Specific capture of phosphopeptides by Zr\textsuperscript{4+}-modified monolithic capillary column

A method to prepare zirconium phosphate (ZrP)-modified monolithic capillary column for highly specific capture of phosphopeptides is presented. In this method, the phosphate monolithic capillary column was prepared by direct copolymerization of the functional monomer containing phosphate group (ethylene glycol methacrylate phosphate) and cross-linker (bis-acrylamide) in a ternary porogenic solvent. Copolymerization of cross-linker and functional monomer simplifies the procedure for the preparation of phosphate chromatographic media. After Zr\textsuperscript{4+} was immobilized, the ZrP-modified monolithic capillary column was evaluated by the analysis of standard phosphoproteins and the excellent selectivity of this approach was demonstrated by analyzing phosphopeptides in the digest mixture of β-casein and BSA with molar ratio of 1:200.

Keywords: Monolithic capillary column / Phosphoproteome analysis / Zirconium phosphate

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1 Introduction

Protein phosphorylation is one of the most important reversible, covalent protein post-translational modifications (PTMs); it plays a key role in eukaryotic signal transduction, gene regulation, and metabolic control in cells [1]. Fundamental processes such as cell proliferation, adaptation, and differentiation are governed by reversible phosphorylation at specific serine, threonine, and tyrosine residues in proteins. However, even phosphorylation being acknowledged as a crucial modification involved in many cellular events, determining the sites of phosphorylation on proteins is still a challenging task. Enrichment of phosphorylated proteins and peptides is generally required before the analysis because of their low abundance. To date, several strategies have been developed to enrich the phosphopeptide prior to mass spectrometric analysis including immobilized metal affinity chromatography (IMAC) [2–5], particles of metal oxide such as TiO\textsubscript{2}, ZrO\textsubscript{2}, and Al(OH)\textsubscript{3}, and Fe\textsubscript{3}O\textsubscript{4}/TiO\textsubscript{2} core/shell nanoparticles [6–10]. The development of new efficient methods for highly specific enrichment of phosphopeptides still is one of the most active research fields in phosphoproteome analysis. Self-assembling monolayer and multilayer thin films of phosphate-containing organic molecules have been prepared for immobilization of oligonucleotides based on the strong interaction between Zr\textsuperscript{4+} and phosphate groups [11–16]. Taking advantage of the strong interaction, we have developed a zirconium phosphate (ZrP)-modified porous silicon wafer and IMAC adsorbent through Zr\textsuperscript{4+} chelating to the phosphate-modified poly(glycidyl methacrylate-co-ethyl- ene dimethacrylate) polymer beads for highly specific capture of phosphopeptides [17–18]. It was observed that the selectivity of the ZrP-modified material was higher than that of conventional Fe\textsuperscript{3+}-IMAC material. As the preparation of the phosphate-modified surface involves several synthesis steps, these approaches are complicated and labor intensive. The modified surface prepared may be also of low density of functional groups because of the limited active sites on the surface and incompletion of these reactions.

Monolithic materials were developed as an alternative to bead-based columns for chromatographic separation. The key advantages of monolithic columns include the ease of preparation and modification, adjustable porosity, fast mass transport, and low column backpressure under high flow rate [19–21]. Considering the small size of biological sample used, nanoliter column is preferable for enrichment of phosphopeptides when only limited sample is available. Recently, we have established procedures for the preparation of phosphate monolithic capil-
lary column by direct copolymerization of an ethylene glycol methacrylate phosphate (EGMP) and bis-acrylamide in a ternary porogenic solvent consisting of DMSO, dodecanol, and $N,N'$-dimethylformamide. It was successfully applied as trap column in the automated sample injection at high loading flow rate for online multidimensional separation of tryptic digest of yeast proteins [22].

In this study, this phosphate monolithic column was applied to specifically isolate phosphopeptides after Zr$^{4+}$ was loaded onto the column to generate ZrP-modified surface. The ZrP-modified monolithic capillary column was evaluated by the analysis of standard phosphoproteins.

2 Experimental

2.1 Materials

DTT, iodoacetamide (IAA), and ammonium bicarbonate were purchased from Sino-American Biotechnology Corporation (Beijing, China). Urea, ammonium acetate, zirconium(IV) oxychloride octahydrate ($\text{ZrOCl}_2\cdot8\text{H}_2\text{O}$), 2,5-dihydroxybenzoic acid (DHB), EGMP, bis-acrylamide, DMSO, dodecanol, $N,N'$-dimethylformamide, and $\gamma$-methacryloxypropyltrimethoxysilane ($\gamma$-MAPS) were obtained from Sigma (St. Louis, MO, USA). Azoisobutyronitrile (AIBN) was obtained from Shanghai Fourth Reagent Plant (Shanghai, China). Formic acid was obtained from Fluka (Buches, Germany). ACN (HPLC grade) was from Merck (Darmstadt, Germany). $\alpha$-Casein, $\beta$-casein, trypsin, and BSA were obtained from Sigma (St. Louis, MO, USA). Fused-silica capillaries with 75 $\mu$m id were purchased from Yongnian Optical Fiber Factory (Hebei, China). All the water used in experiments was purified using a Milli-Q system (Bedford, MA, USA).

2.2 Preparation of ZrP-modified monolithic capillary column

Prior to the polymerization, the capillary was pretreated with $\gamma$-MAPS as described elsewhere [23]. The phosphate monolithic capillary column was prepared with following procedures. Briefly, the reaction mixture consisting of EGMP (80 $\mu$L, ~100 mg), bis-acrylamide (60 mg), DMSO (270 $\mu$L), dodecanol (200 $\mu$L), $N,N'$-dimethylformamide (50 $\mu$L), and AIBN (2 mg) was sonicated for 20 min to obtain a homogeneous solution and then purged with nitrogen for 10 min. After the pretreated capillary was completely filled with the mixture, it was sealed at both ends with rubber stoppers. The sealed capillary was submerged into a water bath and allowed to react for 12 h at 60°C. The resultant monolithic capillary column was washed with methanol for 2 h using an HPLC pump to remove unreacted monomers and porogens. Scanning electron microscopic (SEM) images of the monolithic column were obtained using a JEOL JSM-5600 scanning electron microscope (JEOL Company, Japan). The porous properties were determined by mercury intrusion porosimetry, and its specific surface area was calculated from nitrogen adsorption/desorption isotherms using BET sorptometer and mercury porosimeter (9310 Mercury Porosimeter, USA). The prepared phosphate monolithic capillary column was subsequently rinsed with 5 $\mu$L 20 mM $\text{ZrOCl}_2$ aqueous solution to yield the ZrP at room temperature. After that, the ZrP-modified monolithic capillary column was rinsed with deionized water for several column volumes and stored in the refrigerator at 4°C for usage.

2.3 Sample preparation

BSA (4 mg) was dissolved in 1 mL denaturing buffer solution containing 8 M urea in 50 mM ammonium bicarbonate for 3 h. The obtained protein solution was mixed with 20 $\mu$L of 50 mM DTT. The disulfide bond of protein was reduced by incubation for 2 h at 37°C. And then 40 $\mu$L of 50 mM IAA was added, and the obtained solution was incubated for an additional 30 min at room temperature in dark. After that, the mixture was diluted with 50 mM ammonium bicarbonate by ten-fold and incubated for 16 h at 37°C with trypsin at an enzyme/substrate ratio of 1:40 w/w to produce proteolytic digest. $\beta$-Casein and $\alpha$-casein (1 mg individual) were dissolved in 1 mL ammonium bicarbonate (50 mM, pH 8.2) and digested for 16 h at 37°C with an enzyme/protein ratio of 1:40 w/w. The digested peptide solution was lyophilized by a vacuum concentrator for usage. For the experiments to evaluate the capture specificity of phosphopeptides by ZrP-modified monolithic capillary column, the peptide mixtures originating from tryptic digestion of $\beta$-casein and BSA at molar ratio of 1:100, and 1:200 by keeping $\beta$-casein concentration at 1 pmol were prepared.

2.4 Enrichment of phosphopeptides

The ZrP-modified monolithic capillary column (75 $\mu$m id $\times$ 150 mm length) was rinsed with 10 $\mu$L of 0.1% formic acid solution containing 100 mM NaCl, and then rinsed by 10 $\mu$L of 0.1% formic acid. Then, 2 $\mu$L of tryptic digest of $\beta$-casein or $\alpha$-casein (2 pmol) in 50% ACN solution containing 0.1% formic acid was loaded. Then the column was washed with 10 $\mu$L 0.1% formic acid solution containing 200 mM NaCl, and 10 $\mu$L of 0.1% formic acid in sequence to remove nonphosphopeptides. The captured phosphopeptides were eluted from the column with 10 $\mu$L of ammoniated water (pH 10) into a tube. After lyophilization to dryness, the eluted phosphopeptides were analyzed by MALDI-TOF MS.
2.5 Instruments

MALDI-TOF-MS was performed on the Bruker AutoflexTM (Bruker Co., Bremen, Germany). The instrument was equipped with a nitrogen laser (λ = 337 nm) and its available accelerating potential was in the range of +20/–20 kV. The MALDI MS used a ground-steel sample target. All mass spectra shown were obtained in the positive ion mode. The eluted phosphopeptides were redissolved in 5 μL of 0.1% TFA, and 5 μL of DHB containing 1% phosphoric acid was added, and then 1 μL of aliquot was deposited onto the target. All CEC experiments were carried out on a Beckman P/ACE 5510 instrument (Beckman, Fullerton, CA, USA).

3 Results and discussion

3.1 Characterization of phosphate monolithic capillary column

Monolithic materials may offer an ideal alternative to columns packed with particles or beads as they have extremely low backpressure in LC. Monolithic columns can be classified into two types: organic polymer-based and silica-based columns. Polymer-based monoliths are usually used in the analysis of biomolecules as its pH stability, absence of deleterious effects from silanol, and facility for modification. In preparation of polymer monolithic capillary column, grafting of pore surface [24–25], modification of reactive monoliths [26], and copolymerization of cross-linker and monomer containing the functional group are most widely used to introduce active groups into the monolithic backbone [27–29]. Among the three methods described above, copolymerization of cross-linker and functional monomer is the most straightforward strategy and the dynamic binding capacity of these columns can be controlled by adjusting the amount of functional monomer in the polymerization mixture. Herein, we describe a simple method to prepare Zr⁴⁺-modified phosphate monolithic material in capillary column as the whole scheme is shown in Fig. 1. EGMP and bis-acrylamide were chosen as monomers to prepare monolithic capillary column. Since bis-acrylamide could be well dissolved in DMSO but hardly in aliphatic alcohols, DMSO was chosen as a good solvent so that all monomers could be dissolved completely. On the contrary dodecanol was chosen as poor solvent. In addition, since N,N’-dimethylformamide proved helpful in improving the dissolution of EGMP, and a ternary porogen system with DMSO, dodecanol, and N,N’-dimethylformamide was taken to prepare the monolithic column. The obtained monolith was characterized by scanning electron micrograph as shown in Fig. 2. It can be seen that the monolithic bed linked to the pretreated capillary wall and macropores are feasibly formed by the ternary porogenic solvent. The surface area of the monolithic polymers is about 49.0 m²/g which can afford enough interaction sites. This type of phosphate monolith is relatively hydrophilic and biocompatible due to the usage of cross-linker bis-acrylamide. The monolithic column was applied for the separation of neutral analytes in CEC mode and the obtained electrochromatogram is shown in Fig. 3. It can be seen that baseline separation for neutral analytes was obtained. Neutral analytes were eluted in the order of toluene < dimethylformamide < formamide < thiourea according to their hydrophilicity on the monolithic columns, which illustrated that the matrix of the monolithic stationary possessed relatively strong hydrophilicity. Phosphopeptides have more chance to access Zr⁴⁺ on the hydrophilic surface which can avoid nonspecific adsorption.

3.2 Performance of ZrP-modified monolithic capillary column for isolation of phosphopeptides

The performance of the prepared ZrP-modified monolithic capillary column was evaluated using β-casein as the test protein sample. Phosphopeptides in the tryptic digest of β-casein were enriched by ZrP-modified monolithic capillary as described in Section 2 and analyzed by MALDI-TOF-MS, and the obtained mass spectra of β-casein...
digest before and after enrichment by ZrP-modified monolithic capillary are presented in Figs. 4a and b, respectively. It can be seen that the spectrum in Fig. 4b is much cleaner than that in Fig. 4a. This means the majority of nonphosphorylated peptides were removed after the enrichment by the ZrP-modified monolithic capillary column. Three phosphopeptides were found in Fig. 4b with their sequences listed in Table 1. The detection of phosphopeptides as major peaks in Fig. 4b indicates the high specificity of ZrP-modified monolithic capillary column for the enrichment of phosphopeptides. To elucidate the capture mechanism, a control experiment was also conducted by loading the same sample onto the phosphate-terminated monolithic capillary column. The obtained mass spectrum is shown in Fig. 4c. No ion signal was observed, which meant no peptide was captured by the phosphate-terminated monolithic capillary column. Therefore, the specific capture of phosphopeptides was based on the strong interaction of phosphopeptides with Zr\(^{4+}\).

Phosphoproteins are low abundant components in complex protein samples. In order to further investigate the capability of ZrP-modified monolithic capillary column to enrich low abundant phosphopeptides, BSA tryptic digest was mixed with \(\beta\)-casein tryptic digest in different molar ratio at 1:100 and 1:200 to simulate complex samples. The obtained results are shown in Fig. 5. It was found that phosphopeptides can still be successfully enriched by the monolithic column even at the ratio of 1:200 from Fig. 5c. And almost no BSA tryptic peptides were detected which indicated that the nonspecific adsorption of nonphosphopeptides by the ZrP-modified monolithic capillary column is very low. The high specificity of the ZrP-modified monolithic capillary column for phosphopeptides was further demonstrated by the analysis of tryptic digest of \(\alpha\)-casein, which has more phosphopeptides than \(\beta\)-casein. Seventeen peaks of phosphopeptides from tryptic digest of \(\alpha\)-casein were detected as the obtained mass spectrum shown in Fig. 6. It can be seen that phosphopeptide peaks dominate the mass spec-

**Table 1. The sequences for phosphopeptide peaks in Fig. 4**

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>(M_H^+(\text{Da}))</th>
<th>Number of phosphorylation</th>
<th>Peptide sequence</th>
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</thead>
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<tr>
<td>1</td>
<td>2061.83</td>
<td>1</td>
<td>FQ[pS]EEQQT[DE]ELQDK</td>
</tr>
<tr>
<td>2</td>
<td>2352.85</td>
<td>4</td>
<td>NVPGEV[pS][pS][pS][pS]EESITR</td>
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<td>3</td>
<td>2966.16</td>
<td>4</td>
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</table>

**Figure 2.** Scanning electron micrograph for the end of the phosphate monolith in a fused-silica capillary column with 75 \(\mu\)m id.

**Figure 3.** Separations of polar solutes in hydrophilic interaction mode by CEC. CEC experiment conditions: the phosphate monolith with effective length of 20 cm (total length 27 cm); mobile phase, 10 mM phosphate buffer (pH 3.0) containing 40% ACN; separation voltage 12 kV; detection wavelength, 214 nm; analyte solutes: (1) toluene, (2) dimethylformamide, (3) formamide, (4) thiourea.
Information for the phosphopeptides from α-casein, including amino acid sequence and phosphorylation sites, is shown in Table 2. The above-mentioned results further proved that the ZrP-modified monolithic capillary column can very efficiently capture the phosphopeptides from the digest of phosphoproteins. The good hydrophilicity and the fast mass transfer process of monolithic material resulted in extremely low nonspecific adsorption and good adsorption dynamics, and thereby led to extremely high specificity for phosphopeptide enrichment.

4 Concluding remarks

In conclusion, the phosphate monolithic capillary column was prepared by direct copolymerization of the functional monomer containing phosphate group (EGMP) and cross-linker (bis-acrylamide) in a ternary porogenic solvent. Copolymerization of cross-linker and functional monomer simplifies the procedure for the preparation of phosphate chromatographic media. Besides, the binding capacity of these columns can be
easily controlled by adjusting the amount of functional monomer in the polymerizing mixture. After Zr\(^{4+}\) was immobilized, the ZrP-modified monolithic capillary column showed superior specificity for isolation of phosphopeptides. As the ZrP-modified monolithic capillary column has the ability to specifically enrich extremely low abundance phosphopeptides from complex mixtures, it has potential application in phosphoproteome analysis and determination of protein phosphorylation sites in microliter biological samples.

**Table 2. The sequences for phosphopeptide peaks in Fig. 6**

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>MH(^+) (Da)</th>
<th>Number of phosphorylation</th>
<th>Peptide sequence</th>
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<td>1</td>
<td>EQU[pS][pS]EENSDK</td>
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<td>NANEEDY[pS][pS][pS][pS][SAEER</td>
</tr>
</tbody>
</table>

**Figure 6.** MS spectra of phosphopeptides isolated by the ZrP-modified monolithic capillary column from tryptic digestion of \(\alpha\)-casein (2 pmol).

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**5 References**