The Highly Selective Capture of Phosphopeptides by Zirconium Phosphonate-Modified Magnetic Nanoparticles for Phosphoproteome Analysis

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The highly selective capture of phosphopeptides from proteolytic digests is a great challenge for the identification of phosphoproteins by mass spectrometry. In this work, the zirconium phosphonate-modified magnetic $\text{Fe}_3\text{O}_4/\text{SiO}_2$ core/shell nanoparticles have been synthesized and successfully applied for the selective capture of phosphopeptides from complex tryptic digests of proteins before the analysis of MALDI-TOF mass spectrometry with the desired convenience of sample handling. The ratio of magnetic nanoparticle to protein and the incubation time for capturing phosphopeptides from complex proteolytic digests were investigated, and the optimized nanoparticle-to-protein ratio and incubation time were between 5:1 to 30:1 and 30 min, respectively. The excellent detection limit of 0.5 fmol β -casein has been achieved by MALDI-TOF mass spectrometry with the specific capture of zirconium phosphonate-modified magnetic Fe_3O_4 nanoparticles. The great specificity of zirconium phosphonate-modified magnetic Fe_3O_4 nanoparticles to phosphopeptides was demonstrated by the selective capture of phosphopeptides from a complex tryptic digest of the mixture of β -casein and bovine serum albumin at a molar ratio of 1 to 1000 in MALDI-TOF-MS analysis. An application of the magnetic nanoparticles to selective capture phosphopeptides from a tryptic digest of mouse liver lysate was further carried out by combining with nano-LC-MS/MS and MS/MS analyses, and a total of 194 unique phosphopeptides were successfully identified. © (J Am Soc Mass Spectrom 2008, 19, 1176–1186) © 2008 American Society for Mass Spectrometry

Post-translational modifications of proteins control many biological processes. The reversible phosphorylation of proteins is recognized as an essential post-translational modification regulating cell signaling and ultimately functions of biological systems [1]. The comprehensive understanding of biological processes requires the characterization of protein phosphorylation at molecular level. Mass spectrometry is a fundamental tool for detecting and mapping the phosphoryl modifications of proteins [2–5]. However, due to the minimal amount of phosphorylation found on proteins and the serious ion suppression phenomenon resulting from the proteolytic complexity [6], selective capture of phosphopeptides from complex proteolytic products has been remaining quite a challenge in the field of proteomics.

Up to now, a variety of approaches have been developed for selective determination of phosphopeptides and

thus improve the detection sensitivity of mass spectrometry, such as chemical derivatization [7–9], immunoprecipitation [10,11], strong ion-exchange [12,13], and metal affinity technique [14–24]. Among these, the metal affinity technique using immobilized metal ions or metal oxides as the affinity moieties to phosphopeptides was the most frequently applied method, and has been widely applied for the selective capture or even purification of phosphopeptides via the specific binding between the metal moieties and the phosphoryl groups of peptides and proteins. For instance, the immobilized metal ions of Fe^{3+} , Ga^{3+} , and Zr^{4+} on chromatographic adsorbents showed the specific binding characteristics to capture phosphopeptides [20,23,24], and the titanium dioxide (TiO_2) and zirconium dioxide (ZrO_2) demonstrated the binding specificity to phosphopeptides of the complex proteolytic digests via the bidentate interactions [17–19].

Magnetic nanoparticles have been extensively applied in chemistry, biochemistry, biology, and medicine, such as drug carriers, bioseparation, sensors, and enzyme immobilization [25–28] because of the great convenience of operations by applying a magnetic field and along with the distinct features resulting from the high surface-to-volume ratio [29]. In phosphoproteome,

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the magnetic nanoparticles have been implemented for the selective capture and/or enrichment of phosphopeptides from the complex proteolytic digests by using the metal affinity technique for mass spectrometry. For example, Chen et al. [17, 18] used the TiO_2 and ZrO_2 -coated iron oxide magnetic nanoparticles as the effective affinity probes for the selective concentration of phosphopeptides from the tryptic protein digests in MALDI-TOF MS with a low-femtomole limit of detection; Tan et al. [30] directly employed the TiO_2 -modified magnetic nanoparticles on MALDI targets, which was also demonstrated the specific capture and confident detection of phosphopeptides in MALDI-TOF-MS.

Recently, a new method for the specific trapping of phosphopeptides from the proteolytic digests was developed using the Zr^{4+} -IMAC adsorbents, which showed higher specificity for phosphopeptides compared with ZrO_2 and TiO_2 beads [15, 19, 23]. Here, the zirconium-phosphate (Zr^{4+} - PO_3)-modified magnetic $\text{Fe}_3\text{O}_4/\text{SiO}_2$ (core/shell) nanoparticles were synthesized for highly selective capture of phosphopeptides from protein digests. The synthesized magnetic nanoparticles were first evaluated with tryptic digests of ovalbumin and α - and β -caseins, and then were further applied for the analysis of phosphopeptides with the tryptic digest of a mouse liver lysate for the phosphoproteome analysis.

Experimental

Materials and Reagents

α -Casein and β -casein (from bovine milk), trypsin, bovine serum albumin (BSA), 2,5-dihydroxybenzoic acid (2,5-DHB), zirconium oxychloride ($\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$), tetraethoxysilane (TEOS), 2,4,6-collidine, and 3-aminopropyltriethoxysilane (APTES) were purchased from Sigma-Aldrich (St. Louis, MO). Urea, ammonium bicarbonate, dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from BioRad (Hercules, CA). Iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), iron (II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), ammonium hydroxide, and phosphorus oxychloride (POCl_3) were obtained from Tianjin Chemical Plant (Tianjin, China). Acetonitrile, ethanol and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore, Milford, MA).

Synthesis of Fe_3O_4 Magnetic Nanoparticles

The Fe_3O_4 magnetic nanoparticles were synthesized using a literature procedure [17] with certain small modifications. Briefly, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (2.0 g) and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (5.4 g) were first dissolved in a 2 M hydrochloric acid solution (25 mL), which was then sufficiently degassed with a nitrogen stream. After that, a 25.0% (vol/vol) ammonium hydroxide solution (30 mL) was added to the solution for a 30 min reaction at 60 °C with vigorous stirring under a nitrogen atmo-

sphere. After cooling to ambient temperature, the resultant magnetic nanoparticles were attracted at the bottom by a magnet. Followed by the rinse with sufficient water for three times, the magnetic nanoparticles were finally resuspended in 50 mL water to give a suspension of ca. 40 mg/mL of magnetic nanoparticle in water.

Preparation of SiO_2 -Shell on Fe_3O_4 Magnetic Nanoparticles

The above-obtained Fe_3O_4 magnetic nanoparticles (40 mg/mL, 5 mL) were washed twice with ethanol, and resuspended in ethanol (80 mL) with 1 h sonication. Followed by a sequential addition of ammonia (25%, 8.95 mL), water (7.5 mL), and TEOS (1.0 mL), the resulting mixture was stirred vigorously for 2 h on a water bath at 40 °C. The resultant magnetic $\text{Fe}_3\text{O}_4/\text{SiO}_2$ core-shell nanoparticles were then rinsed with ethanol (30 mL) three times and resuspended in ethanol (30 mL), and subsequently followed by a 12 h reflux at 60 °C to strengthen the Si-O-Fe bonds. The above procedures were repeated once more to ensure the sufficient cover of SiO_2 shell on Fe_3O_4 magnetic nanoparticles.

Functionalization of Magnetic $\text{Fe}_3\text{O}_4/\text{SiO}_2$ Core/Shell Nanoparticles

The above produced $\text{Fe}_3\text{O}_4/\text{SiO}_2$ core/shell magnetic nanoparticles were dried under vacuum at 60 °C for 12 h and dispersed in dry toluene (45 mL) with sonication. Followed by an addition of APTES (1.5 mL), the mixture was refluxed at 110 °C for 12 h under a nitrogen atmosphere. After cooled to ambient temperature, the resultant nanoparticles were washed with dry toluene, water and ethanol twice, respectively. With a further drying process in a vacuum oven at room temperature, the dried aminopropyl-modified magnetic nanoparticles were incubated in an anhydrous acetonitrile solution (50 mL) containing 40 mM POCl_3 and 40 mM 2,4,6-collidine at ambient temperature for 12 h. After rinsing with ACN and water, respectively, the resultant phosphonate-modified nanoparticles were incubated in a 50 mM ZrOCl_2 solution with gentle stirring overnight for the loading of Zr^{4+} cations. Finally, the prepared zirconium-phosphonate-modified nanoparticles were rinsed with deionized water to remove the nonspecifically adsorbed Zr^{4+} cations, and dried under vacuum at 30 °C for 12 h.

Tryptic Digestion of Proteins

α -Casein and β -casein (1 mg) were respectively dissolved in a 1 mL of ammonium bicarbonate buffer (50 mM, pH 8.2), and digested at 37 °C for 16 h with trypsin at the ratio of enzyme-to-substrate of 1:40 (wt/wt). BSA (6.6 mg) and ovalbumin (4 mg) were, respectively, dissolved in 1 mL denaturing buffer containing 8 M

urea and 50 mM ammonium bicarbonate; after the addition of 20 μ L of DTT (50 mM), the mixtures were incubated at 60 °C for 1 h to reduce the disulfide bonds of proteins; subsequently, 40 μ L of IAA (50 mM) was added and the mixtures were then incubated at room temperature in dark for 30 min; finally, the mixtures were diluted 10-fold with 50 mM ammonium bicarbonate buffer (pH 8.2) and digested at 37 °C for 16 h with trypsin at the ratio of enzyme-to-substrate of 1:40 (wt/wt). The proteins from the mouse liver were obtained according to the procedure described in a literature [4], and the Bradford protein assay was used to quantify the concentration of the extracted proteins. The tryptic digestion of the proteins from mice liver was as the same as that of BSA. All of the obtained proteolytic digests were stored in a –30 °C freezer before MALDI-TOF MS analysis.

The Capture of Phosphopeptides Using Zr^{4+} - PO_3 -modified Fe_3O_4/SiO_2 Magnetic Nanoparticles

Protein digests were diluted with loading buffer (pH 0.85) containing 6% TFA and 80% (vol/vol) ACN. A protein digest solution (1 pmol, 2 μ L) was added into a 10 μ L suspension of $ZrPO_3$ -modified magnetic Fe_3O_4 nanoparticles (15 mg/mL) in loading buffer, and incubated at room temperature for 30 min. After that, the magnetic nanoparticles were held by a magnet and the supernatant was removed by Eppendorf pipette, and then the magnetic nanoparticles were rinsed with 20 μ L of loading buffer solutions containing 6% TFA and 80% (vol/vol) ACN with and without 200 mM NaCl, respectively. After the evaporation of solvent, 5 μ L of 25 mg/mL 2, 5-DHB solution containing 70% ACN and 1% H_3PO_4 were added and mixed homogeneously with these dried nanoparticles, where the addition of H_3PO_4 in matrix solution was for the enhancement of phosphopeptide signals on MALDI-TOF MS analysis [31]. Finally, a 0.5 μ L aliquot of the matrix-nanoparticle suspension was directly deposited on MALDI-target for MALDI-TOF MS analysis. For phosphoproteome analysis of mouse liver lysate, the magnetic nanoparticles (100 μ L, 15 mg/mL) were incubated with the tryptic digest of 200 μ g mouse liver lysate. Then the nanoparticles were rinsed with 200 μ L of loading buffer solutions containing 0.5% TFA and 80% (vol/vol) ACN with and without 200 mM NaCl, respectively. Subsequently, the trapped phosphopeptides were eluted from the nanoparticles as followings: (1) by an 80% (vol/vol) ACN solution containing 6.0% TFA and with 10 min incubation, and (2) by an NH_4OH solution (pH 11.5, 70 μ L) with a 15 min sonication. By holding the magnetic nanoparticles with a magnetic field, the eluted supernatant was respectively collected, lyophilized, and then redissolved in 4 μ L of 0.1% formic acid for nano-LC-MS/MS and MS/MS/MS analysis.

Mass Spectrometry

All MALDI-TOF mass spectra were acquired by a Bruker Autoflex time-of-flight mass spectrometer (Bruker, Bremen, Germany) equipped with a delayed ion-extraction device and a 337-nm pulsed nitrogen laser. The MALDI uses a ground-steel sample target with 384 spots. The range of laser energy was adjusted to slightly above the threshold for obtaining good resolution and signal-to-noise ratio. All measurements were carried out in linear positive-ion mode with delayed ion extraction. The delay time for ion extraction and the extraction voltage were set at 90 ns and 20 kV, respectively. Each MS spectrum was acquired by the accumulation of 30 laser shots.

All nano-LC-MS analyses were performed on a ThermoFinnegan LTQ linear ion trap mass spectrometer with a nanospray ion source and a Surveyor HPLC system (ThermoFinnegan, San Jose, CA). The flow rate of HPLC pump was split to 200 nL/min by a cross for capillary separation. A bare fused silica capillary (75 μ m i.d. $\times$ 120 mm length) was manually pulled to form a MS emitter tip (ca. 5 μ m i.d.) at one end, and then was in-house packed with C18 AQ particles (5 μ m, 120 Å; Michrom BioResources, Auburn, CA) using a pneumatic packing device. The packed capillary column was directly coupled to the LTQ mass spectrometer; 0.1% formic acid in H_2O was used as the mobile phase A, and 0.1% formic acid in acetonitrile was used as the mobile phase B. The LTQ mass spectrometer was operated at positive ion mode. A voltage of 1.8 kV was applied to the cross. About 1 μ L (50 μ g) of redissolved peptides was loaded onto a C18 capillary column as sample loop and separated on the capillary column with a linear gradient where the mobile phase B was programmed from 5 to 35% in 75 min. For the detection of phosphopeptides, the mass spectrometer was set to perform a full scan MS followed by three data-dependant MS/MS (MS^2). Subsequently, an MS/MS/MS (MS^3) (spectrum was automatically triggered when the most three intense peaks from the MS/MS spectrum corresponded to the neutral loss event of 98, 49, and 33 ± 1 Da for the precursor ion with 1+, 2+, 3+ charge states, respectively).

Database Search and Data Analysis

The peak lists for MS^2 and MS^3 spectra were generated from the raw data by Bioworks (Thermo-Electron, Boston, MA) with parameters: mass range, 600 to 3500; intensity threshold, 1000; minimum ion count, 10. The MS^2 and MS^3 spectra were searched using SEQUEST [32] (version 2.7) against a composite database including both original database and the reversed version of the forward one with parameters: enzyme, trypsin (KR/P); enzyme limits, fully enzymatic (cleaves at both ends); precursor-ion mass tolerance, 2 Da; fragment-ion mass tolerance, 1 Da; missed cleavages, 2; static modification, Cys (+57). For search with MS^2 data, the

dynamic modifications were set for oxidized Met (+16), and phosphorylated Ser, Thr, and Tyr (+80). For search with MS³ data, besides the parameters for MS², dynamic modifications were also set for water loss on Ser, Thr (−18). The original database is the non-redundant mouse protein database of the International Protein Index (ipi.MOUSE.3.17.fasta), which includes 51,446 entries. For the identification of nonphosphopeptides by MS², the criteria were set at: cross-correlation value (Xcorr) ≥ 1.9, 2.2, 3.75 for single, double, triple charges, and Delta Cn ≥ 0.1 [33–35]. For the identification of phosphopeptides by matching the assigned sequences derived from MS² and MS³ data, the homemade software named APIVASE [36] was used to validate the identification. With this software, the MS²/MS³ target-decoy database search approach (or called MS²/MS³ approach in short) was applied to identify the most likely phosphorylation sites via following four steps: (1) extraction of the valid MS²/MS³ pairs by removal of the MS²/MS³ pairs with incorrect charge states, MS³ not triggered, and intensity of the peak corresponding to neutral loss less than 50% of the base peak in MS²; (2) performing MS² and MS³ target-decoy database search with the valid MS² and MS³ spectra, respectively; (3) reassignment of peptide scores in SEQUEST output to generate the peptide lists with the reassigned scores for MS² and MS³; (4) filtering the candidate phosphopeptides with new defined parameters (Rank'm, DCn'm, and Xcorr's) to achieve phosphopeptide identification with specific FDR. In this study, to achieve the false discovery rate (FDR) of 1%, the following filter criteria were used: Rank'm = 1, DCn'm ≥ 0.1, and Xcorr's ≥ 0.5350. The phosphoproteins identified by the same phosphopeptide(s) were grouped, if the group contained more than one phosphoprotein then only one was kept according to the method described by He et al. [37] as all proteins in each group are highly homologous, generally belonging to the same superfamily, or just different alternative splicing isoforms. Finally, to investigate the reliability of the peptide identification, the PhosphoSite (<http://www.phosphosite.org>) was used to distinguish the known from novel phosphorylation sites.

Results and Discussion

Characteristics of Zr⁴⁺-PO₃-Modified Magnetic Fe₃O₄/SiO₂ (Core/Shell) Nanoparticles

To demonstrate the preparation of the Zr⁴⁺-PO₃-modified magnetic Fe₃O₄/SiO₂ (core/shell) nanoparticles, a synthesis procedure is illustrated in Scheme 1. Briefly, the magnetic Fe₃O₄ nanoparticles were first prepared via a solvothermal method, and coated with a layer of SiO₂ generated from the hydrolysis and condensation of TEOS. Subsequently, the surface silanol groups on the SiO₂ layer were derivatized to aminopropyl groups by APTES and further transferred to phosphonate groups by POCl₃. Lastly, after the reaction with ZrOCl₂, the

Scheme 1. Synthesis of Zr⁴⁺-PO₃-modified magnetic nanoparticles. TEOS: tetraethoxysilane; APTES: 3-aminopropyl-triethoxysilane.

Zr⁴⁺ cations were immobilized on the phosphonated magnetic nanoparticles for selective capture of phosphopeptides from proteolytic digests due to the coordination of zirconium ion and the phosphate moieties from phosphopeptides. The TEM image (Supplementary Figure 1, which can be found in the electronic version of this article) of the Zr⁴⁺-PO₃-modified magnetic nanoparticles was recorded at a high magnification for nanoparticle characterization. The scale bar represents 50 nm. The obtained nanoparticles were aggregated, and the estimated average diameter of nanoparticles was ~16 nm.

Enrichment of Phosphopeptides from Trypsin Digests of Standard Phosphoproteins

To examine the specific selectivity of the prepared Zr⁴⁺-PO₃-modified magnetic Fe₃O₄ nanoparticles for trapping phosphopeptides, the proteolytic digests of bovine β -casein that possess five known phosphorylation sites have been extensively selected as the test standard phosphoprotein with detection of MALDI-TOF mass spectrometry [15, 24, 38]. First, the tryptic digest of bovine β -casein (1 pmol) was incubated with certain amount (150 μ g) of Zr⁴⁺-PO₃-modified magnetic Fe₃O₄ nanoparticles at room-temperature for 30 min. By holding the magnetic nanoparticles with a magnet, the supernatant was removed by a pipette. Followed by a series of rinsing steps for removing nonphosphorylated peptides and salts from the nanoparticles, the MALDI matrix of 2,5-DHB was added and mixed with the peptide-bound magnetic nanoparticles and deposited on MALDI target for mass spectrometry analysis. For comparison, the same amount of the tryptic digest of β -casein (1 pmol) was directly analyzed by MALDI-TOF MS without the trapping steps by Zr⁴⁺-PO₃-magnetic nanoparticles. Figure 1a represents the direct MALDI-TOF mass spectrum of the tryptic digest of β -casein (1 pmol) without the treatment of the nanoparticles, while Figure 1b represents the obtained MALDI-TOF mass spectrum of the tryptic digest of the same amount of β -casein (1 pmol) after treated by Zr⁴⁺-PO₃-magnetic nanoparticles. In Figure 1a, the peaks of the nonphosphorylated peptides and phosphorylated peptides arising from the tryptic digest of β -casein were all

Figure 1. MALDI-TOF-MS analysis of tryptic digests of (a) β -casein (1 pmol, 2 μ L) by direct analysis; (b) β -casein (1 pmol, 2 μ L) treated by Zr^{4+} - PO_3 -modified magnetic nanoparticles; (c) β -casein (1 pmol, 2 μ L) mixed with standard phosphotyrosine (pY) at ratio of 1:1 and subsequently treated by Zr^{4+} - PO_3 -modified magnetic nanoparticles; (d) analysis of β -casein (1 pmol, 2 μ L) treated by PO_3 -magnetic nanoparticle.

detected in the direct MALDI MS analysis manner, where the phosphorylated peptides were marked as β_1 , β_2 , and β_3 at m/z 2061.94, 2556.93, and 3122.56, respectively; however, the nonphosphorylated peptide peak at 1384.30 dominates the mass spectrum and lots of nonphosphopeptides appear in the mass spectrum. In contrast, Figure 1b shows a much clearer mass spectrometric pattern with only four phosphopeptide peaks from the tryptic digest of β -casein after the specific capture by Zr^{4+} - PO_3 -magnetic nanoparticles. The identified phosphopeptides in amino acid sequence pattern were listed in Table 1. One (S50) of the five known phosphorylation sites (S30, S32, S33, S34, and S50) of bovine β -casein was founded in both phosphopeptides of β_1 (48-FQ[L]S]EEQQQTEDELQK-63, m/z 2061.94) and β_2 (48-FQ[L]S]EEQQQTEDELQDKIHPF-67, m/z 2556.93). Other four phosphorylation sites of bovine β -casein were identified in phosphopeptide β_3 (16-RELEELN-VPGEIVE[L]S]L[L]S]L[L]S]EESITR-40, m/z 3122.56). And β_3^{2+} represents the doubly charged ion derived from β_3 . As can be seen in Figure 1b, the peak intensities of these isolated phosphorylated peptides were significantly enhanced ~ 5 -fold. This is because of the elimination of ion

suppression from nonphosphorylated peptides and salts after the selective capture of phosphopeptides using the Zr^{4+} - PO_3 -modified magnetic nanoparticles. Two other standard phosphoproteins (α -casein and ovalbumin) were also used to examine the selectivity of Zr^{4+} - PO_3 -modified magnetic nanoparticles; the identified phosphopeptides are listed in Table 1. These results indicate that the Zr^{4+} - PO_3 -modified magnetic nanoparticles have the specific selectivity for capturing phosphopeptides and thus as the promising substrate for isolation of phosphorylated peptides from a proteolytic digest mixture to increase the detection sensitivity of phosphopeptides with the detection of MALDI-TOF MS.

The above used phosphoproteins of α -casein and β -casein all possess phosphorylation sites at serine residues. To evaluate the capture performance of Zr^{4+} - PO_3 -modified magnetic nanoparticles towards other peptides phosphorylated at different amino acid residues, a singly tyrosine phosphorylated peptide of RRLIEDAE[L]Y]AARG (where pY represents phosphotyrosine; molecule weight, 1599) was added to a tryptic digest of β -casein at a 1:1 M ratio, and followed by a trapping process of phosphopeptides by Zr^{4+} - PO_3 -modified magnetic nanoparticles and the analysis of MALDI-TOF MS. As shown in Figure 1c, four dominant peaks representing three phosphoserine peptides from β -casein (β_1 , β_2 , β_3) and one phosphotyrosine peptide (pY) can be clearly observed. This result indicated that Zr^{4+} - PO_3 -modified magnetic nanoparticles do possess the promising ability to selectively trap other types of phosphopeptides without bias.

To further confirm the interacting moiety of the prepared Zr^{4+} - PO_3 -magnetic nanoparticles for selectively isolating phosphopeptides, the tryptic digest of β -casein (1 pmol) were also analyzed by MALDI-TOF mass spectrometry by using PO_3 -modified magnetic nanoparticles without loading of zirconium cations. Figure 1d represents the obtained MALDI mass spectrum of the tryptic digest of β -casein using the PO_3 -modified magnetic nanoparticle instead of the Zr^{4+} - PO_3 -modified magnetic nanoparticles. As expected, no any peaks representing the phosphorylated peptides from β -casein was observed. Based on this result, it can be confirmed that the selective capture of phosphopeptides is from the strong specific interaction between the immobilized zirconium cations and the phosphoryl groups of phosphopeptides.

Inspired by the satisfactory enrichment of phosphopeptides derived from β -casein, we further applied the Zr^{4+} - PO_3 -modified magnetic nanoparticles on the selective capture of phosphopeptides from the proteolytic digest of ovalbumin, a diphosphorylated protein containing a disulfide bond. For ovalbumin, the addition of urea, DTT, and IAA are necessary for denaturing, opening disulfide bond and carbamidomethylating cysteine groups before tryptic digestion. It is of interest to investigate the impacts of urea, DTT, and IAA for tryptic digestion on the selective capture of phos-

Table 1. Identification of phosphopeptides from α -casein, β -casein and ovalbumin tryptic digests by using Zr^{4+} - PO_3 -modified magnetic Fe_3O_4 nanoparticles as affinity probes in MALDI-TOF MS analysis

Protein	No.	Amino acid sequence ^a	Number of phosphoryl groups	Measured m/z
α -Casein	α_1	TVDME[_p S]TEVF	1	1237.08
	α_2	TVD[Mo]ME[_p S]TEVF ^b	1	1253.11
	α_3	EQL[_p S]T[_p S]EENSK	2	1411.90
	α_4	TVDME[_p S]TEVFTK	1	1466.97
	α_5	TVD[Mo]E[_p S]TEVFTK ^b	1	1482.61
	α_6	EQL[_p S]T[_p S]EENSKK	2	1538.31
	α_7	VPQLEIVPN[_p S]AEER	1	1660.15
	α_8	YLGEYLIVPN [_p S]AEER	1	1832.70
	α_9	DIGSE[_p S]TEDQAMEDIK	1	1847.95
	α_{10}	DIG[_p S]E[_p S]TEDQAMEDIK	2	1927.89
	α_{11}	DIG[_p S]E[_p S]TEDQA[Mo]EDIK ^b	2	1943.89
	α_{12}	YKVPQLEIVPN[_p S]AEER	1	1951.09
	α_{13}	KKYKVPQLEIVPN[_p S]AEERL	1	2080.00
	α_{14}	NTMEHV[_p S][_p S][_p S]EESII[_p S]QETYK	4	2618.93
	α_{15}	VNEL[_p S]KDIG[_p S]E[_p S]TEDQAMEDIK	3	2678.02
	α_{16}	Q*MEAE[_p S]I[_p S] [_p S] [_p S]EEIVPN[_p S]VEAQK ^c	5	2703.75
	α_{17}	QMEAE[_p S]I[_p S][_p S][_p S]EEIVPNPN[_p S]VEQK	5	2720.05
	α_{18}	KEKVNEL[_p S]KDIG[_p S]E[_p S]TEDQAMEDIKQ	3	2935.87
	α_{19}	NANEEYSIG[_p S][_p S][_p S]EE[_p S]AEVATEEVK	4	3008.22
	α_{20}	NANEEY[_p S]IG[_p S][_p S][_p S]EE[_p S]AEVATEEVK	5	3087.93
	α_{21}	KNTMEHV[_p S] [_p S] [_p S]EE[_p S]ISOQETYKQEK	4	3132.23
β -Casein	β_1	FQ[_p S]EEQQQTEDELQDK	1	2061.94
	β_2	FQ[_p S]EEQQQTEDELQDKIHFP	1	2556.93
	β_3	RELEELNVPGEIVE[_p S]L[_p S][_p S]EESITR	4	3122.56
Ovalbumin	O ₁	EVVG[_p S]AEAGVDAASVSEEF	1	2090.62
	O ₂	FDKLPFGGD[_p S]IEAQCGTSVNVHSSLR	1	2903.46

^pS: phosphorylated site.^aThe amino acid sequence are cited from Larsen et al. [15] and Kweon et al. [16].^bOxidation on methionine.^cPyroglutamylation on the N-terminal Q*.

phopeptides by Zr^{4+} - PO_3 -modified magnetic nanoparticles. Figure 2a and b, represent MALDI mass spectra of the tryptic digest of ovalbumin (1 pmol, 2 μL) before and after phosphopeptide captured by Zr^{4+} - PO_3 -modified magnetic nanoparticles. By comparing Fig. 2a and b, it clearly shows that the phosphopeptides

at m/z 2090.62 and 2903.46 can be distinctly isolated from the strong background of nonphosphopeptides without the loss of peak intensity by Zr^{4+} - PO_3 -modified magnetic nanoparticles. The amino acid sequences of detected phosphopeptides of ovalbumin with marked phosphorylation sites are illustrated in Table 1. It can be concluded that the use of urea, DTT, and IAA for tryptic digestion will not impact the specific capture of phosphopeptides with Zr^{4+} - PO_3 -modified magnetic nanoparticles. This is a very interesting merit for using Zr^{4+} - PO_3 -modified magnetic nanoparticles because the denaturing processes are routinely applied in proteome analysis.

Figure 2. MALDI-TOF mass spectra of a tryptic digest of ovalbumin (1 pmol, 2 μL) (a) by direct analysis; (b) treated by Zr^{4+} - PO_3 -modified magnetic nanoparticles.

Effects of Incubation Conditions on Capture of Phosphopeptide

To evaluate the effect of the amount of Zr^{4+} - PO_3 -modified magnetic nanoparticles on the specific capture of phosphopeptides, the Zr^{4+} - PO_3 -modified magnetic nanoparticle suspensions at different concentrations with a certain volume were used for isolating phosphopeptides and followed by the detection of MALDI-TOF MS. Figure 3 shows the peak intensities of the isolated phosphorylated peptides from the tryptic digests of β -casein (1 pmol) at the different ratios of

Figure 3. The effect of the ratio of nanoparticle-to-protein (mg/mL)/pmol on signal intensities (averaged, $n = 3$) of phosphopeptides from a tryptic digest of β -casein (1 pmol, 2 μ L).

nanoparticle-to-protein (mg/mL of nanoparticle versus pmol of protein) ranged from 1:1 to 500:1. As shown in Figure 3, at low ratio of nanoparticle-to-protein of 1:1, only two dominant peaks (β_3 and β_3^{2+}) representing multiphosphopeptides from the tryptic digest of β -casein can be observed, whereas other monophosphopeptides in the tryptic digest could not be detected, possibly due to stronger chelating interaction between multiphosphopeptide and Zr^{4+} cations on nanoparticles than that for monophosphopeptides, which resulted in the competitive occupation of the Zr^{4+} sites by multiphosphopeptides. When the ratio increased to 5:1, the other two peaks (β_1 and β_2) of monophosphopeptides were then observed as well as the increase of the peak intensity of β_3 and β_3^{2+} . By further increasing the ratio to 10:1, 15:1, the intensities of detectable peaks of phosphopeptides were also increased, and β_3^{2+} , β_1 , and β_3 reached the summit at the ratio of 15:1 with the expected highest capacity for phosphopeptides enrichment. After this summit, the peak intensities of β_3^{2+} , β_1 , and β_3 started to decline when the ratio increased to 30:1, while the peak intensity of the monophosphopeptides of β_2 increased to the summit. Interestingly, when the ratio kept on increasing to 100:1 and 500:1, the peak intensities of phosphopeptides then declined. This phenomenon clearly indicated that the phosphopeptides in digestion solution have not been maximally adsorbed when the ratio of 15:1 of nanoparticle-to-protein was less than 15:1. So, the more nanoparticles used, the more phosphopeptides were adsorbed onto the nanoparticles, and the peak intensities of phosphopeptides were thus proportionally increased in MALDI-TOF MS. However, after the ratio of 15:1, the more nanoparticles used would result in the decrease of the specific concentrations of the phosphopeptides adsorbed on the surface of the nanoparticles and consequently caused the decrease of the peak intensities of the phosphopeptides. Based on the above observation, the ratio of nanoparticle-to-protein of 15:1 was selected to capture phosphopeptides from tryptic digests, which corresponded to the concentration of nanoparticle suspension of 15 mg/mL for final use.

Besides the selection of the ratio of nanoparticle-to-protein, the incubation time for phosphopeptides enrichment was also examined. Figure 4 displays the MALDI mass spectra of the phosphopeptides isolated by Zr^{4+} - PO_3 -modified magnetic nanoparticles with different incubation time ranged from 0.5 min to 60 min. As seen in Figure 4, four intensive phosphopeptide peaks can be observed even the incubation time is only 0.5 min, when the incubation process remained longer than 30 min, the best peak intensity could be achieved, it can be assumed that the chelating interaction between the zirconium cations and the phosphoryl groups of phosphopeptides have achieved equilibrium. These results indicated that the affinity nanoparticles have the good selectivity to capture phosphopeptides even in short interacting time. For obtaining better analysis results of phosphopeptides, incubation time of 30 min was selected.

Detection Sensitivity for Phosphopeptides Using Zr^{4+} - PO_3 -Magnetic Nanoparticles

After going through the same trapping procedure by using Zr^{4+} - PO_3 -modified magnetic nanoparticles as the affinity probes to phosphopeptides with MALDI-TOF MS analysis, the detection sensitivity of phosphopeptides on this approach was evaluated by using 5 fmol and 0.5 fmol of β -casein with the ratio of nanoparticle-to-protein at 15:1 and the incubation time at 30 min. The resulting MALDI-TOF mass spectra for 5 fmol and 0.5 fmol of β -casein were shown in Figure 5a and b, respectively. It can be seen that the three phosphopeptide peaks of β_1 , β_2 , and β_3 were well detected at these low femtomole levels by mass spectrometry after the enrichment of nanoparticles. The signal-to-noise (S/N) of detected phosphopeptides of β_1 , β_2 , and β_3 derived from 5 fmol β -casein were 16, 6, and 7, respectively. For even lower level of 0.5 fmol β -casein, these three β_1 , β_2 , and β_3 phosphopeptides peaks were still able to be detected with S/N of ca. 3. This low fmol level was considered as the detection limit of this approach for

Figure 4. The effect of the incubation time on peak intensities of phosphopeptides captured by Zr^{4+} - PO_3 -magnetic Fe_3O_4/SiO_2 nanoparticles from a tryptic digest of β -casein (1 pmol, 2 μ L).

Figure 5. MALDI-TOF mass spectra of a tryptic digest of β -casein of (a) 5 fmol, 6 μ L; and (b) 0.5 fmol, 8 μ L, treated by Zr^{4+} - PO_3 -magnetic $\text{Fe}_3\text{O}_4/\text{SiO}_2$ nanoparticles, respectively.

detecting phosphopeptides, which is lower than that of other approaches by using TiO_2 beads, ZrO_2 beads and Fe^{3+} -immobilized affinity chromatography as the affinity probes in conjunction with MALDI-TOF MS analysis [15, 17, 18, 31]. The lower limit of detection for phosphopeptide could be attributed to large surface-to-volume ratio of the magnetic nanoparticle, which results in the high loading capacity of Zr^{4+} cations and consequently large trapping capacity towards phosphopeptides of proteolytic digests.

Validating the Selective Capture of Phosphopeptides from Other Proteolytic Digests

Phosphoproteins are typically at low abundance in real biological proteins samples. The specific isolation of phosphopeptides with the interference of a large amount of nonphosphorylated peptides is an important technique for phosphoproteome analysis. However, Fe^{3+} -IMAC adsorbents showed not enough specificity for selective capture of phosphopeptides from a complex peptides mixture. Using Fe^{3+} -IMAC to capture phosphopeptides, Larsen et al. [15] found that the number of nonphosphopeptide peaks increased significantly, while the number of detected phosphopeptides decreased dramatically when the ratio of phosphoproteins (α -casein, β -casein, and ovalbumin) to nonphosphoproteins (serum albumin, β -lactoglobulin, and carbonic anhydrase) in a protein sample decreased to 1:10; and when the ratio further decreased to 1:50, the peaks of phosphopeptides could hardly be detected. This phenomenon was also observed by Zhou et al. [39]. To examine the performance of Zr^{4+} - PO_3 -modified magnetic nanoparticles for selective capturing phosphopeptides from a complex protein sample, the mixture of

standard proteins of α -casein and BSA was used. Figure 6 displays the obtained MALDI mass spectra of captured phosphopeptides from the tryptic digest of the mixture with different molar ratios of 1:0, 1:10, and 1:100 of α -casein to BSA. In Figure 6a, many nonphosphopeptides were observed in mass spectrum. However, in Figure 6c, 20 phosphopeptides including mono- and multiphosphorylated peptides were clearly detected at the molar ratio of α -casein to BSA of 1:10. Although the peak intensities of phosphopeptides were decreased due to the increased interference from nonphosphorylated peptides at higher ratio of protein-to-BSA, this result is still as good as that of α -casein without mixed with BSA, as shown in Figure 6b, where 21 phosphopeptides were observed. The identified phosphopeptides from α -casein, including amino acid sequence and phosphorylation sites, are listed in Table 1. By further decreasing the molar ratio to 1:100, 17 phosphopeptides still could be detected with the similar peak pattern of phosphopeptides with the disappearance of three phosphopeptide the peaks at m/z 1411.90, 1538.31, and 2678.02 as shown in Figure 6d. But, considering the level of α -casein is two orders of magnitude lower than that of BSA in sample, the specificity of Zr^{4+} - PO_3 -modified magnetic nanoparticles is good enough for the selective capture of phosphopeptides. This could be contributed by the enhanced surface area of nanoparticles with the increased loading capacity of Zr^{4+} cations for binding phosphopeptides.

Application of Zr^{4+} - PO_3 -Magnetic Nanoparticles for Phosphoproteome Analysis of Mouse Liver

To further evaluate the performance for selective capture of phosphopeptides in real complex biological samples, Zr^{4+} - PO_3 -magnetic nanoparticles were also applied to analyze the phosphoproteome of mouse liver. Based on a further investigation on the effect of TFA content in washing solution, we found that some phosphopeptides could be lost when 6% TFA was used in washing step, which could be avoided by using a washing solution containing lower TFA content of 0.5% (see Supplementary Figure 2). Thus, a two-step elution protocol was applied for eluting the phosphopeptides from nanoparticles based on the pre-removal of nonphosphopeptides by the loading solution containing 0.5% TFA. Namely, the eluents were collected by rinsing the nanoparticles with the 80% acetonitrile solution containing 6% TFA (first eluting step) and the NH_4OH solution (second eluting step), respectively. Then, both collected eluents were analyzed by nano-LC-MS/MS and MS/MS, respectively, as described in the Experimental section. The MS^2 and MS^3 spectra obtained were searched against database following the procedures described in the Experimental section, and an automatic validation approach, i.e., MS^2/MS^3 approach [36] was used to process these data. Phosphopeptide identification by matching the assigned sequences de-

Figure 6. MALDI-TOF mass spectra of (a) α -casein tryptic digest (1 pmol, 2 μ L) by direct analysis; the mixtures of α -casein (1 pmol, 2 μ L) and BSA at ratios of (b) 1:0, (c) 1:10, and (d) 1:100, respectively, treated by Zr^{4+} - PO_3 -modified magnetic nanoparticles.

rived from MS^2 and MS^3 data were described in the Experimental section. As a result, the total of 397 unique peptides including 203 nonphosphorylated peptides and 194 phosphorylated peptides were successfully identified. Among the 194 unique phosphorylated peptides, 39 (29 singly phosphorylated and 10 multi phosphorylated) were identified in the first eluting step and 155 (21 singly phosphorylated and 134 multi phosphorylated) were identified in the second eluting step, by the automatic validation approach. The sequences of the identified phosphopeptides and their Xcorr scores are listed in Supplemental Table 1. The majority of these peptides have very high Xcorr scores. In the first eluting step, the monophosphopeptides constitute 74.36% of all identified phosphopeptides, whereas they accounted for only 13.54% in the second eluting step. This might be ascribed to that fact the monophosphorylated peptides are supposed to have lower binding affinities with Zr^{4+} - PO_3 -magnetic nanoparticles, and nearly eluted by loading solution containing 6% TFA. On the other hand,

the multiphosphorylated peptides had higher binding affinities and were eluted in the second step by the solution of NH_4OH , which is beneficial to the analysis of multiphosphorylated peptides by MS spectrometry because they could be easily suppressed by the presence of singly phosphorylated peptides owing to their poorer ionization efficiency.

Finally, to investigate the reliability of the results, PhosphoSite (<http://www.phosphosite.org>) was further used to distinguish the known from novel phosphorylation sites for the mouse liver lysate. For the 194 identified phosphorylated peptides with 318 phosphorylated sites, it was found 88.99% (283 sites) were identified as the known phosphorylation sites (see Supplemental Table 1). These results indicated the phosphopeptides identified both by MS^2 and MS^3 spectra are of high confidence. These results also showed that the Zr^{4+} - PO_3 -magnetic nanoparticles do have high specificity for the capture of phosphopeptides in real complex biological samples and are of very promising

in the purification of phosphopeptides for phosphoproteome analysis.

Conclusions

In this work, the zirconium phosphonate-modified magnetic $\text{Fe}_3\text{O}_4/\text{SiO}_2$ (core/shell) nanoparticles have been successfully prepared and applied as the affinity probes for the specific capture of phosphopeptides from complex tryptic digests with the detection of MALDI-TOF mass spectrometry. The affinity interactions between the zirconium phosphonate-modified magnetic nanoparticles and the phosphopeptides resulted from the strong chelating interactions between the zirconium cations and the phosphoryl groups of phosphopeptides. The specificity of the $\text{Zr}^{4+}\text{-PO}_3^-$ magnetic nanoparticles has been confirmed by the selective capture of phosphopeptides from the tryptic digests of standard phosphoproteins, and the mixture of standard protein with BSA at the molar ratio of standard protein-to-BSA up to 1:100. The performance of the magnetic nanoparticles was also investigated by applying the nanoparticles to a tryptic digest of a mouse liver lysate, followed by nano-LC-MS/MS and MS/MS/MS analysis for phosphoproteome analysis, which confirmed that the magnetic nanoparticles did show their specificity in selective capture of phosphopeptides even from a complex real sample. In addition, the isolation procedure of phosphopeptides from the complex tryptic digests of proteins was very convenient by applying an extra magnetic field to hold the $\text{Zr}^{4+}\text{-PO}_3^-$ magnetic nanoparticles, which significantly reduced the analysis time and the loss of sample.

Acknowledgments

The authors gratefully acknowledge financial supports from the National Natural Sciences Foundation of China (no. 20675081, 20735004), the China State Key Basic Research Program Grant (2005CB522701, 2007CB914104), the China High Technology Research Program Grant (2006AA02A309), the Knowledge Innovation program of CAS (KJCX2.YW.HO9, KSCX2-YW-R-079), and the Knowledge Innovation program of DICP (to H. Zou) and the Hundred Talent Program of Chinese Academy of Sciences (to R. Wu).

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