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Enrichment of Phosphopeptides by Fe^{3+}-Immobilized Mesoporous Nanoparticles of MCM-41 for MALDI and Nano-LC−MS/MS Analysis

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Fe^{3+}-immobilized mesoporous molecular sieves MCM-41 with particle size of ca. 600 nm and pore size of ca. 3 nm is synthesized and applied to selectively trap and separate phosphopeptides from tryptic digest of proteins. For the capture of phosphopeptides, typically 10 μL of tryptic digest solution was first diluted to 1 mL by solution of ACN/0.1% TFA (50:50, v/v) and incubated with 10 μL of 0.1% acetic acid dispersed Fe^{3+}-immobilized MCM-41 for 1 h under vibration. Fe^{3+}-immobilized MCM-41 with trapped phosphopeptides was separated by centrifugation. The deposition was first washed with a volume of 300 μL of solution containing 100 mM NaCl in ACN/0.1% TFA (50:50, v/v) and followed by a volume of 300 μL of solution of 0.1% acetic acid to remove nonspecifically bound peptides. The nanoparticles with trapped phosphopeptides are mixed with 2,5-dihydroxybenzoic acid (2,5-DHB) and deposited onto the target for analysis by matrix-assisted laser desorption/ionization mass spectrometry (MALDI−MS). It was found that phosphopeptides from tryptic digest of α-casein and β-casein are effectively and specifically trapped on Fe^{3+}-immobilized MCM-41 with few peptides nonspecifically adsorbed. After the extraction by Fe^{3+}-immobilized MCM-41, the suppression to the detection of phosphopeptides caused by abundant nonphosphopeptides from tryptic digest is effectively eliminated, and the detection of phosphopeptides by MALDI is greatly enhanced with the value of signal-to-noise (S/N) increased by more than an order of magnitude. It is demonstrated that the mechanism of the adsorption of phosphopeptides on Fe^{3+}-immobilized MCM-41 is based on the interaction between the Fe^{3+} and the phosphate group. Finally, Fe^{3+}-immobilized MCM-41 is applied to extract phosphopeptides from tryptic digest of the lysate of mouse liver for phosphoproteome analysis by nano-LC−MS/MS.

Keywords: phosphopeptides • MALDI • Fe^{3+}-immobilized MCM-41 • nano-LC−MS/MS • enrichment

Introduction

Phosphorylated proteins and peptides are one of the most important post-translational modifications in regulation of biological functions. Generally, mass spectrometry (MS)-based methods,[1−5] including electrospray ionization mass spectrometry (ESI−MS) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI−MS)[6−11] are demonstrated to be the most powerful tool for identification of phosphorylated proteins and peptides by the analysis of enzymatic digest. However, as the phosphopeptides are always in a low abundance, signals of phosphopeptides are easily suppressed by other abundant peptides in MS analysis. Many efforts[12−14] have been made to enhance the signals of phosphopeptides in enzymatic digest mixtures. For example, Asara et al.[12] found that the addition of ammonium salts to the matrix/analytes solution substantially enhances the detection of phosphopeptides. Thus, the phosphorylated peptide peaks become the dominant peaks in the spectrum, which allows the identification of phosphopeptides in an unfractionated proteolytic digestion mixture. Recently, Kjellstrom et al.[13] used phosphoric acid as a matrix additive in combination with the matrix of 2,5-DHB, to enhance significantly ion signals of phosphopeptides in MALDI mass spectra of crude peptide mixtures derived from the phosphorylated proteins α-casein and β-casein. However, separation and enrichment of the phosphopeptides is still a preferable choice for the identification of phosphopeptides. Nowadays, immobilized metal-ion affinity chromatography (IMAC)[15−17] is one of the most widely applied techniques for enrichment[18,19] of phosphopeptides and proteins. Because of the problems usually occurred in the elution step for off-bead analysis, such as the incomplete elution and the possible loss of phosphate group, another strategy of on-bead analysis is utilized, in which IMAC beads with selectively trapped phosphorylated peptides and proteins were transferred onto the target for direct detection by MALDI.[20,21] For example, Hart et al.[22] presented the factors governing the solubilization

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of phosphopeptides retained on ferric IMAC beads and their analysis by MALDI-TOF−MS. However, because of the unavoidable "shadow effect"[22] of the porous resin beads at the micron scale, most of the phosphopeptides bound in the pores would be probably inaccessible during the direct laser desorption. Recently, some materials in nanoscale have been introduced as a new kind of IMAC beads for the separation, enrichment and direct detection of selectively trapped phosphorylated peptides and proteins by MALDI. For example, Fe^{3+}-immobilized zeolite nanoparticles[23] have been applied to isolate phosphopeptides from tryptic β-casein digest and the phosphopeptides enriched on the modified zeolite nanoparticles are detected by MALDI-TOF−MS/MS. Chen et al.[24] employed TiO_2 coated magnetic (Fe_3O_4/TiO_2 core/shell) nanoparticles to selectively concentrate phosphopeptides from protein digest products.

Mesoporous molecular sieves MCM-41[25−27] has been widely studied in chemistry of materials because of its structural simplicity and ease in preparation. The prominent features of MCM-41, and in general of most periodic mesoporous materials, are as follows: well-defined pore shapes (hexagonal/cylindrical); narrow distribution of pore sizes; negligible pore networking or pore blocking effects. For its large pore volumes and very high surface area, MCM-41 shows the adsorptive capacity[28−32] more than an order of magnitude higher than that of conventional adsorbents. Furthermore, the surface of MCM-41 with a large amount of silicate hydroxyl (silanol) groups (about 40−60%) can be easily modified with chemical reaction. MCM-41 therefore shows a great potential as a selective adsorbent in separation techniques through the modification of the surface hydroxyl groups in the pore wall.[33,34]

Herein, we first synthesized the highly ordered MCM-41 with particle size of ca. 600 nm and pore size of ca. 3 nm, and then immobilized Fe^{3+} on its surface through the modification of silicate hydroxyl (silanol) groups. The Fe^{3+}-immobilized MCM-41 is used as the adsorbent to selectively trap phosphopeptides from the tryptic digest. Thus, abundant nonphosphopeptides from tryptic digest are effectively removed, and the on-bead detection of phosphopeptides by MALDI is greatly enhanced with the value of signal-to-noise (S/N) increased by more than an order of magnitude after the extraction by Fe^{3+}-immobilized MCM-41. At last, Fe^{3+}-immobilized MCM-41 is applied to phosphoproteome analysis of mouse liver by nano-LC−MS/MS with database searching for identification in batch.

Experimental Section

Reagents. Trifluoroacetic acid (TFA, CAS No. 76-05-1), 2,5-dihydroxybenzoic acid (2,5-DHB, CAS No. 490-79-9), tetra-ethyl orthosilicate (TEOS, CAS No. 78-10-4), hexadecyltrimethylammonium bromide (CTAB, CAS No. 57-09-0), 3-glycidoxypropyltrimethoxysilane (GLYMO, CAS No. 2530-83-8), iminodiacetic acid (IDA, CAS No. 142-73-4), α-casein, β-casein and BSA, urea (CAS No. 57-13-6), 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS, CAS No. 75621-03-3), dithiothreitol (DTT, CAS No. 3483-12-3), ammonium bicarbonate (NH_4HCO_3, CAS No. 1066-33-7), and iodoacetamide (IAA, CAS No. 144-48-9) were all purchased from Bio-Rad (Hercules, CA); tris(hydroxymethyl)aminomethane (Tris, CAS No. 77-86-1), guanidine hydrochloride(CAS No. 50-01-1), sodium orthovanadate (Na_3VO_4, CAS No. 13721-39-6), and sodium fluoride (NaF, CAS No. 7681-49-4) were obtained from Sigma (St. Louis, MO); trypsin was purchased from Promega (Madison, WI); acetic acid (HAc, CAS No. 64-19-7), ferric chloride ($FeCl_3$, CAS No. 7705-08-0), sodium chloride (NaCl, CAS No. 7647-14-5), formic acid (FA, CAS No. 64-18-6), and acetonitrile (CAN, CAS No. 75-05-8) were obtained from Aldrich (Milwaukee, WI). Ethylenediaminetetraacetic acid (EDTA, CAS No. 60-00-4), [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA, CAS No. 67-42-5), and phenylmethylsulfonyl fluoride (PMSF, CAS No. 329-98-6) were purchased from Amresco (Solon, OH). POROS 20 MC beads were purchased from PerSeptive Biosystems (Framingham, MA). All the chemicals were of analytical grade except TFA and acetonitrile (ACN), which are of HPLC grade. Adult female C57 mice were purchased from Dalian Medical University (Dalian, China). Water was prepared using a Milli-Q system (Millipore, Bedford, MA). No specific care of safety to all chemical reagents, proteins and methodologies are required during normal operation.

Procedures. Synthesis of Mesoporous Nanoparticle of MCM-41. The synthesis of a mesoporous nanoparticle of MCM-41 is very similar to the method reported by Cai et al.[35] The synthetic procedures were briefly described as follows: 2.0 g of CTAB were added into the solution containing 205 mL of NH_4OH (25 wt % solution) and 270 mL of deionized water with stirring and heating. When the solution became homogeneous, 10 mL of TEOS were introduced. After a reaction of 2 h, the resulting product was filtered, washed with deionized water, dried at vacuum overnight, and followed by acid extraction to remove the template. For a typical acid extraction,[36] 6.0 g of the as-synthesized MCM-41 was added to a mixture of 450 mL of dry ethanol and 6.0 mL of 1 M HCl. Then, the resulting mixture was stirred at room temperature for 6 h. After this extraction, the solid was filtered, washed with dry ethanol, and then dried at vacuum overnight.

Preparation of Fe^{3+}-Immobilized MCM-41. The scheme of immobilization of Fe^{3+} onto the MCM-41 was inspired by our previous work,[37] in which Cu^{2+} was immobilized onto the inner wall of the fused-silica capillary. First of all, the metal chelating agent of IDA was reacted with GLYMO before its immobilization onto the MCM-41. The rate of this reaction is slow, and the only byproduct is water, so it is quite easy to control the reaction. IDA (4.25 g) was dissolved in 50 mL of deionized water. The obtained solution was adjusted to pH 11.0 by the powder of NaOH and then transferred into a flask. To minimize the hydrolysis of GLYMO, 1.4 mL of GLYMO was slowly added into the IDA solution with stirring when the temperature of solution was decreased to 0 °C at the ice-bath. The mixed solution was heated to 65 °C for reaction of 6 h with stirring, subsequently placed into an ice-bath for 5 min to decrease the temperature to 0 °C again, and 1.6 mL of GLYMO was added and mixed again. Then the solution was raised to 65 °C for reaction of another 6 h with stirring. Similarly, another 1.7 mL of GLYMO was added to the solution, which was further reacted at 65 °C overnight with stirring. Finally, the prepared GLYMO−IDA−silane solution was adjusted to pH 6 by concentrated HCl and stored in a refrigerator with the vessel sealed for usage.

Second, the prepared 100 mg of MCM-41 were mixed with 2.0 mL GLYMO−IDA−silane solution in a 3 mL flask for reaction at 75 °C for 1 h with stirring, and then the solution was centrifugated at 15 000 g for 3 min. After the supernatant was removed, 2 mL of fresh GLYMO−IDA−silane solution was added to the flask to restart the reaction. This procedure as described above was repeated 3 times to prepare the product of GLYMO−IDA immobilized MCM-41, which is designated as MCM−GLYMO−IDA.

At last, after the prepared MCM−GLYMO−IDA were rinsed by 10 mL of deionized water, 50 mM EDTA, and deionized

water in turn, 5 mL of 100 mM $FeCl_3$ in 0.1% acetic acid was introduced into the flask. After stirring at room temperature for 2 h, the Fe^{3+}-immobilized MCM-41 solution was centrifuged at 15 000 g for 5 min, and the deposition was rinsed by 0.1% acetic acid and finally dispersed in 10 mL of 0.1% acetic acid at concentration of 10 mg/mL for further usage.

Preparation of Fe^{3+}−IMAC Beads. The 10 mg POROS 20 MC beads were rinsed by 10 mL of deionized water, 50 mM EDTA, and deionized water in turn, 5 mL of 100 mM $FeCl_3$ in 0.1% acetic acid was introduced into the flask. After stirring at room temperature for 2 h, the Fe^{3+}−IMAC beads solution was centrifuged at 15 000 g for 5 min, and the deposition was rinsed by 0.1% acetic acid and finally dispersed in 1 mL of 0.1% acetic acid at a concentration of 10 mg/mL for further usage.

Preparations of Tryptic Digest of α-Casein/β-Casein/BSA. Protein of α-casein/β-casein/BSA was dissolved in appropriate volumes of reducing solution (6 M guanidine hydrochloride, 100 mM ammonium bicarbonate, pH 8.3) with the protein concentration adjusted to 10 μM. Then, 1 mL of this protein sample was mixed with 10 μL of 1 M DTT. The mixture was incubated at 37 °C for 2.5 h, and then 50 μL of 1 M IAA was added and incubated for an additional 30 min at room temperature in the dark. The protein mixtures were exchanged into 100 mM ammonium bicarbonate buffer, pH 8.5, and incubated with trypsin (25:1) at 37 °C overnight. The digested peptide mixture was stored in the refrigerator under −30 °C for further usage.

Preparation of the Lysate of Mouse Liver. Briefly, mice were sacrificed and the livers were promptly removed and placed in ice-cold homogenization buffer consisting of 8 M urea, 4% CHAPS (w/v), 65 mM DTT, 1 mM EDTA, 0.5 mM EGTA, and a mixture of protease inhibitor (1 mM PMSF) and phosphatase inhibitors (0.2 mM Na_3VO_4, 1 mM NaF) and 40 mM Tris−HCl at pH 7.4. After mincing with scissors and washing to remove blood, the livers were homogenized in 10 mL the homogenization buffer per 2 g of tissue. The suspension was homogenized for approximately 1 min, sonicated for 30 s at 100 W, and centrifuged at 25 000 g for 1 h. The supernatant contained the total liver proteins. Appropriate volumes of protein sample were lyophilized to dryness and redissolved in reducing solution (6 M guanidine hydrochloride, 100 mM ammonium bicarbonate, pH 8.3) with the protein concentration adjusted to 1.0 mg/mL. The digestion of lysate of mouse liver was performed with the same procedures for the digestion of α-casein or β-casein as described above.

Extraction of Phosphopeptides. For sample of tryptic digest of protein (α-casein /β-casein/BSA/lysate of mouse liver), 10 or 100 μL pf tryptic digest solution was first diluted to 1 mL by solution of ACN/0.1% TFA (50:50, v/v). Then, 10 μL of 0.1% acetic acid-dispersed Fe^{3+}-immobilized MCM-41 (0.1 mg) or Fe^{3+}−IMAC beads (0.1 mg) were added. After incubation for 1 h with vibration, Fe^{3+}-immobilized MCM-41 or Fe^{3+}−IMAC beads with trapped phosphopeptides was separated by centrifugation at 30 000 g for 5 min. To remove nonspecifically adsorbed peptides, the deposition was first washed with a volume of 300 μL of a solution containing 100 mM NaCl in ACN/0.1% TFA (50:50, v/v) and followed by a volume of 300 μL of a solution of 0.1% acetic acid.

Sample Preparation for MALDI-TOF−MS. For all samples with direct analysis by MALDI,[12] 2 μL of a solution of tryptic digest were mixed with 2 μL of 25 mM diammonium citrate $(NH_4)_2HCit$ and 4 μL of a matrix of 2,5-DHB in ACN/0.1% TFA (50:50, v/v) at a concentration of 20 mg/mL, and 0.5 μL of the mixture was applied onto the MALDI plate for the MS analysis. For on-bead analysis of samples after extraction, the deposition of Fe^{3+}-immobilized MCM-41 or Fe^{3+}−IMAC beads with trapped phosphopeptides were first redispersed in 10 μL of a solution of ACN/0.1% TFA (50:50, v/v). Then, 2 μL of the dispersed solution were mixed with 2 μL 25 mM diammonium citrate $(NH_4)_2HCit$ and 4 μL matrix of 2, 5-DHB in ACN/0.1% TFA (50:50, v/v) at concentration of 20 mg/mL to form a purple slurry. Finally, 0.5 μL of the purple slurry was deposited onto the MALDI plate for the MS analysis. For off-bead analysis of samples after extraction, phosphopeptides captured by Fe^{3+}-immobilized MCM-41 or Fe^{3+}−IMAC beads are both eluted by 100 μL of 1% NH_4OH, lyophilized to dryness, redissolved in 10 μL solution of ACN/0.1% TFA (50:50, v/v), and 2 μL of solution were performed the MALDI analysis as the direct analysis described above.

Table 1. Phosphopeptides Identified in α-Casein[19,43]

symbol	AA[a]	sequence	[M + H]$^+$ *m/z*	S/N (a)[b]	S/N (b)[c]
α1	153−162	TVDME[pS]TEVF	1237.5	12	13
α2	153−164	TVDME[pS]TEVFTK	1466.61	8	25
α3	121−134	VPQLEIVPN[pS]AEER	1660.79	25	226
α4	58−73	DIG[pS]E[pS]TEDQAMETIK	1927.69	6	39
α5	119−134	YKVPQLEIVPN[pS]AEER	1951.95	36	374
α6	58−73	DIG[pS]E[pS][pT]EDQAMETIK	2007.65	−[d]	52

[a] AA denotes amino acid residue number. [b] Direct MALDI analysis. [c] On-bead analysis after extraction by Fe^{3+}-immobilized MCM-41. [d] Not detected.

Sample Preparation for Nano-LC−MS/MS Analysis. After the extraction of phosphopeptides from tryptic digest by Fe^{3+}-immobilized MCM-41, 100 μL of 1% NH_4OH were added to redisperse Fe^{3+}-immobilized MCM-41. After vibration of 30 min for the dispersed solution, it was centrifugated at 30 000 g for 10 min. The supernatant containing phosphopeptides was collected and lyophilized to dryness, and finally redissolved in 5 μL 0.1% formic acid for nano-LC−MS/MS analysis.

Apparatus. MALDI spectrometry was performed on the Bruker Autoflex (Bruker Daltonics, Germany). The instrument was equipped with a nitrogen laser (λ = 337 nm) and its available accelerating potential is in the range of ±20 kV. The MALDI uses a ground-steel sample target with 384 spots. The analytical range of laser energy was adjusted to slightly above the threshold to obtain good resolution and signal-to-noise ratios. External mass calibration was obtained by using two points that bracketed the mass range of interest.

Nano-LC−MS/MS and MS/MS/MS were performed on the μRPLC−MS/MS system. A Finnigan surveyor MS pump (ThermoFinnigan, San Jose, CA) was used to deliver mobile phase. The pump flow rate was split by a cross to achieve the flow rate at 200 nL/min. For the capillary separation column, one end of the fused-silica capillary (75 μm i.d. × 120 mm length) was manually pulled to a fine point ~5 μm with a flame torch. The columns were in-house packed with C18 AQ particles (5 μm, 120 Å) from Michrom BioResources (Auburn, CA) using a pneumatic pump. The μRPLC column was directly coupled to a LTQ linear ion trap mass spectrometer from ThermoFinnigan (San Jose, CA) with a nanospray source. The mobile phase consisted of mobile phase A, 0.1% formic acid in H_2O, 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. A 50 min gradient elution was applied for the separation of phosphopeptides enriched from

Table 2. Phosphopeptides Identified in β-casein[24]

symbol	AA[a]	sequence	$[M + H]^+$ m/z	S/N (a)[b]	(b)[c]	(c)[d]	(d)[e]	(e)[f]
β1	33−48	FQ[pS]EEQQQTEDELQDK	2061.83	4	66	30	140	69
β2	30−48	IEKFQ[pS]EEQQQTEDELQDK	2432.08	−[g]	7	−[g]	5	−[g]
β3	33−52	FQ[pS]EEQQQTEDELQDKIHPF	2556.09	13	32	4	65	24
β4	1−25	RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR	3122.27	40	42	48	425	369

[a] AA denotes amino acid residue number. [b] Direct analysis. [c] On-bead analysis after extraction by Fe^{3+}-immobilized MCM-41. [d] On-bead analysis after extraction by Fe^{3+}−IMAC beads. [e] Off-bead analysis after extraction by Fe^{3+}-immobilized MCM-41. [f] Off-bead analysis after extraction by Fe^{3+}−IMAC beads. [g] Not detected.

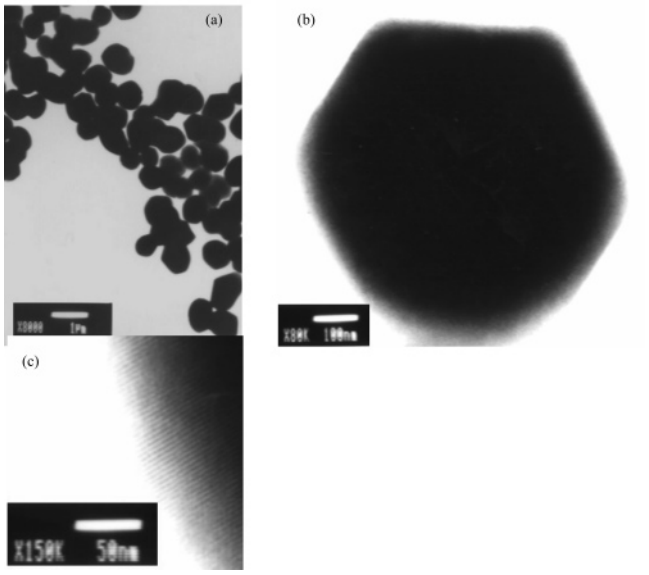

Figure 1. TEM image of the synthesized Fe^{3+}-immobilized MCM-41 (a) in low magnification (scale bar, 1 μm; 8000×), (b) in medium magnification (scale bar, 100 nm; 80 000×), and (c) in high magnification (scale bar, 50 nm; 150 000×).

α-casein and mouse liver protein extract, respectively. All MS and MS^n spectra were acquired in data-dependent mode, and the mass spectrometer was set as a full scan MS followed by three data-dependent MS/MS events. A subsequent MS^3 event was triggered upon detection of neutral loss of −98, −49, or −32.7 from the precursor (corresponding to the neutral loss of phosphoric acid from 1+, 2+, and 3+ charge states, respectively) during any of the MS/MS scans.

Database Searching and Manual Validation. The obtained MS^2 and MS^3 spectra were searched with the SEQUEST algorithm against the mouse protein database from IPI (ipi.mouse.v.3.08, including 43, 343 proteins), respectively. In MS^2 searching, differential modifications of 80 Da to Ser, Thr, and Tyr residues were selected, and for MS^3 database searching, besides above modifications, −18 Da were added to Ser, Thr, and Tyr residues. The obtained result of database searching was first filtrated by setting Xcorr as 1.5, 2.0, and 2.5 corresponding to 1+, 2+, and 3+ charge states, respectively. Furthermore, phosphorylated peptides identified by SEQUEST were manually validated using the following criteria. First, the MS/MS spectrum must be of good quality, with fragment ion peaks clearly above baseline noise with at least three sequential members of the b- or y-ion series observed. Second, the phosphoric acid neutral loss peak must be detected in the MS/MS spectrum and be the dominant signal for peptides that are singly phosphorylated and doubly charged. Third, the MS/MS/MS spectrum should be consistent with the determined peptide sequence. Peptides with only MS/MS data but no MS/MS/MS data were confirmed as phosphorylated peptides only if their phosphoric acid neutral loss peak was clearly defined.

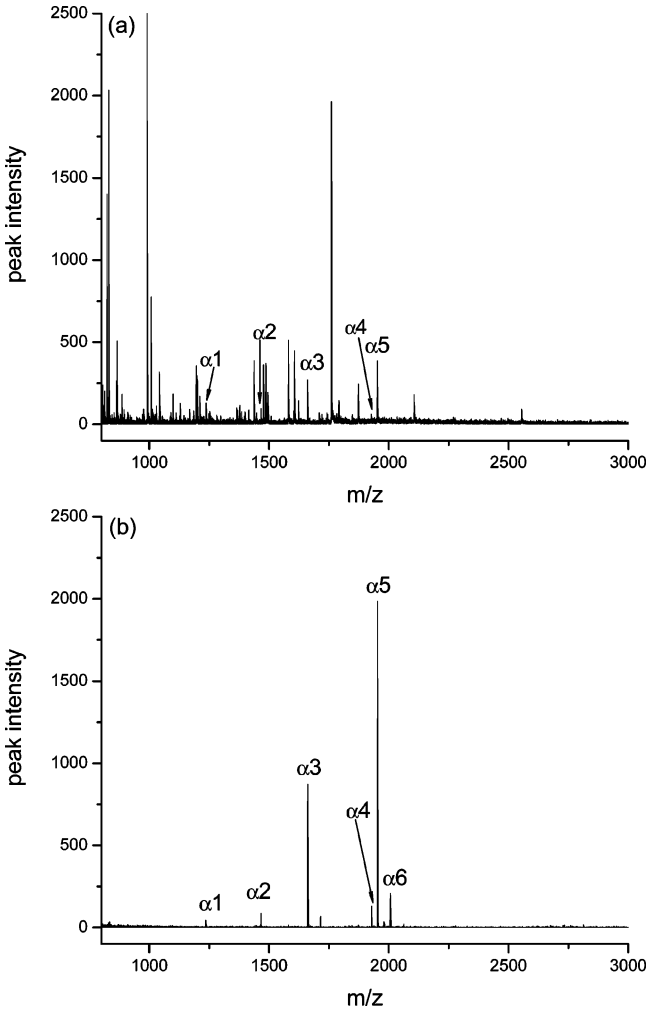

Figure 2. MALDI mass spectra of the tryptic digest of α-casein (10 μM) (a) Direct analysis and (b) on-bead analysis, in which 0.1 mg Fe^{3+}-immobilized MCM-41 were added into the 10 μL tryptic digest of α-casein. Peaks for identified phosphopeptides are designated as α1, α2, α3, α4, α5, and α6. The detailed information is listed in Table 1.

Results and Discussion

Figure 1a shows the low magnification (scale bar, 1 μm; 8000×) TEM image of Fe^{3+}-immobilized MCM-41, which indicates that nanoparticles of Fe^{3+}-immobilized MCM-41 are not agglomerated. Furthermore, it is estimated that the particle size of our synthesized Fe^{3+}-immobilized MCM-41 is ca. 600 nm

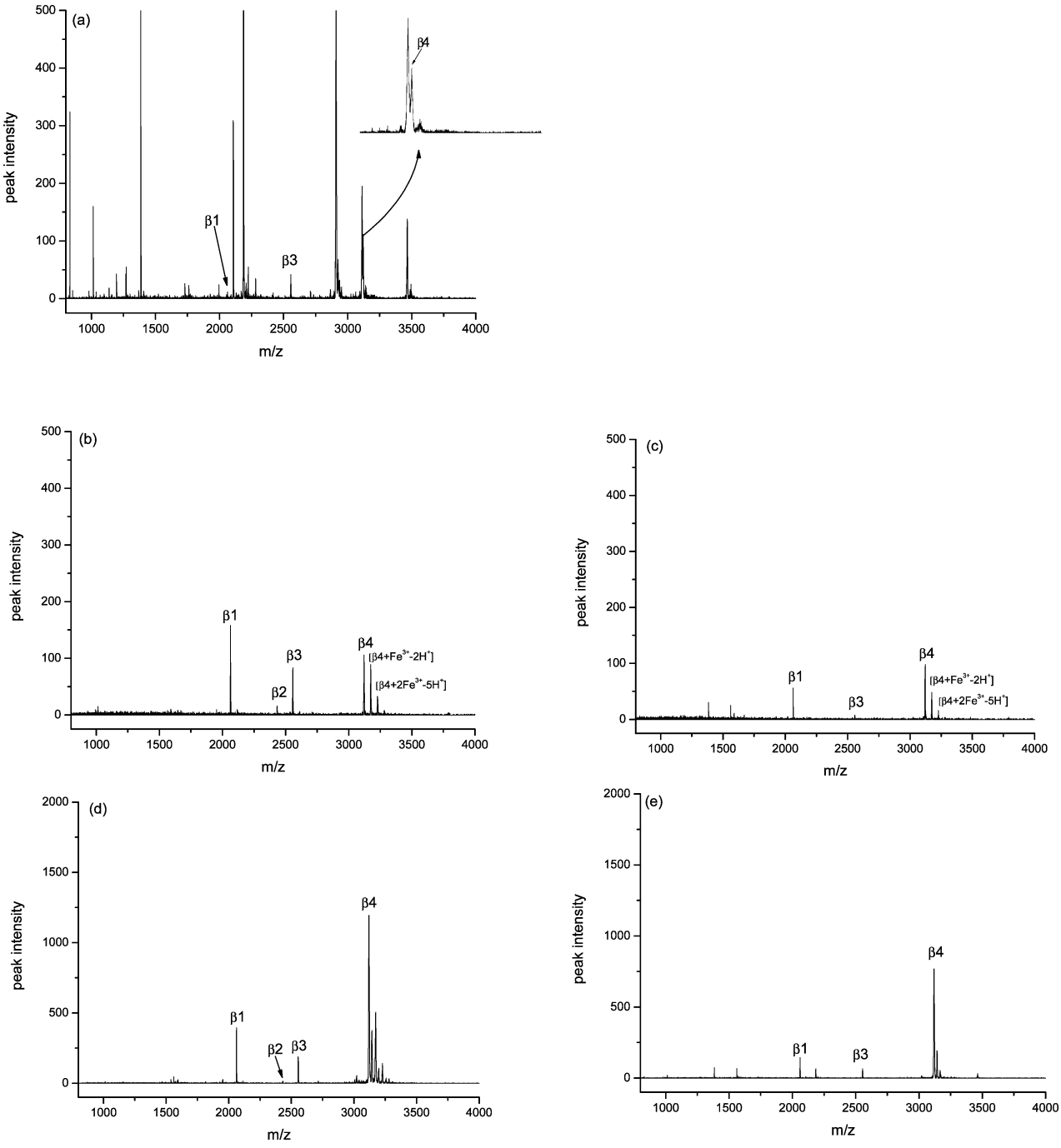

Figure 3. MALDI mass spectra of the tryptic digest of β-casein (10 μM). (a) Direct analysis; (b) and (c) on bead analysis after extraction by adding 0.1 mg Fe^{3+}-immobilized MCM-41 and Fe^{3+}−IMAC beads into the 10 μL tryptic digest of β-casein; (d) and (e) off-bead analysis after extraction by Fe^{3+}-immobilized MCM-41 and Fe^{3+}−IMAC beads. Peaks for identified phosphopeptides are designated as β1, β2, β3, and β4. The detailed information is listed in Table 2.

from the TEM image at a medium magnification (scale bar, 100 nm; 80 000×) as shown in Figure 1b. The TEM image at a high magnification (scale bar, 50 nm; 150 000×) of Fe^{3+}-immobilized MCM-41 is shown in Figure 1c. Apparently, the pores of prepared Fe^{3+}-immobilized MCM-41 are very highly ordered, without intergrowth or twinning, with the pore size of ca. 3 nm. So, it is believed that the prepared Fe^{3+}-immobilized MCM-41 have a well-defined hexagonal particle shape with particles size of ca. 600 nm and pore sizes of ca. 3 nm as reported by Cai et al.[35]

Figure 2 shows the MALDI mass spectra for (a) direct analysis of the tryptic digest of α-casein (10 μM) and (b) on-bead analysis after extraction by Fe^{3+}-immobilized MCM-41 from 10 μL of tryptic digest of α-casein (10 μM). As shown in Figure 2a, peaks for phosphopeptides, designated as α1, α2, α3, α4, and α5, are detected with a large amount of peaks for nonphosphopeptides, which indicates that the detection of phosphopeptides is strongly suppressed by those dominant peaks from nonphosphopeptides in mass spectrum. After the extraction by Fe^{3+}-immobilized MCM-41, apparently, α1, α2, α3, α4, and α5 are

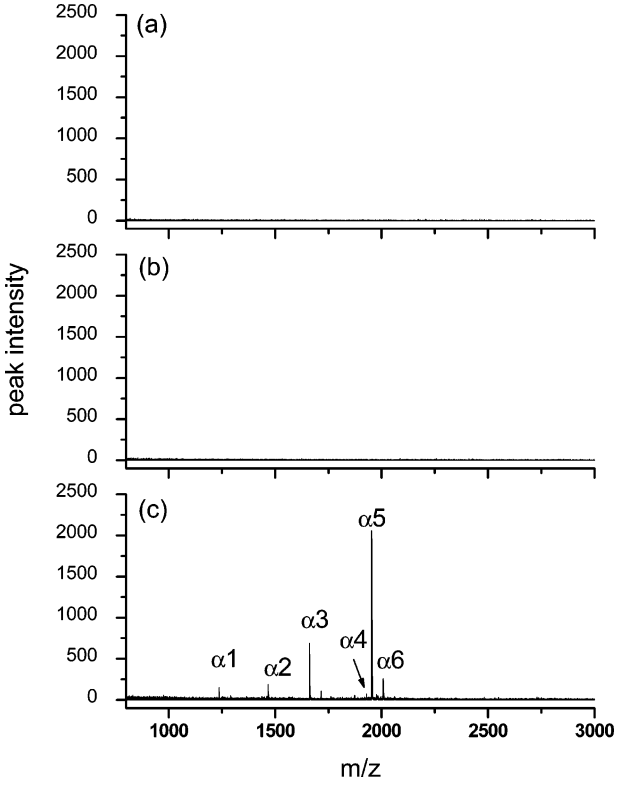

Figure 4. MALDI mass spectra for on-bead analysis of trapped peptides by Fe^{3+}-immobilized MCM-41 from the 10 μL of tryptic digest product of α-casein (10 μM) by (a) MCM-41, (b) MCM−GLYMO−IDA and (c) Fe^{3+}-immobilized MCM-41, respectively. Peaks for identified phosphopeptides are designated as α1, α2, α3, α4, α5, and α6. The detailed information is listed in Table 1.

all detected with an additional peak for phosphopeptides, designated as α6, as shown in Figure 2b. Peaks for phosphopeptides become dominate in the mass spectrum with a clean background of few peaks for nonphosphopeptide. The detailed information of identified phosphopeptides in α-casein, including their signal-to-noise (S/N) values obtained in direct MALDI analysis and MALDI analysis after extraction, is listed in Table 1. From Table 1, it can be seen that, phosphopeptides from tryptic digest of α-casein are effectively trapped and separated from nonphosphopeptides after the extraction by Fe^{3+}-immobilized MCM-41; that is to say, phosphopeptides from α-casein are trapped and detected by MALDI with a clean background of few peaks for nonphosphopeptide. In addition, the detection for α3 and α5 by MALDI is greatly enhanced because the S/N of peaks for α3 and α5 are both increased by more than an order of magnitude higher after the extraction by Fe^{3+}-immobilized MCM-41.

It has been reported that IMAC beads can also be transferred onto the target for on-bead analysis[20−22] of selectively trapped phosphorylated peptides and proteins by MALDI, so Fe^{3+}−IMAC beads were applied to selectively trap phosphopeptides from tryptic digests of β-casein for a direct comparison with Fe^{3+}-immobilized MCM-41. Figure 3a shows the MALDI MS spectrum of direct analysis of the tryptic digest of β-casein (10 μM), in which three phosphopeptides, designated as β1, β3, and β4, are detected with a large amount of peaks for nonphosphopeptides. After the extraction from 10 μL of tryptic digest of β-casein (10 μM) by Fe^{3+}-immobilized MCM-41, it can be seen that four phosphopeptides in tryptic digest of β-casein are all detected in on-bead analysis with a clean background and designated as β1, β2, β3, and β4 in Figure 3b, whereas only

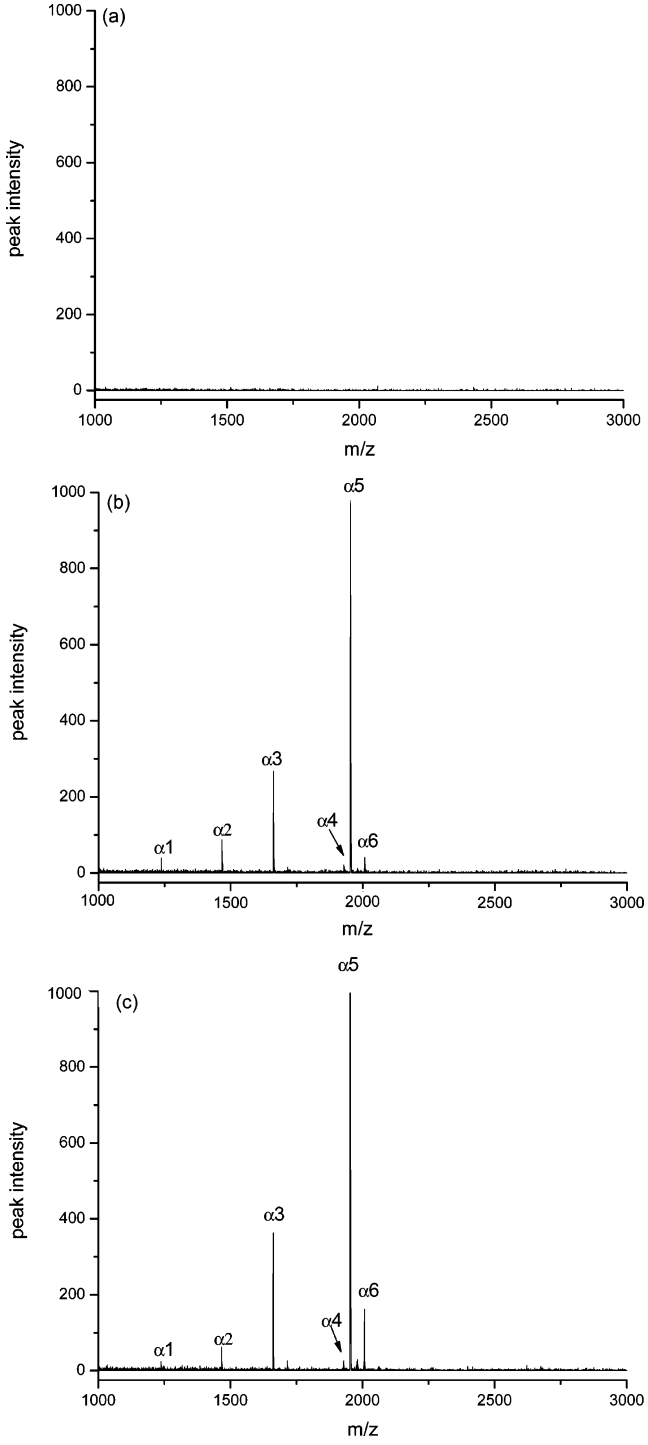

Figure 5. MALDI mass spectra for on-bead analysis of trapped peptides by Fe^{3+}-immobilized MCM-41 from (a) 10 μL of tryptic digest product of BSA (10 μM), (b) tryptic digest of α-casein (10 μM) mixed with tryptic digest product of BSA (10 μM) at a volume ratio of 1:1 and (c) 1:10, respectively. Peaks for identified phosphopeptides are designated as α1, α2, α3, α4, α5, and α6. The detailed information is listed in Table 1.

β1, β3, and β4 were observed in on-bead analysis after the extraction by Fe^{3+}−IMAC beads with some peaks of nonphosphopeptides in Figure 3c. To eliminate the possible "shadow effect" of IMAC beads, phosphopeptides selectively captured by Fe^{3+}-immobilized MCM-41 and Fe^{3+}−IMAC beads are both eluted to perform an off-bead analysis by MALDI. The spectra

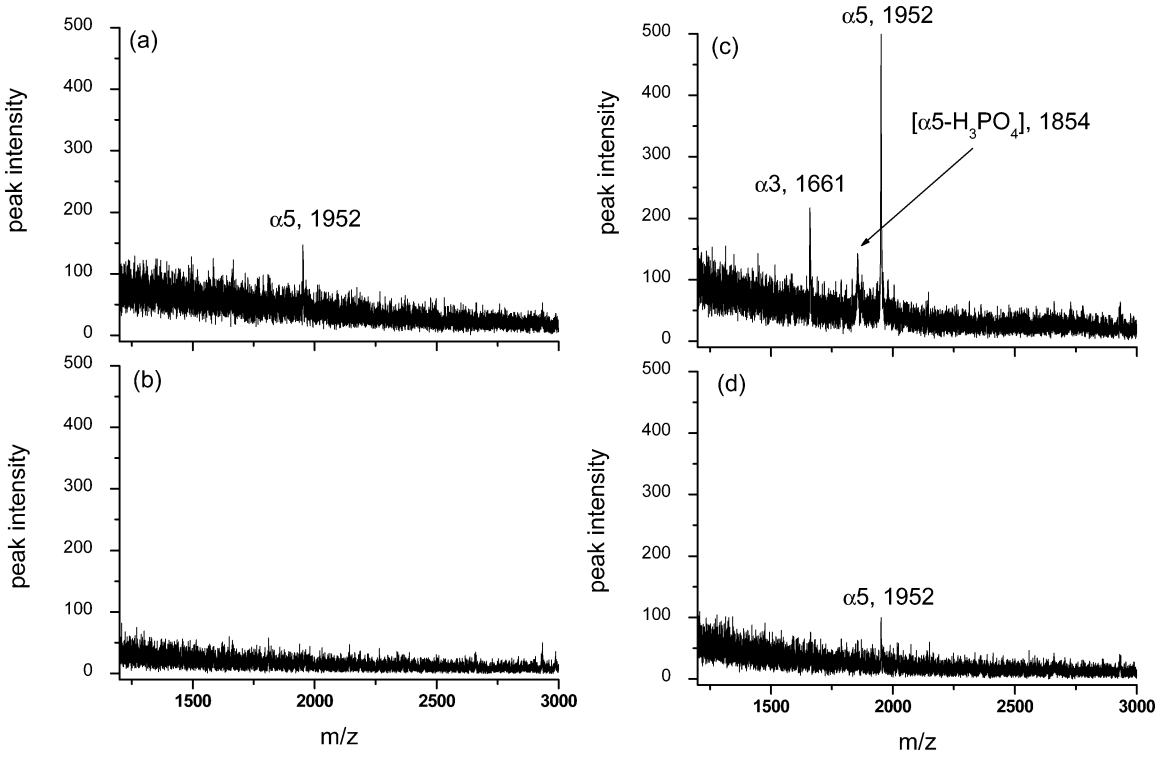

Figure 6. MALDI mass spectra for on-bead analysis of the trapped phosphopeptides by Fe^{3+}-immobilized MCM-41 from the tryptic digest product of α-casein at the concentrations and volumes of (a) 0.1 μM, 10 μL; (b) 0.01 μM, 10 μL; (c) 0.1 μM, 100 μL; and (d) 0.01 μM, 100 μL.

in off-bead analysis of phosphopeptides eluted from beads of Fe^{3+}-immobilized MCM-41 and Fe^{3+}−IMAC beads are shown in Figure 3d,e, respectively. The detailed information of identified phosphopeptides in β-casein, including their S/N values obtained in direct MALDI analysis and MALDI analysis after extraction, is listed in Table 2. It is notable that phosphopeptides are detected with higher values in S/N after extraction by Fe^{3+}-immobilized MCM-41 in comparison with Fe^{3+}−IMAC beads, no matter they are obtained in on-bead analysis or off-bead analysis. These results indicate that Fe^{3+}-immobilized MCM-41 has higher capacity and selectivity in capturing phosphopeptides in comparison with Fe^{3+}−IMAC beads. It is supposed that the higher capacity and selectivity of Fe^{3+}-immobilized MCM-41 for phosphopeptides is resulted from its higher surface area in comparison with Fe^{3+}−IMAC beads, because Fe^{3+}-immobilized MCM-41 have well-defined hexagonal particle shape with particles size of ca. 0.6 μm, whereas the particle size of Fe^{3+}−IMAC beads is larger than 20 μm.

All results above demonstrated again that the detection of phosphopeptides both in α-casein and β-casein by MALDI are greatly enhanced due to the fact that phosphorylated peptides are selectively trapped on Fe^{3+}-immobilized MCM-41 and separated from other nonphosphopeptides. In addition, it is interestingly found that phosphopeptides β4 are not only detected as its peak in proton adduct ($[M + H]^+$, 3122), but also as a series of Fe^{3+} adduct ($[M + nFe^{3+} - (3n - 1) H^+]^+$, n = 1, 2) with an interval of 54 Da as shown both in Figure 3b and c. This phenomenon of Fe^{3+} adducts were also found in the work reported by Hart et al.[22] when 2, 5-DHB was used as matrix for MALDI analysis. In addition, such a phenomenon was not observed for all phosphopeptides. It is assumed that proton adducts of phosphopeptides are more easily formed and more stable than their Fe^{3+} adducts in the process of desorption/ionization of MALDI. Only when the concentration of phosphopeptides is high enough, peaks of Fe^{3+} adducts ($[M + nFe^{3+} - (3n - 1) H^+]^+$ may appear.

Control tests are performed to investigate the mechanism for adsorption of phosphopeptides on the Fe^{3+}-immobilized MCM-41 due to the interaction between Fe^{3+} and phosphate group on phosphopeptides. Figure 4 shows the MALDI mass spectra in on-bead analysis of trapped peptides from the 10 μL of tryptic digest of α-casein (10 μM) by (a) MCM-41, (b) MCM-GLYMO−IDA, and (c) Fe^{3+}-immobilized MCM-41, respectively. When MCM-41 is used as the adsorbent, no peaks are detected for trapped peptides as shown in Figure 4a. Because MCM-41 contains a large amount of silicate hydroxyl on its surface,[25−27] it is supposed that analytes are adsorbed on MCM-41 due to the interaction of hydrophilic/hydrophobic force. In that case, it is believed that few peptides could be adsorbed on MCM-41 through hydrophilic/hydrophobic force in solution of ACN/0.1% TFA (50:50, v/v), and further, those peptides adsorbed on MCM-41 could be rinsed off by the washing solution containing 100 mM NaCl in ACN/0.1% TFA (50:50, v/v) too. For MCM−GLYMO−IDA, because every IDA contains two carboxylic groups, ionic force may be involved to adsorb peptides besides the hydrophilic/hydrophobic force. However, the dissociation of carboxylic groups in IDA would be strongly suppressed under a condition of strong acid (pH = 2−3). Thus, few peptides could be trapped on MCM−GLYMO−IDA through ionic force in solution of ACN/0.1% TFA (50:50, v/v), which is a strong acidic solution (pH = 2−3). Further those peptides trapped on MCM−GLYMO−IDA could be rinsed off by the washing solution containing 100 mM NaCl in ACN/0.1% TFA (50:50, v/v), which is also a strong acidic solution (pH = 2−3) with additional salt of NaCl at concentration of 100 mM. As a result, no peaks for peptides are detected either when MCM−GLYMO−IDA is used as adsorbent in Figure

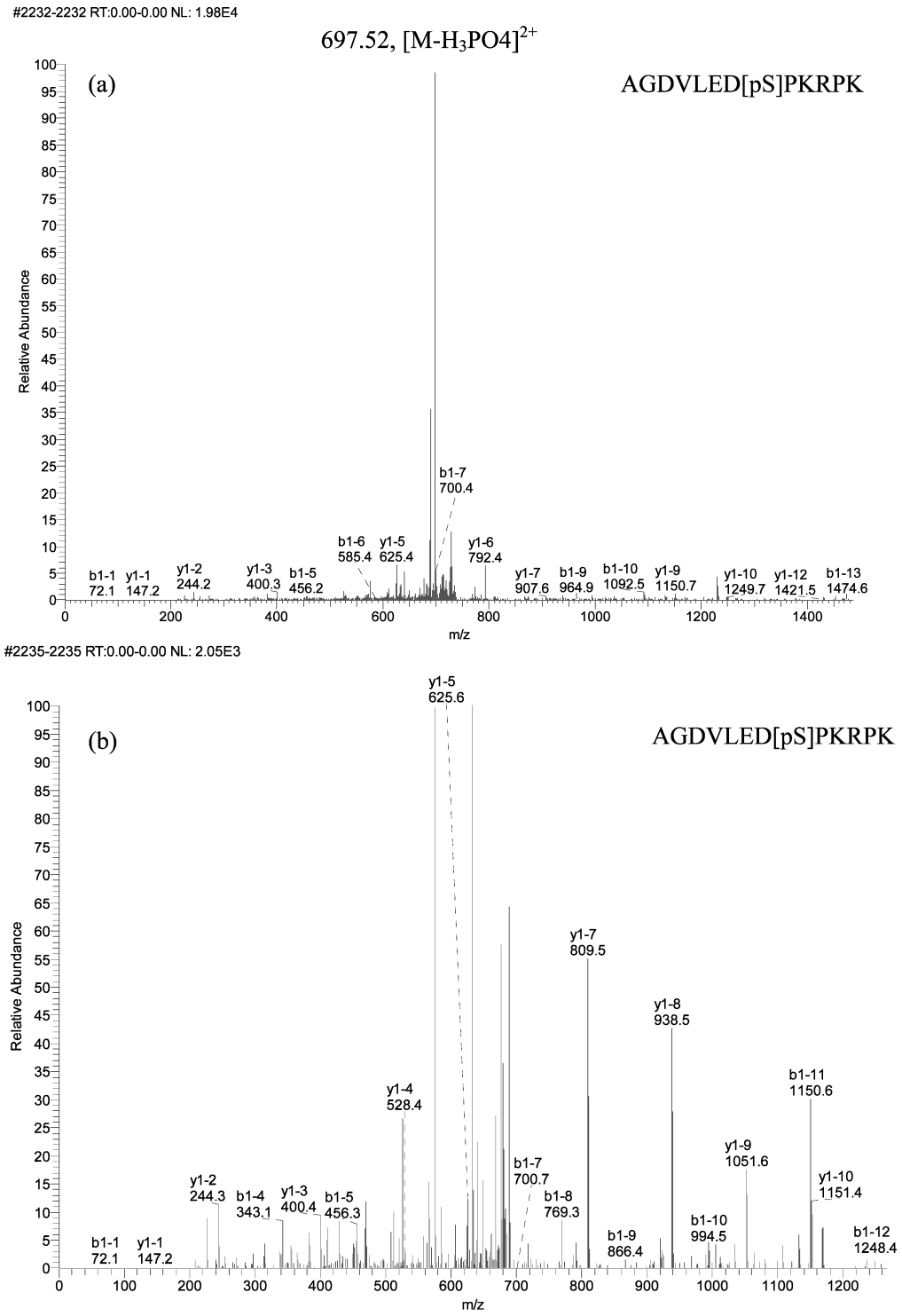

Figure 7. (a) MS/MS spectrum of the doubly charged form of a phosphopeptide AGDVLED[pS]PKRPK identified from the tryptic digest of the lysate of mouse liver (1 mg/mL). The dominant peak at *m*/*z* 697.52 represents the doubly charged neutral loss peak (after loss of the H_3PO_4 group) for its selected precursor ion (*m*/*z* 746.69). (b) MS/MS/MS spectrum of the doubly charged neutral loss peak at *m*/*z* 697.52 for phosphopeptide AGDVLED[pS]PKRPK. The b- and y-ion series shown both in (a) and (b) are indicated as to verify the identification of the peptide.

4b. Finally, when the Fe^{3+}-immobilized MCM-41 is used as adsorbent to extraction of phosphopeptides from tryptic digest of α-casein, 6 peaks for phosphopeptides (α1, α2, α3, α4, α5, and α6) detected by MALDI as shown in Figure 4c. It is demonstrated that those peptides are selectively trapped on Fe^{3+}-immobilized MCM-41 due to the interaction between Fe^{3+} and phosphate group.

Comparing to methods[22,23] reported before, in which some nonphosphopeptides are still detected as dominant peaks through nonspecific adsorption, Fe^{3+}-immobilized MCM-41 shows a better selectivity and specificity to phosphopeptides. When Fe^{3+}-immobilized MCM-41 is applied to extract peptides from the 10 μL of tryptic digest of BSA (10 μM), no peak for peptides is detected by MALDI as shown in Figure 5a, because

Table 3. Singly Phosphorylated Peptides Identified after Manual Validation in the Digest of Lysate of Mouse Liver

no.	sequence[a]	$[M + H]^+$ m/z	charge	Xcorr	MS^2	MS^3
1	R.SAS[pS]DTSEELNSQDSPKR.Q	2018.94	2	2.10	√[b]	√[b]
2	R.AGDVLED[pS]PKRPK.E	1492.57	2	2.44	√[b]	√[b]
3	R.TASF[pS]ESRADEVAPAKK.A	1874.94	2	2.06	√[b]	√[b]
4	R.KN[pS]ASLHVLK.T	1177.29	2	2.23	√[b]	√[b]
5	K.FAGLLQDMQVTGVEDD[pS]R.A	2062.16	2	3.03	√[b]	√[b]
6	R.RG[pS]VSLGNK.V	998.03	2	2.68	√[b]	√[b]
7	R.TGEPDEEEGTFR[pS]SIR.R	1890.86	2	2.44	√[b]	√[b]
8	-.MANLND[pS]AVTNGTLHNPK.T	1978.09	2	2.32	−[c]	√[b]
9	R.[pS]GELLVWDLLGAKVSER.I	1953.14	2	2.41	−[c]	√[b]
10	R.PRRTGEA[pS]GSHLGPPGGALHLHCATAAFGR.A	3120.32	3	2.74	√[b]	−[c]
11	K.IEDVG[pS]DEEDDSGKDKK.K	1946.87	2	2.80	√[b]	−[c]
12	R.HETYLC[PY]VVKRR.D	1704.84	2	2.12	√[b]	−[c]
13	R.P[pS]PVTLRRM*ASQLSGLPR.R[d]	2063.33	2	2.09	√[b]	−[c]
14	R.SRLHNGKLTD[pT]TAMTLGQSR.E	2268.46	3	2.57	√[b]	−[c]
15	R.ETCFAFALTPQQVQQISS[pS]M*DISGTK.C[d]	2972.16	3	2.51	√[b]	−[c]
16	K.VEIVAI[pS]DPFIDLNYM*VYM*FQSDSTHGK.F[d]	3333.65	3	2.51	√[b]	−[c]
17	K.IQM*LEKELEDVKAE[pT]AR.K[d]	2100.30	2	2.15	√[b]	−[c]
18	K.LLKEGEEP[pT]VYSDDEEPKDETAR.K	2731.78	3	3.33	√[b]	−[c]
19	R.PFAIEQV[pT]GMLLAKLKETSENALK.K	2713.10	3	2.79	√[b]	−[c]
20	K.MIEENSHC[pS]YVIEMLK.S	2064.26	2	2.76	√[b]	−[c]
21	K.VEEVLEEEEEE[pY]VVEK.V	2062.09	2	2.82	√[b]	−[c]
22	K.IEE[pS]SANVIVIYADIVSVQGLM*R.H[d]	2603.89	3	2.51	√[b]	−[c]
23	-.METAGATADA[pT]AGPQKLSRK.K	2085.24	2	2.01	√[b]	−[c]
24	K.[pY]EEEDEVIVR.V	1361.35	2	2.11	√[b]	−[c]
25	K.ASLESLGLK[pS]LFNESTSDFSGMSETK.G	2847.02	3	2.55	√[b]	−[c]
26	R.GSPQGSAAELGSGLLA[pS]AAASSR.A	2126.19	3	2.69	√[b]	−[c]
27	K.PCVD[pT]M*AHSVKVMLDK.W[d]	1928.15	2	2.29	√[b]	−[c]
28	K.LFPNM*LPS[pT]FETQSIK.E[d]	1950.16	2	2.32	√[b]	−[c]
29	K.NMPSATI[pS]KAPSTPEPNK.N	1951.11	2	2.37	√[b]	−[c]
30	R.DQDSGENGRM*M*C[pS]IEDK.L[d]	2085.06	2	2.04	√[b]	−[c]
31	R.ES[pT]M*QLLSLVQHGQGARK.A[d]	2080.27	3	2.67	√[b]	−[c]
32	R.LDDTWPV[pS]PHSRAKEDK.D	2062.14	2	2.38	√[b]	−[c]
33	K.NWVM*TTRSVSNHF[pS]NLPSQR.A[d]	2458.61	3	2.66	√[b]	−[c]

[a] Sequence and site of phosphorylation were identified by SEQUEST (see text). [b] Identified. [c] Not identified. [d] * denotes the methionine oxidation site with +16 Da both in MS^2 and MS^3.

it is believed that there is no phosphorylated sites in BSA. Figure 5b,c shows the MALDI mass spectra for the extracted phosphopeptides by Fe^{3+}-immobilized MCM-41 from the mixture of tryptic digest of α-casein (10 μM) with the tryptic digest of BSA (10 μM) at a volume ratio of 1:1 and 1:10, respectively. It can be seen that 6 peaks for phosphopeptides (α1, α2, α3, α4, α5, and α6) are successfully detected both in Figure 5b and c. It is demonstrated that phosphopeptides still can be efficiently extracted from a solution with the existence of a large amount of nonphosphopeptides by Fe^{3+}-immobilized MCM-41.

Phosphopeptides also can be extracted from digestion solution at a larger volume and finally concentrated into the 10 μL of Fe^{3+}-immobilized MCM-41 dispersed solution. In that case, it not only increases the signal intensity for phosphopeptides but also lowers the detection limit for phosphopeptides. Figure 6 shows MALDI mass spectra in on-bead analysis of trapped phosphopeptides from α-casein at the different concentration and volume of (a) 0.1 μM, 10 μL, (b) 0.01 μM, 10 μL, (c) 0.1 μM, 100 μL, and 0.01 μM, 100 μL, respectively. It can be seen that only phosphopeptide α5 is detected after extraction from 10 μL of tryptic digest of 0.1 μM α-casein, whereas phosphopeptide α3 and α5 are detected after extraction from 100 μL of tryptic digests in Figure 6 (c). It is supposed that the peak at m/z 1854 is the fragment of α5 after the loss of H_3PO_4 group for its difference in 98 Da comparing to α5. When concentration of α-casein was decreased to 0.01 μM, a peak for phosphopeptide α5 is still detected after extraction from 100 μL of the sample, as shown in Figure 6d, whereas no peaks are detected in Figure 6b after extraction from 10 μL of digest solution. By keeping α-casein at the same concentration, the signal intensity for phosphopeptides α3 and α5 shown in Figure 6 are increased apparently when the volume increased from 10 to 100 μL. Furthermore, phosphopeptides α5 digested from 0.01 μM α-casein still can be detected with extraction by Fe^{3+}-immobilized MCM-41 from 100 μL of digest solution, but cannot be detected with extraction from 10 μL of digest solution. It can be estimated that the minimum amount of digest for detection of phosphopeptides by the MALDI target is about 10 fmol in this method with extraction by Fe^{3+}-immobilized MCM-41.

Fe^{3+}-immobilized MCM-41 was applied to trap the phosphopeptides from tryptic digest of lysate of mouse liver for phosphoproteome analysis through nano-LC−MS/MS, MS/MS/MS and database searching. Dispersed solution (20 μL) of Fe^{3+}-immobilized MCM-41 was mixed with 100 μL of tryptic digest of the lysate of mouse liver (1 mg/mL) to trap and enrich phosphopeptides, and then 100 μL of 1% NH_4OH was added to disperse Fe^{3+}-immobilized MCM-41. After vibration of 30 min for the dispersed solution, it was centrifugated at 30 000 g for 10 min. The supernatant containing phosphopeptides was collected, lyophilized to dryness, and finally redissolved in 5 μL of 0.1% formic acid for the analysis by nano-LC−MS/MS and MS/MS/MS.

Figure 7a shows a typical MS/MS spectrum for identification of phosphopeptide (AGDVLED[pS]PKRPK) with doubly charged form. The dominant peak at m/z 697.52 represents the doubly charged neutral loss fragment (after loss of the H_3PO_4 group) for its selected precursor ion (m/z 746.69). Figure 7b shows the MS/MS/MS spectrum of the doubly charged neutral loss fragment (AGDVLEDSPKRPK) at m/z 697.52. The b- and y-ion

series matched with the database are indicated as shown in Figure 7a,b to verify the identification of the peptide. It is commonly observed that the doubly charged singly phosphorylated peptide almost keeps intact after the loss H_3PO_4 group in the MS/MS spectrum and does not produce more fragment ions until further collision induced dissociation is introduced. Thus, the peak for neutral loss fragment of doubly charged singly phosphorylated peptide is always the dominant peak with a few peaks for fragment ions, whereas the MS^3 spectrum could provide enough fragment ion peaks for the identification and confirmation of phosphopeptides. There are also a few publications[38,39] that reported using MS^3 to confirm the identification of phosphopeptides derived from the MS^2 spectrum.

In total, 514 unique peptides including 413 phosphopeptides and 101 nonphosphopeptides (data not shown) from the SEQUEST search result of MS^2 remain and are identified after the filtration by setting Xcorr as 2.0 no matter what the charge state is. This result indicates that nonspecific interaction is still taking place as the tryptic digest of the lysate of mouse liver is too complicated. It was reported that the methyl esterification of carboxylic acid group prior to IMAC can significantly minimize the nonspecific binding[40−42]. Because the adsorption of phosphopeptides on the surface of Fe^{3+}-immobilized MCM-41 and Fe^{3+}−IMAC beads are both based on the coordination interaction between the P in phosphopeptides and Fe^{3+} on the beads surface, it is expected that a successful methyl esterification can also improve the selectivity and specificity of Fe^{3+}-immobilized MCM-41 for phosphopeptides. When the filtration threshold is increased by setting Xcorr as 1.5, 2.0, and 2.5 corresponding to 1+, 2+, and 3+ charge states, respectively, there are still 134 unique peptides left, including 105 phosphopeptides and 29 nonphosphopeptides. Among 105 phosphopeptides, 31 singly phosphorylated peptides were identified after manual validation based on the criteria described above and listed in Table 3 with the detailed information including their sequences, molecular weights, charge states and Xcorr values. In addition, among the SEQUEST search result of MS,[3] 27 singly phosphorylated peptides are remained and identified after the filtration by setting Xcorr as 1.5, 2.0, and 2.5 corresponding to 1+, 2+, and 3+ charge states, respectively, and 9 singly phosphorylated peptides were identified after manual validation, in which 7 singly phosphorylated peptides were matched the results derived from MS.[2] Thus, totally 33 singly phosphorylated peptides from the lysate of mouse liver are identified by the manual validation based on the criteria described above. It is demonstrated that the application of Fe^{3+}-immobilized MCM-41 in phosphoproteome analysis for lysate of the mouse liver is successful by comparing with previously reported result of phosphoproteome analysis by commercial IMAC,[38] in which about 150 phosphopeptides were identified after the filtration by setting Xcorr as 2.0 no matter what the charge state is, and 26 singly phosphorylated peptides are identified by the manual validation based on the same conservative criteria as in this work.

Conclusions

The Fe^{3+}-immobilized MCM-41 with particle size of ca. 600 nm and pore size of ca. 3 nm through the modification of silicate hydroxyl group on surface was prepared, and then the Fe^{3+}-immobilized MCM-41 was used as the adsorbent to selectively trap, separate, and enrich phosphopeptides from the tryptic digest of proteins. Because of its higher surface area, the Fe^{3+}-immobilized MCM-41 has an advantage of higher capacity and selectivity for phosphopeptides in comparison with Fe^{3+}−IMAC beads. After the extraction by Fe^{3+}-immobilized MCM-41, the suppression to detection of phosphopeptides caused by abundant nonphosphopeptides from tryptic digest is effectively eliminated, and the detection of trapped phosphopeptides by MALDI is greatly enhanced with the S/N value increased by more than an order of magnitude. It is demonstrated that the mechanism of the adsorption of phosphopeptides on Fe^{3+}-immobilized MCM-41 is based on the interaction between the Fe^{3+} and the phosphate group. Fe^{3+}-immobilized MCM-41 is also successfully applied to phosphoproteome analysis of mouse liver by nano-LC−MS/MS. Thirty-three singly phosphorylated peptides are identified by manual validation using a series of conservative criteria.

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