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Capture of Phosphopeptides Using α-Zirconium Phosphate Nanoplatelets

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α-Zirconium phosphate nanoplatelets (α-ZrPN) were studied as a binding agent for phosphopeptides. Nanoplatelets of α-zirconium phosphate were incubated overnight with zirconium oxychloride, followed by centrifugation, and washed twice with water followed by an aqueous solution of 80% acetonitrile to form the binding agent. α-ZrPN were able specifically to capture phosphoserine-containing peptides from a tryptic digest of a complex peptide mixture in which its abundance was only 0.05%. α-ZrPN also bound peptides containing phosphothreonine and phosphotyrosine. The limit of detection for phosphopeptides is ~2 fmol, based on using matrix-assisted laser desorption/ionization mass spectrometry. α-ZrPN were applied for the analysis of tryptic digests of mouse liver and leukemia cell phosphoproteomes and succeeded in identifying 158 phosphopeptides (209 phosphorylation sites) from 101 phosphoproteins in mouse liver lysate and 78 phosphopeptides (104 phosphorylation sites) from 59 phosphoproteins in leukemia cell extract. For these two tryptic digests, the α-ZrPN approach is able to capture more phosphopeptides than that obtained from TiO_2 particles or from Fe^{3+}-IMAC beads, but each method is able to bind some phosphopeptides that the others do not.

Protein phosphorylation is among the most important post-translational modifications in eukaryotic cells involved in regulation of cell division, growth, migration, differentiation, and intercellular communication.[1–3] A fundamental understanding of these biological processes at the molecular level requires a characterization of the phosphorylated sites of the proteins. It is therefore important to develop sensitive and selective methods for this task. However, characterization of these post-translationally modified proteins is an analytical challenge in proteomics. There have been numerous efforts to study protein phosphorylation and sequence alignment by the development of varying technologies and biochemical and immunological methods. For example, antibodies have been developed that can be used to specifically purify phosphoproteins, as well as detect the phosphoproteins by Western blots, flow cytometry, or immunohistochemistry.[4,5] However, generation and validation of such phosphopeptide-specific antibodies is a rather time-consuming process.[6] Another widely used method is the Edman degradation procedure combined with ^{32}P labeling to obtain the sequence of the peptide and site of phosphorylation,[7] but this method is only suitable for single phosphoprotein studies, and the use of ^{32}P requires special handling.

Mass spectrometry (MS)-based analysis of the phosphoproteome is a potentially powerful approach for the global profiling and quantification of protein phosphorylation.[8] The low abundance of phosphopeptides present in complex mixtures of phosphoproteins and proteins results in reduced ion signals of phosphopeptides, which are often easily suppressed by the high abundance of nonphosphopeptides in the MS analysis. Consequently, it is advantageous to purify and enrich the phosphopeptides from complex mixtures of peptides prior to obtaining the MS sequence information. Immobilized metal affinity chromatography (IMAC), using metal ions such as Fe^{3+}, Ga^{3+}, and Zr^{4+} as the capture agent, is a commonly used method for the enrichment of phosphorylated peptides from peptide mixtures.[9–21] However, the specificity of IMAC for phosphopeptides has not been high

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because some non-phosphopeptides with multiple acidic residues can also bind to IMAC metal ions.[12] To overcome this problem, several investigators have chemically converted the acidic groups to methyl esters,[11,18,19] but this procedure requires an extra step with the potential for more loss of starting material. Chemical modification by β-elimination of phosphoric acids from phosphopeptides and concurrent Michael addition has also been used to specifically purify phosphopeptides.[22,23] This approach generally suffers from side reactions and increased sample complexity, which may lead to a loss of phosphopeptides and poor reproducibility.[20–22] Metal oxide particles of titanium, zirconium, and aluminum have also been used to selectively enrich phosphopeptides and phosphoproteins from complex mixtures.[25–32] TiO_2 particles, as affinity agents, have attracted attention as particles to selectively bind phosphopeptides from complex peptide solutions. Compared with conventional IMAC-Fe^{3+} beads, it was demonstrated that TiO_2 particles have a higher affinity and better selectivity for binding phosphopeptides.[26]

Crystalline α-zirconium phosphate (α-ZrP) was first discovered in 1964 by Clearfield and Stynes[33] via refluxing amorphous α-ZrP with phosphoric acid. α-ZrP is a layered structure consisting of sheets of Zr atoms connected to O atoms, which are part of the phosphate groups. The layered structure of α-ZrP exhibits excellent ion exchange capacity, high mechanical strength, and biocatalytic performance.[33–40] Kumar and Chaudhari[35] first used crystalline α-ZrP to immobilize proteins, which are believed to intercalate between the layers of the crystalline α-ZrP. Interestingly, it was found that the presence of DNA and high concentrations of urea can stabilize the activity of enzymes and proteins in ZrP nanomaterials.[36,38] Herein, we present a new application to selectively bind and enrich phosphopeptides from complex mixtures using α-zirconium phosphate nanoplatelets (α-ZrPN) that Sun et al.[41] prepared by the refluxing method. To examine the characteristics of α-ZrPN for this purpose, we employed as model samples the tryptic digest products of standard phosphoproteins, individual phosphopeptides, and a mouse liver and leukemia cell protein extract. We believe that the application of α-ZrPN for the selective binding of phosphopeptides provides a new modality for phosphoproteomic research.

EXPERIMENTAL SECTION

Reagents and Materials. All reagents were obtained from Sigma (St. Louis, MO) and used as received, unless otherwise specified. Porous 20-μm IMAC beads were purchased from Applied Biosystems Inc. (San Jose, CA). Titanium dioxide beads were obtained from GL Science Inc. (Tokyo, Japan). Standard phosphopeptides TRDIYETD***pY***YRK (pY), TRDI***pY***ETD***pY******pY***RK (pYpYpY), and RRREEE***pT***EEEAA (pT) were obtained from AnaSpec, Inc. (San Jose, CA). Deionized water used for all experiments was purified with a Milli-Q water system (Millipore, Milford, MA). ZipTip-C4 was also obtained from Millipore (Millipore). We use the common abbreviations of ACN for acetonitrile and TFA for trifluoroacetic acid.

Preparation of Protein Samples. α-Casein and β-casein (1 mg/mL) were dissolved in 1 mL of ammonium bicarbonate (50 mM, pH 8.0) and digested in trypsin for 18 h at 37 with a 1:40 (w/w) enzyme-to-protein ratio. Bovine serum albumin (BSA; 10 mg/mL) was dissolved for 3 h in 1 mL of denaturing buffer solution containing 8 M urea in 100 mM ammonium bicarbonate. This protein solution was then mixed with 50 μL of 50 mM dithiothreitol and incubated for 2 h at 37 °C. Iodoacetic acid (100 μL of 50 mM) was then added, and the incubation continued for an additional 30 min at room temperature in the dark. The mixture was then diluted 10-fold with 100 mM ammonium bicarbonate and incubated for 18 h at 37 °C with trypsin at a 1:25 (w/w) enzyme-to-substrate ratio. The protein extract from mouse liver was prepared according to a procedure described in detail elsewhere.[42] The Bradford assay was used to quantify the concentration of the extracted proteins. Tryptic digestion of the protein extract was the same as that of BSA.

Leukemia Cell Culture and Lysis. The REH human leukemia cell line were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C. Cells were collected by centrifugation and washed three times in phosphate-buffered saline. A cell lysate was obtained by incubating 50×10^6 cells in 1.0 mL of lysis solution (1% triton X-100 containing 50 mM KCl and 5 mM $MgCl_2$) on ice for 30 min with occasional mixing. A soluble fraction was obtained by using the supernatant solution following centrifugation at 15000*g* for 20 min at 4 °C.

Preparation of α-ZrPN. α-ZrPN were made through a refluxing method at Texas A&M University using the following

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procedure. A 20.0-g sample of $ZrOCl_2 \bullet 8\ H_2O$ was refluxed in 200 mL of 3.0 M H_3PO_4 in a flask with stirring at 100 °C for 24 h. After the reaction, the products were washed and collected by centrifugation three times. Then, The isolated α-ZrPN were dried at 65 °C in an oven for 24 h. The dried α-ZrPN were ground with a set of mortar and pestle into a fine powder. Scanning electron microscopy (SEM) of α-ZrP nanoplatelets was obtained using a Zeiss 1530 VP FE-SEM operated at 1 kV.

We rinsed 10 mg of α-ZrPN in 1.0 mL of water and centrifuged it at 15000*g* for 3 min. This procedure was repeated, and the resulting α-ZrPN were then resuspended in (1:1, v/v) ACN/water once, collected by centrifugation, and then suspended again in 1.0 mL of water with shaking to form a uniform α-ZrPN suspension. The α-ZrPN solution was then incubated with 20 mg/mL $ZrOCl_2$ at room temperature overnight with shaking. The α-ZrPN were collected by centrifugation, washed with water twice, then washed with (80:20, v/v) ACN/water with 0.1% TFA once, and resuspended in (80:20 v/v) ACN/water with 0.1% TFA for further use.

Capture of Phosphopeptides Using α-ZrPN, TiO_2, and IMAC-Fe^{3+}. A 10-μL volume of the tryptic digest of either α-casein or β-casein (0.1 mg/mL) was diluted with a 90-μL solution of 80% ACN containing 5% TFA as a loading solution. The loading solution (8 μL) was mixed with 10 μL of the α-ZrPN suspension (5 mg/mL in 80% ACN containing 0.1% TFA), and then 200 μL of 80% acetonitrile containing 5% TFA was added. After incubation for 30 min with shaking, the mixture was centrifuged at 15000*g* for 3 min, and the supernatant removed. The pelleted α-ZrPN were further washed with 100 μL of 80% ACN/5% TFA once, followed by 80% ACN/0.1% TFA twice. Finally, the trapped phosphopeptides on the α-ZrPN were released by eluting with 10 μL of dihydroxybenzoic acid (DHB) solution (20 mg/mL) containing either 1.5% H_3PO_4 or ammonia hydroxide in a bath sonicator. The DHB solution was prepared by dissolving DHB in a (50/50 v/v) ACN/water solution containing 1.5% H_3PO_4 at a concentration of 20 mg/mL. Then 1 μL of the eluted solution was spotted onto the NP20 ProteinChip array (Bio-Rad) for mass spectrometric analysis.

An optimized method for capturing phosphopeptides by TiO_2 beads[45] was used for the pretreatment of the tryptic digest of standard lysates of mouse liver and REH cells. Briefly, the tryptic digest of the lysate of mouse liver and REH cells were incubated with TiO_2 beads (10 mg mL^{-1} in 30% ACN, 0.1% TFA). After incubation for 30 min with vibration, the TiO_2 beads was first washed with 300 μL of a 50% ACN, 6% TFA solution, followed by 300 μL of a 30% ACN, 0.1% TFA solution for three times, respectively. The bound peptides were eluted with 100 μL of 10% NH_4OH.

We also investigated the capture of phosphopeptides using IMAC-Fe^{3+}.[42,45] The tryptic digest of standard lysates of mouse liver and REH cells were incubated with the Fe^{3+}-IMAC beads (10 $mg.mL^{-1}$) for 30 min with vibration. Then, the beads were washed with 300 μL of acetonitrile/water/glacial acetic acid (25:74:1, v/v/v) for three times. The bound phosphopeptides on the beads were eluted with 100 μL of 10% NH_4OH.

Mass Spectrometric Analysis. MALDI-TOF mass spectra were obtained using a PCS4000 mass spectrometer (Bio-Rad, Fremont, CA). Mass spectra were acquired using 2.1−2.5 μJ laser power. The mass spectra were externally calibrated using a standard mixture of low molecular weight peptides (Bio-Rad). Data were acquired in the positive ion mode from m/z 1000 to 10 000, focused at 2500 Da.

Nano-LC-MS^2 and MS^3 were performed on a nano-RPLC-MS/MS system.

Finnigan surveyor MS pump (Thermo-Electron Finnigan, San Jose, CA) was used to deliver the mobile phase. The fused-silica capillary column (75 μm i.d. × 120 mm length), having at its exit a ∼5-μm aperture, was packed with C18 AQ beads (5 μm, 120 Å) from Michrom BioResources (Auburn, CA) using a pneumatic pump. The mobile phase consisted of mobile phase A, 0.1% formic acid (v/v) in H_2O, and mobile phase B, 0.1% (v/v) formic acid in acetonitrile. The samples were manually loaded onto the C18 capillary column using a 75 μm i.d. × 220 mm long empty capillary as a sample loop, and the separation of trapped phosphopeptides was performed using a 75-min linear gradient elution at ∼200 nL/min. A Finnigan LTQ linear ion trap mass spectrometer equipped with an ESI nanospray source (operated at 1800 V) with an ion-transfer capillary at 180 °C was used for the MS study. The LTQ instrument was operated in the positive ion mode. For the detection of phosphopeptides, the mass spectrometer was set at a full-scan MS followed by three data-dependent MS^2. Subsequently, MS^3 spectra were automatically triggered when the most three intense peaks from the MS^2 spectrum corresponded to a neutral loss event of 98, 49, and 33 ± 1 Da for the precursor ion with 1+, 2+, and 3+ charge states, respectively.

Database Searching and Data Analysis. The peak lists for MS^2 and MS^3 spectra were generated from the raw data by Bioworks (Thermo-electron) with the following parameters: mass range was 600−3500; intensity threshold was 1000; minimum ion count was 10. The MS^2 and MS^3 spectra lists were searched by SEQUEST (version 2.7) against a composite database including both original database (ipi.MOUSE.3.21.fasta) and the reversed version of the forward one with the following 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; and static modification, Cys (+57). For searching the MS^2 data, dynamic modifications were set for oxidized Met (+16), and phosphorylated Ser, Thr, and Tyr (+80). For searching the MS^3 data, besides the above settings, dynamic modifications were also set for water loss on Ser(−18) and Thr(−18).

For phosphopeptide identification by matching the assigned sequences derived from MS^2 and MS^3 data, a newly developed software named APIVASE[43] (The Automatic Phosphopeptide Identification Validating Algorithm for SEQUEST, APIVASE) was applied. This procedure is called the MS^2/MS^3 target-decoy database search or the MS^2/MS^3 approach. Briefly, there are four steps in the MS^2/MS^3 approach: (1) evaluation of charge state to remove invalid MS^2/MS^3 pairs; (2) performing MS^2 and MS^3 target-decoy database searches, separately; (3) reassignment of the peptide scores in SEQUEST output to generate a list of peptide identifications for pairs of MS^2/MS^3 spectra; and (4) filtering the candidate phosphopeptides with criteria ($Rank'_m = 1$, $\Delta Cn'_m \geq 0.1$, and $Xcorr'_s \geq 0.6$) to achieve phosphopeptide identification

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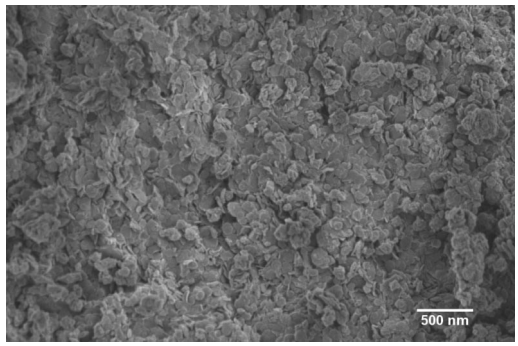

Figure 1. SEM of α-zirconium phosphate nanoplatelets (α-ZrPN).

Table 1. Observed Phosphopeptides Derived by Tryptic Digestion of α-Casein and β-Casein

no.	relative molecular weight	phosphorlation site(s)y	amino acid sequence
			α-Casein
P1	1237	1	TVDME*p*STEVF
P2	1466	1	TVDME*p*STEVFTK
P3	1660	1	VPQLEIVPN*p*SAEER
P4	1832	1	YLGEYLIVPN*p*SAEER
P5	1847	1	DIGSE*p*STEDQAMEDIK
P6	1927	2	DIG*p*SE*p*STEDQAMEDIK
P7	1951	1	YKVPQLEIVPN*p*SAEER
P8	2080	1	KKYKVPQLEIVPN*p*SAEERL
P9	2618	4	NTMEHV*p*S*p*S*p*SEESII*p*SQETYK
P10	2720	5	QMEAE*p*SI*p*S*p*S*p*SEEIVPNPN*p*SVEQK
P11	2935	3	EKVNEL*p*SKDIG*p*SE*p*STEDQAMEDIK
P12	3008	4	NANEEEYSIG*p*S*p*S*p*SEE*p*SAEVATEEVK
P13	3088	5	NANEEEY*p*SIG*p*S*p*S*p*SEE*p*SAEVATEEVK
			β-Casein
P14	2062	1	FQ*p*SEEQQQTEDELQK
P15	2556	1	FQ*p*SEEQQQTEDELQDKIHPF
P16	3122	4	RELEELNVPGEIVE*p*SL*p*S*p*S*p*SEESITR

with low false positive rate (≤1). For more information about this procedure, the original paper should be consulted.[43]

RESULTS AND DISCUSSION

In our study, α-ZrPN were used as affinity probes to capture the phosphopeptides from complex mixtures. The TEM image of α-ZrPN, seen in Figure 1, shows that these nanoplatelets are flattened spheres with a diameter of ~50−100 nm. The α-ZrPN with a hydrophilic surface can be uniformly dispersed in aqueous and aqueous/polar organic solvents.

The tryptic digest of the model phosphoprotein α-casein was used to evaluate the performance of α-ZrPN for the selective binding of phosphopeptides. Table 1 lists the tryptic phosphorylated peptides derived from α-casein. Figure 2A presents the mass spectrum of the tryptic digest of α-casein (500 fmol/spot) using DHB as a MALDI matrix.[44] The mass spectrum is dominated by the peaks of nonphosphopeptides, and only lower intensity peaks representing the phosphopeptides at *m/z* 1660, 1927, 2720, and 3008 can be observed. Figure 2B shows the mass spectrum of the material that was eluted from α-ZrPN that had been resuspended and washed without prior treatment with $ZrOCl_2$. It is clear that, after thorough washing, essentially all of the nonphosphopeptides were removed from α-ZrPN and only phosphopeptides

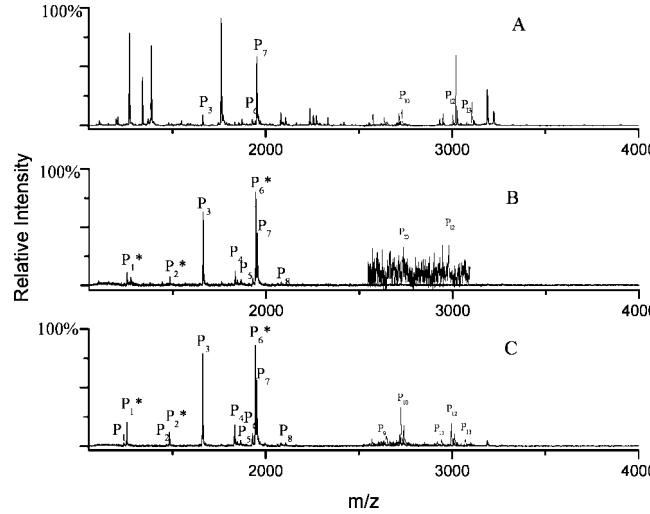

Figure 2. MALDI mass spectra of the tryptic digest of α-casein (400 fmol): (A) direct analysis; (B) analysis after using 10 μL of original α-ZrPN(10 mg/mL); and (C) analysis after using 10 μL of α-ZrPN(10 mg/ml) pretreated with $ZrOCl_2$. An asterisk marks a phosphopeptide resulting from an oxidized Met residue.

can be extracted from α-ZrPN. A total 10 phosphopeptides (*m/z*, 1237, 1466, 1660, 1832, 1847, 1927, 1951, 2080, 2720, 3008) from the tryptic digest of α-casein were observed. Figure 2C shows that more phosphopeptides could be specifically extracted from the nanoplatelets when α-ZrPN were first pretreated with $ZrOCl_2$ (20 mg/mL) solution prior to incubation with the tryptic digest of α-casein. We observed 13 phosphopeptides (*m/z*, 1237, 1466, 1660, 1832, 1847, 1927, 1951, 2080, 2618, 2720, 2935, 3008, 3088). It is apparent that α-ZrPN pretreated with $ZrOCl_2$ has a higher affinity for the phosphopeptides than the original α-ZrPN without pretreatment with $ZrOCl_2$. For all subsequent experiments, we used α-ZrPN pretreated with $ZrOCl_2$ as the affinity probes for the binding of phosphopeptides.

The α-zirconium phosphate nanoplatelets were synthesized from $ZrOCl_2$ and phosphoric acid to form a layered structure. We believe that the nanoplatelet surface contains not only zirconium atoms but also some free phosphate groups. We therefore treated the nanoplatelets with additional $ZrOCl_2$ to bind to the phosphate on the surface. These results demonstrate that α-ZrPN effectively bind phosphopeptides from the tryptic digest of single phosphoprotein α-casein in a specific manner. However, phosphoproteins are often present in low abundance in complex biological samples. To measure the ability to capture phosphopeptides from a more complex sample, we used α-ZrPN to isolate phosphopeptides from the tryptic digest of a mixture of α-casein, plus BSA (which is not a phosphoprotein). Figure 3A and B present the MALDI mass spectra of the tryptic digest of α-casein/BSA in the ratios of 1:100 and 1:500, respectively. Peaks of all 13 phosphopeptides from the α-casein digest mixture were detected in the mass spectra. It is clear that α-ZrPN can specifically and selectively bind phosphopeptides from the complex sample solution, as almost all the nonphosphopeptides can be removed by thoroughly washing the α-ZrPN. When the weight ratio of α-casein to BSA was increased to 1:2000, the ion signal of phosphopeptides was still observable (see Figure 3C). Ion signals of some nonphosphopeptides also appeared at a α-casein/BSA ratio of 1:2000, but as shown in Figure

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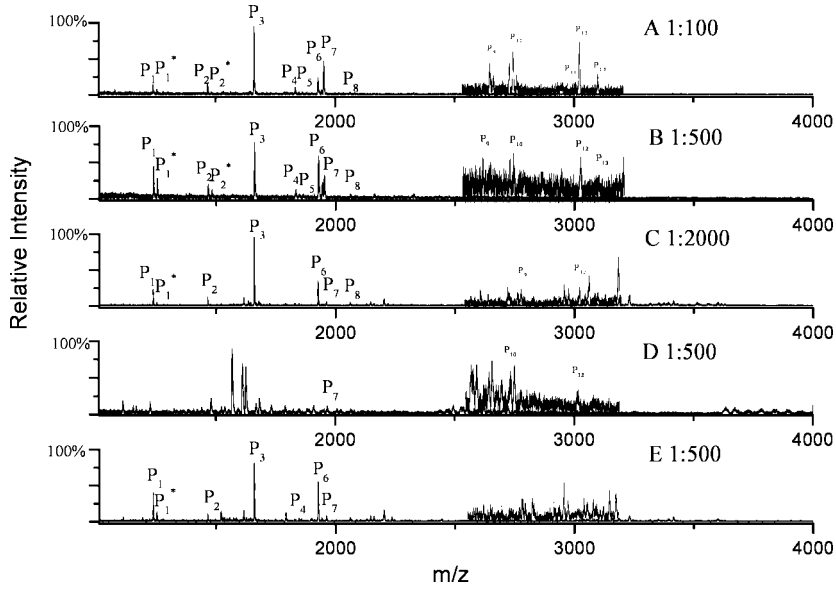

Figure 3. MALDI mass spectra of the selective binding of the phosphopeptides from a tryptic digest of a mixture of α-casein, and BSA using 10 μL of α-ZrPN (10 mg/mL) as an affinity probe at the weight ratio of α-casein to BSA: (A) 1:100; (B) 1:500; and (C) 1:2000. In (D) we use IMAC-Fe^{3+} and (E) TiO_2 as the affinity probe for a casein mixture to BSA of 1:500. An asterisk indicates a phosphopeptide resulting from an oxidized Met residue.

3C, the intensity of ion signals of nonphosphopeptides was very weak. These results show that α-ZrPN have strong specificity for selectively binding phosphopeptides in these complex mixtures of phosphopeptides and nonphosphopeptides.

For comparison purposes, we evaluated the selectivity of phosphopeptide binding using commercial IMAC beads that contain Fe^{3+} as a binding agent.[42,45] At an α-casein/BSA ratio of 1:1, the IMAC-Fe^{3+} beads selectively bound the phosphopeptides (data not shown). However, when the ratio of α-casein/BSA was increased to 1:50 (data not shown) and 1:500 (Figure 3D), peaks of nonphosphopeptides were observed with the concomitant decrease in the signal of peaks of phosphopeptides in the mass spectrum. At an α-casein/BSA ratio of 500, only four peaks of multiphosphopeptides could be detected amid numerous nonphosphopeptide peaks, in comparison to all α-casein phosphopeptides observed using the α-ZrPN (compare Figure 3B to Figure 3D).

We also compared the performance of metal oxide TiO_2 particles for the binding of phosphopeptides[45] to that of α-ZrPN. At ratios of α-casein/BSA of 1:1 and 1:100, TiO_2 effectively bound the phosphopeptides from the digest mixture (similar to the result from Lasen et al.[26]). Figure 3E presents that mass spectra of TiO_2 particles for the isolation of phosphopeptides from the mixture of α-casein/BSA at a ratio of 1:500. The results show that, at this ratio, TiO_2 can only bind several phosphopeptides from the peptide mixture, and there were more nonphosphopeptides detected in the mass spectrum (Figure 3E) compared with those found in Figure 3B. These results show that α-ZrPN have much higher selectivity for the enrichment of phosphopeptides than the conventional IMAC-Fe^{3+} beads and TiO_2 particle techniques previously described.

It is well-known that the IMAC-Fe^{3+} bead method has been plagued by nonspecific retention of peptides rich in acidic amino acids, effectively inhibiting the application of this approach to many complex mixtures. Moreover, it has been shown that the chemical conversion of acidic groups to methyl esters significantly decreases the capture of nonphosphorylated peptides.[22] It is not expected that esterification increases the number of retained phosphorylated peptides. Therefore, we suggest that methyl esterification of the lysates will not significantly change the comparisons presented above.

The present results demonstrate that α-ZrPN have high specificity for the binding of α-casein-derived phosphopeptides. However, phosphorylation sites in α-casein are only on serine residues, and phosphoproteins may also be phosphorylated at threonine and tyrosine residues and, less commonly, on other amino acid residues.[1] Therefore, we evaluated the ability of α-ZrPN to bind phosphotyrosine- and phosphothreonine-containing peptides. Two phosphotyrosine-containing peptides (abbreviated it as pY and pYpYpY, containing one and three phosphotyrosines, respectively), one phosphothreonine-containing peptide (abbreviated pT), and the tryptic digest of β-casein were mixed and bound to α-ZrPN. Figure 4A shows the MALDI mass spectrum of the material extracted from these α-ZrPN. Peaks corresponding to the pY phosphopeptide (m/z 1705), pYpYpY phosphopeptide (m/z 1862), pT phosphopeptide (m/z 1584), and the three peaks of β-casein phosphopeptides P_{14}, P_{15}, P_{16} (see Table 1) were detected in the mass spectrum. To test the specificity of the α-ZrPN for binding these different types of phosphopeptides, a mixture of pY, pT, and pS phosphopeptides was incubated with tryptic peptides of BSA as above at ratios of 1:100 and 1:500 (Figures 4B and Figure 4C, respectively). The ion signals of all six phosphopeptides were clearly detected in the same mass spectra whereas only a few low-signal nonphosphopeptide peaks can be detected, as shown in Figure 5B and C). These results further demonstrate that α-ZrPN have a high affinity and specificity for the phosphoric acids group of phosphopeptides and display no bias for the enrichment of different phosphopeptides.

Phosphatase inhibitors are often used to reduce the activity of phosphatases of the cells and tissues in the biological samples.

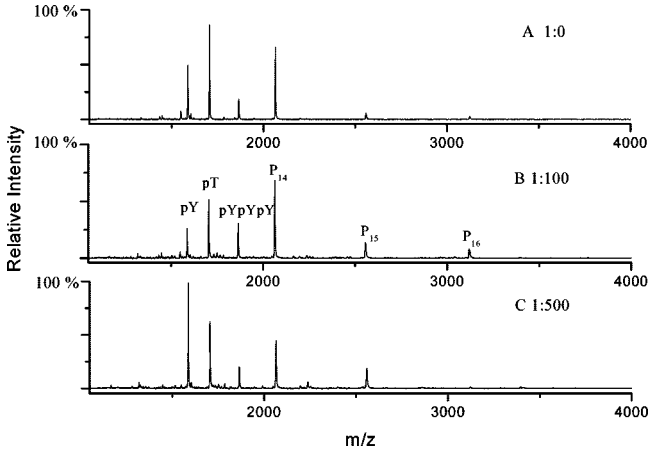

Figure 4. Binding of phosphopeptides from the mixtures of the tryptic digest of β-casein (400 fmol), a singly tyrosine-phosphorylated peptide, TRDIYETD***pY***YRK (pY, 400 fmol), triply tyrosine-phosphorylated peptide, TRDI***pY***ETD***pYpY***RK (pYpYpY, 400 fmol), singly threonine-phosphorylated peptide, RRREEE***pT***EEEAA (pT, 400 fmol), and the tryptic digest of BSA using 10 μLof α-ZrPN as affinity probe at the weight ratio of phosphopeptide to BSA of (A) 1:0; (B) 1:100; and (C) 1:500. An asterisk indicates phosphopeptides resulting from oxidized Met residues.

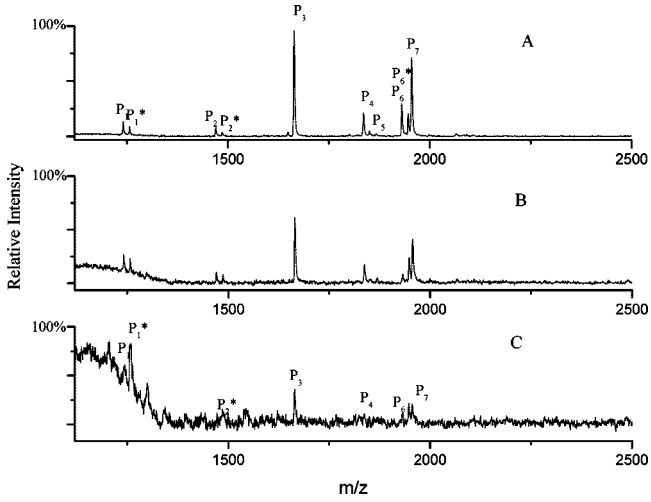

Figure 5. MALDI mass spectra for the phosphopeptides captured after using α-ZrPN as the affinity probe to selectively bind the tryptic digest with differing amounts of α-casein: (A) 200; (B) 20; and (C) 2 fmol. An asterisk indicates a phosphopeptide resulting from an oxidized Met residue.

To determine whether the phosphatase inhibitor, vanadate, has any influence on the binding of phosphopeptides by α-ZrPN, we evaluated the effects of 5, 25, and 50 mM vanadate on the affinity of phosphopeptides in the tryptic digest solution by the α-ZrPN. Sixteen peaks of phosphopeptides from an α-casein mixture were observed, and similar results were obtained at each vanadate concentration. Thus, up to 50 mM vanadate has no effect on the binding of phosphopeptides to α-ZrPN.

The sensitivity of phosphopeptide ion signals captured after using α-ZrPN was investigated for the tryptic digest of α-casein. In this work, we chose the phosphopeptides P_1, P_2, P_3, P_4, P_5, P_6, and P_7 (Table 1) from α-casein as targets to test the sensitivity of the α-ZrPN method. Figure 5 shows MALDI mass spectra of varying amounts of the phosphopeptides: (A) 200, (B) 20, and

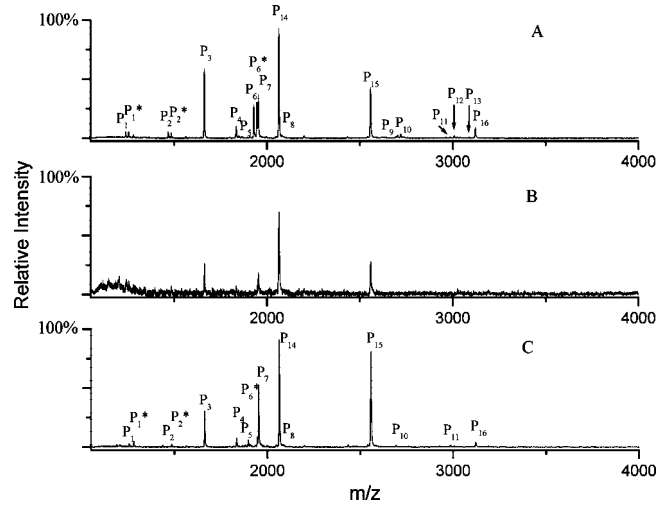

Figure 6. MALDI mass spectra of the tryptic digest of casein mixture (α-casein and β-casein) after using (A) 8 μL of α-ZrPN, (B) 8 μL of α-ZrPN and 8 μL of 20 mM $ZrOCl_2$, and (C) 8 μL of α-ZrPN and 30 μL of 100 mM $FeCl_3$. An asterisk indicates a phosphopeptide resulting from an oxidized Met residue.

(C) 2 fmol. It can be seen that when the amount of tryptic digest of α-casein is 20 fmol, the ion signals from the phosphopeptides can be easily detected. When the amount of tryptic digest of α-casein was decreased to 2 fmol, the phosphopeptides can still be found in the mass spectrum but with low signal-to-noise ratios, and we regard this amount as the limit of detection.

We have found that the presence of ZrO^{2+} ions in solution interferes with the binding of phosphopeptides to α-ZrPN whereas the presence of Fe^{3+} does not. Evidence for this behavior is presented in Figure 6. Figure 6A shows the mass spectrum of the tryptic digest of α-casein and β-casein mixture using α-ZrPN. Figure 6B is the mass spectrum obtained when 20 mM $ZrOCl_2$ is added to the tryptic digest prior to incubation with α-ZrPN, and Figure 6C is the mass spectrum when 100 mM $FeCl_3$ is added instead. The presence of 20 mM ZrO^{2+} in the α-ZrPN solution bound most of the phosphopeptides so that very few phosphopeptides were trapped by α-ZrPN, whereas the presence of 100 mM Fe^{3+} had essentially no effect. Thus, ZrO^{2+} is a much better binding agent than Fe^{3+} for phosphopeptides, and we suggest that this fact explains why α-ZrPN is superior to Fe^{3+}-IMAC.

We applied α-ZrPN to isolate and enrich phosphopeptides from the tryptic digest of mouse liver protein extract, followed by analysis and identification of the captured phosphopeptides using LC−MS^2 (MS^3). For the analysis of these captured phosphopeptides, one-tenth of the enriched phosphopeptides from 100 μg of mouse liver protein extract digest was loaded onto a capillary C_{18} column and analyzed by LTQ MS. For each sample, three replicates of the enriched phosphopeptides underwent LC−MS analysis. Based on the MS^2/MS^3 target-decoy search,[34] the analysis of the peptide samples from α-ZrPN isolation of phosphopeptides yielded 158 phosphopeptides with 113 single (71%), 38 double (24%), 6 triple (4%), and 1 quadruple (1%) phosphorylated phosphopeptides derived from 101 phosphoproteins. Totally, 211 phosphorylation sites—188 on serine (90%) and 21 on threonine (10%)—were found (see Supporting Information 1, and Table

Table 2. Overview of Data from Phosphoproteome Analysis of Mouse Liver and Leukemia Cell Lysate after Using IMAC-Fe^{3+} Particle, TiO_2 Particle, and α-ZrPN Nanoplatelet Methods for the Isolation of Phosphopeptides[a]

	number of phosphopeptides and distribution					identified phosphorylation site		
isolation method	total	single	double	triple	quad-ruple	pS	pT	number of phospho-proteins
Mouse Liver Lysate								
IMAC-Fe^{3+}	47	13 (28%)	11 (23%)	23 (49%)	0 (0%)	97 (93%)	7 (7%)	36
TiO_2	101	80 (79%)	17 (17%)	4 (4%)	0 (0%)	116 (92%)	10 (8%)	68
α-ZrPN	158	114 (72%)	38 (24%)	5 (3%)	1 (1%)	188 (90%)	21 (10%)	101
Leukemia Cell Lysate								
IMAC-Fe^{3+}	12	5 (42%)	5 (42%)	2 (16%)	0 (0%)	19 (93%)	2 (7%)	9
TiO_2	41	16 (39%)	23 (56%)	2 (5%)	0 (0%)	63 (93%)	5 (7%)	34
α-ZrPN	78	54 (69%)	22 (28%)	2 (3%)	0 (0%)	92 (88%)	12 (12%)	59

[a] All data was analyzed by the MS^2/MS^3 target-decoy searching approach with criteria $Rank'_m = 1$, $\Delta Cn'_m \geq 0.1$, and $Xcorr'_s \geq 0.6$.

2). This ratio is similar to the ratio of phosphoserine/phosphothreonine (9:1) found previously.[37] These results demonstrate that α-ZrPN has no significant bias for the nature of the phosphorylated amino acid.

In this target-decoy search data searching method, no phosphopeptides containing tyrosine were observed. It was reported that the frequency of phosphoserine/phosphothreonine/phosphotyrosine in general is 1800:200:1.[46,47] Given these ratios, it is reasonable that it would be unlikely to find a phosphopeptide with tyrosine from the identified 158 phosphopeptides.

To compare these results with the TiO_2 particle method, an optimized protocol for TiO_2, LC−MS/MS analysis[45] was used for the isolation of phosphopeptides from the tryptic digest of the mouse liver protein extract. The result shows that 101 phosphopeptides with 82 single (80%), 16 double (16%), and 4 triple (4%) phosphopeptides from 68 phosphoproteins were identified in the LC−MS/MS experiments (see Supporting Information 2, and Table 2). The corresponding identified phosphorylation sites were 126 with 118 on serine (94%) and 8 on threonine (6%).

When IMAC-Fe^{3+} [42,45] was used to isolate the phosphopeptides from mouse liver protein extract, 47 phosphopeptides with 12 single (28%), 11 double (23%), and 24 triple (49%) phosphopeptides were identified from 36 phosphoproteins (see Supporting Information 3, and table 2). The distribution of phosphorylation sites from the IMAC-Fe^{3+} method was 99 on serine (93%), and 7 on threonine (7%).

These results show that the α-ZrPN method yields the largest number of identified phosphopeptides from the tryptic digest of a mouse liver protein extract. We conclude that α-ZrPN has the highest affinity for phosphopeptides, compared with TiO_2 particles and IMAC-Fe^{3+} beads, for the protocols used. It should be noted that some optimized Fe^{3+}-IMAC bead studies are able to detect a much larger number of phosphopeptides using alternative protocols.[18,46]

We also used a leukemia cell protein extract as an additional model to evaluate the performance of α-ZrPN, IMAC-Fe^{3+}, and TiO_2 for the binding of phosphopeptides. For α-ZrPN, we can identify 78 phosphopeptides derived from 59 phosphoproteins and 104 phosphorylation sites (see Supporting Information 4 and Table 2). However, when TiO_2 and IMAC-Fe^{3+} methods were used, the number of the identified phosphopeptides was only 41 and 12, respectively. The corresponding phosphorylation sites were 68 and 21, respectively (see Supporting Information 5 and 6, Table 2).

Table 3. Overlap of the Identified Phosphopeptides of the Tryptic Digests of Mouse Liver and REH Leukemia Cell Protein Extracts

identification method	number of identified phosphopeptides from mouse liver extract	number of identified phosphopeptides from REH cell extract
only found with α-ZrPN	85	52
only found with TiO_2	35	16
only found with IMAC-Fe^{3+}	22	5
found with α-ZrPN and TiO_2	66	25
found with α-ZrPN and IMAC-Fe^{3+}	25	7
found with TiO_2 and IMAC-Fe^{3+}	18	6
found with α-ZrPN, TiO_2, and IMAC-Fe^{3+}	18	6

Table 3 shows the number of identified phosphopeptides of the tryptic digests of mouse liver and REH leukemia cell protein extracts using each of the three methods (see Supporting Information 7 and 8). We find that for both protein extracts, α-ZrPN identifies the most phosphopeptides, but some phosphopeptides are unique to each procedure.

In summary, α-ZrPN, obtained by the refluxing synthesis and treatment with $ZrOCl_2$, can be successfully applied for the selective binding of phosphopeptides from complex biomixtures. α-ZrPN were demonstrated to have high selectivity for the phosphopeptides in the presence of a 2000-fold excess albumin over α-casein in the tryptic digest. We also find that α-ZrPN have better sensitivity in mouse liver and leukemia cell protein extracts compared with that of IMAC-Fe^{3+} beads and TiO_2 particles. The limit of detection for phosphopeptides from phosphoprotein α-casein is about ~2 fmol. The studies carried out to date show

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that α-ZrPN has promise for the purification of phosphopeptides, and we recommend its use in conjunction with either MALDI MS/MS or LC-MS/MS for the further identification and quantification of phosphopeptides.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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