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Organic−Inorganic Hybrid Silica Monolith Based Immobilized Titanium Ion Affinity Chromatography Column for Analysis of Mitochondrial Phosphoproteome

Chunyan Hou,[†,‡,§] Junfeng Ma,[†,‡,§] Dingyin Tao,[†,‡] Yichu Shan,[†] Zhen Liang,[†] Lihua Zhang,[*,†] and Yukui Zhang[†]

Key Laboratory of Separation Science for Analytical Chemistry, National Chromatographic Research and Analysis Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China, and Graduate School of the Chinese Academy of Sciences, Beijing 100039, China

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A novel kind of immobilized metal affinity chromatography (IMAC) column based on organic−inorganic hybrid silica monolith has been developed. The monolithic support was prepared in a 250 μm i.d. capillary by the sol−gel method with tetraethoxysilane (TEOS) and 3-aminopropyltriethoxysilane (APTES) as precursors. Subsequently, amine groups were functionalized by glutaraldehyde, and then activated with (aminomethyl) phosphonic acid, followed by Ti^{4+} chelation. By such a hybrid silica monolithic Ti^{4+}-IMAC column, 15 phosphopeptides were effectively isolated from the digest mixture of α-casein and BSA with the molar ratio as low as 1:200, illustrating its superior selectivity. With a synthetic phosphorylated peptide, YKVPQLEIVPNSpAEER, as the sample, the loading capacity and recovery of the Ti^{4+}-IMAC monolithic column were measured to be 1.4 μmol/mL and 69%, respectively. Such an IMAC monolithic column was further applied to enrich phosphopeptides from rat liver mitochondria. In total, 224 unique phosphopeptides, corresponding to 148 phosphoprotein groups, were identified by duplicate nanoRPLC-LTQ MS/MS/MS runs with a false-positive rate of less than 1% at the peptide level. These results demonstrate that the hybrid silica monolith based Ti^{4+}-IMAC column might provide a promising tool for large-scale phosphopeptide enrichment, facilitating the in-depth understanding of the biological functions of phosphoproteomes.

Keywords: immobilized metal ion affinity chromatography • organic−inorganic hybrid silica monolith • titanium ion • phosphopeptide enrichment • mitochondrial phosphoproteome

Introduction

As one of the most biologically relevant and ubiquitous post-translational modifications, phosphorylation is a reversible event affecting both the folding and function of proteins, regulating essential functions such as cell division, signal transduction, and enzymatic activity. It is estimated that one-third of all proteins are phosphorylated *in vivo* in mammalian cells at any time point.[1,2] Besides the whole cell phosphoproteomics, the subcellular/organelle phosphoproteomics has drawn much attention in recent years.[3]

Mass spectrometry (MS) has emerged as a key technology for mapping protein phosphorylation sites due to its high sensitivity and high throughput.[4,5] However, the selective enrichment of phosphopetides is still an indispensable step prior to MS analysis because of the highly dynamic nature of proteomes and low stoichiometry of phosphoproteins.[6,7] One appealing enrichment method is immobilized metal ion affinity chromatography (IMAC),[8] in which Fe^{3+} or Ga^{3+} are immobilized on adsorbents via chelating ligands such as iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) typically.[9,10] However, by such IMAC materials, the enrichment specificity is limited,[11] since acidic peptides and peptides containing histidine residues can also be enriched, which might suppress the detection of phosphopeptides. To solve this problem, the chelate interaction between Zr^{4+} or Ti^{4+} and phosphate groups have been used for phosphopeptide enrichment in recent years,[12,13] which could offer strong interaction and unique coordination specificity to greatly improve the selectivity for phosphopeptides.

Besides metal ions, the supporting materials also greatly affect the performance of IMAC column.[8] To date, several materials, including the inner wall of capillaries,[13,14] polymer beads,[11,15] and monoliths (organic polymer-[16] and silica-monolith[17]) have been employed for metal ion chelate immobilization, among which monoliths have exhibited striking features, such as large loading capacity and fast mass transfer for phosphopeptide enrichment due to their unique porous structures. However, the preparation of conventional silica-based monoliths is time-consuming, difficult to control,[18] and the poor chemical stability further inhibits its wide

* To whom correspondence should be addressed. E-mail: lihuazhang@dicp.ac.cn. Tel.: +86-411-84379720. Fax: +86-411-84379560.
† Dalian Institute of Chemical Physics, Chinese Academy of Sciences.
‡ Graduate School of the Chinese Academy of Sciences.
§ These authors contributed equally to this work.

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acceptance as enrichment matrix. Although organic polymer monolithic materials possess good biocompatibility and excellent pH stability, shrinkage and swelling might occur. This is unfavorable for the enrichment of phosphopeptides. To overcome these drawbacks, organic−inorganic hybrid silica monoliths have been introduced into bioanalysis quite recently. It combines the merits of silica with organic polymer monolithic material, such as good biocompatibility, excellent pH tolerance, high permeability, and has been used as the matrices of separation columns[19] and immobilized enzymatic reactors (IMERs) successfully.[20,21] However, to the best of our knowledge, hybrid silica monoliths have not been exploited as candidate matrices for phosphopeptide enrichment yet.

In this study, based on our recent success in the preparation of hybrid silica monoliths,[20,21] a novel Ti^{4+}-IMAC column was prepared by the sol−gel method and Ti^{4+} chelate immobilization. Furthermore, it was successfully applied to the analysis of mitochondrial phophosproteome, which is important for the regulation of respiratory activity in the cell and to signaling pathways leading to apoptosis, as well as other vital mitochondrial processes.[22] The results demonstrate that the novel hybrid silica monolithic Ti^{4+}-IMAC column might provide a very promising tool for large-scale phosphoproteome analysis.

Experiment

Reagents and Materials. Fused-silica capillaries (250 μm i.d. × 375 μm o.d.) were obtained from Sino Sumtech Co., Ltd. (Handan, China). Cetyltrimethyl ammonium bromide (CTAB) was supplied by Beijing Chemical Reagent Company (Beijing, China). Tetraethoxysilane (TEOS, 95%), 3-amino-propyltriethoxysilane (APTES, 99%), sodium cyanoborohydride ($NaCNBH_3$), and trifluoroacetic acid (TFA) were purchased from Acros Organics (Geel, Belgium). Fe^{3+}-IMAC column (300 μm × 10 cm) was home packed with POROS 20 MC media (Applied Biosystems Co., Ltd., Foster, CA), and activated with $FeCl_3$ following the manufacturers' instructions. TiO_2 beads (5 μm) were obtained from GL Sciences, Inc. (Tokyo, Japan). A synthetic phosphopeptide (YKVPQLEIVPNSpAEER, 95%) was ordered from GL Biochem Ltd. (Shanghai, China). TPCK-treated trypsin (bovine pancreas), bovine serum albumin (BSA), α-casein (bovine milk), (aminomethyl) phosphonic acid (AMPA, 99%), 2,5-dihydroxybenzoic acid (DHB), and $NaVO_3$ were obtained from Sigma (St. Louis, MO). Calbiochem protease inhibitor cocktail set 1 was purchased from Merck (Darmstadt, Germany). 1,4-Dithio-D,L-threitol (DTT) and iodoacetamide (IAA) were purchased from Amresco Inc. (Solon, OH). $Ti(SO_4)_2$ was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). NaF was purchased from Sangon Biological Engineering & Services Co., Ltd. (Shanghai, China). C18 silica particles (5 μm, 200 Å) were from Agela Technologies Inc., (Tianjin, China). Organic solvents were all of HPLC grade. Water was purified by a Milli-Q system (Millipore, Molsheim, France).

Preparation of Hybrid Silica Monolithic Ti^{4+}-IMAC Column. The monolithic material was prepared according to our previously reported procedure.[21] In brief, the capillary with inner diameter of 250 μm was filled with polymerization solution containing TEOS (112 μL), APTES (118 μL), anhydrous ethanol (215 μL), CTAB (8 mg), and water (32 μL) and kept at 40 °C for 24 h to form organic−inorganic hybrid silica monolith. Subsequently, the monolithic support was activated by flushing the capillary with 10% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH 8.0). Then, the activated support was modified with AMPA by continuously pumping 2 mg/mL AMPA in 100 mM phosphate buffer (pH 8.0) containing 10 mg/mL $NaCNBH_3$ through the column for 6 h. Finally, 100 mM $Ti(SO_4)_2$ was flushed through the monolithic capillary column for 6 h. All of the modification processes were performed at room temperature.

Sample Preparation. α-Casein was dissolved in 50 mM ammonium bicarbonate buffer (pH 8.0) to a concentration of 0.2 mg/mL, and digested for 12 h at 37 °C with protein/trypsin ratio of 40/1 (w/w). BSA (1 mg) was dissolved in 100 μL denaturing buffer containing 8 M urea and 20 mM DTT. The protein solution was incubated for 1 h at 56 °C. Then, 20 μL of 200 mM IAA was added and the obtained solution was incubated for an additional 30 min at 37 °C in the dark. After that, the mixture was diluted by 10-fold with 50 mM ammonium bicarbonate buffer (pH 8.0) and digested by trypsin with a substrate/trypsin ratio of 40/1 (w/w) for 12 h at 37 °C. The digests were lyophilized by a vacuum concentrator for further usage.

A fraction highly enriched in mitochondrial proteins was isolated from rat liver as described previously,[22] with minor modifications. Briefly, 2 g of rat liver tissue were dissected and then sliced into small tissue pieces about 1 cube mm each. After washed three times with PBS, tissue pieces were transferred into individual Eppendorf tubes containing 6 mL of 40 mM Tris-HCl buffer (pH 7.4) together with 250 mM sucrose, 1 mM EDTA, 5 mM DTT, 0.2 mM Na_2VO_3, 1 mM NaF, and 1% (v/v) protease inhibitor cocktail set 1. The suspension was homogenized on ice for 1 min, ultrasonicated for 30 s and centrifuged at 800× *g* for 10 min to remove nuclei and undissolved components. Subsequently, the supernatant was further centrifuged at 10 000× *g* for 15 min, and at 100 000× *g* for 1 h to separate cytosolic and microsomal proteins. The pellet containing the mitochondrial proteins was collected and suspended in sample buffer consisting 40 mM Tris-HCl (pH 7.4), 7 M urea, 2 M thiourea, 10 mM DTT, 1 mM EDTA, and 1% (v/v) protease inhibitor cocktail set 1, and centrifuged at 150 000× *g* for 45 min to obtain the soluble fraction. The protein concentration was measured with a Bradford assay kit using BSA as a standard. The tryptic digestion protocol was almost the same as that of BSA, except that a 24-h digestion time was adopted for the complex sample.

Phosphopeptide Enrichment. The enrichment of phosphopeptides was conducted with hybrid silica monolithic Ti^{4+}-IMAC column as follows. For enrichment of standard protein digests, 2 pmol of α-casein digests were dissolved in loading buffer (80% ACN, 6% TFA) at a concentration of 8 μM, then pumped through the Ti^{4+}-IMAC column. To remove the nonphosphopeptides, two washing steps were performed respectively with 25 μL of solution 1 (50% ACN/6% TFA/200 mM NaCl) and solution 2 (30% ACN/0.1% TFA). Finally, 25 μL of 12.5% ammonia solution flushed through the column to elute the phosphopeptides. In all the steps above, constant flow rate of 5 μL/min was adopted. For enrichment of phosphopeptides in mitochondrial samples, the same buffers were used. Peptides were dissolved at a concentration of 2 mg/mL in 250 μL loading buffer and pumped through the column at 2 μL/min. Then, 250 μL of washing and eluting solutions were pumped through the column at 4 μL/min respectively. All experiments were carried out at room temperature. All of the percentages refer to volume ratios unless otherwise specified.

For comparison, phosphopeptides were also enriched with commercial TiO_2 beads and columns packed with Fe^{3+}-IMAC

resin, mainly according to the manufacturers' instructions. For the TiO_2 enrichment method, 25 μL of 0.08 μM tryptic digests of α-casein was incubated with 100 μg TiO_2 beads. The loading, washing, and eluting solution were the same as those used on the monolithic Ti^{4+}-IMAC column, and an incubation time of 30 min was adopted in each step. For the Fe^{3+}-IMAC column enrichment method, peptides were dissolved in 0.1% acetic acid and loaded on the column. Two steps of washing were sequentially performed with solution 1 (25% ACN solution containing 1% TFA and 100 mM NaCl) and solution 2 (0.1% acetic acid). Then, the phosphopeptides were eluted with 12.5% ammonia solution. A 30 min of incubation time was used in each step. The conditions for the enrichment of phosphopeptides from mitochondrial protein sample with these two commercial materials are specified in Supporting Information Figure 3.

For standard protein or protein mixture analysis, the eluted phosphopeptides were lyophilized and then dissolved in 25 mg/mL DHB solution containing 50% ACN and 1% phosphate acid for MALDI-TOF MS analysis. For the mitochondrial protein sample, the eluted phosphopeptides were lyophilized and then dissolved in 2% ACN containing 0.1% formic acid for nanoRPLC−ESI−MS/MS/MS analysis.

MALDI-TOF MS Analysis. MALDI-TOF MS analysis was performed on a Bruker Autoflex time-of-flight mass spectrometer (Bruker, Bremen, Germany) combining a delayed ion-extraction device, a 337-nm pulsed nitrogen laser and a ground-steel sample target with 384 spots. The linear positive-ion mode was employed with delayed ion extraction. The delayed time for ion extraction was 90 ns, and the extraction voltage was 20 kV. Each MS spectrum was typically summed with 30 laser shots.

Nano-RPLC−ESI−MS/MS Analysis. NanoRPLC−ESI−MS/MS/MS analysis was performed on an LTQ linear ion trap XL mass spectrometer with a nanospray ion source and a Surveyor HPLC system (Thermo Fisher Scientific, San Jose, CA). The peptide mixture was loaded on a RP trap column (150 μm i.d. × 2 cm) at a flow rate of 10 μL/min and then separated by a C18 capillary column (75 μm i.d. × 14 cm). The trap column and the analytical column were both packed in-house with C18 silica particles. Two kinds of buffer solution were H_2O with 2% ACN and 0.1% formic acid (A), ACN with 2% H_2O and 0.1% formic acid (B). The elution conditions contained 10 min of elution with 0% B for desalting. Then, the linear gradients was 0−10% buffer B for 15 min, 10−40% B for 85 min, 40−80% for 5 min then maintained at 80% B for 15 min, at last re-equilibrated with buffer A for 15 min, and the flow rate after splitting was about 200 nL/min.

The LTQ instrument was operated in positive mode. The heated capillary temperature and the spray voltage were respectively 180 °C and 1.8 kV. The collision energy for MS/MS scanning was 35%. An automated gain control function was used to manage the number of ions injected into ion trap. One microscan was set for each MS and MS/MS scan. All MS and MS/MS spectra were acquired in the data dependent mode. One full scan MS was followed by three data-dependent MS/MS (MS^2) scans. A MS/MS/MS (MS^3) spectrum was automatically triggered when the 10 most intense peaks from the MS^2 spectrum corresponded to the neutral loss event of 98.00, 49.00, and 32.67 Da for the precursor ion with 1+, 2+, and 3+ charge states, respectively. The dynamic exclusion function was set as follows: repeat count, 1; repeat duration, 30 s; exclusion duration, 180 s. Total ion current chromatograms and mass spectra ranging from m/z 400 to 2000 were recorded with Xcalibur software (Version 2.0.7).

Database Searching. The spectra searching of MS^2 and MS^3 were performed with SEQUEST algorithm incorporated in BioWorks software 3.3.1 against a composite database, ipi.RAT.V3.26, including both original and reversed database, respectively. The following search criteria were employed: full tryptic specificity was required, and two missed cleavages were allowed. The mass tolerances were 2 Da for parent masses and 1 Da for fragment masses. Cysteine residues were searched as static modification of 57 Da. The dynamic modifications for search with MS/MS data were oxidized Met (+16), and phosphorylated Ser, Thr, and Tyr (+80). The dynamic modifications for search with MS^3 were water loss on Ser, Thr (−18), besides the parameters for MS^2. The APIVASE software was used to validate the identification results.[23] The false-positive rate was set less than 1% with criteria ($Rank'_m = 1$, $\Delta Cn'_m \geq 0.1$, and $Xcorr'_s > 0.6$) for phosphopeptide identification. The apparent redundancy in protein identification was reduced.

Results and Discussion

Preparation of Hybrid Silica Monolithic Ti^{4+}-IMAC Column. The preparation procedure of the hybrid silica monolithic Ti^{4+}-IMAC column is illustrated in Figure 1. The

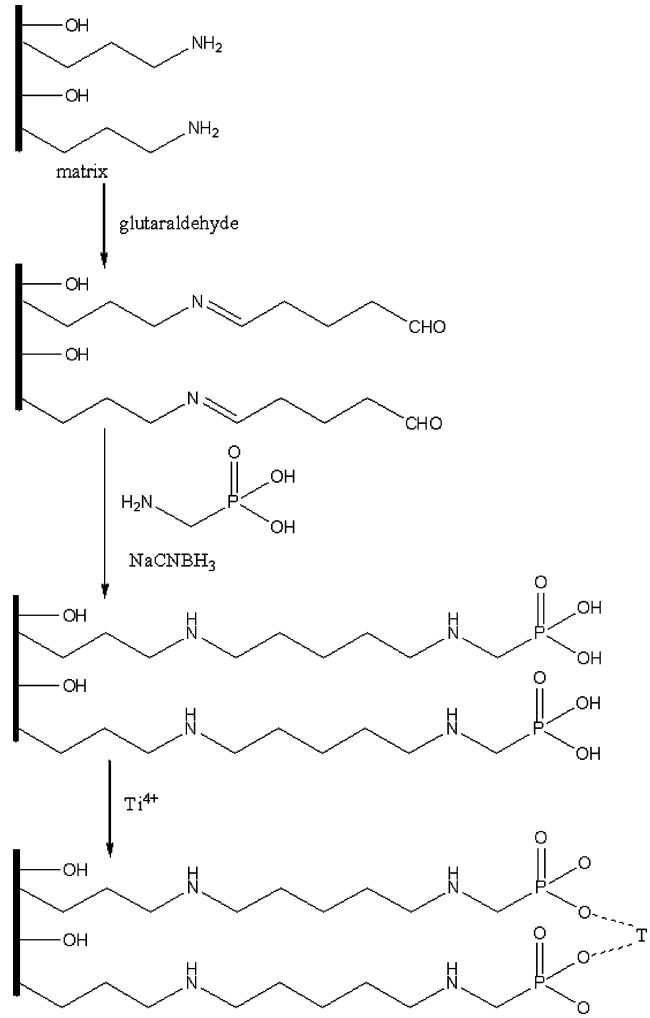

Figure 1. Preparation procedure for hybrid silica monolithic Ti^{4+}-IMAC column.

Table 1. Phosphopeptides Identified from α-Casein Tryptic Digest Enriched by Various Materials

no.	amino acid sequence[a]	measured *m/z*	no. of phosphoryl groups	monolithic Ti^{4+}-IMAC	Fe^{3+}-IMAC	TiO_2 beads
α_1	TVDMESpTEVF	1237.40	1	Y		
α_2	TVDMoMESpTEVF[b]	1254.00	1			Y
α_3	TVDMESpTEVFTK	1466.53	1	Y	Y	Y
α_4	TVDMoESpTEVFTK[b]	1480.59	1			Y
α_5	EQLSpTSpEENSKK	1539.62	2	Y		
α_6	VPQLIVPNSpAEER	1660.59	1	Y	Y	Y
α_7	YLGEYLIVPNSpAEER	1832.48	1	Y	Y	Y
α_8	DIGSpESpTEDQAMEDIK	1928.29	2	Y		
α_9	DIGSpESpTEDQAMoEDIK[b]	1945.19	2	Y		
α_{10}	YKVPQLEIVPNSpAEER	1951.65	1	Y	Y	Y
α_{11}	KKYKVPQLEIVPNSpAEERL	2079.63	1	Y	Y	
α_{12}	NTMEHVSpSpSpEESIISpQETYK	2618.03	4	Y	Y	
α_{13}	Q*MEAESpISpSpSpEEIVPNSpVEAQK[c]	2702.88	5	Y		
α_{14}	QMEAESpISpSpSpEEIVPNSpVEAQK	2721.17	5	Y		
α_{15}	KEKVNELSpKDIGSpESpTEDQAMEDIKQ	2933.72	3	Y		
α_{16}	NANEEEYSIGSpSpSpEESpAEVATEEVK	3006.22	4	Y		
α_{17}	NANEEEYSpIGSpSpSpEESpAEVATEEVK	3086.77	5	Y		

[a] Sp, phosphorylated site. The amino acid sequences were cited from Zhao L., et al.[24] [b] Oxidation on methionine. [c] Pyroglutamylation on the N-terminal Q*.

support was prepared by sol−gel method with TEOS and APTES as precursors, similar to our previously reported method.[21]

A novel approach for the facile immobilization of metal ions on the hybrid silica monolith was employed. The monolith was first activated with glutaraldehyde, to provide a spacer-arm with suitable length, to improve the binding flexibility between IMAC materials and phosphopeptides. Then, to introduce chelating group $-PO_3^{2-}$ onto the supporting material, a heterofunctional reagent, AMPA, was used to react with glutaraldehyde. Since Schiff reaction could be completed quickly under mild conditions (i.e., in aqueous solution at room temperature), the efficient and facile functionalization of hybrid silica monoliths could be achieved. The *in situ* modification procedure adopted herein was of considerable advantages, compared with some previous methods where the chelating group was introduced by incubating supports with $POCl_3$ and 2,4,6-collidine in anhydrous acetonitrile for 12 h or with phosphorous acid, concentrated HCl, and formaldehyde solutions at 100 °C for 24 h.[12,15] In the modification solution, $NaCNBH_3$ was added to reduce C=N to C−N to enhance the stability of the immobilized metal ions. By such a procedure, chelate groups with strong affinity to Ti^{4+} were modified onto the matrix, while Ti^{4+} was further immobilized via chelation.

Evaluation of Hybrid Silica Monolithic Ti^{4+}-IMAC Column. The tryptic digest of α-casein was employed to examine the specific selectivity of the hybrid silica monolithic Ti^{4+}-IMAC column. When analyzed directly without enrichment, many nonphosphopeptide peaks with high intensity were observed, largely inhibiting the detection of phosphopeptides (Figure 2a). However, phosphopeptides in the digest of α-casein were isolated selectively after treated with the Ti^{4+}-IMAC column (Figure 2b). For comparison, a monolithic column without immobilized metal ions was used for phosphopeptide enrichment, and no obvious signals were recorded (Figure 2c), illustrating the little nonspecific adsorption of peptides. These results demonstrated the high selectivity of the hybrid silica monolithic Ti^{4+}-IMAC column.

As a control, Fe^{3+}-IMAC and TiO_2 microspheres were used for the enrichment of phosphopeptides (Figure 2d−e). In comparison to these materials, hybrid silica monolithic Ti^{4+}-IMAC column captured even more phosphopeptides (as listed in Table 1), and most of the exclusively identified phosphopeptides were recognized with two or more phosphorylation sites. The results suggest that single−phosphorylated peptides as well as multiphosphorylated ones can be identified without bias by the Ti^{4+}-IMAC.

To further evaluate the selectivity of the monolithic Ti^{4+}-IMAC column, a series of tryptic digest mixture of α-casein and a nonphosphoprotein BSA was enriched with such a column (Figure 3). Even with the presence of 200-fold excess of BSA digest, 15 phosphopeptide peaks were still identified with few nonphosphopeptides, indicating the high selectivity of the monolithic Ti^{4+}-IMAC column. It was mainly due to the extremely strong interaction between chelating Ti^{4+} and phosphate groups in phosphopeptides.

To evaluate the run-to-run reproducibility, the capture of phosphopeptides from α-casein digests was repeated on the hybrid silica monolithic Ti^{4+}-IMAC column (250 μm i.d. × 10 cm) for seven times. As can be seen from the Supporting Information Figure 1, the number of phosphopeptides captured was almost the same, suggesting the good reproducibility and stability of the monolithic Ti^{4+}-IMAC column for phosphopeptide enrichment. Since the buffer pH values spanned from ∼0.5 (loading buffer) to 11.8 (elution buffer), excellent pH stability of the hybrid silica monolithic column was thus demonstrated. This feature is of much superiority when compared to the pure silica monolithic matrix by which limited buffer conditions could be applied.[17] In addition, in comparison to polymer monolithic supports,[16] no shrinkage of this hybrid matrix could be anticipated especially when large amount of organic solvent (i.e., 50% ACN) was used, rendering it a preferable candidate for the capture of phosphopeptides.

The loading capacity of the hybrid monolithic Ti^{4+}-IMAC column was measured by loading a synthetic phosphopeptide (YKVPQLEIVPNSpAEER, 2 mg/mL) onto a 2 cm-long hybrid silica monolithic Ti^{4+}-IMAC column at the flow rate of 1 μL/min. The void time, estimated by flushing 50 mM thiourea through the column, was subtracted from the total time consumed for saturating the monolithic column. The average time for saturation was 1.3 min (RSD = 7.7%; n = 3). Therefore, the dynamic binding capacity was calculated

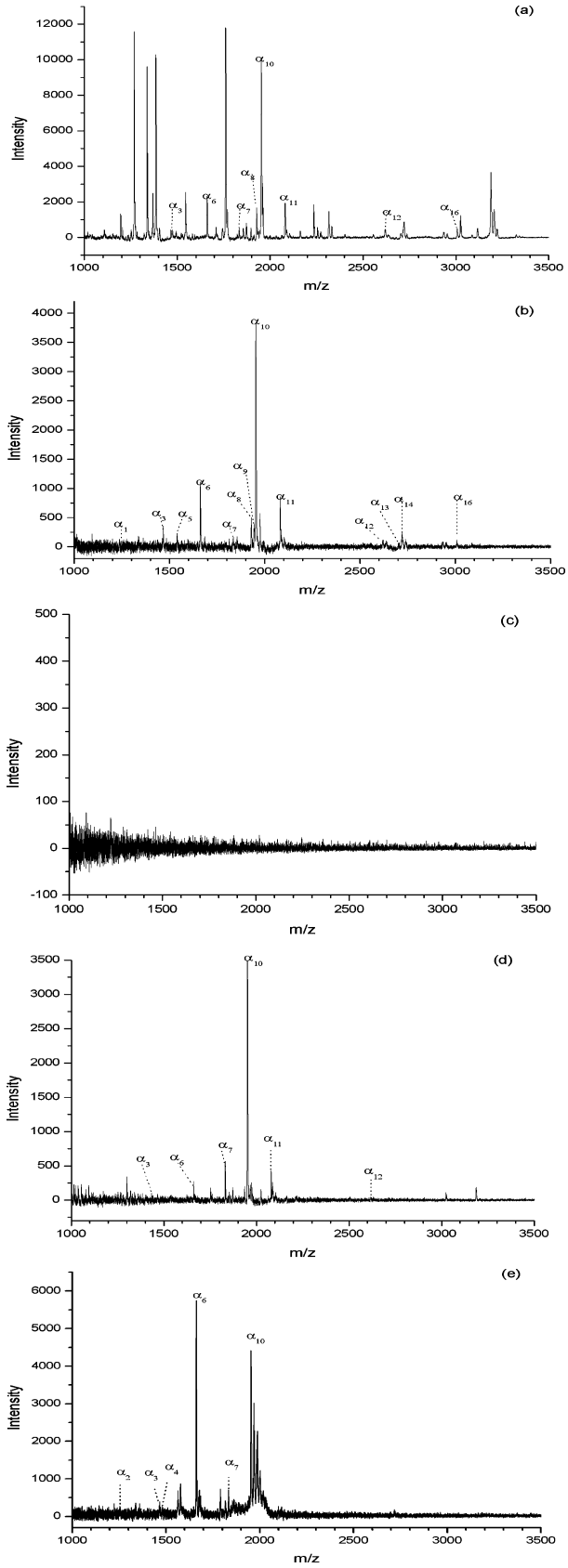

Figure 2. MALDI-TOF mass spectra of tryptic digest of α-casein. (a) Direct analysis; (b) analysis after treatment by the monolithic Ti^{4+}-IMAC column; (c) analysis after treatment by the monolithic column without Ti^{4+}; (d) analysis after treatment by Fe^{3+}-IMAC column; (e) analysis after treatment by TiO_2 beads. The loading amount of tryptic digests of α-casein was 2 pmol.

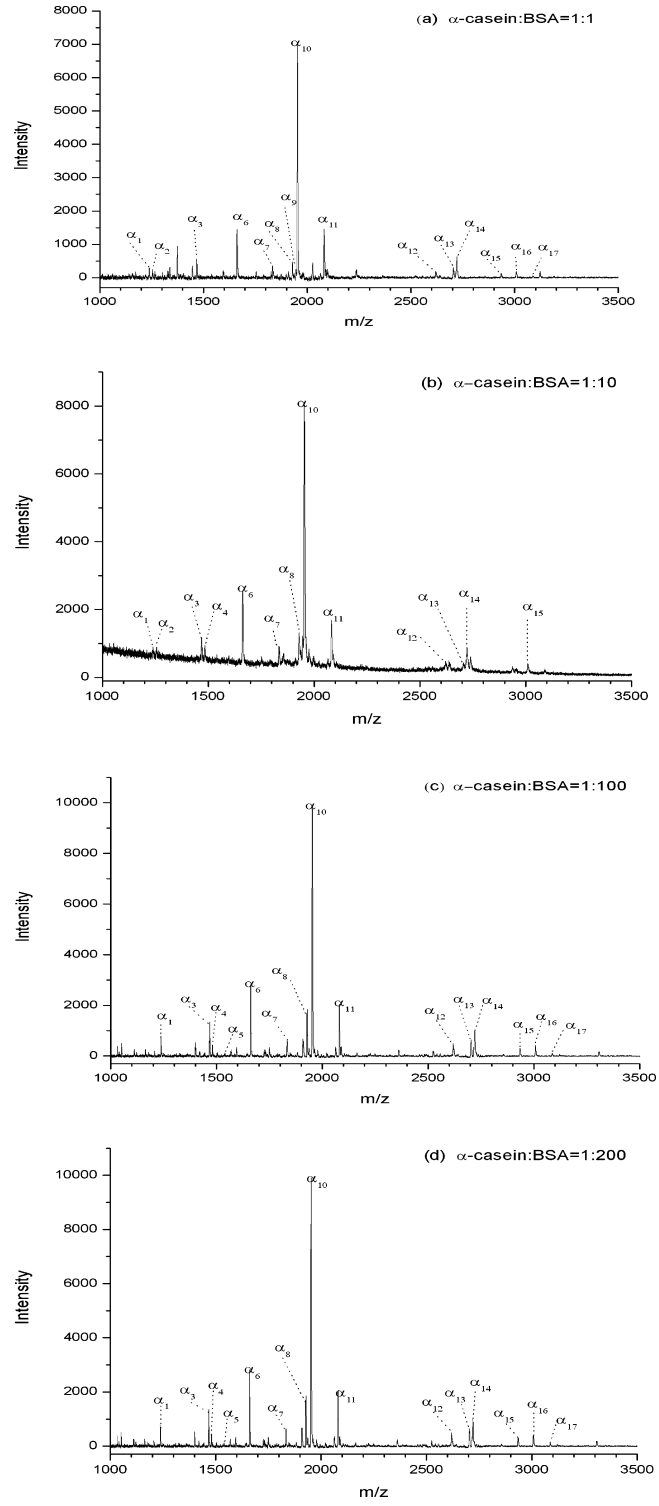

Figure 3. Selective enrichment of phosphopeptides by hybrid silica monolithic Ti^{4+}-IMAC column from the mixture of tryptic digests of α-casein and BSA in different molar ratios: (a) 1:1; (b) 1:10; (c) 1:100; (d) 1:200. The loading amount of tryptic digests of α-casein was 2 pmol.

to be 2.6 mg/mL, corresponding to 1.4 μmol/mL. In contrast to open tubular capillary column coated with zirconium phosphonate,[13] the monolithic column showed about 100-fold increase in binding capacity, which might attribute to the unique porous structure and large surface area of the matrix.

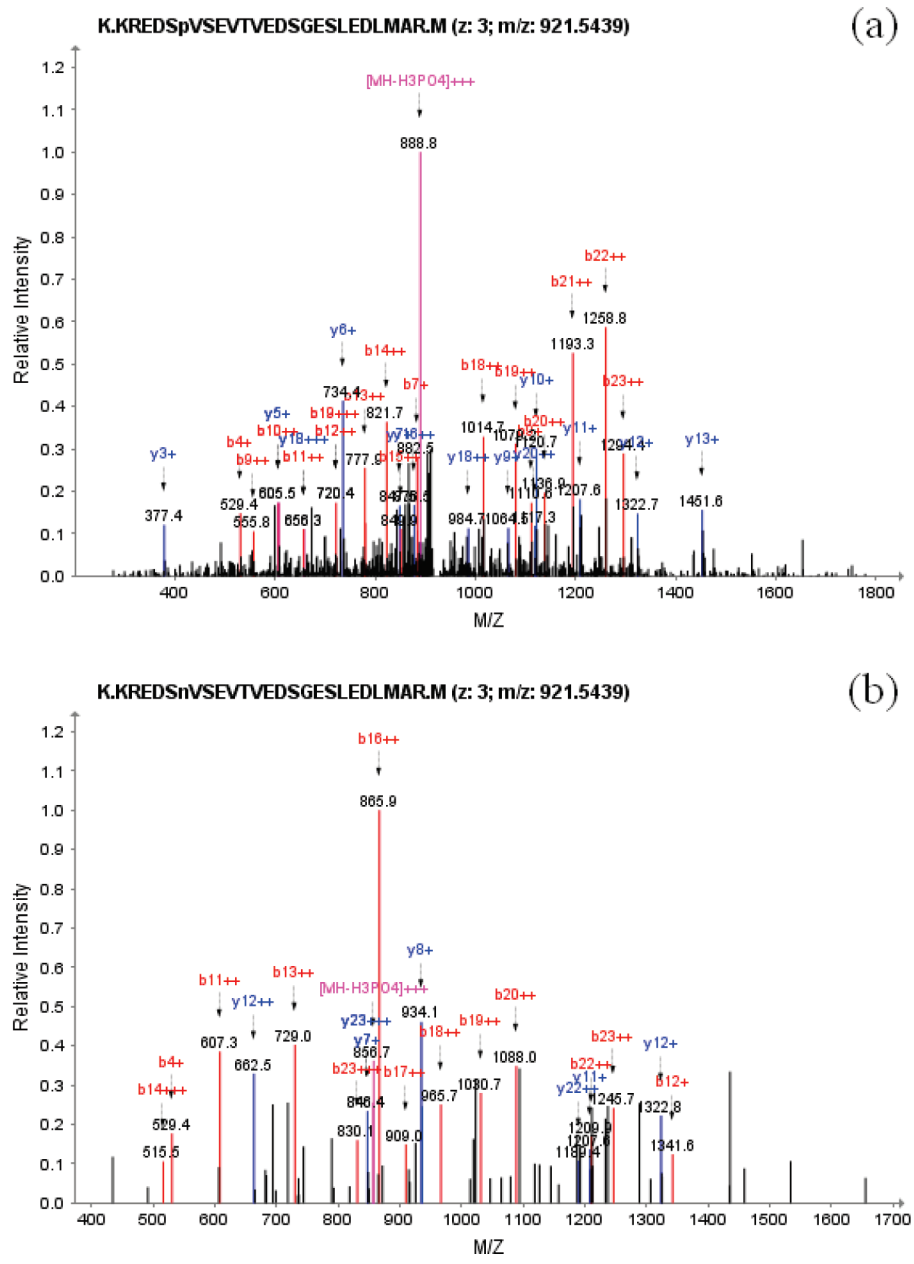

Figure 4. (a) MS^2 spectrum of the triply charged form of a singly phosphorylated peptide identified from the tryptic digests of proteins extracted from rat liver mitochondria. The dominant peak at *m/z* 888.8 represents the triply charged neutral loss peak (after loss of the H_3PO_4 group) for its selected precursor ion (*m/z* 921.5). The identified phosphopeptide was KREDSpVSEVTVEDSGESLEDLMAR. (b) MS^3 spectrum of the triply charged neutral loss peak at *m/z* 888.8. The *b*- and *y*-ion series shown are indicated with red and blue marks to verify the identification of the peptide.

The same synthetic phosphopeptide was chosen to evaluate the recovery efficiency of the monolithic Ti^{4+}-IMAC column. After 20 μL of 0.5 mg/mL phosphopeptide was loaded on a 10 cm-long monolithic Ti^{4+}-IMAC column, the bound phosphopeptides were eluted and the quantity was measured by peak area with a RPLC-UV system. The average recovery efficiency was calculated to be 69% (RSD = 1.1%, $n = 3$), which was comparable to the previous result obtained with monodisperse beads based Ti^{4+}-IMAC.[15]

Phosphoproteome of Mitochondrial Sample from Rat Liver. To demonstrate the applicability of the developed hybrid silica monolithic Ti^{4+}-IMAC column, it was applied to the phosphoproteome study of mitochondrial sample from rat liver. By duplicate runs, totally 148 phosphoprotein groups, corresponding to 224 phosphopeptides with 240 phosphorylation sites, as listed in Supporting Information Table 1, were identified from 1 mg tryptic digests of proteins in mitochondrial sample. The identification was validated by the APIVASE software followed by manual checking to confirm the results. As an example, the MS^2 and MS^3 spectra of a singly phosphorylated peptide KREDSpVSEVTVEDSGESLEDLMAR, are shown in Figure 4 (with the MS^2 and MS^3 spectra of other phosphopeptides in Supporting Information Figure 2). The high quality MS^2 and MS^3 spectra with neutral loss peaks, and *b*-, *y*-ion series that were consistent with the theoretically predicted peaks, imply the high confidence of the phosphopeptide identification.

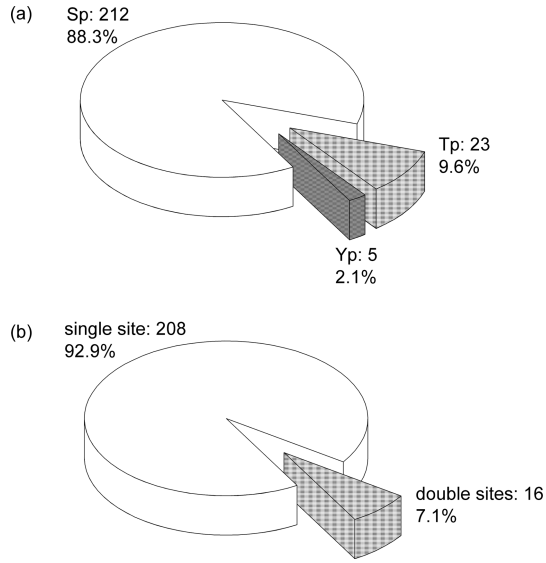

Figure 5. (a) Percentage of phosporylated amino acids. Sp, phosphorylated serine; Tp, phosphorylated threonine; Yp, phosphorylated tyrosine. (b) Percentage of singly-, doubly-, and multiply phosphorylated peptides.

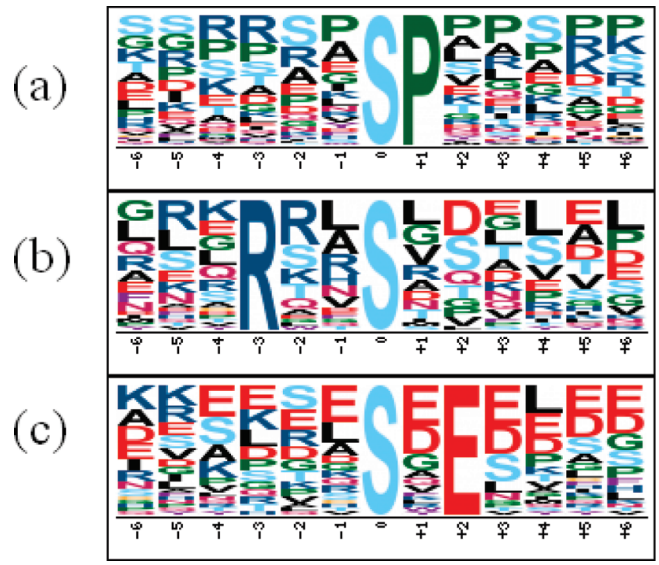

Figure 6. Motifs extracted from phosphorylated serines identified in this study. (a) Proline-directed kinase motif; (b) Calcium/Calmodulin-dependent protein Kinase II motif; (c) Golgi Casein Kinase motif.

For comparison, the mitochondrial samples were also analyzed with Fe^{3+}-IMAC and TiO_2. From duplicate runs of nanoRPLC−MS/MS/MS, only 28 distinct phosphopeptides were identified with Fe^{3+}-IMAC and 72 were recognized with TiO_2 (FDR < 1%). In addition, about 50% of the phosphopeptides by Fe^{3+}-IMAC or TiO_2 were included in the data set obtained with Ti^{4+}-IMAC (as shown in Supporting Information Figure 3), further demonstrating the superiority of Ti^{4+}-IMAC for complex samples.

The distribution of phosphorylated amino acids in the identified phosphopeptides with Ti^{4+}-IMAC was analyzed. It was found that 88.3% of the phosphorylate sites were Ser, 9.6% were Thr, and 2.1% were Tyr (Figure 5a). The distribution of phosphorylated site number on each peptide was further investigated (Figure 5b). A high percentage (92.9%) of singly phosphorylated peptides was observed, and the ratio of multiple phophopeptides was relatively small, partially due to low ionization efficiency of mutli-phosphate groups.[25]

To predict the relationship between the phosphorylated serine sites and various kinases, specific motifs were extracted from the Ti^{4+}-IMAC data set using the Motif-X algorithm (http://motif-x.med.harvard.edu).[26] The candidate sequences were centered at the phosphorylated serine and extended 6 residues on each side. The IPI Rat Proteome was used as the background. The minimum reported number of occurrences for a given motif was set at 5, which was about 2% of the total number of phosphorylated serines, and the significance was set at 10^{-6}. Despite the relatively small number of sites, three known motifs were successfully extracted (Figure 6). The proline-directed protein kinase motif gave a Motif Score of 16, while the other two motifs also showed a Motif Score above 11. The high scores represented high probability values, and were consistent with those previously reported.[27] Therefore, the identification of our data set was of high confidence. The proline-directed kinases were the vast majority of the serine kinases,[28] and some of them were related to the mitochondrial energy-linked function, for example, the classical mitogen-activated protein (MAP) kinases ERK1 and 2 (extracellular signal-regulated kinases).[29,30] The motif of Calcium/Calmodulin-dependent protein Kinase II, which was involved in calcium-regulated signaling pathway controlling mitochondrial biogenesis in mammalian cells,[31] was also recognized.

Besides the properties of phosphopeptides, the physiochemical and bioinformatical properties of the identified phosphoproteins were also analyzed. As shown in Figure 7a, the distribution of the identified proteins was in a molecular weight (M_w) range of about 11 kDa to 295 kDa, and a p*I* value range of about 4 to 12, which were wider than those obtained by 2D-PAGE. About 77% of proteins were in the range of 10 kDa to 100 kDa, while about 10% of them were larger than 200 kDa. Meanwhile, the p*I* values of about 95% of proteins were between 4 and 10 and about 5% of them were of p*I* values above 10. Moreover, the gene ontology analysis with the Software Tool for Researching Annotations of Proteins (STRAP) was used to categorize the function of the identified phosphoproteins in cell process.[32] As shown in Figure 7b and c, a major part of proteins have antioxidant activity, binding, and transcription regulator activity.

Conclusions

To achieve selective phosphopeptide enrichment, a novel approach to modify phosphate groups on organic−inorganic hybrid silica monolithic column followed by Ti^{4+} immobilization was developed. Compared with the commercial Fe^{3+}-IMAC or TiO_2 beads, the hybrid silica monolithic Ti^{4+}-IMAC showed high specificity to phosphopeptides. Moreover, it exhibited large loading capacity, high recovery efficiency, and good reproducibility. Such a monolithic IMAC column was successfully utilized to selectively capture phosphopeptides in digest of mitochondrion-enriched component extracted from rat liver, and 148 phosphoprotein groups were recognized with high confidence. The results demonstrate great potential of the hybrid silica monolithic Ti^{4+}-IMAC column in large-scale phosphoproteome analysis.

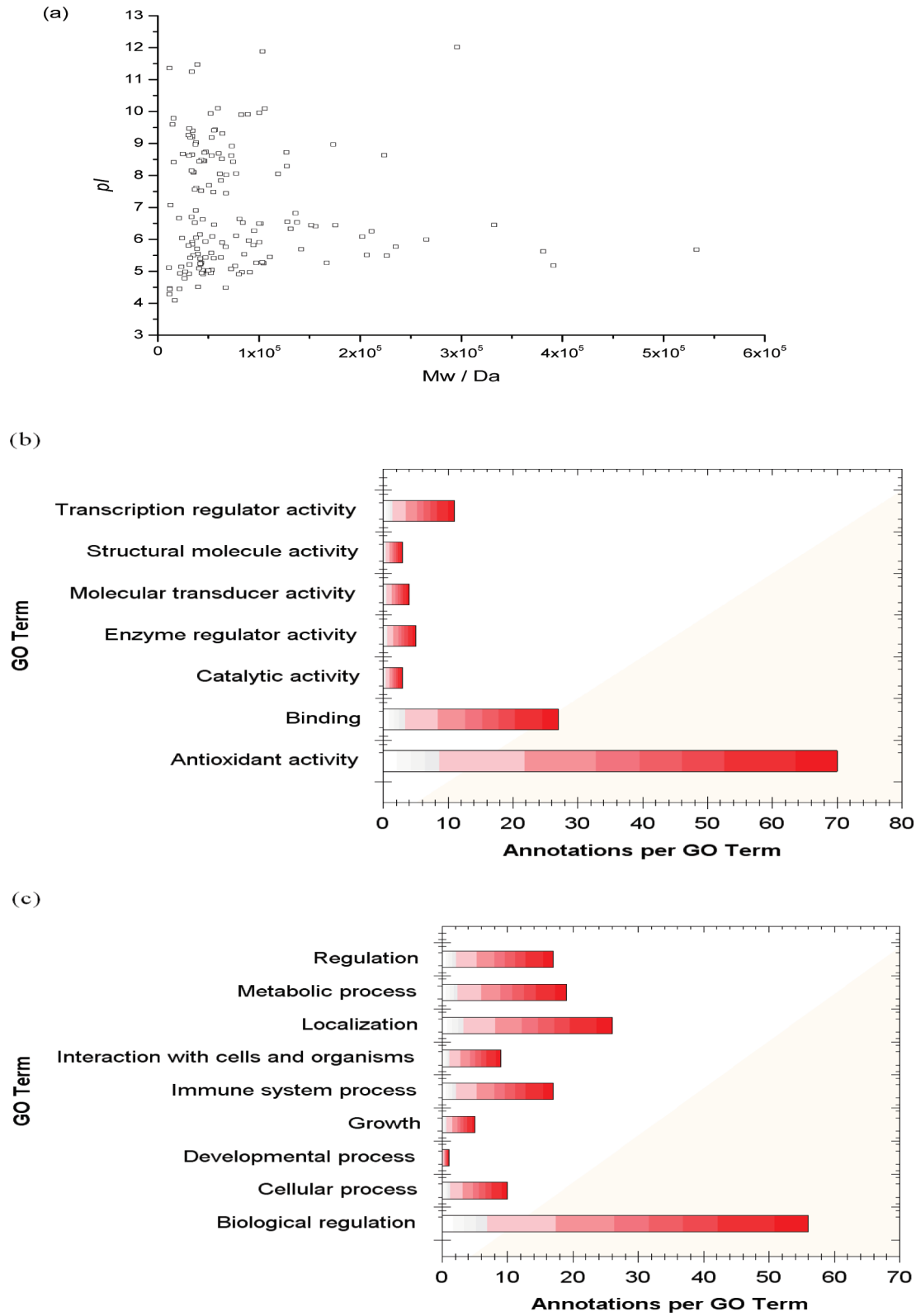

Figure 7. Distribution for identified proteins of: (a) molecular weights (M_w) and pI values; (b) GO annotation of molecular function; (c) biological process.

It is anticipating that with the combination of Ti^{4+}-IMAC enrichment and some fractionation and quantification techniques, in-depth analysis of mitochondrial phosphoproteome would be obtained. In addition, the hybrid silica monolithic Ti^{4+}-IMAC column, which shows good permeability and high pressure resistance, can be readily coupled with micro/nano-RPLC−ESI−MS/MS for online analysis. Relevant experiments are being performed in our lab.

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Supporting Information Available: The reproducibility of enrichment performance of the hybrid silica monolith based Ti^{4+}-IMAC column, mass spectra of all the phosphopeptides, and the overlap status of phosphopeptides enriched by three materials, are shown in Supporting Information Figure 1, 2, and 3, respectively. The identified phosphoproteins are listed in Supporting Information Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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