Immobilized Zirconium Ion Affinity Chromatography for Specific Enrichment of Phosphopeptides in Phosphoproteome Analysis

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Running Title: GMA-EDMA phosphonate zirconium for phosphoproteome analysis
The abbreviations used are:

MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight; LC-MS/MS, liquid chromatography tandem mass spectrometry; i.d., inner diameter; iodoacetamide, IAA; dihydroxybenzioic acid, DHB; IPI, International Protein Index; IMAC, immobilized metal affinity chromatography; GMA-EDMA, poly(glycidyl methacrylate-co-ethylene dimethacrylate); MW, molecular weight.
Summary

Large scale characterization of phosphoproteins requires highly specific methods for purification of phosphopeptides because of the low abundance of phosphoproteins and substoichiometry of phosphorylation. Enrichment of phosphopeptides from complex peptide mixtures by immobilized metal affinity chromatography (IMAC) is a popular way to perform phosphoproteome analysis. However, conventional IMAC adsorbents with iminodiacetic acid as chelating group to immobilize Fe$^{3+}$ lack enough specificity for efficient phosphoproteome analysis. Here we reported a novel IMAC adsorbent through Zr$^{4+}$ chelating to the phosphate modified poly(glycidyl methacrylate–co-ethylene dimethacrylate) polymer beads. The high specificity of Zr$^{4+}$-IMAC adsorbent was demonstrated by effectively enriching phosphopeptides from the digest mixture of phosphoprotein ($\alpha$ or $\beta$-casein) and bovine serum albumin with molar ratio at 1:100. Zr$^{4+}$-IMAC adsorbent was also successfully applied for the analysis of mouse liver phosphoproteome which resulted in the identification of 153 phosphopeptides (163 phosphorylation sites) from 133 proteins in mouse liver lysate. Significantly more phosphopeptides were identified than that of conventional Fe$^{3+}$-IMAC approach, which indicated the excellent performance of Zr$^{4+}$-IMAC approach. The high specificity of Zr$^{4+}$-IMAC adsorbent was found to be mainly resulted from the strong interaction between chelating Zr$^{4+}$ and phosphate group on phosphopeptides. Enrichment of phosphopeptides by Zr$^{4+}$-IMAC provides a powerful approach for large scale phosphoproteome analysis.

Keywords: IMAC, phosphonate modified GMA-EDMA beads, Zr$^{4+}$, phosphopeptides, phosphoproteomics, proteomics
Introduction

Organisms use reversible phosphorylation of proteins to control many cellular processes including signal transduction, gene expression, cell cycle, cytoskeletal regulation and apoptosis. Although phosphorylation is observed on a variety of amino acid residues, by far the most common and important sites of phosphorylation in eukaryotes occur on serine, threonine and tyrosine residues(1, 2). Owing to the importance of protein phosphorylation in cellular signaling, various methods for protein phosphorylation site mapping have been developed through the years. However this task remains a technical challenge, and there is an intense interest in development of technologies and methods for studying phosphorylation events.

Mass spectrometry (MS) has been widely applied as a powerful tool to characterize protein modifications including phosphorylation due to its high sensitivity and capability of rapid sequencing by tandem mass spectrometric (MS^n) technique(3-6). For the phosphoproteome analysis, satisfied results often can not be obtained by direct mass spectrometric analysis of protein digest. This is because phosphopeptides are present at low abundance in the digest and the mass spectrometric response of a phosphopeptide is seriously suppressed by unphosphorylated peptides. To reduce the suppression, it is crucial to purify the phosphorylated peptides from complex peptide mixtures. A number of techniques have been developed to enrich phosphorylated peptides from peptide mixtures. These techniques are immunoprecipitation(7, 8), which is less reliable and more costly; chemical reaction of the phosphate group with an enrichable tag(9, 10) or covalent linking the phosphopeptide to beads and releasing(11, 12), which is labor intensive and the performance is compromised by side reaction and incomplete reaction; strong cation exchange chromatography (SCX)(13, 14), which often results in large scale phosphoproteins identifications but lack enough specificities; and immobilized metal affinity chromatography (IMAC)(15-19), which is the most frequently used enrichment technique. Recently, metal oxide particles, such as TiO_2 and ZrO_2, were also reported to have high specificity for phosphopeptides(20-22).

In the technique of using IMAC, metal ions such as Fe^{3+}, Ga^{3+} were bound to beads typically using the immobilized iminodiacetic acid as chelating group (16, 17), phosphopeptides are selectively retained because of the affinity of the metal ions for the phosphate moiety. However the specificity of those IMAC adsorbents is still not high enough. Some unphosphorylated peptides (typically acidic peptides) are also strongly bound to the adsorbents, which results in serious interference for the analysis of target
phosphopeptides. The poor specificity for phosphopeptides by IMAC may be partially overcome by esterification of the acidic side chains of glutamate and aspartate residues prior to IMAC purification(23), however, it may also increase sample complexity and interfere with subsequent mass spectrometry analysis because of incomplete reactions. A better and simpler solution for this problem is to develop new type of IMAC with higher specificity for phosphopeptides. It was reported that self-assembling monolayer and multilayer thin films of phosphate-containing organic molecules can be prepared based on the strong interaction between phosphate groups on target molecules with zirconium (Zr$^{4+}$) phosphonate on the surface of solid matrix (24-26). Taking advantage of the strong interaction, we have developed a porous silicon wafer with surface chemically modified with zirconium phosphonate for highly specific capture of phosphopeptides followed with direct MALDI-TOF MS analysis(26). It was observed that the selectivity of the zirconium phosphonate modified surface was higher than that of conventional Fe$^{3+}$-IMAC beads. Although the chip based technique is very convenient for MALDI analysis, the amount of captured phosphopeptides is not adequate for LC-MS/MS analysis in phosphoproteomics research. Here, a novel Zr$^{4+}$-IMAC adsorbent was prepared by modifying poly(glycidyl methacrylate–co-ethylene dimethacrylate) (GMA-EDMA) beads with POCl$_3$ followed with immobilization of Zr$^{4+}$ using ZrOCl$_2$ solution. The obtained new IMAC beads were first evaluated by using tryptic digests of α- and β-casein as samples, and then they were applied for phosphoproteome analysis of mouse liver.

**Experiment procedures**

**Reagents and materials**

α-casein, β-casein, trypsin (from bovine pancreas, TPCK-treated), bovine serum albumin (BSA), 2,4,6-collidine, zirconyl chloride and POCl$_3$ were from Sigma (St. Louis, MO, USA); adult female C57 mice were purchased from Dalian Medical University (Dalian, China).

**Preparation of protein samples**

For standard proteins, α-casein, β-casein, and bovine serum albumin (BSA), were digested in 100 mM NH$_4$HCO$_3$ (pH 8.1) buffer with trypsin at a protein/enzyme ratio of 50:1 by weight, and incubated at 37 °C for 16 h. Before the digestion, BSA was reduced with DTT and carboxamidomethylated with IAA. Other standard proteins were digested directly. The protein extract from mice livers was prepared according to
procedure described in detail in our previous reports(3, 26). The Bradford protein assay was used to quantify the concentration of the extracted proteins. The trypsin digestion of the protein extract was the same to that of BSA.

**Preparation of Zr$^{4+}$-IMAC beads**

GMA-EDMA polymer beads were synthesized according to previous report(27). The GMA-EDMA polymer beads were aminated by reaction with 29% ammonium hydroxide solution for 3 h at 40 °C. Then the resulted beads were incubated in a solution of 40 mM POCl$_3$ and 40 mM 2,4,6-collidine in anhydrous acetonitrile for 12 h at ambient temperature to prepare phosphonate modified beads. After rinsing with CH$_3$CN and water, the beads were incubated in 50 mM ZrOCl$_2$ solution to charge GMA-EDMA polymer beads with Zr$^{4+}$ ion overnight under gentle stirring. At last, the Zr$^{4+}$-IMAC beads were washed with 200 mM NaCl in 10% HAc and deionized water to remove nonspecifically adsorbed Zr$^{4+}$ cation, and dried in vacuum at 60 °C.

**Enrichment of phosphopeptides by Zr$^{4+}$-IMAC beads**

For enrichment of phosphopeptides from standard protein digest, 1 μL (2 pmol) sample solution was mixed with an aliquot of 10 μL Zr$^{4+}$-IMAC bead suspension (10 mg/mL in 100% ACN solution), and then 10% HAc was added to reach a volume of 100 μL. After incubation for 30 min with vibration, beads was centrifuged at 35 000 g for 10 min and washed with 100 μL solution of 10% HAc containing 200 mM NaCl and 100 μL solution of 10% HAc, respectively. At last 100 μL NH$_3$·H$_2$O (12.5%) were added to elute the captured phosphopeptides. The supernatant containing phosphopeptides was collected and lyophilized to dryness. For all samples by direct analysis with MALDI-TOF-MS, 5 μL DHB solution (25 mg/mL) containing 1% H$_3$PO$_4$ was mixed with lyophilized phosphopeptides, and 0.5 μL of the resulting mixture was deposited on the MALDI target for MALDI MS analysis. For phosphoproteome analysis of the mouse liver lysate, 20 mg Zr$^{4+}$-IMAC beads were mixed with digest of 100 μg mouse liver lysate, and then the total volume was adjusted to 1 mL by adding 10% HAc solution. Except the volume was 1 mL, the washing and elution steps were the same as for standard protein digests. After lyophilization, 5 μL of 0.1% formic acid was added to redissolve the captured phosphopeptides.
**Mass Spectrometric Analysis**

MALDI-TOF MS experiments were performed on a Bruker Autoflex time-of-flight mass spectrometer (Bruker Co) in the positive ion linear mode, and each mass spectrum was typically summed with 30 laser shots. LTQ linear ion trap mass spectrometer (Thermo-electron) with a nanospray source was used with a Finnigan surveyor MS pump (Thermo-electron). The pump flow rate was split by a cross to achieve a flow rate at 200 nL/min. The columns were in-house packed with C18 AQ beads (5 µm, 120 Å) from Michrom BioResources (Auburn, CA, USA) using a pneumatic pump. The separation of phosphopeptides enriched from the tryptic digest of mouse liver lysate was performed using 75 min linear gradient elution. The mobile phase consisted of mobile phase A, 0.1% formic acid in H₂O, and mobile phase B, 0.1% formic acid in acetonitrile. The LTQ instrument was operated at positive ion mode. A voltage of 1.8 kV was applied to the cross. About 1 µL (20 µg) redissolved peptides was loaded onto the C18 capillary column using a 75 µm i.d. × 220 mm length capillary column as sample loop. The detection of phosphopeptides was performed in which the mass spectrometer was set as a full scan MS followed by three data-dependant MS/MS (MS²). Subsequently MS/MS/MS (MS³) spectrum was automatically triggered when the most three intense peaks from the MS/MS spectrum corresponded to a neutral loss event of 98, 49 and 33±1 Da for the precursor ion with 1+, 2+, 3+ charge states, respectively.

**Database searching and data analysis**

The peak lists for MS² and MS³ spectra were generated from the raw data by Bioworks v 3.2 (Thermo-electron) with following parameters: mass range was 600 – 3500; intensity threshold was 1000; minimum ion count was 10. The generated peak lists were searched by Sequest program included in the Bioworks against the non-redundant mouse protein database of Mouse International Protein Index (ipi.MOUSE.3.21.fasta) including 51446 entries. The MS/MS spectra were searched with a precursor-ion mass tolerance of 2 Da and fragment-ion mass tolerance of 1 Da; full tryptic specificity was applied; 2 missed cleavages were allowed; static modification was set for alkylation of Cys with iodoacetamide (+57). For the searching with MS/MS data, dynamic modifications were set for oxidized Met (+16), and phosphorylated Ser, Thr and Tyr (+80). For the searching with MS/MS/MS data, besides above settings, dynamic modifications were also set for water loss on Ser, Thr (-18). For the identification of phosphopeptides based only on MS/MS or MS/MS/MS spectra, the following criteria were used for
filtering the database searching results: Cross-correlation value (Xcorr) >1.9, 2.2, and 3.75 for singly,
doubly, and triply charged ions, respectively; Delta Cn value (dCn) > 0.1. For the phosphopeptide
identifications derived from MS/MS/MS spectra used to validate the MS/MS identifications, the following
criteria were used: Xcorr >1.5, 2.0 and 2.5; dCn>0.1. Manual validation was further carried out for
peptides passing the above criteria. Criteria used for manual validation included: (a) the phosphoric acid
neutral loss peak to phosphoserine and phosphothreonine must be the dominant peak; (b) the spectrum
must be of good quality with fragment ion clearly above the baseline noise; (c) sequential members of the
b- or y- series were observable in the mass spectra; (d) for multiply phosphorylated peptides, the peptides
derived from MS² must be confirmed by MS³ spectra in the same MS cycle. 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 (28) as all proteins
in each group are highly homologous, generally belonging to the same superfamily, or just different
alternative splicing isoforms.

Results

Preparation and evaluation of Zr⁴⁺-IMAC Beads

Poly(glycidyl methacrylate–co-ethylene dimethacrylate) (GMA-EDMA) polymer beads has neutral
hydrophilic surface and so the non-specific adsorption of biomolecules is very weak. Surface of
GMA-EDMA beads also possesses chemically active sites, i.e. epoxide groups, for chemical modification
which make it easily to be derivatized with other functional groups. In our previous studies, GMA-EDMA
monolithic polymers prepared in HPLC column or capillary column were used as support for affinity
chromatography (29, 30) and enzyme reactor(31). Here GMA-EDMA beads were used to preparation of
Zr⁴⁺-IMAC adsorbents for phosphopeptide enrichment. As shown in Fig. 1, epoxide groups on the polymer
surface were first transferred to amino groups by incubation in ammonium hydroxide solution, and then
phosphonate groups were introduced by reaction of amino groups with POCl₃, and finally Zr⁴⁺ was
immobilized by incubation of the modified beads in ZrOCl₂ solution. Compared with inorganic supports
such as silica beads, GMA-EDMA polymer beads have the advantage of good chemical stability in wider
pH range. They are inert at extreme pH value. This is important for phosphopeptide enrichment since
samples are typically loaded at low pH and the captured phosphopeptides are eluted at high pH.
Due to its well-characterized five phosphorylation sites at serine residues, bovine β-casein, was used to evaluate the performance of prepared Zr\(^{4+}\)-IMAC beads for enrichment of phosphopeptides. Tryptic digest from 2 pmol β-casein was pretreated by the Zr\(^{4+}\)-IMAC beads. After thoroughly washing, the captured phosphopeptides were eluted for MALDI-TOF MS analysis. For comparison, direct analysis of β-casein digest was also performed by MALDI-TOF-MS. The obtained spectra are presented in Fig. 2. The direct analysis resulted in detection of many intensive peaks of nonphosphopeptides besides 2 phosphopeptide peaks with weak intensity (Fig. 2A), while the analysis of the peptides eluted from Zr\(^{4+}\)-IMAC beads yielded only 5 dominant phosphopeptide peaks (Fig. 2B). β1 (m/z 2061) and β2 (m/z 2556) are singly phosphorylated peptides, and β3 (m/z 3122) is a quadruply phosphorylated peptide. The other 3 peaks represent β3 losing one H\(_2\)PO\(_4\), two H\(_2\)PO\(_4\), and its doubly charged ion, respectively. The sequences of phosphopeptides are given in Supplementary Table 1. The nonphosphopeptides were not observed in Fig. 2B, which indicated the high specificity of Zr\(^{4+}\)-IMAC beads for phosphopeptides. The same amount of sample were also pretreated by GMA-EDMA beads and phosphonate modified GMA-EDMA beads with the same procedures as by Zr\(^{4+}\)-IMAC beads, respectively, and the eluted samples were also analyzed by MALDI-TOF-MS to investigate the non-specific adsorption of peptides on the raw and intermediate beads. No peaks were observed for the eluted fractions in both cases (Fig. 2C and 2D), which also indicated that obvious non-specific adsorption of peptides on either GMA-EDMA beads or phosphonate modified GMA-EDMA beads not existed. Obviously neither GMA-EDMA nor phosphonate modified GMA-EDMA beads have affinity for phosphopeptides, the capture of phosphopeptides by Zr\(^{4+}\)-IMAC beads is mainly resulted from the strong interaction between chelating Zr\(^{4+}\) and phosphate groups on the phosphopeptides.

Tryptic digest of α-casein were also pretreated by Zr\(^{4+}\)-IMAC beads with above procedures, and the obtained MALDI mass spectra before and after Zr\(^{4+}\)-IMAC enrichment are shown in Fig. 3 A and 3B. Totally 12 phosphopeptides from tryptic digest of α-casein were observed after Zr\(^{4+}\)-IMAC enrichment (Fig. 3B), while only 9 phosphorylated peptides with weak signals were observed by direct analysis (Fig. 3A). This further demonstrated that the enrichment of phosphopeptides by Zr\(^{4+}\)-IMAC is very effective. For the comparison, enrichment of phosphopeptides from tryptic digest of α-casein was also performed by
using the commercial IMAC resin POROS MC20 chelating with Fe$^{3+}$. The enrichment of phosphopeptides by Fe$^{3+}$-IMAC was performed according to the procedures by Ficarro et al.(23) and improved protocol by Kokubu et al(19) except NH$_3$·H$_2$O (12.5%) was used as elution buffer instead of 50 mM Na$_2$HPO$_4$ (pH 9.0) and 0.1% phosphoric acid. Only 6 phosphopeptides were detected by MALDI in former case (data not shown). When in latter case with the improved protocol, the performance of Fe$^{3+}$-IMAC was improved dramatically, 10 phosphopeptides were observed as shown in Fig. 3C. But compared with the mass spectra of phosphopeptides by using Zr$^{4+}$-IMAC beads, less phosphopeptide peaks were detected and the selectivity of Fe$^{3+}$-IMAC for phosphopeptides is much lower than that of Zr$^{4+}$-IMAC.

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Purification of phosphopeptides from peptide mixture using Zr$^{4+}$-IMAC beads

The previous enrichment of phosphopeptides by using Zr$^{4+}$-IMAC beads were performed with peptides derived from a single protein, but phosphoproteins are often low abundant components in real biological protein samples. In order to further investigate the capability of the Zr$^{4+}$-IMAC beads to enrich low abundant phosphopeptides, α-casein or β-casein tryptic digest was mixed with BSA tryptic digest in different molar ratio of 1:0, 1:1 and 1:100 (α- or β-casein vs. BSA) as semicomplex samples. The phosphopeptides were enriched by the beads and then analyzed by MALDI-TOF MS. Peaks of 14 phosphopeptides varied from singly to multiply phosphorylated peptides were found when the molar ratio of α-casein and BSA was at 1:1 (Fig. 4B), the results is similar to the results obtained from using only α-casein as sample (Fig. 4A). When the molar ratio of α-casein and BSA further decreased to 1:100, the data obtained (Fig. 4C) do not differ drastically from those obtained at 1:0 and 1:1, totally 13 peaks were observed, only 1 phosphopeptide YLGEYLVPNpSAEER disappeared, which is a weak peak in mass spectra shown in Fig. 4A and 4B. Considering the concentration of α-casein digest is 100 times lower than that of BSA, the specificity of Zr$^{4+}$-IMAC beads for isolation of phosphopeptides is very high.

(Here we would like to put Fig. 4)

As phosphorylation sites in α-casein and β-casein are all at serine residues, the previous results demonstrated that Zr$^{4+}$-IMAC beads have high specificity to phosphoserine peptides. In order to evaluate whether the Zr$^{4+}$-IMAC beads can selectively enrich other type of phosphopeptides, a singly tyrosine phosphorylated peptide, RRLIEDAEpYAARG (MW 1599, Upstate Co), was added to a tryptic digest of β-casein in a 1:1 molar ratio. The phosphopeptides were enriched by the Zr$^{4+}$-IMAC beads and then
analyzed by MALDI-TOF MS. When only mixture of β-casein digest and pY was pretreated, 5 dominant peaks, represented 4 phosphoserine peptides from β-casein (β1, β3, β4, β5) and 1 phosphotyrosine peptide (pY), can be observed (Fig. 5A). For the complex sample with the addition of BSA digest in a 1:100 molar ratio, the 5 peaks including pY also can be easily observed (Fig. 5B). The results further demonstrate that Zr⁴⁺-IMAC beads do have high specificity for enrichment of different type of phosphopeptides without bias.

(Here we would like to put Fig. 5)

The ability to specifically enrich phosphopeptides in the presence of huge amount of unphosphorylated peptides is a key issue for phosphoproteome analysis. However, Fe³⁺-IMAC lacks enough specificity to selectively capture phosphopeptides from complex peptide mixture. Larsen et al(20) investigated the performance of Fe³⁺-IMAC to enrich phosphopeptides from the digest mixture of phosphoproteins (α-casein, β-casein, ovalbumin) with nonphosphoproteins (serum albumin, β-lactoglobulin, carbonic anhydrase) at different ratios. It was found that phosphopeptides could be selectively captured in the digest mixture of phosphoproteins to nonphosphoproteins at a molar ratio of 1:1. However, when the ratio decreased to 1:10, significantly number of non-phosphorylated peptides were observed and the number of detected phosphopeptides decreased quickly. When the ratio decreased to 1:50, the peaks of phosphopeptides could hardly be observed. Similar results were also observed in our previous works(26). Phosphopeptides can not be specifically captured by Fe³⁺-IMAC from digest mixture of β-casein and BSA with ratio of 1:10. However, as it can be seen from Fig. 4 that phosphopeptides in a complex peptide mixture can be specifically isolated and well detected by Zr⁴⁺-IMAC beads prepared in this study. Those results demonstrated that Zr⁴⁺-IMAC has much higher selectivity for enrichment of phosphopeptides than conventional Fe³⁺-IMAC. The ability of Zr⁴⁺-IMAC to specifically enrich phosphopeptides from the peptide mixture, therein the concentration of non-phosphorylated peptides were several orders of magnitude higher than that of phosphorylated peptides, is largely resulted from the extremely strong interaction between chelating Zr⁴⁺ and phosphate groups in phosphopeptides. As was mentioned before that the interaction is so strong that stable self-assembling monolayer or multilayer thin films of phosphate-containing organic molecules could be prepared by this way(24-26).

*Application of Zr⁴⁺-IMAC beads for phosphoproteome analysis of mouse liver*
To further evaluate its performance for the capture of phosphopeptides, Zr$^{4+}$-IMAC beads were applied to analyze the phosphoproteome of mouse liver. Phosphopeptides from 100 μg mouse liver lysate digest were enriched and one fifth were loaded onto capillary C18 column and analyzed by LTQ mass spectrometer. Three replicate LC-MS runs were conducted for each sample. The acquired MS/MS spectra were searched by Sequest program and the searching results were filtered with Xcorr (>1.9, 2.2, 3.75 for 1+, 2+, 3+ charged peptides) and dCn (>0.1) criteria. For the three replicate analysis, totally 1681 unique peptides were identified, with an average of 109 nonphosphopeptides and 539 phosphopeptides including 137 singly, 174 doubly and 227 triply phosphopeptides each run. Among 1681 unique peptides, 87.2% were phosphopeptides and only 12.8% were nonphosphopeptides. Very low percentage of identified nonphosphopeptides indicated that the nonspecific adsorption of non-phosphopeptides on Zr$^{4+}$-IMAC beads is weak.

The criteria used to filter MS$^2$ database search results in this study were originally used for processing unmodified peptide data (28, 32), and they were also used to processing phosphorylated peptide data recently(33). To the best of our knowledge, the criteria are the highest for processing phosphorylated peptide data searched by Sequest program. However, because of the poor quality of the spectra for phosphopeptides, the scores of a spectrum passing the criteria does not necessarily mean true identification. Other confirmation is required to increase the confidence of the identification. The fragment ion generated by phosphate-loss in MS/MS stage can be further fragmented to generate MS/MS/MS (MS$^3$) spectrum. It was observed previously that MS$^3$ spectra were useful for the validation of phosphopeptides identified from MS$^2$ spectra(3, 33, 34). In this study, the acquired MS$^3$ spectra were also searched by Sequest program. As the peptide identifications derived from MS$^2$ were used to confirm the identifications derived from MS$^3$, relative poor spectra should be allowed. Therefore, relative low criteria with Xcorr (>1.5, 2.0 and 2.5 for 1+, 2+, 3+ charged peptides) and dCn (>0.1) were used to filter the MS$^3$ Database searching results which resulted in the identification of 244 unique phosphopeptides and 3 nonphosphopeptides. Comparing the phosphopeptides identified from MS$^2$ and MS$^3$ spectra, it was found that the sequences of 50 unique phosphopeptides were the same in both cases (34 singly phosphorylated, and 16 multiply phosphorylated). The sequences of these peptides and their Xcorr scores are listed in Supplemental Table 2. Majority of these peptides have very high Xcorr scores. After manually checking, it was found that their spectra were of high quality and so their identifications were considered as positive. These results
indicated that confirmation of phosphopeptide identifications by MS\textsuperscript{3} data is very effective. Fig. 6 is an example for identification of triply charged phosphopeptides from Septin-2, IYHLPDAEpSDEDEDFKEQTR, in which the S\textsuperscript{218} is phosphorylated. From the spectra it can be seen that b- and y-ion series are consistent with the theoretically predicted peaks in both MS\textsuperscript{2} and MS\textsuperscript{3} spectra, and in MS\textsuperscript{2} spectrum the peak at m/z 807.13 represents the triply charged form of the selected precursor ion at m/z 839.86 by losing a H\textsubscript{3}PO\textsubscript{4} group. This phosphorylated site has not been previously reported in the literature, the high quality of MS/MS and MS/MS/MS spectra showed the high performance of Zr\textsuperscript{4+}-IMAC beads for the capture of phosphopeptides.

(Here we would like to put Fig. 6)

Besides above 50 phosphopeptides, there should be more phosphopeptides identified only from MS\textsuperscript{2}. Without the confirmation with MS\textsuperscript{3} data, these identifications should be manually validated carefully. Many multiply phosphorylated peptide identifications were also observed after filtering with Xcorr and dCn criteria. However till now strictly universal validation criteria have not been established and defined, it has too many subjective factors for interpretation of the spectra of multiply phosphorylated peptides, therefore only singly phosphorylated peptide identifications were validated manually in this study. After manual validation, additional 92 singly phosphorylated peptides were finally identified from MS\textsuperscript{2} spectra. Some MS\textsuperscript{3} spectra may yield significantly more structural information than the corresponding MS\textsuperscript{2} spectra that is usually dominated by the phosphate-loss. It was reported that phosphopeptides can also be confidently identified by MS\textsuperscript{3} spectra (3, 33, 34). The MS\textsuperscript{3} database search results were also filtered with the strict criteria, i.e., Xcorr (>1.9, 2.2, 3.75 for 1+, 2+, 3+ charged peptides) and dCn (>0.1). The singly phosphorylated peptides were further manually validated which resulted in the identification of 11 phosphopeptides. The sequences and Xcorr scores for the phosphopeptides finally identified from MS\textsuperscript{2} and MS\textsuperscript{3} were given in Supplemental Table 3 and 4, respectively. Combining with the phosphopeptides identified by MS\textsuperscript{2} and confirmed by MS\textsuperscript{3} (Supplemental Table 2), totally 153 phosphopeptides (163 phosphorylated sites) corresponding to 133 phosphorylated proteins from mouse liver were identified. Among them, 137 peptides were singly phosphorylated, 13 doubly phosphorylated and 3 triply phosphorylated. (The MS\textsuperscript{2} and MS\textsuperscript{3} spectra with labeled fragment peaks for each peptide are listed in supplemental material 2.).
To characterize the phosphorylation sites identified in our study, we used Scansite (http://scansite.mit.edu) (35) to define possible phosphorylation motifs in our data set. By searching the data with a high stringency cutoff filter, 38 of the confidently localized sites could be assigned with kinase motifs. Most of them are Basophilic serine/threonine kinase group (Supplemental Table 5). To further investigate the reliability of the results, PhosphoSite (http://www.Phosphosite.org) was used to distinguish known from novel phosphorylation sites. Among the 163 phosphorylated sites identified, 73.0% (119) have not been reported previously, and 27.0% (44) were known phosphorylation sites which including 33 sites reported in mouse liver and 11 phosphorylated sites reported in other mammalian such as human, and rat (See Supplemental Table 2~4). There were 64 phosphorylation sites in 50 phosphopeptides determined by both the MS² and MS³ spectra. It was found 42.2% (27) of these sites are known phosphorylation sites. For the phosphorylation sites determined by only MS/MS spectra, the percentage of known phosphorylation sites was down to 16.3%. And that determined by only MS/MS/MS spectra, the percentage was 18.2%. Obviously 42.2% is a very high percentage which indicated that the phosphopeptides identified both by MS² and MS³ spectra are high confident. Based on the fact that high quality MS² and MS³ spectra could be acquired for these phosphopeptides, their concentrations in the sample may be higher than others. In other words, their parent phosphoproteins may be relatively high abundant ones. That probably is one of important reasons for many of these phosphorylation sites were already reported in literatures. Many new phosphorylation sites from low abundance phosphoproteins identified by only MS² or MS³ should be also very useful for related biological research in the future.

**Discussion**

The specificity of phosphopeptide enrichment is crucial for successful phosphoproteome analysis. The less presence of non-phosphopeptides, the higher sensitivity for the detection of phosphopeptides. Phosphopeptide enrichment by Zr⁴⁺-IMAC beads followed with LC-MS/MS (MS/MS/MS) analysis allowed the identification of 153 phosphopeptides from 133 phosphoproteins from 100 μg lysate of mouse liver tissue by three separate runs. Among the 153 identified phosphopeptides, 137 peptides were singly phosphorylated peptides. Besides using of Zr⁴⁺-IMAC, different type of Fe³⁺-IMAC adsorbents were also applied for phosphoproteome analysis of mouse liver in our lab. Those include capillary column packed with commercial IMAC beads (POROS 20 MC beads)(3), self-prepared Fe³⁺-IMAC silica monolithic
capillary column(33), and self-prepared Fe$^{3+}$ IMAC mesoporous molecular sieves MCM-41(36). Correspondingly, a total of 26, 29 and 33 singly phosphorylated peptides were identified with single LC-MS run after manual validation, respectively. The number of identified singly phosphorylated phosphopeptides by Zr$^{4+}$-IMAC approach (average of 61 singly phosphorylated peptides for each run) was significantly higher than that by any of above mentioned Fe$^{3+}$-IMAC approaches. The high performance is largely because Zr$^{4+}$-IMAC beads can highly specifically enrich phosphopeptides which effectively avoiding the suppression of nonphosphopeptides during MS detection. As many as 50 unique phosphopeptides were both identified from MS/MS and MS/MS/MS spectra with Zr$^{4+}$-IMAC approach, which also indicated that phosphopeptides could be efficiently enriched by Zr$^{4+}$-IMAC beads and so high quality MS/MS/MS spectra were acquired.

The specificity of Fe$^{3+}$-IMAC could be improved by esterification of acidic groups on peptide molecules prior to IMAC purification(23, 37). Moser and White(18) have applied this approach to phosphoproteome analysis of rat liver phosphoproteome. It was found that only about 30% of identified peptides were phosphorylated if no esterification was performed, and the percentage increased to 85% when esterification was conducted. In our case by using Zr$^{4+}$-IMAC beads, 87.2% of the peptides identified from MS$^2$ spectra were phosphorylated peptides. The percentage of identified phosphopeptides by using Zr$^{4+}$-IMAC beads was much higher than the equivalent method of Fe$^{3+}$-IMAC beads, and was similar to the method by combination of peptide esterification with Fe$^{3+}$-IMAC beads. This means the specificity of Zr$^{4+}$-IMAC beads was much higher than that of Fe$^{3+}$-IMAC, and was similar to that of Fe$^{3+}$-IMAC beads combined with peptides esterification. The disadvantage of peptides esterification prior to IMAC purification is that the products resulted from side reaction and incomplete reaction may increase sample complexity and interfere with subsequent mass spectrometry analysis. The use of Zr$^{4+}$-IMAC beads is much simpler as no additional sample pretreatment is required.

Metal oxides such as TiO$_2$ and ZrO$_2$ beads were introduced as affinity materials to selectively enrich phosphopeptides(20, 21). The microcolumns or microtips packed with these metal oxides beads have been proved to have higher selectivity for trapping of phosphopeptides than those packed with conventional Fe$^{3+}$-IMAC beads. The mechanism of ZrO$_2$ and Zr$^{4+}$-IMAC beads for selective capture of phosphopeptides are similar. Both of them are based on the strong interaction between Zr atom and phosphate groups on phosphopeptides. We also demonstrated that ZrO$_2$ nanobeads have high specificity for
ZrO₂ nanobeads were also used to enrich phosphopeptides from the digest of mouse liver lysate in our lab. After LC-MS (MS² and MS³) analysis of the enriched phosphopeptides and manual validation of database search results, totally 141 phosphorylated peptides including 48 singly phosphorylated phosphopeptides were identified. The number of validated singly phosphorylated peptides was much less than that in this study using Zr⁴⁺-IMAC. In order to further compare the specificities of ZrO₂ and Zr⁴⁺-IMAC, the percentage of phosphopeptides in the MS/MS database search results (filtered with Xcorr and dCn criteria but without manual validation) obtained by using ZrO₂ nanobeads was calculated to be 72% which was lower than that of Zr⁴⁺-IMAC (87%). Above results demonstrated that Zr⁴⁺-IMAC has higher selectivity for phosphopeptides than that of ZrO₂ nanobeads. The different performance between ZrO₂ beads and GMA-EDMA phosphonate Zr⁴⁺ beads (Zr⁴⁺-IMAC beads) may be caused from two reasons. First, GMA-EDMA beads do not have strong Lewis acid sites as ZrO₂ beads on the surface; therefore surface of GMA-EDMA beads is more biocompatible and less nonspecific adsorption. Second, there is spacer arm between polymer support and Zr⁴⁺, which makes the phosphate groups on phosphopeptides have more chance to access Zr⁴⁺ on GMA-EDMA beads.

Recently, titanium dioxide (TiO₂) has been used as an alternative to IMAC for the selective enrichment of phosphopeptides both from simple protein samples to complex bio-samples(21, 39-42). Larsen et al(20) probed the performance of TiO₂ microcolumn to enrich phosphopeptides form the mixture of phosphoproteins (α-casein, β-casein, ovalbumin) with non-phosphoproteins (serum albumin, β-lactoglobulin, carbonic anhydrase) at different molar ratios. It was found that the molar ratio of phosphoprotein to non-phosphoprotein decreased to 1:10, the number of non-phosphorylated peptides would be well detected; when it decreased to 1:50, a number of non-phosphorylated peptides were also detected. However, when the ratio even decreased to 1:100 in our case, peaks of non-phosphorylated peptides could be hardly observed (as shown in Fig. 4 and 5), this indicates a much more selective binding of the phosphorylated peptides on the Zr⁴⁺-IMAC beads than on the TiO₂ beads.

Kweon and Hakansson(21) investigated the performance of ZrO₂ and TiO₂ microtips for trypsin and Glu-C proteolytic digests of α-casein and β-casein and compared binding specificity and recovery of phosphopeptide by ZrO₂ and to TiO₂. It was demonstrated that ZrO₂ microtips display similar overall performance as TiO₂ microtips. However, more selective isolation of singly phosphorylated peptides was observed with ZrO₂ compared to TiO₂, whereas TiO₂ preferentially enriched multiply phosphorylated
peptides. Since Zr$^{4+}$-IMAC has higher selectivity than ZrO$_2$ does in our case, Zr$^{4+}$-IMAC may also have higher selectivity than TiO$_2$. However further experiments were needed to prove this allegation. As there is no spacer arm on metal oxide beads, one disadvantage of using metal oxide beads for phosphopeptide enrichment is the presence of steric hindrance.

In conclusion, a new generation of IMAC absorbent was presented for high specific enrichment of phosphopeptides for phosphoproteome analysis. The IMAC beads were prepared by immobilization of Zr$^{4+}$ on the phosphonate-modified GMA-EDMA polymer beads. Zr$^{4+}$-IMAC beads were demonstrated to have high specificity to phosphopeptides by using standard phosphoproteins as well as real biological sample. Esterification prior to IMAC purification is not necessary for Zr$^{4+}$-IMAC because of its excellent specificity. Zr$^{4+}$-IMAC beads prepared in this study have both the high selectivity of Zr$^{4+}$ ion and high biocompatibility and chemical stability of GMA-EDMA polymer, and are ideal IMAC adsorbents for purification of phosphopeptides in phosphoproteome analysis.
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Supplemental data

The supplemental tables are included in supplemental material 1, the labeled Spectral for identified phosphopeptides are given in supplemental materials 2, the raw data for the three LC-MS/MS runs obtained by LTQ are available on web: http://www.bioanalysis.dicp.ac.cn/proteomics/Publications/Zr-IMAC/Zr-IMAC-data.htm.
References

1. Mann, M., and Jensen, O. N. (2003) Proteomic analysis of post-translational modifications. Nat. Biotechnol. 21, 255-261.

2. Mann, M., Ong, S. E., Gronborg, M., Steen, H., Jensen, O. N., and Pandey, A. (2002) Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. Trends Biotechnol. 20, 261-268.

3. Jin, W. H., Dai, J., Zhou, H., Xia, Q. C., Zou, H. F., and Zeng, R. (2004) Phosphoproteome analysis of mouse liver using immobilized metal affinity purification and linear ion trap mass spectrometry. Rapid Commun. Mass Spectrom. 18, 2169-2176.

4. Wolschin, F., Lehmann, U., Glinski, M., and Weckwerth, W. (2005) An integrated strategy for identification and relative quantification of site-specific protein phosphorylation using liquid chromatography coupled to MS2/MS3. Rapid Commun. Mass Spectrom. 19, 3626-3632.

5. Giorgianni, F., Beranova-Giorgianni, S., and Desiderio, D. M. (2004) Identification and characterization of phosphorylated proteins in the human pituitary. Proteomics 4, 587-598.

6. Zhang, H., Li, X. J., Martin, D. B., and Aebersold, R. (2003) Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. Nat. Biotechnol. 21, 660-666.

7. Rush, J., Moritz, A., Lee, K. A., Guo, A., Goss, V. L., Spek, E. J., Zhang, H., Zha, X. M., Polakiewicz, R. D., and Comb, M. J. (2005) Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat. Biotechnol. 23, 94-101.

8. Conrads, T. P., and Veenstra, T. D. (2005) An enriched look at tyrosine phosphorylation. Nat. Biotechnol. 23, 36-37.

9. Oda, Y., Nagasu, T., and Chait, B. T. (2001) Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. Nat. Biotechnol. 19, 379-382.

10. Arrigoni, G., Resjo, S., Levander, F., Nilsson, R., Degerman, E., Quadrioni, M., Pinna, L. A., and James, P. (2006) Chemical derivatization of phosphoserine and phosphothreonine containing peptides to increase sensitivity for MALDI-based analysis and for selectivity of MS/MS analysis. Proteomics 6, 757-766.

11. Zhou, H. L., Watts, J. D., and Aebersold, R. (2001) A systematic approach to the analysis of protein phosphorylation. Nat. Biotechnol. 19, 375-378.

12. Tao, W. A., Wollscheid, B., O’Brien, R., Eng, J. K., Li, X. J., Bodenmiller, B., Watts, J. D., Hood, L., and Aebersold, R. (2005) Quantitative phosphoproteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry. Nat. Methods 2, 591-598.

13. Peng, J., Elias, J. E., Thoreen, C. C., Licklider, L. J., and Gygi, S. P. (2003) Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. J. Proteome Res. 2, 43-50.

14. Beausoleil, S. A., Villen, J., Gerber, S. A., Rush, J., and Gygi, S. P. (2006) A probability-based approach for high-throughput protein phosphorylation analysis and site localization. Nat. Biotechnol.
15. Brill, L. M., Salomon, A. R., Ficarro, S. B., Mukherji, M., Stettler-Gill, M., and Peters, E. C. (2004) Robust phosphoproteomic profiling of tyrosine phosphorylation sites from human T cells using immobilized metal affinity chromatography and tandem mass spectrometry. *Anal. Chem.* 76, 2763-2772.

16. Posewitz, M. C., and Tempst, P. (1999) Immobilized gallium(III) affinity chromatography of phosphopeptides. *Anal. Chem.* 71, 2883-2892.

17. Nuhse, T. S., Stensballe, A., Jensen, O. N., and Peck, S. C. (2003) Large-scale analysis of in vivo phosphorylated membrane proteins by immobilized metal ion affinity chromatography and mass spectrometry. *Mol. Cell. Proteomics* 2, 1234-1243.

18. Moser, K., and White, F. M. (2006) Phosphoproteomic analysis of rat liver by high capacity IMAC and LC-MS/MS. *J. Proteome Res.* 5, 98-104.

19. Kokubu, M., Ishihama, Y., Sato, T., Nagasu, T., and Oda, Y. (2005) Specificity of immobilized metal affinity-based IMAC/C18 tip enrichment of phosphopeptides for protein phosphorylation analysis. *Anal. Chem.* 77, 5144-5154.

20. Larsen, M. R., Thingholm, T. E., Jensen, O. N., Roepstorff, P., and Jorgensen, T. J. D. (2005) Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell. Proteomics* 4, 873-886.

21. Kweon, H. K., and Hakansson, K. (2006) Selective zirconium dioxide-based enrichment of phosphorylated peptides for mass spectrometric analysis. *Anal. Chem.* 78, 1743-1749.

22. Chen, C. T., and Chen, Y. C. (2005) Fe3O4/TiO2 core/shell nanoparticles as affinity probes for the analysis of phosphopeptides using TiO2 surface-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* 77, 5912-5919.

23. Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F., and White, F. M. (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat. Biotechnol.* 20, 301-305.

24. Nixon, C. N., Le Claire, K., Odobel, F., Bujoli, B., and Talham, D. R. (1999) Palladium porphyrin containing zirconium phosphonate Langmuir-Blodgett films. *Chem. Mat.* 11, 965-976.

25. Nonglaton, G., Benitez, I. O., Guisle, I., Pipelier, M., Leger, J., Dubreuil, D., Tellier, C., Talham, D. R., and Bujoli, B. (2004) New approach to oligonucleotide microarrays using zirconium phosphonate-modified surfaces. *J. Am. Chem. Soc.* 126, 1497-1502.

26. Zhou, H. J., Xu, S. Y., Ye, M. L., Feng, S., Pan, C., Jiang, X. G., Li, X., Han, G. H., Fu, Y., and Zou, H. (2006) Zirconium phosphonate-modified porous silicon for highly specific capture of phosphopeptides and MALDI-TOF MS analysis. *J. Proteome Res.* 5, 2431-2437.

27. Gong, B. L., Zhu, J. X., Li, L., Qiang, K. J., and Ren, L. (2006) Synthesis of non-porous poly(glycidylmethacrylate-co-ethylenedimethacrylate) beads and their application in separation of biopolymers. *Talanta* 68, 666-672.
Wang, K., Zhang, J.-S., Wang1, H.-X., Song, Z.-M., Zhang, H.-R., Zeng, R., and Zhao, X. (2005) The human plasma proteome: Analysis of Chinese serum using shotgun strategy. Proteomics 5, 3442–3453.

29. Luo, Q., Mao, X., Kong, L., Huang, X., and Zou, H. (2002) High-performance affinity chromatography for characterization of human immunoglobulin G digestion with papain. J. Chromatogr. 776, 139-147.

30. Luo, Q., Zou, H., Xiao, X., Guo, Z., Kong, L., and Mao, X. (2001) Chromatographic separation of proteins on metal immobilized iminodiacetic acid-bound molded monolithic rods of macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate). J. Chromatogr. A 926, 255-264.

31. Feng, S., Ye, M., Jiang, X., Jin, W., and Zou, H. (2006) Coupling the immobilized trypsin microreactor of monolithic capillary with muRPLC-MS/MS for shotgun proteome analysis. J. Proteome Res. 5, 422-428.

32. P.Washburn, M., Wolters, D., and R.Yates, J. (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat. Biotechnol. 19, 242-247.

33. Feng, S., Pan, C., Jiang, X., Xu, S., Zhou, H., Ye, M., and Zou, H. (2007) Fe³⁺ immobilized metal affinity chromatography with silica monolithic capillary column for phosphoproteome analysis. Proteomics 7, 351-360.

34. Lee, J., Xu, Y., Chen, Y., Sprung, R., Kim, S. C., Xie, S., and Zhao, Y. (2007) Mitochondrial phosphoproteome revealed by an improved IMAC method and MS/MS/MS. Mol. Cell. Proteomics In press,

35. Obenauer, J. C., Cantley, L. C., and Yaffe, M. B. (2003) ScanSite 2.0: proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res. 31, 3635-3641.

36. Pan, C., Ye, M., Liu, Y., Feng, S., Jiang, X., Han, G., Zhu, J., and Zou, H. (2006) Enrichment of phosphopeptides by Fe³⁺-immobilized mesoporous nanoparticles of MCM-41 for MALDI and nano-LC-MS/MS analysis J. Proteome Res. 5, 3114-3124.

37. Ndassa, Y. M., Orsi, C., Marto, J. A., Chen, S., and Ross, M. M. (2006) Improved immobilized metal affinity chromatography for large-scale phosphoproteomics applications. J. Proteome Res. 5, 2789-2799.

38. Zhou, H., Tian, R., Ye, M., Songyun, X., Feng, S., Pan, C., Jiang, X., Li, X., and Zou, H. Highly specific enrichment of phosphopeptides by zirconium dioxide nanoparticles for phosphoproteome analysis. Electrophoresis, In press.

39. Bodenmiller, B., Mueller, L. N., Mueller, M., Domon, B., and Aebersold, R. (2007) Reproducible isolation of distinct, overlapping segments of the phosphoproteome. Nat. Methods 4, 231-237.

40. Liang, S. S., Makamba, H., Huang, S. Y., and Chen, S. H. (2006) Nano-titanium dioxide composites for the enrichment of phosphopeptides. J. Chromatogr. A 1116, 38-45.

41. Pocsfalvi, G., Cuccurullo, M., Schlosser, G., Scacco, S., Papa, S., and Malorni, A. (2007) Phosphorylation of B14.5a subunit from bovine heart complex I identified by titanium dioxide selective enrichment and shotgun proteomics. Mol. Cell. Proteomics 6, 231-237.
42. White, M. Y., Hambly, B. D., Jeremy, R. W., and Cordwell, S. J. (2006) Ischemia-specific phosphorylation and myofilament translocation of heat shock protein 27 precedes alpha B-crystallin and occurs independently of reactive oxygen species in rabbit myocardium. *J. Mol. Cell. Cardiol.* 40, 761-774.
Figure captions:

Fig. 1. Scheme for the preparation of Zr$^{4+}$-IMAC beads

Fig. 2. MALDI mass spectra of tryptic digest of β-casein. (A) Direct analysis; (B) analysis after pretreated by GMA-EDMA polymer beads; (C) analysis after pretreated by GMA-EDMA phosphonate polymer beads; (D) analysis after pretreated by Zr$^{4+}$-IMAC beads. The loading amount of tryptic digest of β-casein is 2 pmol.

Fig. 3. MALDI mass spectra of tryptic digest of α-casein. (A) Direct analysis; (B) analysis after enriched by Zr$^{4+}$-IMAC beads; (C) analysis after enriched by F3$^{+}$-IMAC. The loading amount of tryptic digest of α-casein is 2 pmol.

Fig. 4. Selective enrichment of phosphopeptides from the mixture of tryptic digest of α-casein and BSA in different molar ratio: (A) 1:0, (B) 1:1, and (C) 1:100. The loading amount of tryptic digest of α-casein is 2 pmol.

Fig. 5. Selective enrichment of phosphopeptides from the mixture of the tryptic digest of β-casein and a standard phosphotyrosine peptides (pY) using Zr$^{4+}$-IMAC beads. (A) β-casein digest with pY (2 pmol respectively); (B) β-casein digest and pY (2 pmol respectively) were mixed with 200 pmol BSA tryptic digest. The sequence for pY was RRLIEDAEpYAARG.

Fig. 6. A) MS$^2$ spectrum of the doubly charged form of a singly phosphorylated peptide identified from the tryptic digest of lysate of mouse liver. The dominant peak at m/z 807.13 represents the doubly charged neutral loss peak (after loss of the H$_3$PO$_4$ group) for its selected precursor ion (m/z 839.86). The identified phosphopeptides was IYHLPDAEpSDEDFKEQTR. (B) MS$^3$ spectrum of the doubly charged neutral loss peak at m/z 807.13. The b- and y-ion series shown both in (A) and (B) are indicated as to verify the identification of the peptide.
Figure 1

GMA-EDMA beads + NH$_3$ (40 °C) → GMA-EDMA beads

GMA-EDMA beads + POCl$_3$, Collidine in CH$_3$CN, 12 h →

GMA-EDMA beads + ZrOCl$_2$ →

GMA-EDMA beads
Figure 2
Figure 4
