification, with 30% of the proteome transiently phosphorylated at any given time.

Proteome wide characterisation of phosphorylation is quite challenging due to the low abundance of phospho-peptides, the low stoichiometry of the modification as well as the physico-chemical properties of phosphorylated peptides, which make them less amenable to analysis. Consequently, it has become imperative to enrich these peptides prior to mass spectrometric analyses.

ReSyn™ is a novel microsphere technology comprising a loosely linked polyethyleneimine matrix that enables penetration of biological and synthetic molecules throughout the volume of the beads, differentiating it from alternate technologies. Along with a high functional group density, these features allow attachment of a broad range and high concentration of adjuvants. Here we report on the comparative assessment of magnetic TiO_2-functionalised ReSyn microspheres (MagReSyn™ TiO_2) for phosphopeptide enrichment.

Methods

TiO_2 enrichment

MagReSyn™ TiO_2 was supplied by ReSyn Biosciences (www.resynbio.com). The Phospho-enrichment was carried out using the method described by Thingholm *et al.* [Nature Protocols 1, 1929 - 1935 (2006)] with few modifications. Briefly, the tryptic digests were dissolved in (80% acetonitrile, 5% TFA 1M glycolic acid,100 µL) . Equal amounts of the different TiO_2 supports were added to each aliquot After a ten minute incubation, the supports were washed with 100 µL of loading buffer to reduce non-specific interactions. The beads were washed twice with (80% acetonitrile, 1% TFA) and (20% acetonitrile, 0.5% TFA). Finally, phosphopeptides were eluted from the supports by incubating for one hour in aqueous ammonia (pH 11.3, 200 µL) .

In vitro phosphorylation and tryptic digestion

CHK1 (3.7 µL) was autophosphorylated by incubation with ^{32}P ATP (1mM, 5 µL) and cold ATP (1mM, 5 µL) at 37°C for 30 minutes. After that, the protein was precipitated using 50 µL of 50% TCA and incubated at 4° C for 20 minutes. The precipitated protein was centrifuged and the pellet washed with 10% TCA and twice with water. The precipitated protein was resolubilised in digestion buffer (8 M urea, 50 mM DTT, 100 mM ammonium bicarbonate, 10 µL) and shaken at room temperature for one hour. The cysteines were then blocked with iodoacetamide (250 mM, 10 µL) and incubated in the dark for 45 minutes. Finally, the urea was diluted 10 fold prior to tryptic digestion.

LC-MS/MS Analysis

All the tryptic hydrolysates were desalted prior to nano LC-MS/MS. 15% of the material was loaded onto RP-C18 column (75 µm x 150 mm, Dionex) and separated on a 40 minute linear gradient (5-40% acetonitrile) using an Ultimate 3000 (Dionex). Data were acquired on an LTQ-orbitrap XL (Thermo Scientific) equipped with a proxeon source under the automatic control of xcalibur 2.0.7 in data dependent mode (FT-MS survey scan with a resolution of 60,000 and top 5 MS/MS with multi stage activation)

Results

Efficiency of Enrichment

MagReSyn™ TiO_2 (D) performs better than MagReSyn™ TiO_2 (A-C)

Four different variants of the MagReSyn™ TiO_2 supports (denoted A-D) were evaluated in comparison to Titansphere (GL Sciences). The key aspects for a good performance are:

1. Binding Efficiency: All four supports bind phosphopeptides to varying degrees. Support B and D were found to have the highest initial binding (less than 4% of the radioactivity lost in the FT and only a further 10 % lost during subsequent washes. MagReSyn™ TiO_2 B bound 86% of phospho-peptides and MagReSyn™ TiO_2 (D) bound 89% (n=5). A and C had lower binding efficiency (66% and 77% respectively). Titansphere has an overall binding eficiency of 95%.

2. Recovery of bound material: The elution profile of the four supports indicates a wide range of recovery. Support A (19%) was lowest in followed by C (32%) then B (50%) and finally D (81%) with the highest recovery. Titansphere has an overall recovery of 75%.

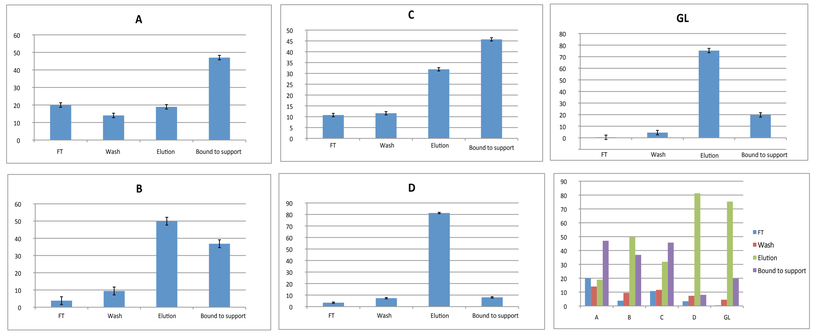


Figure 1: Distribution of ^{32}P labelled phosphopeptides (as a percentage of total phosphopeptides) in the different fractions during the enrichment process for the different titania supports.

MagReSyn™ TiO_2 (D) performs better than GL Sciences Titansphere

Although the recoveries are comparable for MagReSyn™ TiO_2 (81% of the starting material) and titansphere (75% of the starting material), the overall losses due to adsorption on the support are more significant with titansphere (20% of the starting material versus 8% for MagReSyn™ TiO_2 (D)

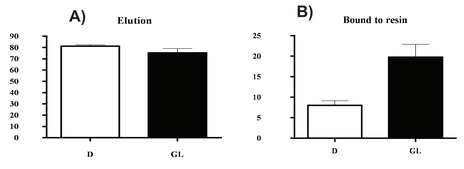


Figure 2: Comparison between A) percentage of phosphopeptides recovery using MagReSyn™ TiO_2 (D) and Titansphere; B) percentage of phosphopeptides that remained bound to the enrichment support after elution.

Reproducibility and Stability tests

Different batches of MagReSyn™ TiO_2 (D) were used for the enrichment of ^{32}P labelled CHK1 digest. The binding characteristics and the perfomance of the three batches are consistent indicating little batch to batch variation. The results are shown in Figure 3.

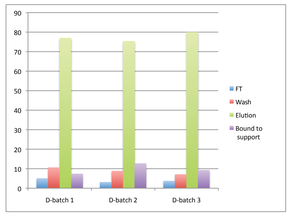
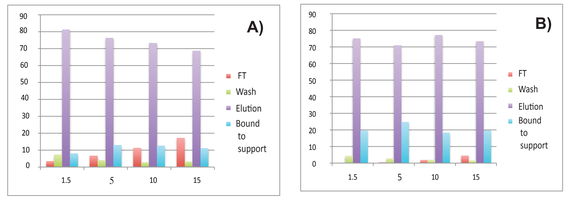


Figure 3: Comparison between different batches of MagReSyn™ TiO_2 (D) showing good reproduciblity in performance.

Capacity of MagReSyn™ TiO_2 (D)

In the experiments described earlier MagReSyn™ TiO_2 (D) below its capacity (1 mg of support per 1.5 µg of digested protein). To determine the capacity of the MagReSyn™ TiO_2 (D) resin was fixed while increasing the amount of tryptic digest and measuring the enrichment of phosphopeptides. The results in Table 1 show that even if the initial binding (total capacity) of titansphere appears higher than that of support D (judged by the FT), the final amount of phospho-peptides recovered in the elution is similar up to 10 µg of tryptic digest. This suggests that the optimal technical capacity (a composite of binding and recovery) for MagReSyn™ TiO_2 up to 10 µg per 1 mg of support.

Figure 4: Elution Profile of A) MagReSyn™ TiO_2; B) Titansphere using increasing amounts of digested ^{32}P labelled CHK1 to determine optimum capacity for each support.



	amount of protein	FT	Wash	Elution	Left on support
A	1.50	0.01	0.07	1.13	0.30
	5.00	0.04	0.15	3.56	1.25
	10.00	0.21	0.21	7.73	1.85
	15.00	0.71	0.27	11.04	2.98
B	1.50	0.05	0.11	1.22	0.12
	5.00	0.34	0.20	3.81	0.65
	10.00	1.12	0.28	7.33	1.27
	15.00	2.57	0.47	10.31	1.65

Table1: Distribution of ^{32}P peptides in the different fractions during the enrichment process using A) Titansphere, B) MagReSyn™ TiO_2 (D)

Specificity of Enrichment

By using ^{32}P labelled tryptic digests, we were able to evaluate the extent of enrichment of phospho-peptides. However, in order to evaluate the extent of non-specific binding, the eluents were analysed by nano LC-MS/MS and the peptides were identified through database searching.

The percentage of non-phosphorylated peptides identified is a good indication of the extent of non-specific binding. With MagReSyn™ TiO_2 (D) all identified peptides in the eluent were phosphorylated while 22% of identified peptides in the eluent of Titansphere were non-phosphorylated suggesting that non-specific binding is higher when using Titansphere. Moreover, there appears to be no bias towards monophophorylated peptides in both cases. The high specificity of binding is particularly important when analysing complex mixtures such as total cell lysate as the time spent analysing phosphopeptides is maximised.

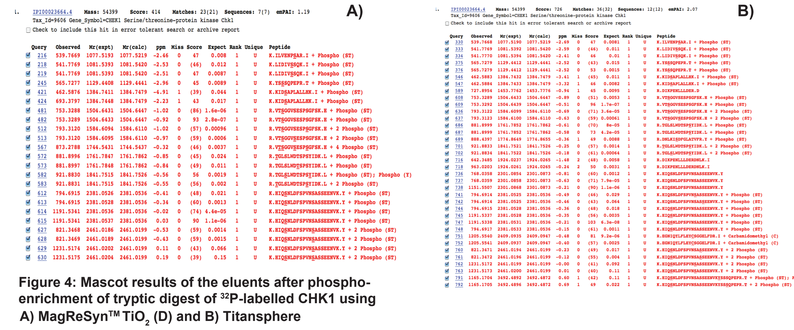


Figure 4: Mascot results of the eluents after phospho-enrichment of tryptic digest of ^{32}P-labelled CHK1 using A) MagReSyn™ TiO_2 (D) and B) Titansphere

Conclusion

A systematic comparison between MagReSyn™ TiO_2 (D) and Titansphere shows that both have similar binding characteristics and afford good enrichment of phosphopeptides. However, MagReSyn™ TiO_2 (D) shows no indication of non-specific interactions and has therefore a higher specificity/selectivity for phosphopeptides.