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Preparation of magnetic polymer material with phosphate group and its application to the enrichment of phosphopeptides

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ABSTRACT

As one of the most important post-translational modifications (PTM), reversible phosphorylation of protein is involved in many cellular processes. Enrichment and separation of phosphopeptides have become essential for large-scale identification of protein phosphorylation by mass spectrometry. In this work, five magnetic polymer materials with different numbers of phosphate groups were fabricated using a simple polymeric method and their abilities to enrich phosphopeptides were investigated. Our results showed that the enrichment efficiency is closely related to the number of phosphate groups attached to magnetic polymer sorbent. Under optimized condition (3% trifluoroacetic acid and 80% acetonitrile), magnetic polymer-particles with appropriate proportion of phosphate groups (Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+}) showed high performance for extracting phosphopeptides from complex peptides mixture of standard protein digestion. In this regard, a total of 988 unique phosphopeptides were successfully identified from proteolytic digestion of HeLa cell extracts by employing magnetic polymer-particles combined with nano-RPLC–MS/MS analysis.

1. Introduction

Magnetic particles have been widely used in separation science due to their ease of operation, uniqueness and nano-scale size [1,2]. In phosphoproteomics, magnetic particles have been used to rapidly separate some target analytes [3,4]. Meanwhile, porous materials and polymers, due to their high-surface area and abundant adsorption sites, have been widely used for enrichment processes [5,6]. The combination of magnetic particles and polymers or porous materials has led to a wide range of applications [7,8].

Reversible phosphorylation of proteins, one of the most important post-translational modifications (PTM) in cells, is involved in many cellular processes, such as proliferation, differentiation and apoptosis [9–11]. For the sake of understanding the molecular basis of these regulatory mechanisms, it is vital to identify and quantify the specific sites of phosphorylation. Mass spectrometry (MS), including matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization-mass spectrometry (ESI-MS), has been widely used in proteomics as well as phosphoproteomics [12–14]. However, due to their low abundance, substoichiometry and low ionization efficiency in mass spectrometry, it remains difficult to analyze phosphorylated peptides from complex samples. Therefore, to establish appropriate methods for the enrichment and separation of phosphopeptides has become highly important [15,16].

Immobilized metal affinity chromatography (IMAC) is one of the most extensively used enrichment methods for phosphoproteomics analysis. In this technique, various metal ions (Fe^{3+}, Ga^{3+}, etc.) were immobilized on supporting substrates via chelating reagents such as iminodiacetic acid (IDA) [17] and nitrilotriacetic acid (NTA) [18]. Zr^{4+} and Ti^{4+} have also been employed to substitute the common metals and they showed a better performance [19]. However, as a result of the existence of non-phosphopeptides adsorption in this method, methyl esterification has been widely used in this strategy [20]. Because of the potential side reactions in chemical modification, a new enrichment method is required for the continual development of affinity chromatography.

Recently, Zou and co-workers, used phosphate groups to replace IDA or NTA as the chelator [21]. Phosphate groups-containing matrices were loaded with metal ions, such as Zr^{4+}, Ti^{4+}, and then

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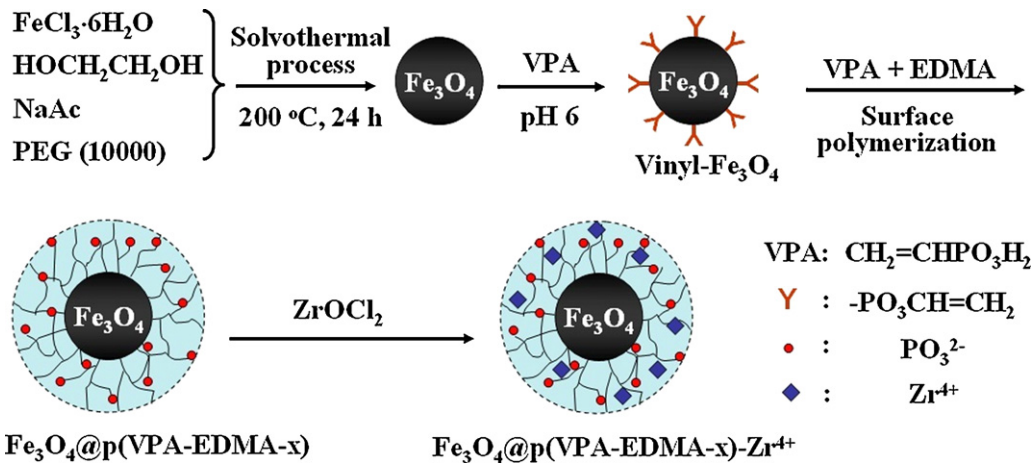

Fig. 1. Schematic diagram for the preparation of magnetic polymer Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+}.

the enrichment performance towards phosphopeptides was investigated [19]. The results showed that phosphate chelators have a higher affinity than IDA and NTA, and more phosphopeptides can be identified. More recently, phosphate has also been introduced into other matrices, such as polymer beads [19,22], magnetic nanoparticles [23–25], porous silicon [26], nanoplatelets [27], monolithic capillary columns [28] and self-assembled monolayer [29]. Nevertheless, so far there have been no attempts to study the influence of phosphate chelator concentration on the enrichment efficiency.

In the current study, by using a simple procedure we synthesized a new phosphate groups-containing magnetic polymer-particle for rapid and efficient enrichment of phosphopeptides. We also investigated the relationship between enrichment efficiency and the number of phosphate chelator groups. Under optimized conditions, magnetic polymer-particles with suitable proportion of phosphate groups (Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+}) showed high performance on selective enrichment of phosphopeptides from complex peptides mixture of standard protein digest and proteolytic digest of HeLa cell extracts. In this respect, a total of 988 phosphopeptides (1291 phosphorylation sites) were identified by employing phosphate groups-containing magnetic polymer-particles combined with LC–MS/MS analysis, which provides a rapid and efficient alternative IMAC strategy for phosphopeptide enrichment.

2. Experimental

2.1. Reagents and materials

Iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), sodium acetate anhydrous (NaAc), polyethylene glycol 10000 (PEG10000), ethylene glycol, ethanol (EtOH), 2,2′-azobid(2-methylpropionitrile) (AIBN), zirconyl chloride octahydrate ($ZrOCl_2{\cdot}8H_2O$) were all of analytical reagent grade and supplied by Shanghai General Chemical Reagent Factory (Shanghai, China). Vinyl phosphonic acid (VPA) and ethylene glycol dimethacrylate (EDMA) were purchased from Aladdin Chemical Reagent Co. (Shanghai, China) and Acros (Sweden), respectively. HPLC grade acetonitrile (ACN) was from Fisher Scientific (Pittsburgh, PA, USA). Ammonia hydrate ($NH_3{\cdot}H_2O$, 25%), phosphoric acid (H_3PO_4), trifluoroacetic acid (TFA), 2, 5-dihydroxybenzoic acid (2, 5-DHB), bovine α-casein, bovine β-casein and bovine serum albumin were from Sigma–Aldrich (St. Louis, MO, USA). Sequencing grade trypsin was from Promega (Madison, WI, USA). Purified water was obtained with a Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.2. Synthesis of magnetic polymer-particles (Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+})

Five magnetic polymer-particles containing different proportions of phosphate groups, which was defined as Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+} (*x* refers to the molar ratio of VPA over EDMA), were synthesized by surface free radical polymerization (Fig. 1). In a typical preparation, Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+} was synthesized using the following procedures. Bare Fe_3O_4 particles were prepared by solvothermal processing as described previously [30]. 4 g of Fe_3O_4 was then dispersed in vinyl phosphonic acid aqueous solution, the pH value of which was adjusted to 6 by alkaline solution at 80 °C for 12 h. The product was then washed with distilled water and ethanol several times and separated magnetically with an external magnet. 0.5 g vinyl-Fe_3O_4 was sonicated in 50 mL of ACN for 30 min. 0.9900 g vinyl phosphonic acid and 1.8170 g EDMA was added to the suspension and the reaction was initiated by the addition of 0.03 g AIBN at 60 °C. After 8 h, the as-prepared magnetic polymer-particle Fe_3O_4@p(VPA-EDMA-1), was washed with distilled water and ethanol under ultrasonicating conditions until the eluent was transparent, and then dried under vacuum at 60 °C. Finally, 100 mg of the magnetic polymer-particle was added to 100 mL of 100 mM $ZrOCl_2$ solution at 30 °C and gently stirred for 12 h. The Zr^{4+}-magnetic polymer-particles were rinsed with distilled water to remove nonspecifically adsorbed Zr^{4+}. The final product was dried in vacuum at 60 °C. The other four magnetic polymer-particles were prepared using similar procedure but with different molar ratio of VPA over EDMA, as described in Table 1.

Table 1
The proportion of raw materials used for production of the five magnetic polymers.

	VPA-EDMA-0.2	VPA-EDMA-0.6	VPA-EDMA-1	VPA-EDMA-1.4	VPA-EDMA-2
vinyl-Fe_3O_4			0.500 g		
CH_2=$CHPO_3H_2$	0.198 g (0.00183 mol)	0.594 g (0.00550 mol)	0.990 g (0.00916 mol)	1.386 g (0.0128 mol)	1.980 g (0.0183 mol)
EDMA			1.82 g (0.00917 mol)		
AIBN			0.0300 g		
ACN			50 mL		

2.3. Characterization

Fourier transform infrared spectroscopy was performed with a Thermo Nicolet 670 FT-IR instrument (Boston, MA, USA). The morphology of the particles was observed with a transmission electron microscope (JEOL, Kyoto, Japan). Shimadzu UV-240 (Kyoto, Japan) was used to determine the phosphate content in the magnetic materials. Magnetization curves were determined with a PPMS-9 vibrating sample magnetometer (Quantum Design, San Diego, CA, USA).

2.4. Determination of phosphorus content

The content of phosphate was determined by the molybdenum blue method [31].

Sample pretreatment: Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+} (10 mg) was added into a crucible, followed by adding 1 mL concentrated sulfuric acid, 4 drops of concentrated nitric acid, and a few grains of zeolite. The mixture was then heated until white smoke was formed. After cooling to room temperature, the material attached to the wall of the crucible was washed to the bottom with 2.0 mL of distilled water, and then re-heated. The resulting solution was transferred to volumetric flask (50-mL) and stored until use.

Calibration curve construction: 0, 0.2, 0.5, 0.7, 1.0, 1.2 mL of standard solution (1 μg/mL) was added to a volumetric flask (50-mL), followed by adding 3 mL of 2.25 mM H_2SO_4, 10 mL 5% ammonium molybdate solution, and 5 mL 10% ferrous ammonium sulfate solution. The absorption of phosphomolybdic acid was determined at 750 nm after 45 min.

Sample determination: 10 mL of the sample solution was added in volumetric flask (50-mL), followed by adding 1.5 mL 2.25 mM H_2SO_4, 10 mL 5% ammonium molybdate solution, 5 mL 10% ferrous ammonium sulfate solution. After diluting to the scale mark, the sample was measured under the same conditions as the standard solution.

2.5. Sample preparation

Bovine α-casein and β-casein were originally made up into stock solutions of 1 mg/mL with purified water. Proteins were digested with trypsin by using an enzyme to substrate ratio of 1:50 (w/w) in 100 mM Tris–HCl pH 8.5, and the digestion was performed at 37 °C for overnight.

BSA (1 mg) was dissolved in 100 μL of denaturing buffer solution (8 M urea in 100 mM Tris–HCl pH 8.5). The protein solution was mixed with 5 μL of 100 mM tri(2-chloroethyl)phosphate (TCEP) and incubated for 20 min at room temperature to reduce protein disulfide bonding. Iodoacetamide (3 μL of 500 mM stock) was added to the solution, and incubated for an additional 30 min at room temperature in the dark. The reduced and alkylated protein mixture was diluted with 100 mM Tris–HCl (pH 8.5). 9 μL of 100 mM $CaCl_2$ was then added to the above solution, and the mixture (~50 μL in volume) was digested with trypsin at an enzyme to substrate ratio of 1:50 (w/w) by incubating at 37 °C for overnight.

HeLa cell culturing, protein extraction and digestion were performed following previously described procedures [32].

2.6. Phosphopeptide enrichment procedure

To optimize the performance of phosphopeptide enrichment, we investigated the five magnetic polymer sorbents for the enrichment of trypsin digested standard proteins, and the use of different loading buffers at different TFA and organic phase concentrations.

A typical process is described below: 30 mg of Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+} was suspended in 1 mL of purified water. 5 μL of magnetic material suspension was added to 30 μL of peptide mixture (in 3% TFA–80% ACN) and incubated at 30 °C for 30 min. Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+} captured phosphopeptides were separated from the sample solution by applying an external magnet. After washing with 50 μL 3% TFA–80% ACN twice, the trapped peptides were eluted with 30 μL 2.5% ammonium hydroxide. The eluted solution was then lyophilized to dryness. Two microliters of matrix solution (mixture of 20 mg/mL 2, 5-DHB in 50% (v/v) ACN, 1% (v/v) phosphoric acid) was introduced into the eluate and 1 μL of the mixture was used for MALDI-MS analysis.

For phosphopeptide enrichment from digested peptides mixtures of HeLa cell lysate, 5 mg magnetic material were mixed with 200 μg lysate in 100 μL 3% TFA–80% ACN, and incubated at 30 °C for 30 min. After washing with 100 μL 3% TFA–80% ACN twice, the trapped peptides were eluted with 100 μL 2.5% ammonium hydroxide twice and then both eluents were analyzed by LC–MS/MS, respectively.

2.7. Mass spectrometry

All MALDI-TOF MS spectra of the peptides were recorded with a Voyager DE STR MALDI-TOF work station mass spectrometer (Applied Biosystems Inc., USA). During a typical analysis, 200 scans were accumulated and performed in positive ion reflector mode with an accelerating voltage of 20 kV and delayed extraction of 280 ns.

RPLC–ESI-MS/MS was used to analyze the real sample. A nano flow multiple dimensional HPLC system (TempoTM nano MDLC system, Applied Biosystems) was coupled online with a QSTAR ELITE mass spectrometer (Applied Biosystems) and used for RPLC–ESI-MS/MS analysis. Peptides were first trapped within a CapTrap column (0.5 mm × 2 mm, MICHROM Bioresources Inc.) and then eluted into an integrated nano-scale analytical column MAGIC C18AQ (100 μm × 150 mm, 3 μm particle size, 20 nm pore size, MICHROM Bioresources Inc.). Mobile phase A (2% ACN, 0.1% formic acid) and mobile phase B (98% ACN, 0.1% formic acid) were used to establish a 130 min gradient, which comprised of: 5 min in 5% B, 25 min of 5–15% B, 55 min of 15–40% B, 15 min of 40–80% B, the gradient was maintained in 80% B for 10 min, followed by 5 min of 80–5% B, and a final step in 5% B for 15 min. A constant flow rate was set at 300 nL/min. MS scans were conducted from 400 to 1800 amu, with a 1 s time span. For MS/MS analysis, each scan cycle consisted of one full-scan mass spectrum (with m/z ranging from 400 to 1800 and charge states from 2 to 5) followed by five MS/MS events. The threshold count was set to 30 to activate MS/MS accumulation and former target ion exclusion was set for 90 s. The mass tolerance was set at 50 mDa, and automatic collision energy and automatic MS/MS accumulation were selected.

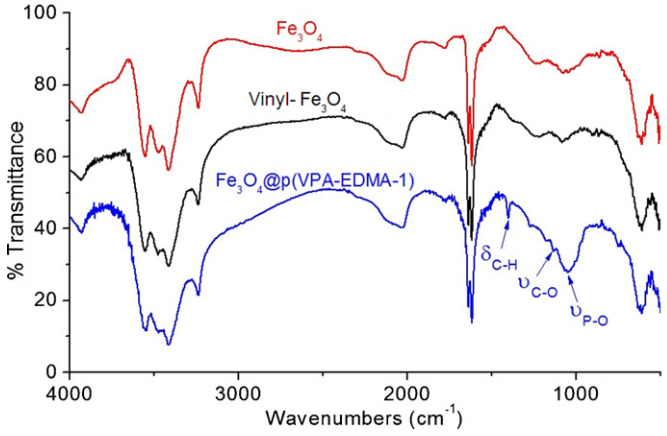

Fig. 2. FTIR spectra of the materials.

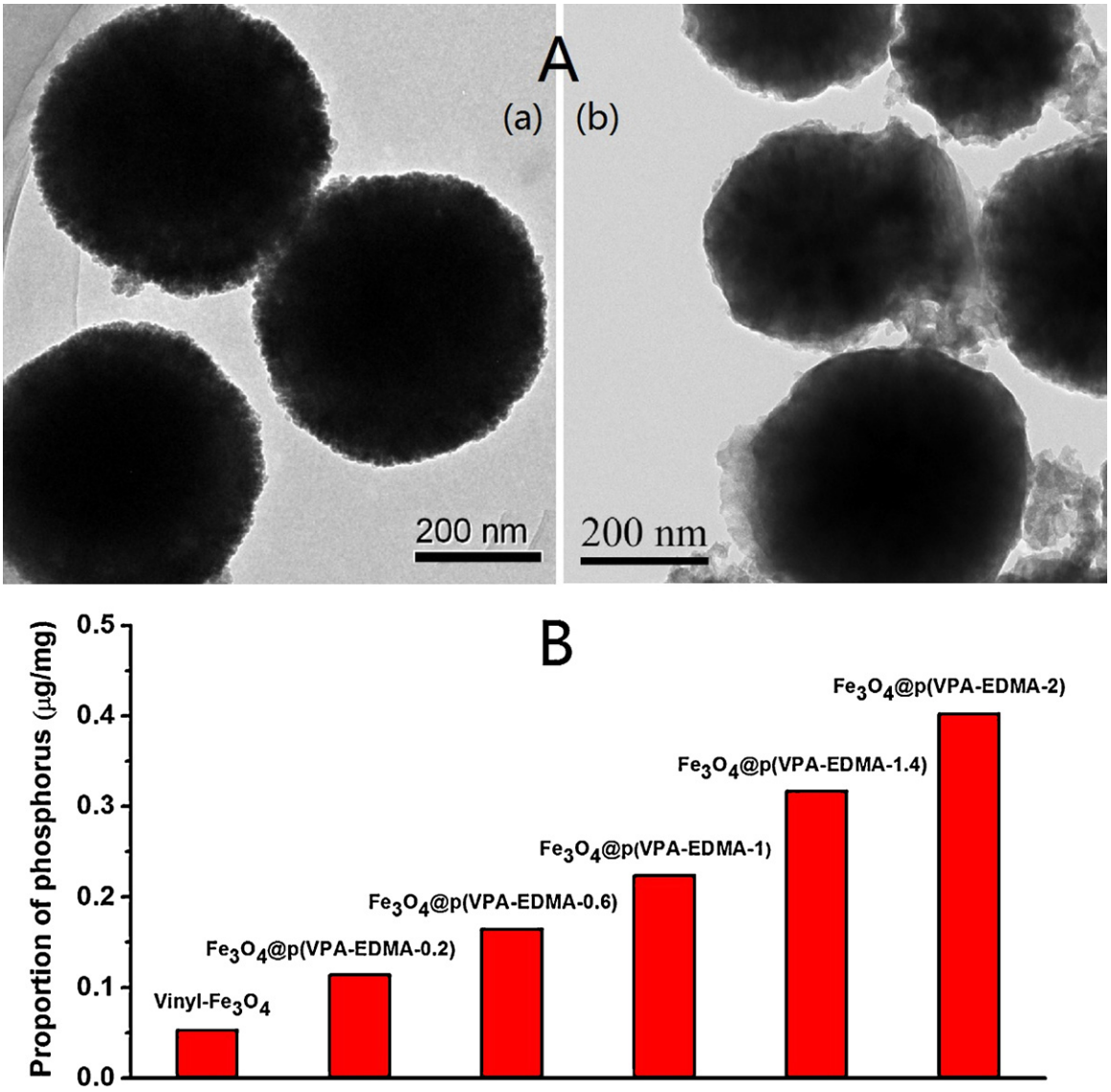

Fig. 3. (A) Transmission electron microscopy images of Fe_3O_4 (a) and Fe_3O_4@p(VPA-EDMA-1) (b). (B) Proportion of phosphate groups attached to the six magnetic polymer sorbents.

2.8. Data analysis

Raw data from QSTAR ELITE were analyzed with ProteinPilot Software 3.0. Data were searched against the SwissProt protein database (version 201011) using the following parameters: sample type was set to identification, Cys alkylation was set to Iodoacetamide, Digestion was set to trypsin, Instrument was set to QSTAR ESI, special factors were set to Phosphorylation emphasis and Urea denaturation, species was set to Homo sapiens, ID focus was set to biological modifications, search effort was set to thorough ID, detected protein threshold was set to >1.3 (95%).

3. Results and discussion

3.1. Characterization of materials

To study the characteristics of Fe_3O_4@p(VPA-EDMA-1), our prepared magnetic polymer sorbent was analyzed by Fourier transform infrared spectroscopy (Fig. 2). The bands at 1060 and 1140 cm^{-1} are bands characteristic for P–O and C–O, respectively, while the bands at 1403 cm^{-1} indicates the presence of C–H. The

Table 2
Detailed information of the observed phosphopeptides obtained from tryptic digests of α-casein and β-casein.

No.	$[M+H]^+$	Phosphorylation site	Amino acid sequence
α_1	1411.5	2	EQLSTSEENSK
α_2	1466.6	1	TVDMESTEVFTK
α_3	1539.7	2	EQLSTSEENSKK
α_4	1660.8	1	VPQLEIVPNSAEER
α_5	1832.9	1	YLGEYLIVPNSAEER
α_6	1847.7	1	DIGSESTEDQAMEDIK
α_7	1927.7	2	DIGSESTEDQAMEDIK
α_8	1952.0	1	YKVPQLEIVPNSAEER
α_9	2619.0	4	NTMEHVSSSEESIISQETYK
α_{10}	2703.5	1	LRLKKYKVPQLEIVPNSAEERL
α_{11}	2720.9	5	QMEAESISSSEEIVPNSVEQK
α_{12}	2747.1	4	NTMEHVSSSEESIISQETYKQ
α_{13}	3008.0	4	NANEEEYSIGSSSEESAEVATEEVK
β_1	2061.8	1	FQSEEQQQTEDELQDK
β_2	2966.2	4	ELEELNVPGEIVESLSSSEESITR
β_3	3122.3	4	RELEELNVPGEIVESLSSSEESITR

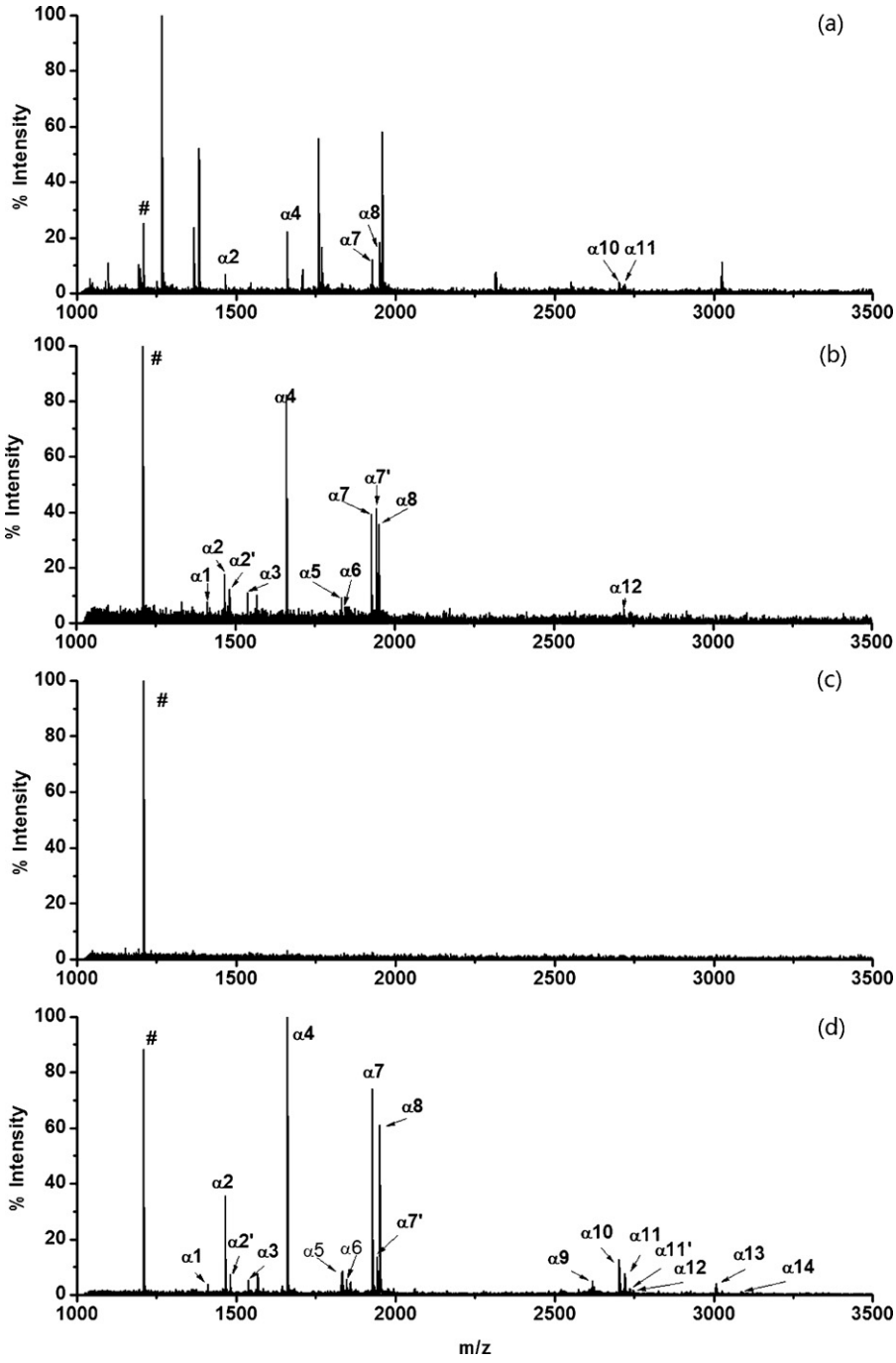

Fig. 4. Mass spectra of trypsin digested α-casein using a concentration of 1.0×10^{-7} M (3 pmol) obtained by (a) direct analysis, or after enrichment using (b) vinyl-Fe_3O_4, (c) Fe_3O_4@p(VPA-EDMA-1) and (d) Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+}. Phosphopeptides are marked with "α*n*" and the internal standard is marked with "#".

morphology of bare Fe_3O_4 and Fe_3O_4@p(VPA-EDMA-1) was analyzed by transmission electron microscopy (Fig. 3A). The Fe_3O_4 particles exhibit monodisperse, uniform and spherical morphologies with diameter of 400–600 nm (Fig. 3A(a)). The transmission electron microscopy image clearly reveals the thickness of the polymer shell of Fe_3O_4@p(VPA-EDMA-1) was around 20 nm. The phosphate content of Fe_3O_4@p(VPA-EDMA-*x*) was determined by the molybdenum blue method, and the results showed that with the increased concentration of vinyl phosphonic acid, proportion of phosphate groups gradually increase in Fe_3O_4@p(VPA-EDMA-*x*) (Fig. 3B). The saturation magnetization values of bare Fe_3O_4, vinyl-Fe_3O_4 and Fe_3O_4@p(VPA-EDMA-1) were 64.0, 67.8 and 29.0 emu/g, respectively (Fig. S1, SI-1). Although the saturation magnetization value decreased after coating with polymer, Fe_3O_4@p(VPA-EDMA-1) was still able to meet the requirement of magnetical separation.

3.2. Investigation of the enrichment efficiency

The efficiencies of vinyl-Fe_3O_4, Fe_3O_4@p(VPA-EDMA-1) and Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+} for phosphopeptide enrichment were evaluated by using trypsin digested α-casein (3 pmol) (Fig. 4). When the trypsin digested α-casein was directly analyzed by MALDI-TOF MS, only a few phosphopeptides were detected due to the signal suppression caused by large amount of non-phosphorylated peptides (Fig. 4(a)). For clarity, details of the observed phosphopeptides are listed in Table 2. Fe_3O_4 has been

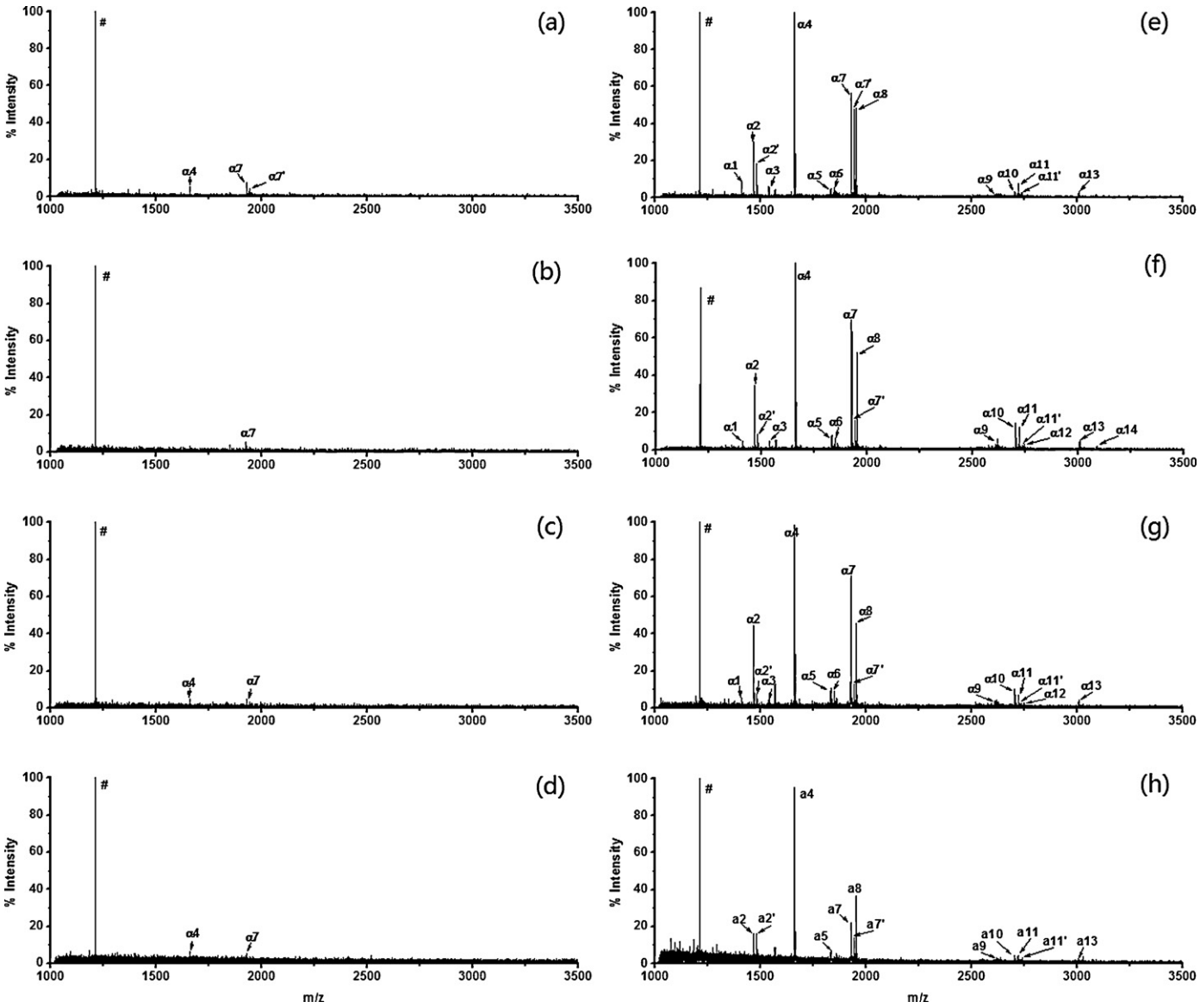

Fig. 5. MALDI-TOF mass spectra obtained using Fe_3O_4@p(VPA-EDMA-*x*) (a–d) and Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+} (e–h) to selectively enrich phosphopeptides from a tryptic digest of α-casein at concentration of 1.0×10^{-7} M (3 pmol). *x* refers to 0.2, 0.6, 1.4 and 2, respectively.

reported as an adsorbent for capturing phosphopeptides [33], but it is not a good phosphopeptides enrichment material because it potentially can be dissolved in the acidic environment as the loading buffer usually is acidic [21,22]. Consistent with previous report regarding the binding affinity of Fe_3O_4 for phosphopeptides [33], here vinyl-Fe_3O_4 also showed enrichment for phosphopeptides, but fewer multi-phosphopeptides were detected due to most of the adsorption sites of Fe_3O_4 had been occupied by VPA (Fig. 4(b)). As shown in Fig. 4(c), Fe_3O_4@p(VPA-EDMA-1) also exhibited no enrichment ability for phosphopeptides, which further proved that the adsorption sites of Fe_3O_4 had been covered by the polymer. When zirconium ions were chelated on the polymer of Fe_3O_4@p(VPA-EDMA-1), Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+} showed satisfactory enrichment ability for phosphopeptides (Fig. 4(d)).

Table 3
Large-scale phosphoproteome analysis of HeLa cell lysate by directly enriched and analyzed by nano-RPLC–MS/MS.

Methods	Identified phosphopeptides	Identified phosphorylation sites	Refs.
NH_4Glu-modified TiO_2 microcolumn nano-RPLC–MS/MS	858	1034	[34]
Aliphatic hydroxy acid-modified MOC tips nano-RPLC–MS/MS	1100 (TiO_2/lactic acid)	983	[35]#
	1181 (ZrO_2/HPA)	1057	
Nanocast MOAC nano-RPLC–MS/MS	619 (Nanocast SnO_2)	726	[37]*
	896 (Nanocast TiO_2)	1048	
magnetic polymer nano-RPLC–MS/MS	988	1276	This work

#: these data were obtained from four replicated experimental results, *: these data were obtained by combining the results of three technical replicates.

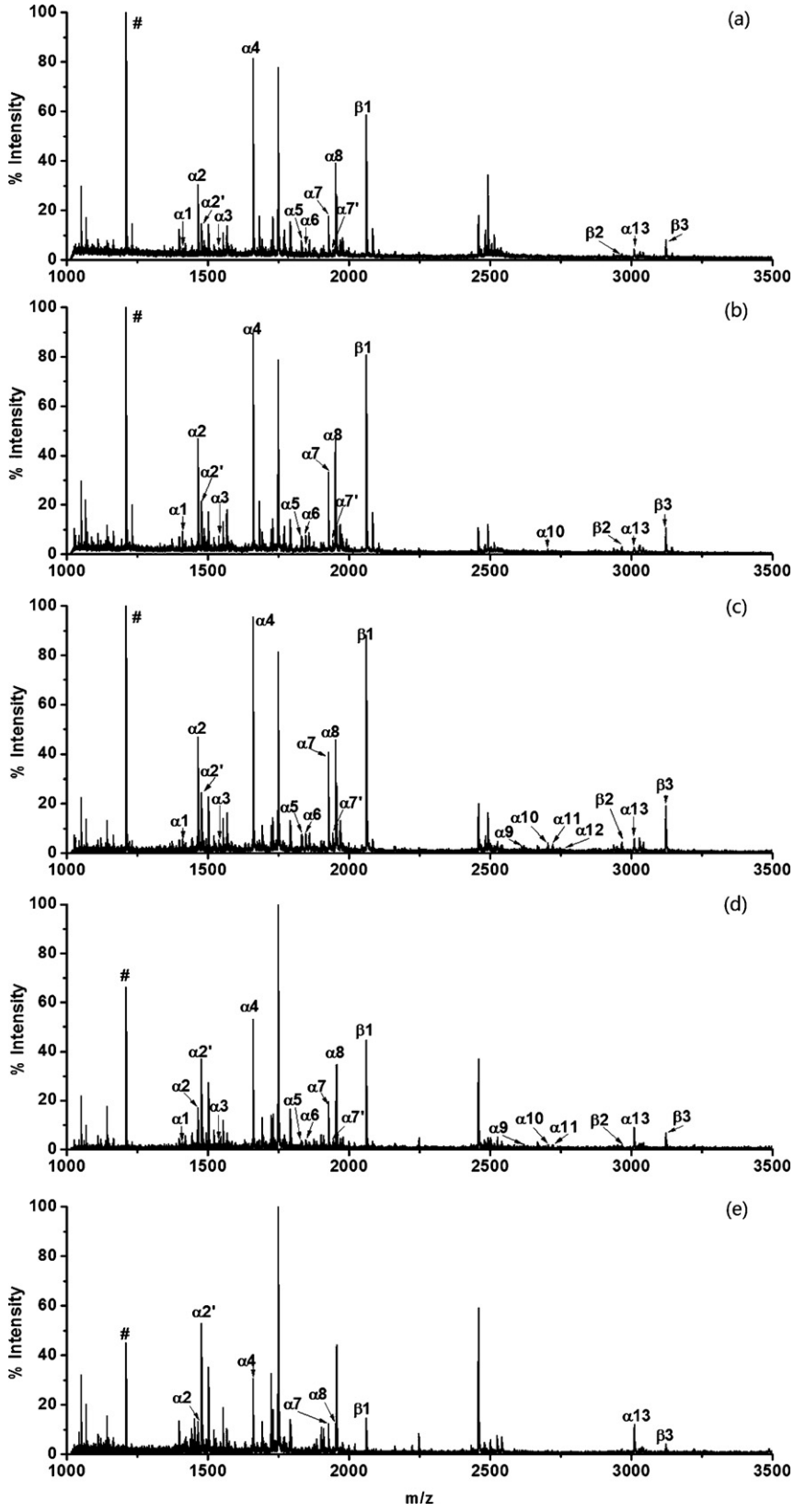

Fig. 6. MALDI-TOF mass spectra obtained using Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+} to selectively enrich phosphopeptides from a tryptic digest of a mixture of α-casein, β-casein and non-phosphoprotein BSA with a ratio of 1:1:50. α-Casein was at a concentration of 1.0×10^{-7} M. *x* refers to 0.2, 0.6, 1, 1.4 and 2, respectively.

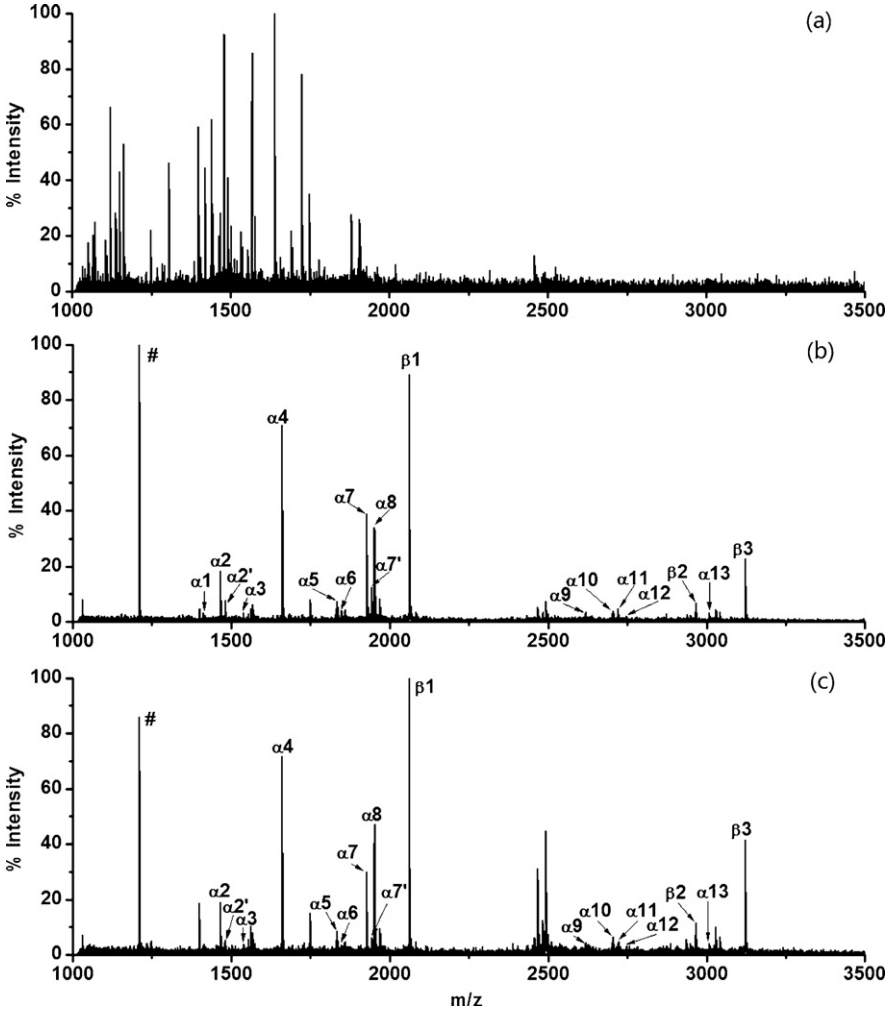

Fig. 7. Mass spectra obtained by direct analysis (a), or after Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+} enrichment (b) from the tryptic digest of a mixture of α-casein/β-casein/BSA with the ratio of 1:1:50. (c) Mass spectra obtained after Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+} enrichment from the tryptic digest of a mixture of α-casein/β-casein/BSA with the ratio of 1:1:100. α-Casein was at concentration of 1.0×10^{-7} M (3 pmol). The phosphopeptides are marked with "α*n*" and "β*n*". The internal standard is marked with "#".

Other zirconium-chelated materials (Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+}, *x* referring to 0.2, 0.6, 1.4 and 2) also provided desirable specificity for phosphopeptides (Fig. 5).

3.3. Relationship of enrichment efficiency and the number of chelating agent

To investigate the enrichment efficiency of Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+} for phosphopeptides with respect to the number of chelating groups, phosphopeptides from a tryptic digest mixture of α-casein, β-casein and BSA (1:1:50) were used as analytes. All sorbents showed specific efficiency for purification of phosphopeptides to some extent (Fig. 6). However, Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+} exhibited the highest performance for the selective capture of the phosphopeptides from the digest mixture (Fig. 6(c)), which implied that the phosphate group content of the adsorbents is related to their capturing ability. This change may be caused by the amount of phosphate groups attached onto the magnetic polymer-particles. When the attached phosphate group content is low, there may be only limited chelating sites for immobilized zirconium ions, leading to poor enrichment efficiency (Fig. 6(a and b)); while the content of phosphate groups is too high, there would be an excess of tridentate anionic ligands sharing oxygen atoms with metal ion. Therefore, most of the metal ions immobilized on the sorbent with a higher proportion of phosphate groups may be unable to chelate with phosphopeptides (Fig. 6(d and e)).

3.4. Optimization of enrichment condition

The factors which contribute to the enrichment efficiency were examined. By increasing acid concentration, the nonspecific binding of non-phosphorylated peptides was reduced. However, the phosphopeptide signals were also inhibited once the concentration of TFA reached 6% (Fig. S2, SI-1). So 3% (v/v) TFA was chosen in the further experiments. The effect of organic solvent on loading was also investigated. Here we showed that reducing the content of organic solvent can result in increased non-specific adsorption of non-phosphopeptides and decreased specific affinity of phosphopeptides. We reason that this effect may be caused by the solubility of peptides, which changes with different content of organic phase. In order to elute phosphopeptides from the sorbent, a certain concentration (2.5%) of ammonia was used in the elution step, therefore the desalting step can be avoided.

Under optimized conditions, the enrichment ability of Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+} for phosphopeptides from more complex samples was further tested. To this end, a mixture of α-casein, β-casein and BSA with a molar ratio of 1:1:50 was analyzed by MALDI-TOF before and after enrichment (Fig. 7(a

and b)). The identification of phosphopeptides from α-casein and β-casein was not achievable before enrichment due to the high abundance of non-phosphorylated peptides interfering with the overall mass spectra. However, all the phosphopeptides were detectable upon Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+} purification, which suggested a high enrichment ability of Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+}. When a mixture of α-casein, β-casein and BSA with a molar ratio of 1:1:100 was directly analyzed by MALDI-TOF, the mass spectrum was complex (data not shown). Whereas, clear mass spectra of phosphopeptides were still obtained after the mixture was enriched with Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+} (Fig. 7(c)).

3.5. Applications in complex protein sample generated from cell lysate

Since the protein mixture (α-casein, β-casein and BSA) contains only a small number of phosphopeptides, we applied Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+} to the protein mixtures generated from HeLa cells, a commonly used cell line for phosphoproteomics studies [34–37]. Among the large-scale phosphoproteomics analysis of HeLa cells, the strategies that the trypsin digested peptides mixture from cell lysate was directly enriched and analyzed by nano-RPLC–MS/MS, were listed in Table 3. In current study, using just two eluents, we identified a total number of 988 unique phosphopeptides and 1276 phosphorylation sites (953 on serine, 318 on threonine and 5 on tyrosine). All phosphopeptides identified are listed in SI-2.

4. Conclusion

In this study, we prepared Fe_3O_4@p(VPA-EDMA-*x*)-Zr^{4+} magnetic polymer-particles, with which we established a new method for the rapid and efficient enrichment of phosphopeptides. Here, five magnetic polymer sorbents with different numbers of phosphate groups attached were examined. Our results showed that the enrichment efficiency of phosphopeptides was closely related to the number of chelators, especially when the peptides mixture is a complex system. Under optimized conditions, magnetic polymer-particles with appropriate proportion of phosphate groups (Fe_3O_4@p(VPA-EDMA-1)-Zr^{4+}) showed good performance on the phosphopeptides enrichment from both complex peptides mixture and proteolytic digest of HeLa cell extracts. Taken together, the above-established method, developed in current study, provided a powerful tool for the phosphopeptides enrichment and large-scale phosphoproteomics analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.04.044.

References

[1] P.S. Doyle, J. Bibette, A. Bancaud, J.L. Viovy, Science 295 (2002) 2237.
[2] J. Ding, Q. Gao, D. Luo, Z.G. Shi, Y.Q. Feng, J. Chromatogr. A 1217 (2010) 7351.
[3] C.T. Chen, Y.C. Chen, Anal. Chem. 77 (2005) 5912.
[4] H.M. Chen, X.Q. Xu, N. Yao, C.H. Deng, P.Y. Yang, X.M. Zhang, Proteomics 8 (2008) 2778.
[5] C.W. Huck, G.K. Bonn, J. Chromatogr. A 885 (2000) 51.
[6] C.A. Nelson, J.R. Szczech, Q.G. Xu, M.J. Lawrence, S. Jin, Y. Ge, Chem. Commun. 42 (2009) 6607.
[7] A. Sarkar, S.K. Biswas, P. Pramanik, J. Mater. Chem. 20 (2010) 4417.
[8] J.H. Wu, X.S. Li, Y. Zhao, Q. Gao, L. Guo, Y.Q. Feng, Chem. Commun. 46 (2010) 9031.
[9] T. Hunter, Cell 100 (2000) 113.
[10] L.N. Johnson, R.J. Lewis, Chem. Rev. 101 (2001) 2209.
[11] M. Bollen, M. Beullens, Trends Cell Biol. 12 (2002) 138.
[12] M. Mann, S.E. Ong, M. Gronborg, H. Steen, O.N. Jensen, A. Pandey, Trends Biotechnol. 20 (2002) 261.
[13] M.J. Chalmers, W. Kolch, M.R. Emmett, A.G. Marshall, J. Chromatogr. B 803 (2004) 111.
[14] F. Meng, A.J. Forbes, L.M. Miller, N.L. Kelleher, Mass Spectrom. Rev. 24 (2005) 126.
[15] G.H. Han, M.L. Ye, H.F. Zou, Analyst 133 (2008) 1128.
[16] A. Leitner, Trends Anal. Chem. 29 (2010) 177.
[17] T.S. Nuhse, A. Stensballe, O.N. Jensen, S.C. Peck, Mol. Cell. Proteomics 2 (2003) 1234.
[18] J.D. Dunn, J.T. Watson, M.L. Bruening, Anal. Chem. 78 (2006) 1574.
[19] Z.Y. Yu, G.H. Han, S.T. Sun, X.N. Jiang, R. Chen, F.J. Wang, R.A. Wu, M.L. Ye, H.F. Zou, Anal. Chem. Acta 636 (2009) 34.
[20] S.B. Ficarro, M.L. McCleland, P.T. Stukenberg, D.J. Burke, M.M. Ross, J. Shabanowitz, D.F. Hunt, F.M. White, Nat. Biotechnol. 20 (2002) 301.
[21] H.J. Zhou, S.Y. Xu, M.L. Ye, S. Feng, C.S. Pan, X.G. Jiang, X. Li, G.H. Han, Y. Fu, H.F. Zou, J. Proteome Res. 5 (2006) 2431.
[22] S. Feng, M.L. Ye, H.J. Zhou, X.G. Jiang, X.N. Jiang, H.F. Zou, B.L. Gong, Mol. Cell. Proteomics 6 (2007) 1656.
[23] J.Y. Wei, Y.J. Zhang, J.L. Wang, F. Tan, J.F. Liu, Y. Cai, X.H. Qian, Rapid Commun. Mass Spectrom. 22 (2008) 1069.
[24] L. Zhao, R.A. Wu, G.H. Han, H.J. Zhou, L.B. Ren, R.J. Tian, H.F. Zou, J. Am. Soc. Mass Spectrom. 19 (2008) 1176.
[25] D. Qi, Y. Mao, J. Lu, C.H. Deng, X.M. Zhang, J. Chromatogr. A 1217 (2010) 2606.
[26] L. Hu, H.J. Zhou, Y.H. Li, S.T. Sun, L.H. Guo, M.L. Ye, X.F. Tian, J.R. Gu, S.Y. Yang, H.F. Zou, Anal. Chem. 81 (2009) 94.
[27] S.Y. Xu, J.C. Whitin, T.T.S. Yu, H.J. Zhou, D.Z. Sun, H.J. Sue, H.F. Zou, H.J. Cohen, R.H. Zare, Anal. Chem. 80 (2008) 5542.
[28] J. Dong, H.J. Zhou, R.A. Wu, M.L. Ye, H.F. Zou, J. Sep. Sci. 30 (2007) 2917.
[29] T. Hoang, U. Roth, K. Kowalewski, C. Belisle, K. Steinert, M. Karas, Anal. Chem. 82 (2010) 219.
[30] H. Deng, X.L. Li, Q. Peng, X. Wang, J.P. Chen, Y.D. Li, Angew. Chem. Int. Ed. 44 (2005) 2782.
[31] R.A. Chalmers, D.A. Thomson, Anal. Chim. Acta 18 (1958) 575.
[32] X. Chen, D. Wu, Y. Zhao, B.H.C. Wong, L. Guo, J. Chromatogr. B 829 (2011) 25.
[33] A. Lee, H.J. Yang, E.S. Lim, J. Kim, Y. Kim, Rapid Commun. Mass Spectrom. 22 (2008) 2561.
[34] L.R. Yu, Z. Zhu, K.C. Chan, H.J. Issaq, D.S. Dimitrov, T.D. Veenstra, J. Proteome Res. 6 (2007) 4150.
[35] N. Sugiyama, T. Masuda, K. Shinoda, A. Nakamura, M. Tomita, Y. Ishihama, Mol. Cell. Proteomics 6 (2007) 1103.
[36] Y. Kyono, N. Sugiyama, K. Imami, M. Tomita, Y. Ishihama, J. Proteome Res. 7 (2008) 4585.
[37] A. Leitner, M. Sturm, O. Hudecz, M. Mazanek, J.H. Smatt, M. Linden, W. Lindner, K. Mechtler, Anal. Chem. 82 (2010) 2726.