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Phosphate-functionalized magnetic microspheres for immobilization of Zr^{4+} ions for selective enrichment of the phosphopeptides

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ABSTRACT

In this work, we developed phosphate functionalized magnetic Fe_3O_4@C microspheres to immobilize Zr^{4+} ions for selective extraction and concentration of phosphopeptides for mass spectrometry analysis. Firstly, we synthesized Fe_3O_4@C magnetic microspheres as our previous work reported. Then, the microspheres were functionalized with phosphate groups through a simple hydrolysis reaction using 3-(trihydroxysilyl)propyl methylphosphate. And the Zr^{4+} ions were immobilized on phosphate-functionalized magnetic microspheres by using phosphate chelator. Finally, we successfully employed Zr^{4+}-phosphate functionalized magnetic microspheres to selectively isolate the phosphopeptides from tryptic digests of standard protein and real samples including rat brain. All the experimental results demonstrate the enrichment efficiency and selectivity of the method we reported here.

1. Introduction

Reversible phosphorylation of serine, threonine, and tyrosine residues in proteins represents a prominent mechanism in eukaryotes for regulating cellular processes involving signal transduction, proliferation, and migration. For the aforementioned reason, characterization of the exact phosphorylated sites of proteins is important to lead a fundamental understanding of the biologic processes [1–3].

Mass spectrometry, which has been proved to be a powerful approach to reveal comprehensive information for biological function, has been widely used for phosphoproteomics analysis [4–7]. However, as a result of low stoichiometry and low ionization efficiency, direct analysis of phosphopeptides by mass spectrometry cannot provide satisfying outcomes. Therefore, specific enrichment of phosphorylated proteins or peptides possibly is the most widely used approach in phosphoproteomics. One of the most prevalent enrichment techniques is immobilized metal ion (Fe^{3+}, and Ga^{3+}) affinity chromatography (IMAC) [8–13]. Recently, Zou group and our group developed IMAC techniques based on new metal ions such as Ce^{4+}, Zr^{4+} and Ti^{4+}, for selective enrichment of phosphopeptides for phosphoproteomics research [14–16].

In IMAC technique, metal ions were immobilized on supporting substrates by using iminodiacetic acid (IDA, a tridentate metal-chelator) or nitrilotriacetic acid (NTA, a tetradentate metal-chelator) as chelator [17]. Recently, Zou group reported that using phosphate groups to replace IDA or NTA for metal ions immobilization has been proved to be a new promising strategy for phosphopeptides enrichment. They found that IMAC based on phosphate chelator could improve the enrichment selectivity for phosphopeptides [18–20]. More recently, Zou et al. immobilized Zr^{4+} ions on the phosphate-modified poly(glycidylmethacrylate-co-ethylene dimethacrylate) polymer beads by using phosphate chelator, and successfully applied for the analysis of the mouse liver phosphoproteome, resulting in the identification of 153 phosphopeptides derived from 133 phosphoproteins [16]. In the tridentate IDA, Zr^{4+} ions will bind to the nitrogen atom and the two carboxylate oxygens, leaving three sites for phosphopeptides. Likewise the tetradentate NTA is supposed to bind the Zr^{4+} ions with extra carboxylate oxygen [17]. Each metal ion only coordinates with one IDA ligand or one NTA ligand. However, immobilizing the metal ions with the phosphate groups can result in a different chelating way. It is well known that the layer structure of metal phosphate can be obtained by MO_6 octahedra, and each Zr^{4+} ion sharing six oxygen atoms with different monohydrogen phosphate groups. So each metal ion coordinates to more than one phosphate molecule. Compared with the traditional IMAC chelator, such as IDA and NTA, the phosphate groups used as chelator for metal ions immobilization can provide superior metal chelating strength. Zou et al. and Qian et al. reported that the unique coordination specificity of Zr^{4+} ions immobilized on the phosphate functionalized magnetic silica

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microparticles may also greatly improve the selectivity of phosphopeptides binding, which prevents from acidic peptides binding [20,21]. However, as we know, in their method [18–21], it takes three steps to implement phosphate modification on various substrates such as mesoporous silica and magnetic silica microspheres, so the modification process is time-consuming. And the reagents, such as phosphorus oxychloride, used in the chemical modification reaction were very poisonous. Therefore, development of a simple, fast and safe method to modify phosphate group on substrate is very important and interesting.

In our previous studies [22–24], functionalized magnetic microspheres were successfully applied to selective enrichment of phosphopetides. More recently, we synthesized biological-compatible C8-functionalized magnetic carbonaceous polysaccharide (Fe_3O_4@C) microspheres through a facile synthesis approach for peptides and proteins enrichment [25]. Herein, we developed a new method for the immobilization of Zr^{4+} onto Fe_3O_4@C microspheres for selective extraction and concentration of phosphopeptides. Briefly, the as-synthesized biological-compatible Fe_3O_4 magnetic microspheres were coated with a thin layer of carbon [25]. Then the Fe_3O_4@C magnetic microspheres were modified with 3-(trihydroxysilyl)propyl methylphosphate, and Zr^{4+} ions were located by chelating with phosphate groups. Finally, enrichment efficiency and selectivity of the Zr^{4+}-phosphate functionalized magnetic Fe_3O_4@C microspheres were demonstrated.

2. Experimental

2.1. Materials and chemicals

Bovine β-casein, bovine serum albumin (BSA), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin (from bovine pancreas), ammonium hydrogen carbonate (NH_4HCO_3), trifluoroacetic acid (TFA) and 3-(trihydroxysilyl)propyl methylphosphate were purchased from Sigma USA (St. Louis, MO). Acetonitrile was purchased from Merck (Darmstadt, Germany). All aqueous solutions were prepared using Milli-Q water by Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals and reagents were of the highest grade commercially available.

2.2. Synthesis of Zr^{4+}-phosphate functionalized magnetic microspheres

The Fe_3O_4@C magnetic microspheres were synthesized through two-step hydrothermal reactions as described in our previous work [25]. Then, the obtained 80 mg Fe_3O_4@C magnetic microspheres and 0.5 mL 3-(trihydroxysilyl)propyl methylphosphate were dispersed into 20 mL toluene and refluxed at 80 °C for 12 h. Next, the microspheres were washed with anhydrous ethanol and deionized water for five times. The resultant phosphate-functionalized magnetic microspheres were incubated in a 0.2 M $ZrOCl_2$ solution with gentle stirring overnight for the loading of Zr^{4+} cations. Finally, the prepared Zr^{4+}-phosphate functionalized magnetic microspheres were rinsed with deionized water to remove the nonspecifically adsorbed Zr^{4+} cations, and dried in vacuum at 50 °C for 12 h.

2.3. Sample preparation

Bovine β-casein and BSA were each dissolved in 25 mM NH_4HCO_3 buffer at pH 8.3 and treated with trypsin (2%, w/w) for 16 h at 37 °C, respectively. Nonfat milk was purchased from Nestle Company. Thirty microliters of nonfat milk was diluted in 900 μL of NH_4HCO_3 aqueous solution at 25 mM. This solution was then centrifuged at 16 000 rpm for 15 min, and the supernatant was saved for tryptic digestion. After denaturation at 100 °C for 5 min, the supernatant was incubated for 12 h at 37 °C with 30 μg of trypsin for proteolysis.

2.4. Preparation of the lysate of rat brain

Rats were killed and their brains were promptly removed and placed in ice-cold homogenization buffer consisting of 7 M urea, 2 M thiourea and a mixture of protease inhibitor (1 mM phenylmethanesulphonylfluoride) and phosphatase inhibitors (0.2 mM Na_3VO_4, 1 mM NaF). After mincing with scissors and washing to remove blood, the brains were homogenized in a Potter-Elvejhem homogenizer with a Teflon piston, using 5 mL of the homogenization buffer per 1 g of tissue. The suspension was homogenized for approximately 2 min, vortexed at 0 °C for 30 min, and centrifuged at 22 000 × *g* for 1.5 h. The supernatant contained the total brain proteins.

Appropriate volumes of protein sample were precipitated as above, lyophilized to dryness, and redissolved in reducing solution (6 M guanidine hydrochloride, 100 mM NH_4HCO_3, pH 8.3) with the protein concentration adjusted to 2 μg/μL. Then, 200 μg of this protein sample (100 μL volume) was mixed with 10 μL of 0.5 M DTT. The mixture was incubated at 37 °C for 1 h, and then 20 μL of 0.5 M 2-iodoacetamide was added and incubated for an additional 30 min at 37 °C in the dark. The protein mixtures were exchanged into 50 mM NH_4HCO_3 buffer, pH 8.5, and incubated with trypsin (40:1) at 37 °C overnight.

2.5. Selectively enrichment of phosphopeptides from tryptic digestion using Zr^{4+}-phosphate functionalized magnetic microspheres

To optimize the performance of the phosphopeptides enrichment, different loading buffers with different TFA concentration were tested. And the final enrichment protocol uses 50% (v/v) acetonitrile and 1.5% (v/v) TFA aqueous solution as loading buffer. If not otherwise stated, the phosphopeptides enrichment was conducted using 50% (v/v) acetonitrile and 1.5% (v/v) TFA aqueous solution as loading buffer. The peptide mixtures originating from tryptic digestions were diluted by loading buffer first, suspension of Zr^{4+}-phosphate functionalized magnetic microspheres (200 μL of 2 μg/μL) was added into 200 μL of diluted peptide mixture. Then the mixed solutions were vibrated at 25 °C for 30 min. After that, with the help of magnet, the phosphopeptides adsorbed to Zr^{4+}-phosphate functionalized magnetic microspheres were collected and washed with loading buffer for three times. Then the obtained phosphopeptides were eluted with 5 μL of 0.4 M ammonia aqueous solution.

The tryptic digests of rat brain were lyophilized and then dissolved in loading buffer. 2 mg Zr^{4+}-phosphate functionalized magnetic microspheres were added into 400 μL of diluted rat brain digests. Then the mixed solutions were vibrated at 25 °C for 30 min. After that, with the help of magnet, the phosphopeptides adsorbed to Zr^{4+}-phosphate functionalized magnetic microspheres were collected and washed with loading buffer for three times. Then the obtained phosphopeptides were eluted with 50 μL of 0.4 M ammonia aqueous solution. The eluate was lyophilized and then dissolved in 40 μL loading phase. Finally, the solution was submitted for LC–ESI-MS analysis.

2.6. Quantitative recovery of phosphopeptides/nonphosphopeptides

In order to estimate the recovery of phosphopeptides/nonphosphopeptides, two phosphopeptides were mixed up with two standard nonphosphopeptides. Then, the peptide mixture was submitted to RPLC analysis using a completely

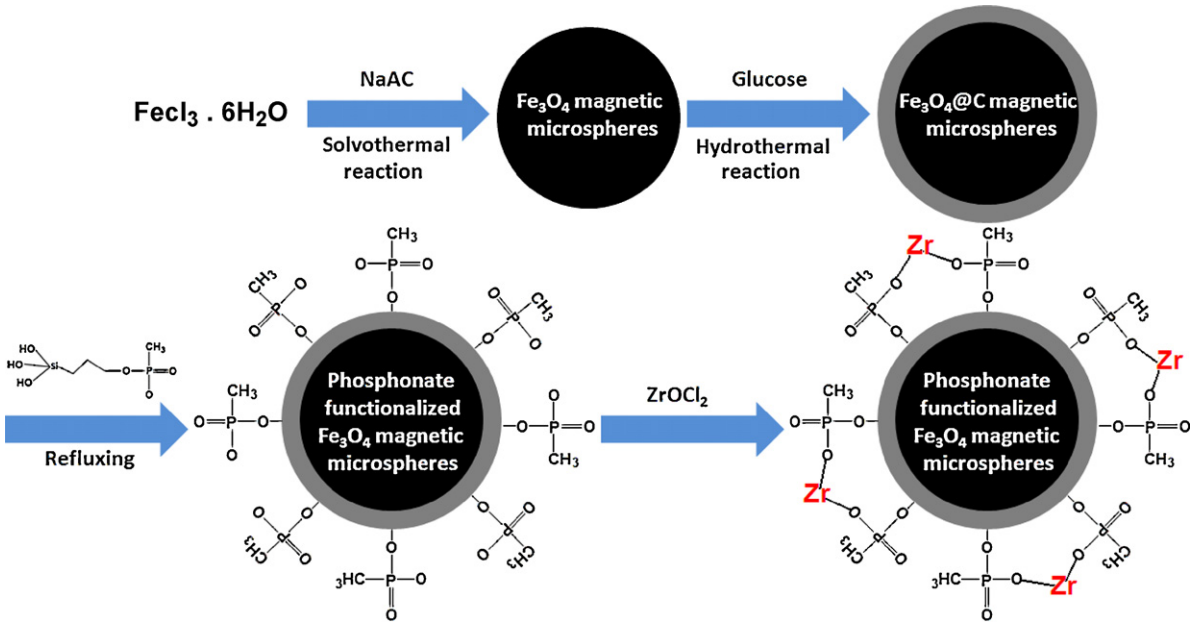

Fig. 1. The synthesis procedure of the Zr^{4+}-phosphate functionalized magnetic microspheres.

automated Shimadzu LC-2010A system (Shimadzu Corp., Japan) and RP-HPLC column (Zorbax 300SB-C18, 4.6 mm × 250 mm, 5-Micron, Agilent Technologies, Waldbronn, Germany). 20 μL peptide mixture was loaded onto the reversed phase column. A flow rate of 1 mL/min was used for separation. Gradient elution was performed with mobile phase A: 5% acetonitrile (v/v) + 0.05% TFA (v/v) and mobile phase B: 95% acetonitrile (v/v) + 0.05% TFA (v/v). The gradient was as follows: firstly, an isocratic elution with 0% B lasted for 5 min, followed by a linear increase to 60% B at 35 min and an abrupt increase to 100% B within 5 min. The 100% B was maintained for 3 min, and then ramped down to 0% for column equilibrium. According to the different retention time, four fractions were collected and analyzed by MLADI-TOF MS. The equivalent peptide mixture was diluted with 180 μL loading buffer, and 400 μg Zr^{4+}-phosphate functionalized magnetic microspheres were used to enrich the phosphopeptides. The peptides adsorbed to the microspheres were eluted with 20 μL 0.4 M ammonia aqueous solution. The eluate was submitted to the RPLC analysis. The supernatant was lyophilized and dissolved in 20 μL mobile phase A, and was analyzed by RPLC. Finally, the recovery was estimated by analyzing the peak area of parallel RPLC runs.

2.7. MALDI MS analysis

The above phosphopeptides eluted from Zr^{4+}-phosphate functionalized magnetic microspheres were deposited on the MALDI target using dried droplet method. 0.5 μL of the washing buffer was deposited on the plate and then another 0.5 μL of a mixture of 20 mg/mL 2,5-dihydroxybenzoic acid in 50% acetonitrile and 1% H_3PO_4 aqueous solution (v/v) was introduced as a matrix. MALDI-TOF MS experiments were performed in positive ion mode on a 4700 Proteomics Analyzer (Applied Biosystems, USA) with the Nd-YAG laser at 355 nm, a repetition rate of 200 Hz and an acceleration voltage of 20 kV.

2.8. Nano-flow LC–ESI-MS

Nano-LC MS/MS experiment was performed on an HPLC system composed by 2 LC-20AD nano-flow LC pumps and 1 LC-20AB micro-flow LC pump (all from Shimadzu Corporation, Tokyo, Japan) connected to an LTQ mass spectrometer (Thermo Electron Corporation, San Jose, CA). Sample injection was done via an SIL-20 AC auto-sampler (Shimadzu Corporation, Tokyo, Japan) and loaded onto a CAPTRAP column (0.5 mm × 2 mm, MICHROM Bioresources Inc., Auburn, CA) for 5 min at a flow rate of 60 μL/min. The sample was subsequently separated by a PICOFRIT C18 reverse-phase column (0.075 mm × 100 mm, New Objective Inc., Woburn, MA) at a flow rate of 300 nL/min. The mobile phases consisted of 5% acetonitrile with 0.1% formic acid (phase A and the loading phase) and 95% acetonitrile with 0.1% formic acid (phase B). To achieve proper separation, a 120-min linear gradient from 5% to 45% phase B was employed. The separated sample was introduced into the mass spectrometer via a 15 μm silica tip (New Objective Inc., Woburn, MA) adapted to a DYNAMIC nano-electrospray source (Thermo Electron Corporation, San Jose, CA). The spray voltage was set at 2.0 kV and the heated capillary at 210 °C. The mass spectrometer was operated in data-dependent mode and each cycle of duty consisted one MS survey scan at the mass range 400–2000 Da, followed by MS2 experiments for 8 strongest peaks and MS3 experiment for the peak corresponding to the neutral loss of 97.97, 48.99, and 32.67 Da among the three most intense fragment ions using the LTQ section. Peptides were fragmented in the LTQ section using collision-induced dissociation with helium and the normalized collision energy value set at 35%. Previously fragmented peptides were excluded for 30 s.

2.9. Peptide sequencing and data interpretation

The peak lists for MS2 and MS3 spectra were generated from the raw data by Bioworks version 3.3 (Thermo Electron) with the following parameters: mass range was 600–3500, intensity threshold was 1400, and minimum ion count was 10. The generated peak lists were searched by the Sequest program included in Bioworks against International Protein Index (IPI) database (IPI rat v3.45 fasta with 40 189 entries). The MS/MS spectra were searched with a precursor ion mass tolerance of 2 Da and fragment ion mass tolerance of 1 Da, full tryptic specificity was applied, two missed cleavages were allowed, and fixed modification, carboxyamidomethylation (C); variable modifications, phosphorylation (S, T, Y) and oxidation (M). For the searching with MS/MS/MS data, besides the above set-

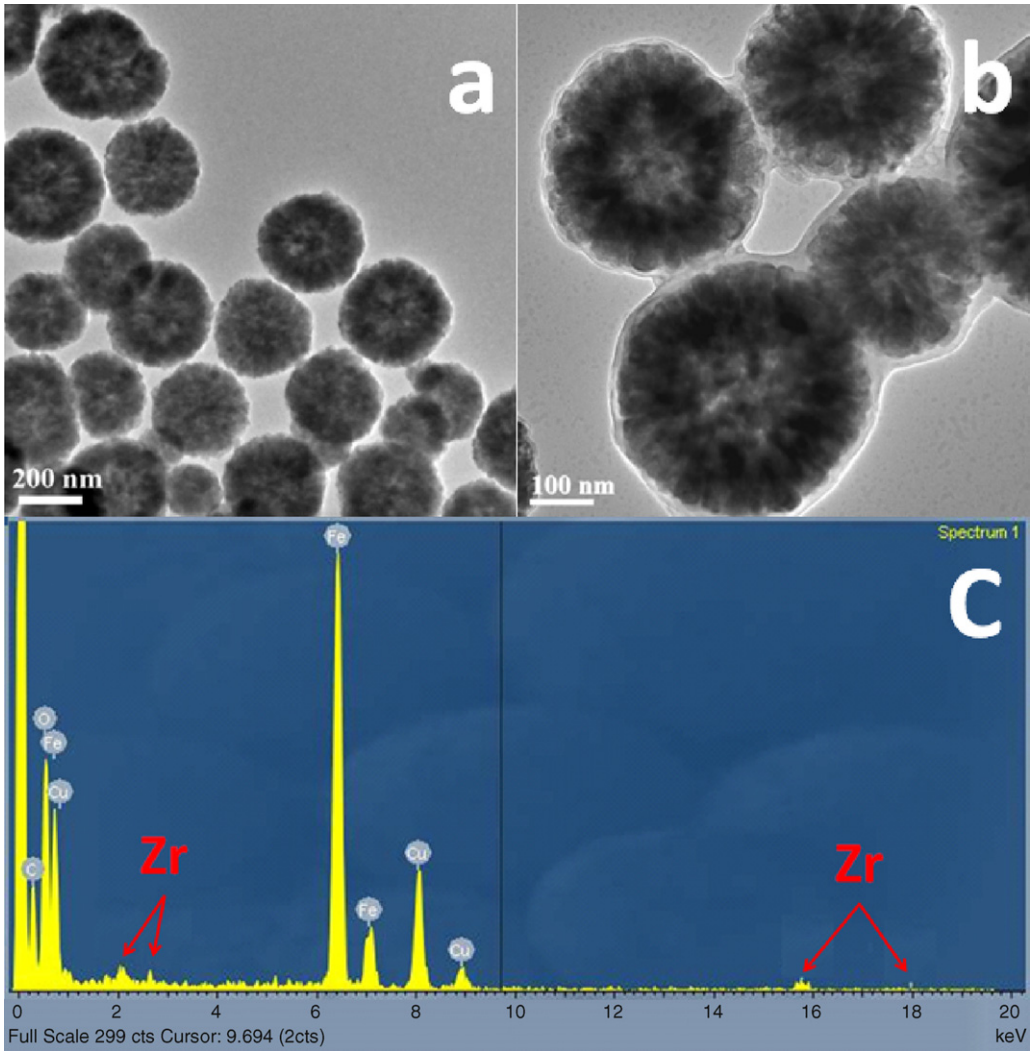

Fig. 2. TEM image of (a) Fe_3O_4 magnetic microspheres, (b) core–shell Fe_3O_4@C magnetic microspheres, and (c) the energy-dispersive X-ray analysis spectrum data of the obtained Zr^{4+}-phosphate functionalized magnetic microspheres.

tings, dynamic modifications were also set for water loss on Ser and Thr (−18 Da). For the identification of phosphopeptides based only on MS/MS or MS/MS/MS spectra, the following criteria were used for filtering the database searching results: cross-correlation value (Xcorr) > 2.0, 2.9, and 3.73 for singly, doubly, and triply charged ions, respectively; delta Cn value (ΔCn) > 0.1. For the phosphopeptide identifications derived from MS/MS/MS spectra used to validate the MS/MS identifications, the following criteria were used: Xcorr > 1.5, 2.4, and 2.96; ΔCn > 0.1. Manual validation was further carried out for peptides passing the above criteria. Criteria used for manual validation included the following. (a) The phosphoric acid neutral loss peak to phosphoserine and phosphothreonine must be the dominant peak. (b) The spectrum must be of good quality with fragment ion clearly above the base-line noise. (c) Sequential members of the b- or y-ion series were observable in the mass spectra. (d) For multiple phosphorylated peptides, the peptides derived from MS2 must be confirmed by MS3 spectra in the same MS cycle.

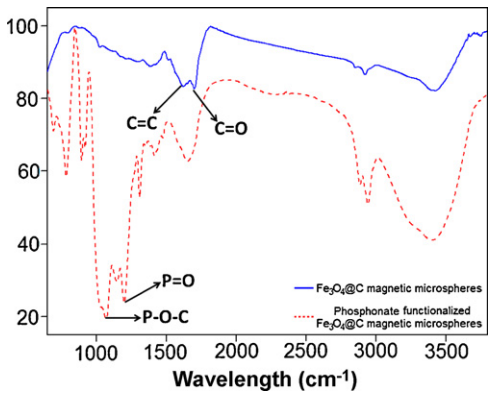

Fig. 3. The FTIR spectra of the phosphate-functionalized magnetic microspheres.

3. Results and discussions

3.1. Synthesis and characterization of Zr^{4+}-phosphate functionalized magnetic microspheres

The synthesis strategy of Zr^{4+}-phosphate functionalized magnetic microspheres is shown in Fig. 1. The morphology of as-prepared Fe_3O_4 and Fe_3O_4@C magnetic microspheres was obtained by transmission electron microscope (TEM). The Fe_3O_4 magnetic microspheres were synthesized by a solvothermal reduction method [26]. Fig. 2a reveals that the obtained Fe_3O_4 magnetic microspheres were monodispersed with a mean diameter of ~250 nm. In order to enhance their dispersibility in aqueous solu-

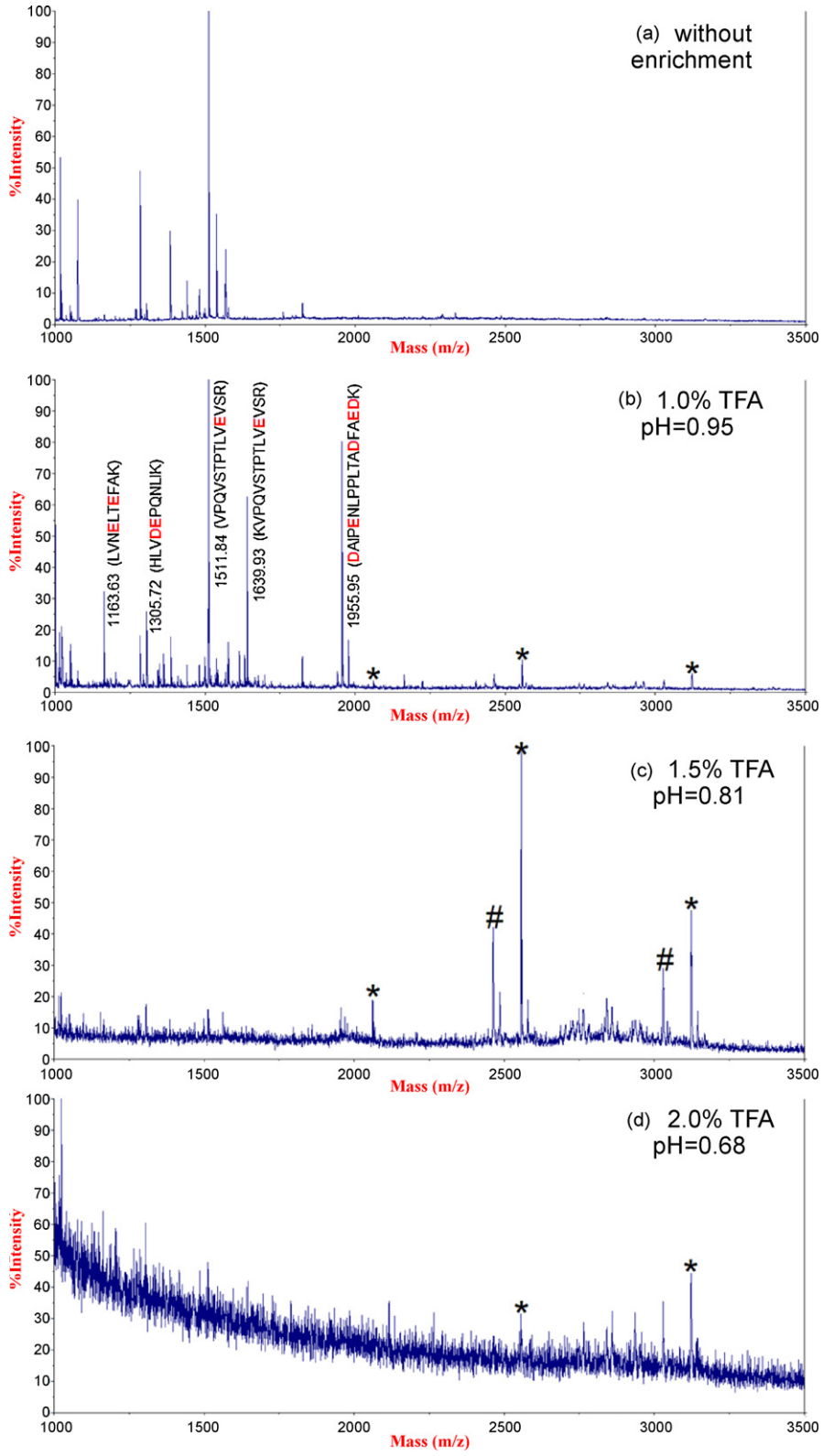

Fig. 4. Effect of the pH value on the selective binding of phosphopeptides to Zr^{4+}-phosphate functionalized magnetic microspheres. The peptide mixture contains β-casein and BSA (ratio of 1:25). (a) Without enrichment, enriched by Zr^{4+}-phosphate functionalized magnetic microspheres using a loading buffer of (b) 1.0% (v/v) TFA, (c) 1.5% (v/v) TFA, and (d) 2.0% (v/v) TFA. *, phosphopeptides; #, dephosphorylated fragment.

tion and prevent them from aggregating in liquid media, the Fe_3O_4 magnetic microspheres were coated with carbon shell as our previous work reported [23]. As seen in Fig. 2b, an obvious carbon shell (~10 nm) was observed, and it indicates that the microspheres have a mean diameter of ~300 nm with narrow size distribution. Fourier transform infrared (FTIR) was employed to investigate the functional groups modified on the Fe_3O_4@C magnetic microspheres. The solid line in Fig. 3 represents the infrared spectra of Fe_3O_4@C magnetic microspheres. The bands at 1700 and 1620 cm^{-1} can be attributed to the C=O and C=C vibrations, respectively, which

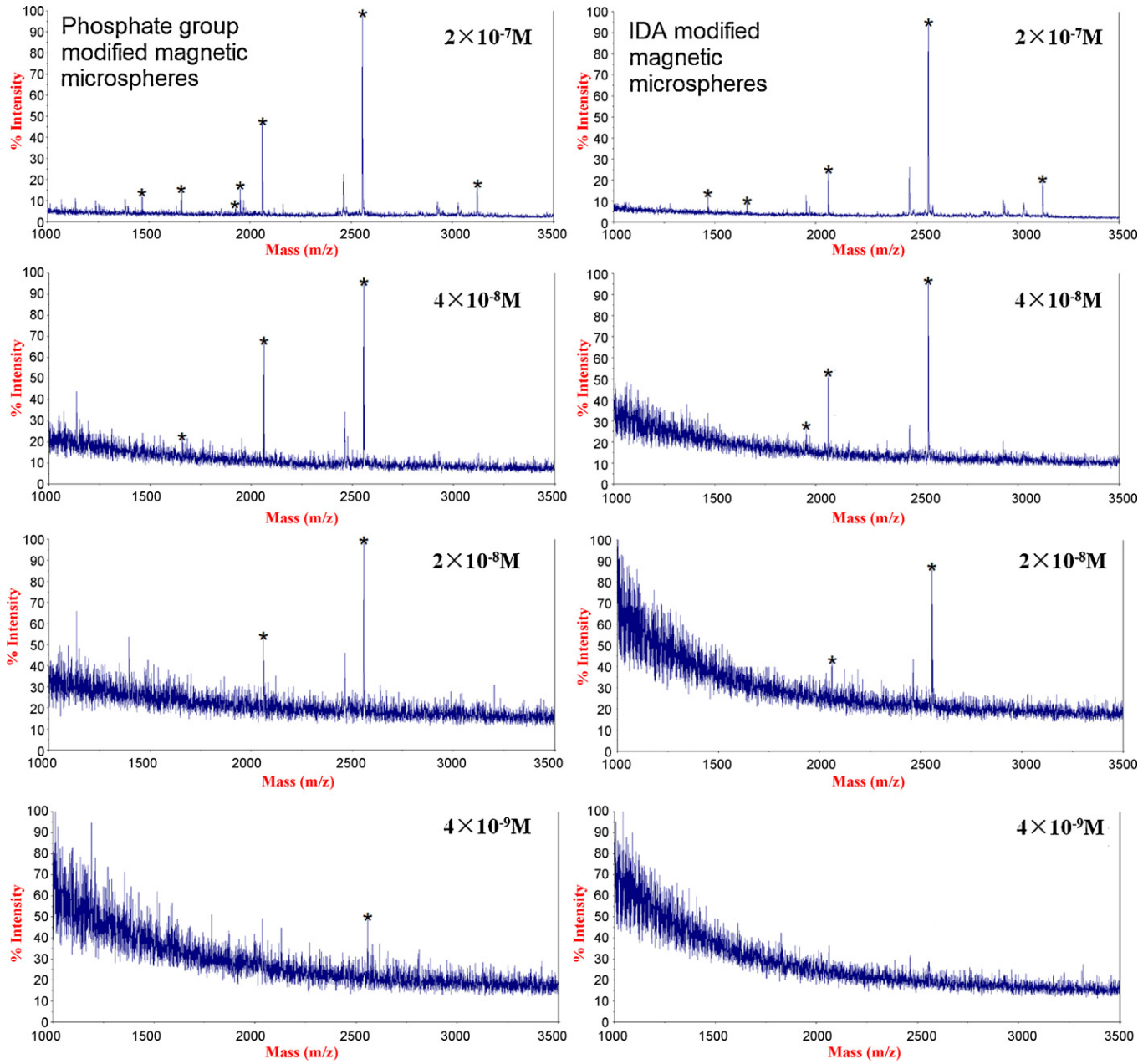

Fig. 5. MALDI mass spectra of phosphopeptides enriched form β-casein with different concentrations using Zr^{4+}-phosphate functionalized magnetic microspheres and Zr^{4+}-IDA modified magnetic microspheres. *, phosphopeptides.

reflect the carbonization of glucose. The bands at 1000–1300 cm^{-1} corresponding to the C–OH stretching and O–H bending vibrations reveal the presence of numerous hydrophilic groups and the incomplete carbonization of glucose. The presence of hydrophilic groups not only enhances the dispersity of Fe_3O_4@C magnetic microspheres, but also provides sufficient chemical groups for the next chemical modification. Finally, the Fe_3O_4@C magnetic microspheres were modified with phosphate groups. From the dashed line in Fig. 3, obvious absorption peaks were observed at 1050 and 1275 cm^{-1}, and can be attributed to the P–O–C and P=O vibrations, respectively. It indicates that the phosphate groups have been successfully modified on the surface of the Fe_3O_4@C magnetic microspheres. The obtained phosphate-functionalized magnetic microspheres were dispersed in $ZrOCl_2$ solution for Zr^{4+} cations immobilization. The energy-dispersive X-ray analysis (EDXA) (Fig. 2c) of the illuminating electron beams on the obtained Zr^{4+}-phosphate functionalized magnetic microspheres reveals the existence of Fe, Zr, C and O elements. It confirms that the strategy we reported here is feasible for phosphate-functionalized magnetic microspheres synthesis, and also suggests that the obtained phosphate-functionalized magnetic microspheres were efficient for ions immobilization. In our method, the phosphate group was modified on the magnetic microspheres through a hydrolysis reaction. Compared with the method reported by Zou and Qian [20,21], the new approach is simple, fast and safe.

3.2. Effect of the pH value on the selective binding of phosphopeptides to Zr^{4+}-phosphate functionalized magnetic microspheres

Next, we employed the Zr^{4+}-phosphate functionalized magnetic microspheres to implement phosphopeptides enrichment.

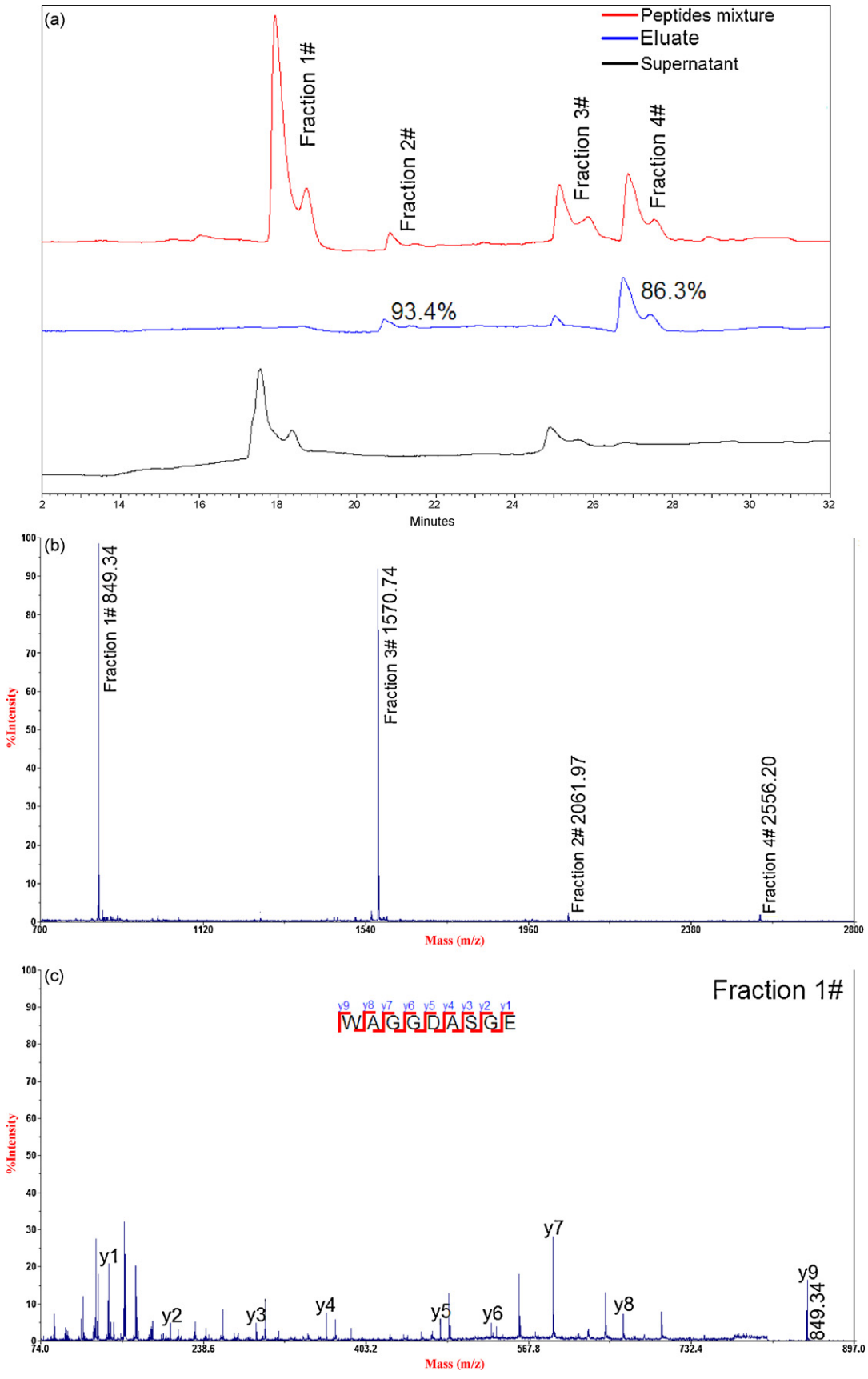

Fig. 6. (a) RPLC spectrum of the peptide mixture. (b) MALDI MS spectrum of the original peptide mixture. (c) MALDI MS/MS spectrum of fractions 1#. (d) MALDI MS/MS spectrum of fractions 2#. (e) MALDI MS/MS spectrum of fractions 3#. (f) MALDI MS/MS spectrum of fractions 4#.

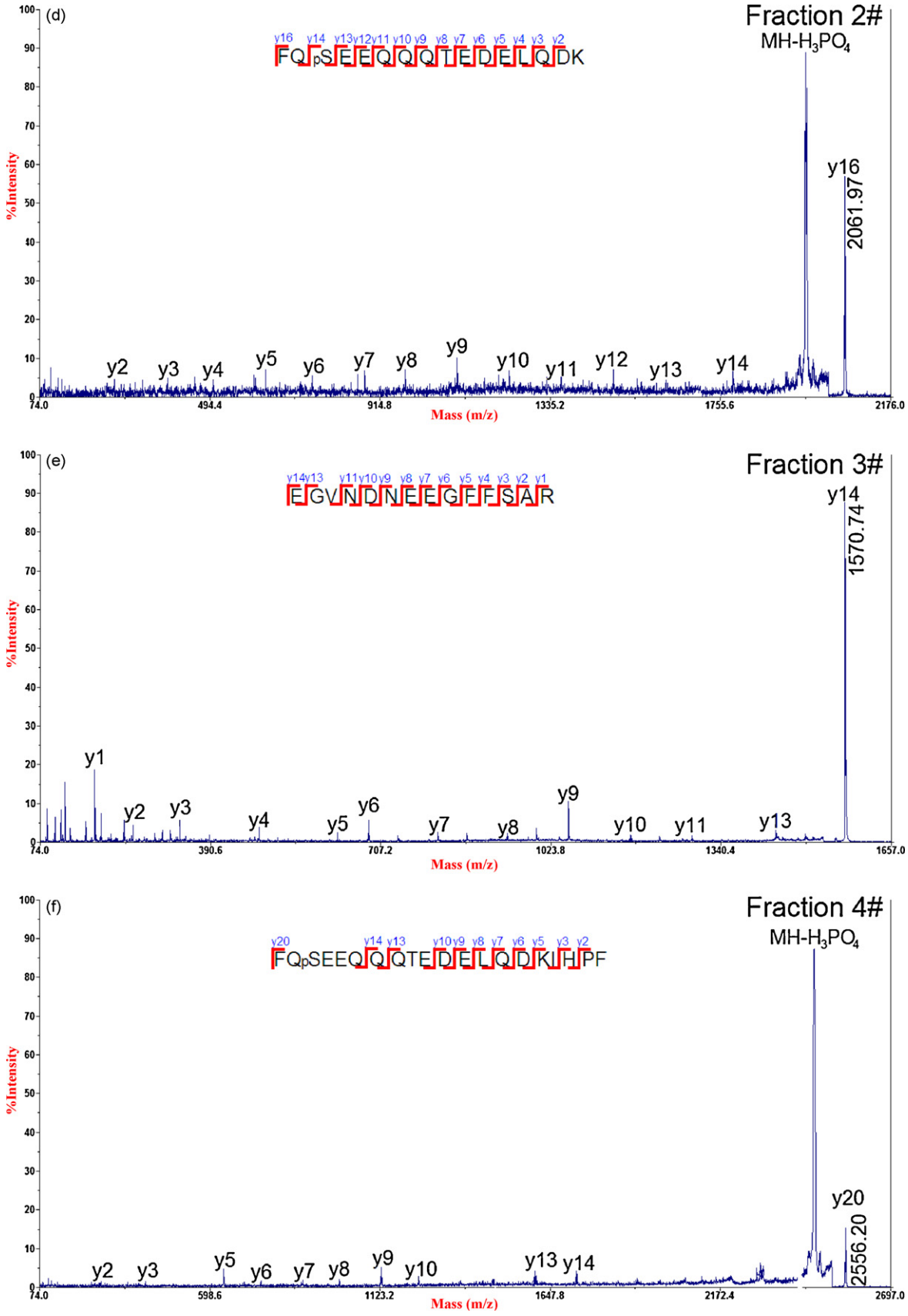

Fig. 6. (*Continued*).

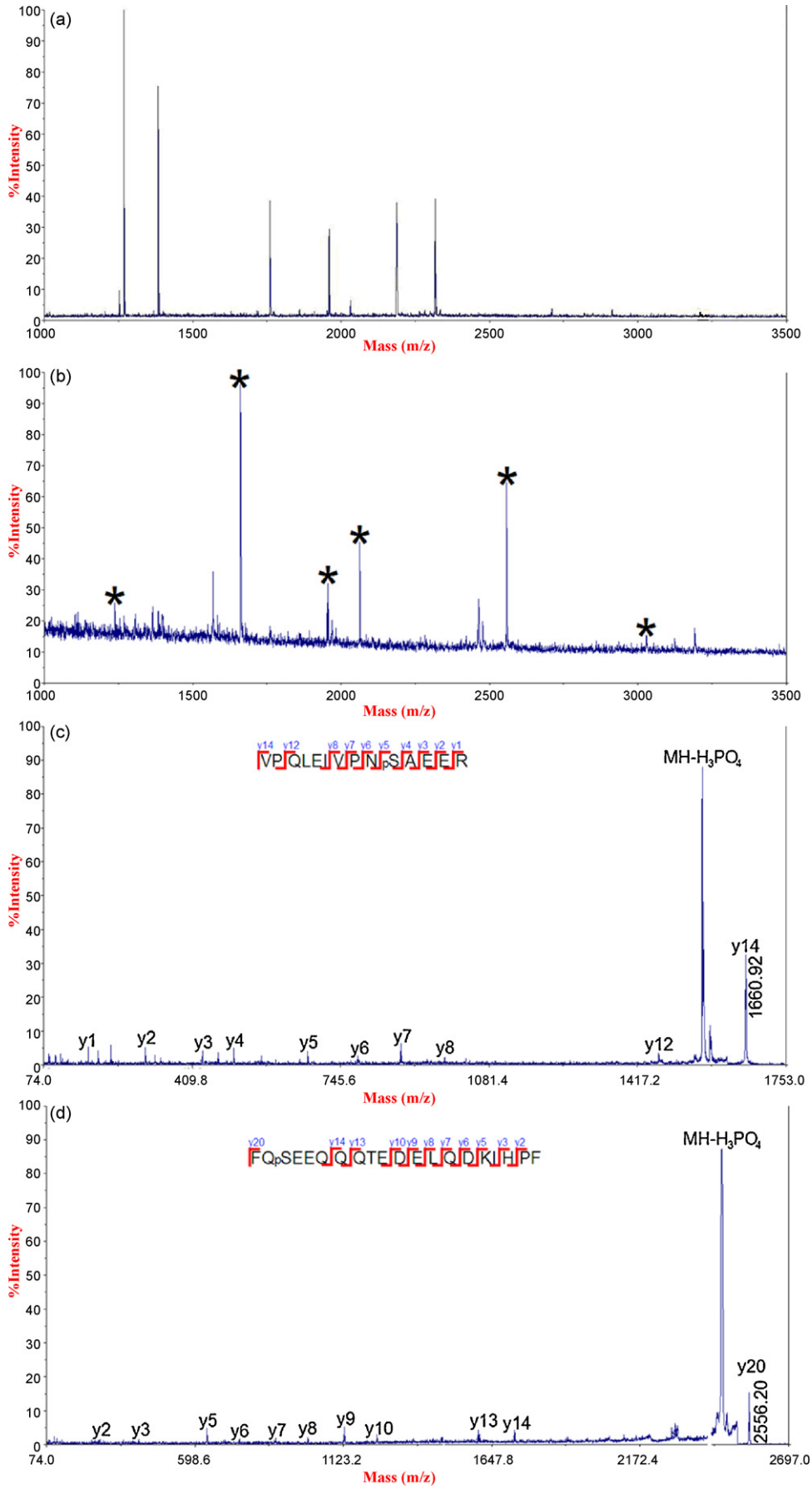

Fig. 7. MALDI mass spectrum of (a) tryptic digests of nonfat milk and (b) phosphopeptides derived from nonfat milk enriched by Zr^{4+}-phosphate functionalized magnetic microspheres. (c) MS/MS spectra of the phosphopeptide at *m*/*z* 1660.92. (d) MS/MS spectra of the phosphopeptides at *m*/*z* 2556.20. *, phosphopeptides; #, dephosphorylated fragment.

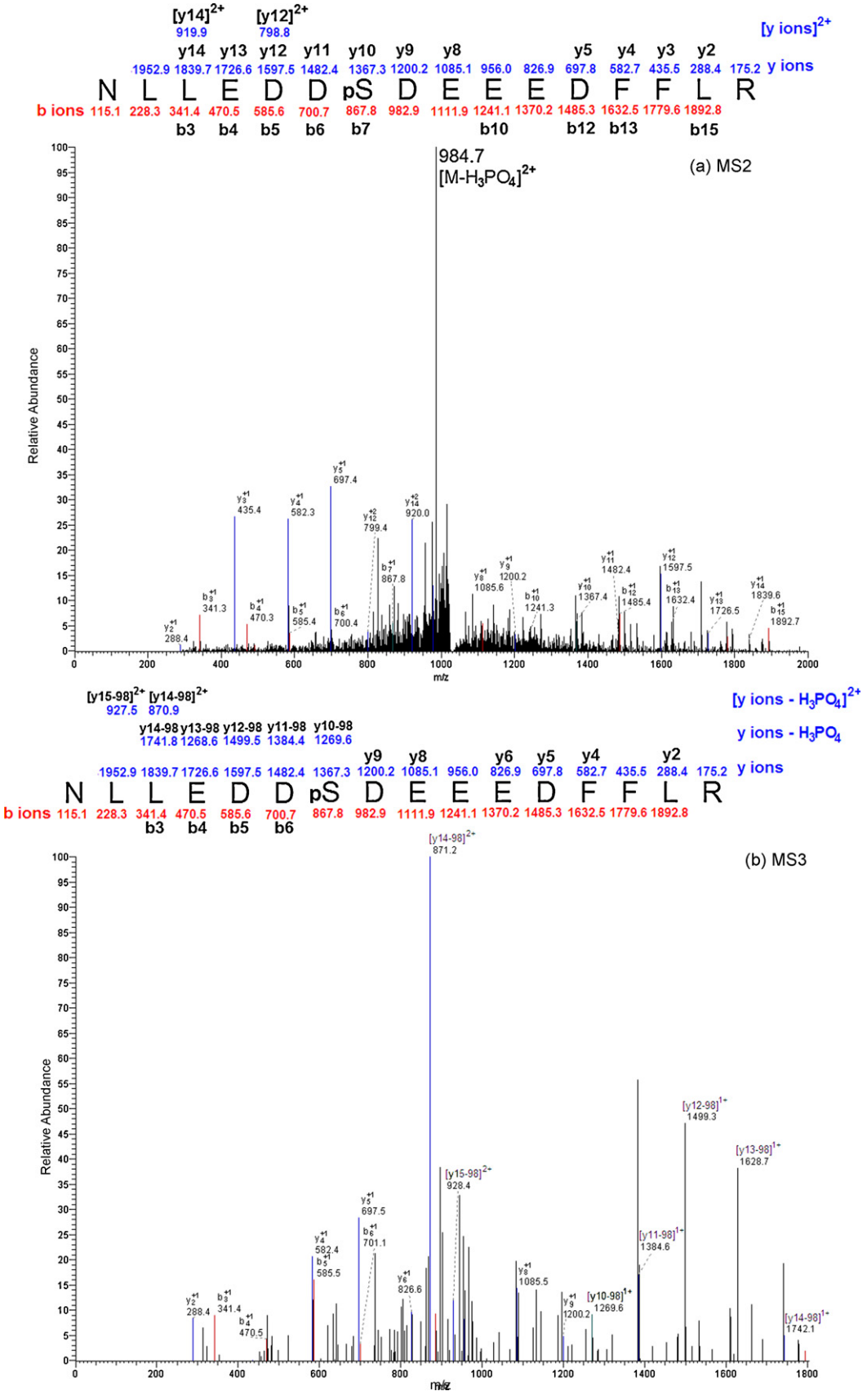

Fig. 8. (a) MS2 spectrum of the doubly charged form of a singly phosphorylated peptide identified from the tryptic digest of rat brain. The identified phosphopeptide was NLLEDDpSDEEEDFFLR. (b) MS3 spectrum of the doubly charged neutral loss peak at m/z 984.7.

Table 1
Phosphopeptides enriched form tryptic digests of nonfat milk using Zr^{4+}-phosphate functionalized magnetic microspheres.

No.	Peptide sequence	$(M+H)^+$	Accession number
1	TVDMES#TEVFTK(α-S2-(153–164))	1466.73	IPI00698843
2	VPQLEIVPNS#AEER(α-S1-(121–134))	1660.92	IPI00706094
3	DIGS#ES#TEDQAMEDIK(α-S1-(58–73))	1927.83	IPI00706094
4	YKVPQLEIVPNS#AEER(α-S1-(119–134))	1952.09	IPI00706094
5	FQS#EEQQQTEDELQDK(β-c-(33–48))	2061.96	IPI00697085
6	FQS#EEQQQTEDELQDKIHPF(β-c-(33–52))	2556.20	IPI00697085
7	RELEELNVPGEIVES#LS#S#S#EESITR(β-c-(14–40))	3122.40	IPI00697085

#, phosphorylation site.

Although the IMAC is the most widely used protocols for the phosphopeptides enrichment, the major disadvantage of IMAC is the nonspecific binding of unphosphorylated peptides, which contained carboxyl residues. The pH value of the loading buffer for the phosphopeptides enrichment has been considered to be the most important factors for the enrichment selectivity. In the most studies, 0.1–0.25 M acetic acid (pH 2.7) has been used for IMAC; however, Saha et al. showed that the pK_a value of phosphoric acid decreased to 1.1 upon methylation [27]. The pK_a values of phosphopeptides would therefore be expected to be significantly lower than that of phosphoric acid due to the organic environment provided by the surrounding amino acid residues. In this work, we investigated the effect of different pH value on the selective binding of phosphopeptides to Zr^{4+}-phosphate functionalized magnetic microspheres. A complex peptide mixture was employed to implement the investigation. The peptide mixture (ratio of 1:25) refers to a mixture of peptides from tryptic digests 0.04 pmol of β-casein and 1 pmol of BSA. The MALDI MS spectra of the peptides eluted from Zr^{4+}-phosphate functionalized magnetic microspheres with different loading buffer are shown in Fig. 4. The direct analysis of the peptide mixture is shown in Fig. 4a, numerous unphosphorylated peptides were detected and no phosphopeptide was observed. Although all the theoretical phosphopeptides were observed with 1.0% TFA as loading buffer, the MS spectra were still dominated by unphosphorylated peptides (Fig. 4b). The top five unphosphorylated peptides were assigned to peptides derived from BSA, and the peptide sequence has been listed in the figure. All the five unphosphorylated peptides contain at least one carboxyl residues which has been considered to be the main reason of nonspecific binding. With the concentration of TFA increasing to 1.5%, phosphopeptides derived from β-casein dominated the MS spectrum, and no unphosphorylated peptides can be perceived (Fig. 4c). Nevertheless, the higher pH value did not enhance the enrichment efficiency as shown in Fig. 3d. Although no unphosphorylated peptides were detected, the intensity of the phosphopeptides signal was also decreased. We supposed that the phosphate groups were protonated when the pH value of the loading buffer decreased to 0.68, and the enrichment efficiency was dramatically reduced. The next experiment was conducted using 50% (v/v) acetonitrile and 1.5% (v/v) TFA aqueous solution as loading buffer.

3.3. Selective enrichment of phosphopeptides using Zr^{4+}-phosphate functionalized magnetic microspheres

As mentioned above, the phosphopeptides usually has low stoichiometry. We investigated the sensitivity of the new method we reported here. The mass spectra of phosphopeptides enriched form β-casein digest with different concentration are shown in Fig. 5. It can be seen that when the concentration of β-casein digest is 4×10^{-9} M, the signals from the phosphopeptides can still be detected, which indicates that the high detection sensitivity of Zr^{4+}-phosphate functionalized magnetic microspheres. In our previous work, we have reported a method that synthesizing IDA modified magnetic microspheres for ions immobilization. And the IDA modified microspheres were used for the phosphopeptides enrichment [11]. Herein, the IDA modified microspheres were immobilized with Zr^{4+} ions and used to compare the enrich sensitivity of the two different methods. As shown in Fig. 5, when the concentration of β-casein digest is 4×10^{-9} M, no phosphopeptides can be observed. It demonstrated that the new method we reported here provide a superior enrichment sensitivity.

RPLC was employed to investigate the quantitative recovery of phosphopeptides/nonphosphopeptides of the Zr^{4+}-phosphate functionalized magnetic microspheres. Firstly, two phosphopeptides were mixed up with two standard nonphosphopeptides and analyzed by RPLC. According to the different retention time, four fractions were collected and analyzed by MLADI-TOF MS. The RPLC spectrum and MS/MS spectra of the four factions are shown in Fig. 6. As shown in MS/MS spectra, the fraction 2# and 4# was identified to be phosphopeptides. The equivalent peptide mixture was enriched by Zr^{4+}-phosphate functionalized magnetic microspheres, and then the peptides eluted from the microspheres were analyzed by RPLC. As shown in LC spectrum, the eluate contains two phosphopeptides and one nonphosphopeptide. Notably, the MS/MS spectrum of the fraction 3# reveals that the nonphosphopeptide contains four carboxyl residues, which have been considered to be the main reason of nonspecific binding. By analyzing the peak area of parallel RPLC runs, the recovery of two phosphopeptides was estimated to be 86.3% and 93.4%, respectively. The recovery of the fraction 3# nonphosphopeptides was calculated to be 0.6%. Compared with the original peptide mixture, no phosphopeptide peaks were observed in the supernatant. All the results demonstrated the efficiency of the method we reported here.

In order to evaluate the performance of the Zr^{4+}-phosphate functionalized magnetic microspheres for the real sample, we employed nonfat milk as a real sample. Nonfat bovine milk contains two main phosphorylated proteins, α-casein and β-casein, which have been widely used as standard proteins for phosphopeptides enrichment. Researchers have employed the nonfat milk as real sample to demonstrate the new phosphopeptides enrichment technique [28]. The mass spectrum of the peptides eluted from the Zr^{4+}-phosphate functionalized magnetic microspheres is shown in Fig. 7b. The MS/MS spectra of the two strongest phosphopeptide peaks are also shown in Fig. 7c and d. Compared with Fig. 7a, the phosphopeptides dominated the mass spectrum and no unphosphorylated peptides were detected. It indicates that Zr^{4+}-phosphate functionalized magnetic microspheres show great capability of specific phosphopeptides enrichment. The detail information of the phosphopeptides enriched by Zr^{4+}-phosphate functionalized magnetic microspheres is listed in Table 1.

To further evaluate the performance of Zr^{4+}-phosphate functionalized magnetic microspheres for phosphopeptides enrichment, the microspheres were employed to enrich phosphopeptides from tryptic digest of rat brain. 4 mg Zr^{4+}-phosphate functionalized magnetic microspheres were used to enrich the phosphopeptides derived from 100 μg of rat brain protein digest. The peptides eluted form Zr^{4+}-phosphate functionalized magnetic microspheres were

analyzed by LC–ESI-MS. Four technical replicate runs were conducted. The acquired MS/MS and MS/MS/MS spectra were searched separately by Sequest program as described in Section 2. The searching results were filtered with Xcorr values obtained by statistical calculation of reverse database searching results. For $p < 0.05$, the Xcorr > 2.0, 2.9, and 3.73 for singly, doubly, and triply charged ions, respectively, and $\Delta Cn > 0.1$ were used to filter the MS/MS database searching results. Due to the poor quality of the spectra for the phosphopeptides, the MS/MS/MS spectra were used to validate the phosphopeptides identified form the MS/MS. For the MS/MS/MS spectra, Xcorr > 1.5, 2.4, and 2.96 and $\Delta Cn > 0.1$ were used as criteria. Comparing the phosphopeptides identified from MS/MS and MSMS/MS/MS spectra, it was found that the sequences of 84 unique phosphopeptides were the same in both cases. Fig. 8 is an example for the identification of doubly charged phosphopeptide NLLEDDpSDEEEDFFLR. From the spectra it can be seen that b- and y-ions series are consistent with the theoretically predicted peaks in both MS/MS and MS/MS/MS spectra, and in MS/MS spectrum that peak at m/z 984.7 represents the doubly charged form of the selected precursor ion at m/z 2065.8 by losing H_3PO_4 group. Besides the above 84 phosphopeptides, more phosphopeptides, including multiple phosphorylated peptides, have been identified by the MS/MS spectra, which could not be validated by MS/MS/MS spectra. However, until now, strictly universal validation criteria have not been established and defined; it has too many subjective factors for interpretation of the spectra of multiple phosphorylated peptides. Therefore, only singly phosphorylated peptide identifications were validated manually in this work. After manual validation, an additional 109 singly phosphorylated peptides were finally identified from MS/MS spectra. Totally, 192 phosphorylation sites – 164 on serine (85.42%), 27 on threonine (14.06%) and 1 on tyrosine (0.52%) – were identified. The peptide sequence and Xcorr value of the obtained phosphopeptides are listed in Table S1.

4. Conclusions

In the work, we successfully modified phosphate group on magnetic carbon microspheres by simple hydrolysis reaction using 3-(trihydroxysilyl)propyl methylphosphate, and immobilized Zr^{4+} ions for phosphopeptides enrichment. Comparisons to the previous method for the modification of phosphate on substrates, the proposed method is simple, safe and fast. In the work, we also demonstrate the enrichment efficiency of the Zr^{4+}-phosphate functionalized magnetic microspheres. Finally, the Zr^{4+}-phosphate functionalized magnetic microspheres were used to isolate the phosphopeptides from the tryptic digests of rat brain, resulting in the identification of 192 phosphopeptides.

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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.2009.10.084.

References

[1] M.J. Hubbard, P. Cohen, Trends Biochem. Sci. 18 (1993) 172.
[2] T. Hunter, Philos. Trans.: Biol. Sci. 353 (1998) 583.
[3] J.D. Graves, E.G. Krebs, Pharmacol. Ther. 82 (1999) 111.
[4] D.E. Kalume, H. Molina, A. Pandey, Curr. Opin. Chem. Biol. 7 (2003) 64.
[5] S.A. Beausoleil, M. Jedrychowski, D. Schwartz, J.E. Elias, J. Villén, J. Li, M.A. Cohn, L.C. Cantley, S.P. Gygi, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 12130.
[6] T.E. Thingholm, O.N. Jensen, M.R. Larsen, Proteomics 9 (2009) 1451.
[7] A. Chi, C. Huttenhower, L.Y. Geer, J.J. Coon, J.E.P. Syka, D.L. Bai, J. Shabanowitz, D.J. Burke, O.G. Troyanskaya, D.F. Hunt, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 2193.
[8] 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.
[9] J. Lee, Y. Xu, Y. Chen, R. Sprung, S.C. Kim, S. Xie, Y. Zhao, Mol. Cell. Proteomics 6 (2007) 669.
[10] A. Gruhler, J.V. Olsen, S. Mohammed, P. Mortensen, N.J. Færgeman, M. Mann, O.N. Jensen, Mol. Cell. Proteomics 4 (2005) 310.
[11] X. Xu, C. Deng, M. Gao, W. Yu, P. Yang, X. Zhang, Adv. Mater. 18 (2006) 3289.
[12] T.S. Nuhse, A. Stensballe, O.N. Jensen, S.C. Peck, Mol. Cell. Proteomics 2 (2003) 1234.
[13] M.C. Posewitz, P. Tempst, Anal. Chem. 71 (1999) 2883.
[14] Y. Li, D. Qi, C. Deng, P. Yang, X. Zhang, J. Proteome Res. 7 (2008) 1767.
[15] H. Zhou, S. Xu, M. Ye, S. Feng, C. Pan, X. Jiang, X. Li, G. Han, Y. Fu, H. Zou, J. Proteome Res. 5 (2006) 2431.
[16] S. Feng, M. Ye, H. Zhou, X. Jiang, X. Jiang, H. Zou, B. Gong, Mol. Cell. Proteomics 6 (2007) 1656.
[17] E.K.M. Ueda, P.W. Gout, L. Morganti, J. Chromatogr. A 988 (2003) 1.
[18] L. Hu, H. Zhou, Y. Li, S. Sun, L. Guo, M. Ye, X. Tian, J. Gu, S. Yang, H. Zou, Anal. Chem. 81 (2009) 94.
[19] H. Zhou, M. Ye, J. Dong, G. Han, X. Jiang, R. Wu, H. Zou, J. Proteome Res. 7 (2008) 3957.
[20] L. Zhao, R. Wu, G. Han, H. Zhou, L. Ren, R. Tian, H. Zou, J. Am. Soc. Mass Spectrom. 19 (2008) 1176.
[21] J. Wei, Y. Zhang, J. Wang, F. Tan, J. Liu, Y. Cai, X. Qian, Rapid Commun. Mass Spectrom. 22 (2008) 1069.
[22] Y. Li, T. Leng, H. Lin, C. Deng, J. Proteome Res. 6 (2007) 4498.
[23] Y. Li, J. Wu, C. Deng, Chem. Commun. 46 (2008) 564.
[24] Y. Li, X. Xu, J. Proteome Res. 7 (2008) 2526.
[25] H. Chen, C. Deng, Y. Li, X. Zhang, P. Yang, Adv. Mater. 21 (2009) 2200.
[26] H. Deng, X. Li, Y. Li, Q. Peng, S. Wang, J. Chen, Y. Li, Angew. Chem. Int. Ed. 44 (2005) 2782.
[27] A. Saha, N. Saha, L.N. Ji, J. Zhao, J. Biol. Inorg. Chem. 1 (1996) 231.
[28] L. Qiao, C. Roussel, J. Wan, P. Yang, J. Proteome Res. 6 (2007) 4763.