Enrichment of Phosphopeptides Using Polyethyleneimine-modified Magnetic Nanoparticles

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Abstract

Determination of low abundance phosphopeptides based on mass spectrometry, usually utilized well-known techniques (e.g. immobilized metal affinity chromatography, IMAC) for enhancing the signal intensity. In this study, we employ polyethyleneimine (PEI) modified magnetic nanoparticles as an affinity probe to isolate phosphopeptides derived from phosphoproteins. The results in MALDI mass spectrometry analysis showed a dramatic efficiency for enriching phosphopeptides containing both mono and multiply-phosphorylated sites.

Introduction

Reversible protein phosphorylation regulates the signal transmitting, growing, differentiating, and metabolism of the cells.¹ Thus, phosphorylation mechanism plays a major role in a variety of biological systems. Furthermore, it’s also estimated that one-third of cellular proteins were regulated through certain phosphorylated sites.² Mass spectrometry (MS) has been widely used in shotgun phosphoproteomics during the past decade³ to understand the relationship of phosphorylation among vital acts and many diseases.⁴

However, detection of phosphopeptides by using the MS-based methods is a challenge due to the following factors. Firstly, phosphorylated site(s) on the proteins is present at a very low stoichiometric ratio. Therefore, non-phosphopeptides signals would generally dominate the spectrum and suppress the signals of phosphopeptides. Secondly, some tryptic peptides contain multiple phosphate groups and these modified regions can significantly reduce the protonation efficiency of the precursor ions. The ionization efficiency can be improved by adding 1% phosphoric acid into the matrix (DHB) for MALDI analysis.⁵ Addition of phosphoric acid also lowers the detection limits for the LC-ESI-MS/MS analysis of multi-phosphorylated peptides.⁶ To detect the low abundance phosphopeptides, many methods have been developed to concentrating the phosphopeptides prior to MS analysis. The enriching strategies can be divided into three categories: (1) immunoprecipitation, (2) chemical derivatization, (3) affinity-based methods.

In general, Western blot is a technique to select target proteins by using specific antibodies which are immobilized on one-dimensional (1-D) gel or 2-D gel. Although some research groups
applied anti-phosphotyrosine antibodies to enrich the tyrosine-phosphorylated proteins,\textsuperscript{7, 8} this immunoassay was feasible for the analysis of phosphotyrosine sites, which only account for 1.8\% of phosphorylation sites in whole proteomes.\textsuperscript{9} Besides, the activity (false positive or negative) and the cost of antibodies are the major concerns for researchers. Another approach to separate phosphoproteins from complicated samples can be through some chemical reactions. The dehydroalanine are obtained through $\beta$-elimination reaction of phosphoserine and phosphothreonine under strong alkaline conditions. The resulting modified residues are reacted with thiolated reagent through Michael's addition and the phosphorylated sites are identified by using tandem mass spectrometry.\textsuperscript{10} The activated phosphate groups may be coupled with cystamine via a covalent bonding to a solid supporting phase. Hence, in the so-called phosphoramidate chemistry (PAC) method, the enrichment and quantitative events are completed simultaneously.\textsuperscript{11, 12} Furthermore, immobilized metal affinity chromatography (IMAC) has been most widely employed to date for the enrichment of phosphopeptide.\textsuperscript{13-18} In addition, metal oxide affinity chromatography (MOAC) has been used to select phosphopeptides via the bidentate binding between the microspheres (or nanospheres) and the phosphate groups on a peptide.\textsuperscript{19, 20} Various materials such as TiO$_2$,\textsuperscript{20-24} ZrO$_2$,\textsuperscript{24-28} and Al$_2$O$_3$\textsuperscript{24, 29-31} have been investigated comprehensively by many research groups.

The negatively charged phosphate groups on phosphopeptides offered another possibility for the enrichment of phosphopeptides through an interaction with positively charged objects. Whereas cationic arginines could form a stable “covalent like” complex with phosphate groups,\textsuperscript{32} Chang et al.\textsuperscript{33} utilized the polyarginine (PA)-nanodiamond composite as a tool to enrich phosphopeptides. Compared to IMAC and MOAC, PA-coated diamond leads to binding with multiply phosphorylated peptides. However, it’s still a challenge to observe mono- and multiply-phosphopeptides simultaneously. Polyethyleneimine has been developed to condense DNA which by exploiting the electrostatic interaction between the positively charged from polyethyleneimine and negatively charged phosphate group.\textsuperscript{34} The PEI-modified MALDI plate has been employed to concentrate DNA and protein digests.\textsuperscript{35, 36}

We use magnetized PEI nanoparticles as a convenient vehicle to select and concentrate phosphopeptides. Based upon the highly protonation ability of polyethyleneimine (approximately 20\% of the nitrogens were protonated under physiological conditions)\textsuperscript{37}, we demonstrate that highly selective enrichment of phosphopeptide can be achieved by a simple approach. Simultaneous enrichment of mono- and multiple phosphopeptides was realized by changing the polarity of solutions.
Methods

Materials. Iron (III) chloride hexahydrate was obtained from Aldrich (Milwaukee, WI). Iron (II) chloride tetrahydrate and phosphoric acid were obtained from Acros Organics (Geel, Belgium). Ethanol, ammonia solution and trifluoroacetic acid were purchased from Reidel-de Haën (Seelze, Germany). Hydrochloric acid was obtained from J. T. Baker (Phillipsburg, NJ). Poly(ethyleneimine) solution, 2,5-dihydroxybenzoic acid, sodium bicarbonate, lysozyme, cytochrome c, α-casein and β-casein were obtained from Sigma Chemical Co. (St. Louis, MO). Tetraethyl orthosilicate (TEOS) was obtained from Fluka (Buchs, Switzerland). Middlebrook 7H11 agar plate was obtained from BioStar (Boulder, Colorado). Porcine trypsin (sequence grade) was obtained from Promega (Madison, WI). Fresh egg was bought from local store. Urea was obtained from Wako Pure Chemical Industries (Osaka, Japan). Water was purified using a Milli-Q system (Millipore, Bedford, MA).

Preparation of Fe$_3$O$_4$@PEI Nanoparticles. The silica coated magnetic nanoparticles were carried out according to the procedure reported by Chen. 10mg polyethyleneimine was added into the stock solution, which was stirred for 8 h. The PEI-coated nanoparticles were rinsed with 2 mL distilled water for three times and redispersed in distilled water by ultrasonication. The concentration of the cationic nanoparticles was estimated to be about 50 µg/µL.

Characteristics of the particle. Zeta potential measurements were made using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The temperature was maintained at 25 °C. Each measurement consisted of a minimum of 30 runs, all measured data were calculated automatically by Dispersion Technology Software Version 4.2. The transmission electron microscope (TEM) image was obtained using a JEM2000-FX (JEOL, Japan).

Phosphopeptides Enrichment and MS analysis. The tryptic peptides were diluted with appropriate volume of solvent for each experiment. Tryptic peptides were acidified with a binding buffer The 0.3 µL suspension of PEI@Fe$_3$O$_4$ nanoparticles was added into protein digest solution (100 µL) and the mixture was incubated for 30 s. Then, the nanoparticles were isolated by a magnet and the nonphosphopeptides were washed out with 100 µL of binding buffer for two times. The particles were mixed with 0.5 µL matrix solution (DHB, 30 mg/mL) containing 1% phosphoric acid and the mixture was loaded on the MALDI plate, allowed to dry at atmosphere prior to analysis. The entire enrichment process scheme is outlined in Figure 1.
Results

The suspension of magnetic PEI@Fe3O4 nanoparticles were deep brown in color. The zeta potential of the nanoparticles was measured by electrophoretic light scattering (ELS). The potential of SiO2@Fe3O4 increased from −42 mv to 23 mv when the particle was modified with PEI (Figure 2a). The adsorption of PEI via strong electrostatic interaction to the nanoparticles results in positive charge on the particle surface and therefore stabilizes the nanoparticles. In order to test whether charges on the particle surface decrease due to the solubility of PEI in water, the zeta potential of particles in various pH conditions was measured as a function of time. The PEI@Fe3O4 particles remained positively charged at the pH value ranging from 3 to 11 two months after the synthesis. (Figure 2b).

Generally, phosphoproteins are present in low abundance in stoichiometry relative to non-phosphoproteins in biological samples. Therefore, enrichment strategies are often required to reduce the analyzing time and enhance the sensitivity for the detection of phosphopeptides. In this work, polyethyleneimine-modified magnetic nanoparticles were used for capturing phosphopeptides from proteolytic digests. Figure 3a shows a MALDI-TOF MS spectrum of two standard phosphopeptides mixed at the same molar ratio. The effect of binding buffers, 50% ACN/0.1% TFA and 100% ACN/0.1% TFA, on the selectivity for phosphopeptides was shown in Figure 3b and 3c. Only tetra-phosphopeptides (4p) were captured when 50%ACN/0.1%TFA was used as a binding solvent (Fig. 3b). On the contrary, both mono and tetra-phosphopeptides were captured when the binding buffer of 100%ACN/0.1%TFA was used (Figure 3c). The water content in the solution may be attributable to the observed buffer dependence. When the binding buffer contains 50% of water, hydrogen binding among water, phosphopeptides and PEI particles may stabilize the binding of phosphopeptides to PEI particles. This stabilizing effect is more obvious for multiply phosphorylated peptides. In the binding buffer of 100%ACN, the hydrogen-binding effect is absent, and both mono- and multi-phosphopeptides are bound to the particles.

We further investigated PEI selectivity for phosphopeptides of bovine α-casein tryptic digests. Figure 4a presents a MALDI spectrum of the α-casein digest without enrichment. In the spectrum, most of the mass peaks correspond to nonphosphopeptides. The large amount of nonphosphorylated peptides suppressed the ionization of phosphopeptides in the MS analysis. After the enrichment with the PEI nanoparticles, we clearly observed dominant mass peaks corresponding to phosphopeptides, as shown in Fig. 4b and c. The sequence and the phosphorylated site(s) are summarized in Table 1. The selective enrichment of multiply phosphorylated peptides was also observed in this experiment. When 50% ACN was used as a binding buffer and the hydrogen bonding effect was favored multiply phosphorylated peptides are dominated as shown in Figure 4b. Monophosphopeptides (α1, α2, α4, and α6) were not
observed. Monophosphopeptides corresponding to mass peaks α1, α2, α3, α4, and α6 were observed (Figure 4c) when a nonhydrous solvent (100% ACN) was used. Although, both mono and multiply phosphorylated peptides were observed under the present conditions, monophosphopeptides remain dominant. To further enhance as well as multi-phosphorylated signals simultaneously, we performed a two-step reaction by using two solvents of different polarity. After the sample was incubated in 100% ACN/0.1% TFA for 30s, 50 µL of 0.1% TFA was mixed with the same volume of the reaction solution to make up a solution of 50% ACN/0.1% TFA and the binding reaction was continued for another 30s. The spectrum was shown in Figure 4d. The 100% ACN solution allowed all the phosphopeptides to bind with the particles. When the solution was changed to 50% ACN, the binding of multiply phosphorylated peptides to particle were further enhanced. Furthermore, this modified strategy not only captured mono- and multiply phosphorylated peptides but also leads to increased ion abundance in MALDI spectra. Nonspecific binding is often the major problem with IMAC, which leads to the complexity in phosphopeptides identification. However, PEI-modified nanoparticles showed excellent selectivity toward phosphopeptides.

**Conclusion**

The use of polytheneimine-modified magnetic nanoparticles was an efficient tool to enrich phosphopeptides from tryptic digests for mass spectrometric analysis. High-abundance amino groups on Fe$_3$O$_4$@PEI allow for strong electrostatic interactions with phosphate groups on phosphopeptides. Interestingly, the constitutions of binding solution affect the enrichment characteristics of the functionalized nanoparticles. This approach should also be suitable for analyzing complex biological samples.

**Acknowledgment.**

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Figure 1. Flow diagram of the phosphopeptide enrichment process.

Figure 2. Characterization of the functionalized magnetic nanoparticles. (a) The zeta potential of Fe₃O₄@SiO₂ before and after modification with PEI. (b) Effect of pH on zeta potential of Fe₃O₄@PEI.
Figure 3. Mass spectra of a mixture of standard phosphopeptides (a) without enrichment and with enrichment using Fe₃O₄@PEI as an affinity probe in the binding buffer of (b) 50% ACN/0.1% TFA, (c) 100% ACN/0.1% TFA. “Mono-” and “tetra-” indicate the number of phosphorylation sites.

Figure 4. Mass spectra of 2 x 10⁻⁸ M (100 µL) tryptic digests of α-casein (a) without enrichment and with enrichment using Fe₃O₄@PEI as an affinity probe in the binding buffer of (b) 50% ACN/0.1% TFA, (c) 100% ACN/0.1% TFA for 30 s. (d) After the sample was incubated in 100% ACN/0.1% TFA for 30s, a 50 µL of 0.1% TFA was mixed with the same volume of the reaction solution and the binding reaction was continued for 30s.
Table 1. List of matched phosphopeptides of α-casein digest that was enriched by using Fe₃O₄@PEI.

<table>
<thead>
<tr>
<th>No.</th>
<th>(M+H)+ Da</th>
<th>Residues</th>
<th>Number of phosphorylation sites</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>α1</td>
<td>1466.6</td>
<td>S2-(153-164)</td>
<td>1</td>
<td>TVDMEpSTEVFTK</td>
</tr>
<tr>
<td>α2</td>
<td>1594.7</td>
<td>S2-(153-165)</td>
<td>1</td>
<td>TVDMEpSTEVFTKK</td>
</tr>
<tr>
<td>α3</td>
<td>1660.8</td>
<td>S1-(121-134)</td>
<td>1</td>
<td>VPQLEIVpSAEER</td>
</tr>
<tr>
<td>α4</td>
<td>1832.8</td>
<td>S1-(104-119)</td>
<td>1</td>
<td>YLGELYIVpSAEER</td>
</tr>
<tr>
<td>α5</td>
<td>1911.9</td>
<td>S2-O(1-16)</td>
<td>1</td>
<td>oMKFFIFpTCLLAVAALAK</td>
</tr>
<tr>
<td>α6</td>
<td>1927.7</td>
<td>S1-(58-73)</td>
<td>2</td>
<td>DIGpSEpSTEDQAMEDIK</td>
</tr>
<tr>
<td>α7</td>
<td>1951.9</td>
<td>S1-(119-134)</td>
<td>1</td>
<td>YKVPOLEIVpSAEER</td>
</tr>
<tr>
<td>α8</td>
<td>2061.8</td>
<td>S2-(40-56)</td>
<td>1</td>
<td>KMAINpSKENLCSTFCK</td>
</tr>
<tr>
<td>α9</td>
<td>2703.9</td>
<td>S1-pyro-(74-94)</td>
<td>5</td>
<td>pyroEMEAEpSpSpSpGElVaPVpSVEQK</td>
</tr>
<tr>
<td>α10</td>
<td>2720.9</td>
<td>S1-(74-94)</td>
<td>5</td>
<td>EMEAEpSpSpSpGElVaPVpSVEQK</td>
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<tr>
<td>α11</td>
<td>2736.9</td>
<td>S1-O(74-94)</td>
<td>5</td>
<td>EoMEAEpSpSpSpGElVaPVpSVEQK</td>
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<tr>
<td>α12</td>
<td>2747.1</td>
<td>S2-(17-37)</td>
<td>4</td>
<td>NTMEHVpSpSpSpSVEQETKYQ</td>
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<tr>
<td>α13</td>
<td>2757.9</td>
<td>S1-(52-73)</td>
<td>4</td>
<td>VNELpSKDIGpSepSpTEDQAMEDIK</td>
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<tr>
<td>α14</td>
<td>2935.1</td>
<td>S1-(50-73)</td>
<td>3</td>
<td>EKVNElpSKDIGpSepSpTEDQAMEDIK</td>
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<tr>
<td>α15</td>
<td>3087.9</td>
<td>S2-(61-85)</td>
<td>5</td>
<td>NANEYeEpSpSpSpSVEpSAEVEFVK</td>
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<tr>
<td>α16</td>
<td>3132.2</td>
<td>S2-(16-39)</td>
<td>4</td>
<td>KNTMEHVpSpSpSpSVEQETYQQEK</td>
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</tbody>
</table>

*The phosphorylation of serine and the oxidation of methionine are indicated by pS and oM.
*PyroE represents a sequence where N-terminal glutamine was cyclized to pyroglutamic acid.
Reference

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