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Rapid Enrichment of Phosphopeptides and Phosphoproteins from Complex Samples Using Magnetic Particles Coated with Alumina as the Concentrating Probes for MALDI MS Analysis

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In this study, we used nanocomposite magnetic particles coated with alumina as the affinity probes to selectively concentrate phosphorylated peptides and proteins from a low volume of sample solution. Tryptic digest products of phosphoproteins including α- and β-caseins, human protein phosphatase inhibitor 1, nonfat milk, egg white, and a cell lysate were used as the samples to demonstrate the feasibility of this approach. In only 30 and 90 s, phosphopeptides and phosphoproteins sufficient for characterization by MALDI-MS were enriched by the particles, respectively. Proteins trapped on the particles could be directly digested on the particles. The same particles in the digest solution were employed for enrichment of phosphopeptides. We estimated the required time for performing the enrichment of phosphopeptides from complex samples and characterization by MALDI MS was within 5 min. A small volume (50 μL) and a low concentration (5×10^{-10} M) of tryptic digest product of a phosphoprotein sample could be dramatically enriched and characterized using this approach.

Keywords: alumina • magnetic particles • phosphopeptides • phosphoproteins • MALDI MS

Introduction

Nanomaterials have been widely employed as affinity probes toward specific target species.[1−12] The composition and the surface modification of a nanomaterial determine the properties of its affinity to a target species. Magnetic materials are the most commonly selected substrates as affinity probes because of the ease of isolation of the magnetic material-target conjugate from the sample solution based on their magnetic properties.[4−7] Target species can be categorized into two major groups: cells and molecules. For example, functionalized nanomaterials,[3] vancomycin-immobilized,[4] and IgG immobilized[5] magnetic nanoparticles have been successfully prepared to probe bacterial cells. Identification of the trapped bacteria can be rapidly achieved using matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) based on the mass spectral fingerprinting generated from the target bacterial cells.[4,5]

Selective enrichment of phosphopeptides from enzymatic digest product of proteins remains an interesting research topic in the field of bioanalytical chemistry. We previously have demonstrated that gold nanoparticles immobilized on magnetic particles can be used as effective concentrating probes for enzymatic digest products of proteins.[6] Phosphorylation as a post-translational modification of proteins plays a critical role in regulating biological functions. Immobilized metal affinity-based enrichment of phosphopeptides for protein phosphorylation analysis has been demonstrated to be an effective approach.[13−15] We recently demonstrated that Fe_3O_4/TiO_2 core/shell magnetic nanoparticles can selectively trap phosphopeptide residues from tryptic digest products of phosphoproteins based on the high binding capacity of TiO_2 toward phosphate groups.[16] In addition to TiO_2, other metal oxides such as Al(III) containing materials have the capacity to interact with phosphate groups. So far, the interaction of Al(III)-containing materials with phosphorylated species has been reported in only a few studies.[17−21] Coletti-Previero and Previero[17] demonstrated that alumina has high specificity for analytes containing phosphate groups within a large range of pH. Koppel et al.[18] employed an alumina column to purify phosphorylated proteins from rabbit serum. Li et al.[19] also generated phosphorylated pepsin to enhance its immobilization onto alumina nanoparticles via the binding of phosphate groups to Al(III)

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ions to improve the enzymatic activity of pepsin. Shin et al.[20] employed aluminum-impregnated mesoporous silicates to remove small molecules such as fertilizers and detergents containing phosphate groups from wastewater. Lately, Wolschin et al.[21] employed $Al(OH)_3$ slurry as adsorbents to extract phosphorylated proteins and peptides from complex mixtures. It is possible to further improve the isolation efficiency by using modified magnetic nanoparticles as the affinity probes. In this study, we alternatively generated iron oxide magnetic nanoparticles coated with sol−gel alumina to generate functional nanocomposite magnetic nanoparticles for the selective-enrichment of phosphoproteins and phosphopeptides from tryptic digest product of proteins. Centrifugation is generally required to isolate the affinity probe-target species conjugates from sample solution after enrichment if the affinity probes having no magnetic properties. However, on the basis of the magnetic property of the affinity probes we fabricated herein, the magnetic probe-target species conjugates could be rapidly isolated from sample solutions by simply positioning an external magnetic field. Affinity probes with magnetic properties lead the isolation of the trapped species from sample solutions very conveniently and rapidly; therefore, the time required for separation can be dramatically reduced. Herein, we proposed a novel method, which only took as short as 30 s to pipet in and out of low volumes (50 μL) of sample solutions in a vial to enrich sufficient target species, followed by rapid aggregation of magnetic beads on the edge of the vial by positioning an external magnetic field. If the affinity probes do not have magnetic properties, centrifugation should be used for isolation, which is time-consuming. Thus, this is a unique advantage by using our magnetic particles as the affinity probes for enrichment. Our results have demonstrated this approach is an effective enrichment means with high sensitivity and specificity for phosphorylated species.

Experimental Section

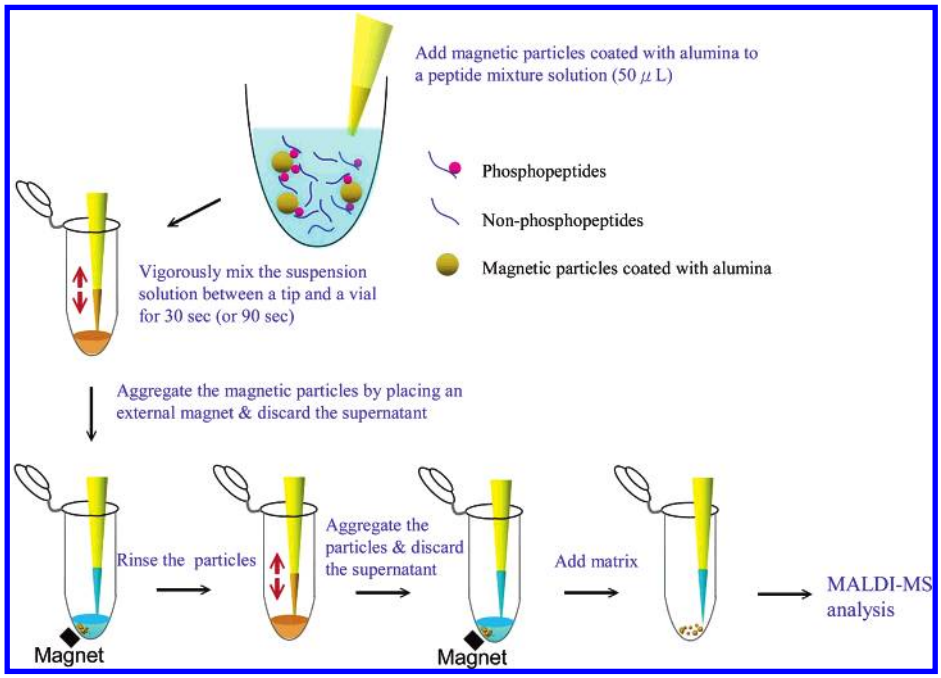
Reagents. Iron(III) chloride hexahydrate, sodium sulfite, ammonium hydrogen carbonate, trifluoroacetic acid, urea, ammonium sulfate, potassium chloride, and phosphoric acid were purchased from Riedel-de Haën (Seelze, Germany), whereas sodium chloride, hydrochloric acid, and acetonitrile were obtained from Merck (Darmstadt, Germany). Sodium silicate and sodium dodecyl sulfate (SDS) were obtained from J. T. Baker (Phillipsburg, NJ), and ammonium hydroxide solution was obtained from Fluka (Seelze, Germany). α-Casein (from bovine milk), β-casein (from bovine milk), cytochrome C, bovine serum albumin (BSA), dithiothreitol (DTT), iodoacetic acid (IAA), disodium phosphate heptahydrate, potassium phosphate monobasic anhydrate, ethylenediamine tetraacetic acid (EDTA), Triton X-100, sodium deoxycholate, leupeptin, pepstatin A, phenylmethyl sulfonyl fluoride (PMSF), sodium orthovanadate, and trypsin (from bovine pancreas, TPCK treated) were purchased from Sigma (St. Louis, MO). Aluminum isopropoxide, α-cyano-4-hydroxycinnamic acid, and 2,5-dihydroxybenzoic acid were purchased from Aldrich (Milwaukee, WI). Dulbecco's modified Eagle medium (DMEM) was obtained from Biowest (Miami, FL). Human protein phosphatase inhibitor 1 (PPI 1) was a gift kindly provided by Dr. H.-B. Huang (National Chung Cheng University, Taiwan). Nonfat milk and chicken eggs were purchased from a local grocery store. Egg white was directly isolated from chicken eggs.

Preparation of Fe_3O_4 Magnetic Nanoparticles. Iron oxide magnetic nanoparticles were prepared by the steps shown as follows. Iron(III) chloride ($FeCl_3 \cdot 6H_2O$, 6.48 g) was dissolved in hydrochloric acid (2 M, 12 mL) and then diluted to 100 mL by water. The mixture was degassed using a pump. Sodium sulfite (50 mL, 0.08M) was slowly added to the above solution under nitrogen with stirring at room temperature. Subsequently, ammonia (5%, 45 mL) was added slowly to the reaction solution with vigorous stirring at room temperature. The whole mixture was left to react for 30 min in a water bath at 70 °C. The magnetic nanoparticles were rinsed with deionized water three times.

Preparation of Nanocomposite Magnetic Particles Coated with Alumina. Iron oxide in water (5 mg/mL, 40 mL) was well suspended in an air-free condition by sonication under nitrogen gas. Sodium silicate aqueous solution (0.6%, pH 9, 40 mL) was added into the above solution and vortex-mixed for 24 h at 35 °C to coat a thin film of SiO_2 onto the surfaces of magnetic particles. The particles were rinsed with deionized water (40 mL) three times, resuspended in deionized water (40 mL), and followed by sonication for 30 min. Aluminum isopropoxide (6 mg) was added to the nanoparticle solution followed by sonication for 30 min at room temperature. The mixture in a reaction vial was reacted at 80 °C in an oil bath with vigorous stirring for 1 h, and then the cap of the vial was opened to remove the generated gas, i.e., 2-propanol. The mixture was reacted at 90 °C for 30 min, followed by the reflux at 90 °C for another 2 h. After the mixture was cooled to room temperature, the particles were isolated, rinsed with water (40 mL × 3), and resuspended in water before use.

Preparation of Cell Lysates. Human epidermoid carcinoma epithelial cells (HEp-2; ATCC CCL-23) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). *Streptococcus pyogenes* strain M29588 was isolated from a patient with necrotizing fasciitis in Tzu-Chi General Hospital, Taiwan. *S. pyogenes* were grown in tryptic soy broth supplemented with 0.5% yeast extract (TSBY). In the infection assay, HEp-2 cells (5×10^6 cells/mL) were seeded on 100-mm diameter Petri dishes, and infected with *S. pyogenes* at MOI (multiplicity of infection) of 100 for 4 h.[22] After infection, the cells were rinsed four times with cold PBS buffer (6 mL). PBS buffer was prepared by dissolving sodium chloride (8 g), potassium chloride (0.2 g), disodium hydrogen phosphate (1.44 g), and potassium phosphate monobasic anhydrous (0.24 g) in sterilized deionized-water (800 mL). The pH of the buffer was adjusted to pH 7.4 by hydrochloric acid, followed by dilution with deionized water to 1 L. The buffer was filtered to remove impurities (filters with 0.22 μm of pore size). A solution (1 mL) containing trypsin (0.05%, w/v)/EDTA (0.02%, w/v) prepared in PBS buffer was added into the Petri dish to cover all the cells. The Petri dish was incubated in an incubator at 37 °C for 3 min. DMEM-containing serum (5 mL) was added into the Petri dish so that the cells can be easily removed to a centrifugation tube, followed by centrifugation at 1000 rpm for 5 min. After centrifugation, the supernatant was pipetted out, and PBS buffer (1 mL × 3) was added into the tube to rinse the cells remaining in the tube. Lysis buffer (1 mL) prepared in PBS buffer (100 mL), which was composed of Triton X-100 (1 mL), sodium dodecyl sulfate (0.1 g), sodium deoxycholate (0.5 g), leupeptin (5 mg/mL, 10 μL), EDTA (0.5 M, 0.2 mL), pepstatin A (1 mg/mL, 100 μL), PMSF (100 mM, 200 μL), and sodium orthovanadate (100 mM, 20 μL) were added into the tube, followed by incubation in an ice bath for 10 min. The cell lysate was centrifuged at 4000 rpm (4 °C) for 10 min.[23] The supernatant was put in an ice bath, followed by

Scheme 1. Procedures of Using Magnetic Nanocomposite Particles Coated with Alumina to Enrich Phosphopeptides from a Sample Solution.



the addition of ammonium sulfate (0.43 g). The mixture was vortex-mixed for 1 h. The sample was centrifuged at 12 000 rpm (4 °C) for 20 min. Precipitation floating on the top of the solution after centrifugation was obtained. A syringe was used to carefully remove the solution, whereas the precipitation remaining in the tube was redissolved in aqueous ammonium bicarbonate solution (50 mM, 0.5 mL) and stored at −20 °C before use.

Procedures for Denaturing Proteins. A protein mixture (β-casein (2×10^{-6} M), cytochrome C (2×10^{-6} M), myoglobin (2×10^{-6} M), and BSA (2×10^{-6} M)), nonfat milk, egg white (10-fold diluted), and a cell lysate obtained above were used as the samples for demonstration of the trapping capacity of our functional particles for their target species. Protein mixtures were prepared in an ammonium bicarbonate solution (0.5 mL, 50 mM) containing urea (8 M), whereas the milk (0. 25 mL) was mixed with the same solution (0.25 mL), followed by the incubation at 38 °C for 30 min. A DTT (50 μL, 100 mM) prepared in an ammonium bicarbonate solution (50 mM) was added into this mixture, which was maintained at 50 °C for 1 h. After cooling to room temperature, the sample solution was mixed an IAA solution (50 μL, 200 mM) prepared in ammonium bicarbonate solution (50 mM) in an ice bath. The sample vial was wrapped with aluminum foil and mixed by vortex-mixing for 2.5 h at room temperature. After 2.5 h, the sample solution was ready for carrying out either the enrichment process by our magnetic particles or tryptic digestion.

Procedures for Enrichment of Phosphoproteins from Protein Mixtures. The protein sample prepared above (5 μL) was mixed with TFA (0.15%, 44.3 μL). A magnetic beads coated with alumina solution (40 mg/mL, 0.7 μL) was added to this acidified solution. The mixture was vigorously mixed by pipeting in and out of a sample vial for 90 s. The particles conjugated with their target species were then aggregated on the sample wall by positioning a magnet to the outside of the sample vial so that the solution could be easily removed by pipet. The isolated particles were rinsed with 0.15% TFA solution (60 μL × 2), in acetonitrile/deionized water (1/1, v/v), mixing vigorously by pipeting in and out of the mixture in the vial, to wash out any unbound impurities. The rinsed particles were mixed with 0.7 μL of sinapinic acid (30 mg/mL), which was prepared in an acetonitrile/deionized water (2/1, v/v) solution containing 1% phosphoric acid. After evaporation of the solvent, the sample was ready for MALDI MS analysis.

On Particle Tryptic Digestion. To obtain more information, the proteins trapped on the particles obtained above were alternatively digested by trypsin (10 μL, 0.5 mg/mL), which was prepared in an ammonium bicarbonate solution (50 mM), under sonication for 15 s. The mixture was incubated at 38 °C for 20 min. After incubation, a 0.15% TFA solution (90 μL) prepared in deionized water was added into the digest product. The suspension was mixed by pipeting in and out of the mixture in the vial to carry out the trapping process. The particles conjugated with their target species were then aggregated on the sample wall by applying a magnet to the outside of the sample vial so that the solution could be easily removed by pipet. The isolated particles were rinsed with 0.15% TFA solution (100 μL × 2), in acetonitrile/deionized water (1/1, v/v), mixing vigorously by pipeting in and out of the mixture in the vial, to wash out any unbound impurities. The rinsed particles were mixed with 0.7 μL of 2,5-DHB (30 mg/mL), which was prepared in an acetonitrile/deionized water (2/1, v/v) solution containing 1% phosphoric acid. The particle suspension (~0.7 μL) was directly deposited on a MALDI plate by pipet. After the solvent evaporated, the sample was ready for MALDI MS analysis.

Procedures for Enrichment of Phosphopeptides. Proteins and trypsin were prepared in aqueous ammonium bicarbonate (50 mM) solution. Proteins (2 mg/mL, 500 μL) and trypsin (2 mg/mL, 10 μL), at a weight ratio of 50:1, were incubated at 37 °C for 18 h. The digest product was then diluted in TFA solution (0.15%) to acidify the solution. To perform an enrichment experiment, the acidified digest solution (49.4 μL) was mixed with the suspension of magnetic particles coated with alumina (40 mg/mL, 0.6 μL) in 0.15% TFA solution. The mixture was vigorously mixed by pipeting in and out of a sample vial for 30

s. The particles conjugated with their target species were then aggregated on the sample wall by positioning a magnet to the outside of the sample vial so that the solution could be easily removed by pipet. The isolated particles were rinsed with 0.15% TFA solution (60 μL × 2), in acetonitrile/deionized water (1/1, v/v), mixing vigorously by pipeting in and out of the mixture in the vial, to wash out any unbound impurities. The rinsed particles were mixed with 0.7 μL of 2,5-dihydroxybenzoic acid (DHB, 30 mg/mL), which was prepared in an acetonitrile/deionized water (2/1, v/v) solution containing 1% phosphoric acid. The particle suspension (~0.7 μL) was directly deposited on a MALDI plate by pipet. After evaporation of the solvent, the sample was ready for MALDI MS analysis.

When MALDI-TOF−TOF MS was employed for characterization of target peptides, the mixture of 2,5-DHB (15 mg/mL) and CHCA (15 mg/mL) containing 1% phosphoric acid was used as the matrix. MS/MS ion searching was carried out by using MASOCT search engine and SwissProt protein database. Missed cleavages were set at "2", whereas carboxymethyl was set as the fixed modification and phosphor (ST) was set as the variable modification. The mass tolerance for peptide ions was set at ±0.4 Da, whereas it was set at ±1.5 (or 2) Da for MS/MS analysis.

The trapping capacity of the alumina-coated magnetic particles for phosphopeptides was estimated using absorption spectroscopy. The phosphopeptides isolated from the tryptic digest of β-casein were used as the sample for examining the trapping capacity. Because we demonstrated that alumina-coated magnetic particles could selectively enrich only phosphopeptides from the tryptic digest of β-casein in this study, we first used the magnetic particles to trap phosphopeptides from the tryptic digest of β-casein. The particles were then isolated by employing an external magnetic field. The remaining particles were rinsed twice with 0.15% TFA solution (1 mL × 2), in acetonitrile/deionized water (1/1, v/v), mixing vigorously by pipeting in and out of the mixture in the vial, to wash out any unbound impurities. The phosphopeptides trapped on the particles were then eluted with aqueous ammonia (~2.5%). The eluted solution was lyophilized. A given amount of lyophilized product (0.12 mg) was prepared in 0.15% TFA aqueous solution (1 mL). The absorption unit of the solution was measured using an absorption spectroscopy. A particle suspension containing alumina-coated magnetic particles was then added into the solution with vigorously mixing by pipeting in and out of the mixture for 30 s. The solution in the suspension was separated from the suspension by aggregating the particles on the edge of the sample vial by applying an external magnetic field. The absorption unit of the separated solution was measured by absorption spectroscopy. The trapping capacity was estimated based on the absorption change before and after incubation with the alumina-coated magnetic nanoparticles.

Instrumentation. All mass spectra were obtained using a Biflex III (Bruker Daltonics, Germany) time-of-flight mass spectrometer equipped with a 337-nm nitrogen laser, a 1.25-m flight tube, and a sample target having the capacity to load 384 samples simultaneously. The accelerating voltage was set at 19 kV, whereas the laser power was carefully adjusted during analysis to obtain the optimized mass resolution. All MS/MS spectra were obtained using an Ultraflex MALDI-TOF−TOF mass spectrometer (Bruker Daltonics, Germany). Absorption spectra were obtained using a Varian Cary 50 spectrophotometer (Melbourne, Australia). The TEM image was obtained using a JEOL 2000FX (Japan) transmission electron microscope (TEM).

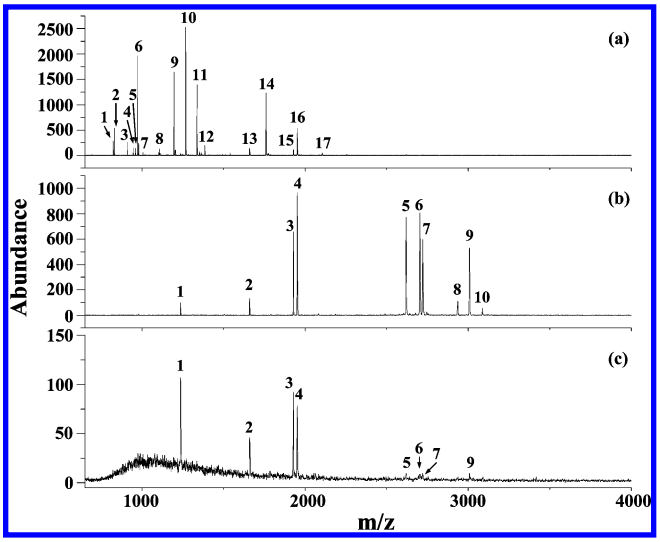


Figure 1. (a) Direct MALDI mass spectrum of the tryptic digest product of α-casein (10^{-5} M, 0.3 μL) prior to enrichment. MALDI mass spectra of (b) the tryptic digest product of α-casein (10^{-6} M, 50 μL) and (c) the tryptic digest product of α-casein (5×10^{-10} M, 50 μL) after enrichment by the magnetic particles coated with alumina.

Results and Discussion

Scheme 1 displays the detailed procedures for phosphopeptide enrichment. We mixed the acidified sample solution (50 μL) containing a tryptic digest of phosphorylated species with the nanocomposite particles, by pipeting the mixture in and out of a sample vial for 30 s (or 90 s). The TEM image of the nanocomposite magnetic particles and the confirmation of alumina coating on the magnetic particles by mass spectrometry are present in the Supporting Information. The particles were then isolated from the solution by applying an external magnet on the edge of the vial. After washing the isolated particles with a solution (60 μL) of 0.15% trifluoroacetic acid (TFA) dissolved in a solution of acetonitrile in distillated water (1/1, v/v), the particles were mixed with MALDI matrix for MALDI MS analysis. The presence of acetonitrile in the washing solution is required in order to effectively remove all the nonspecific binding peptides.[24] Figure 1a presents the direct MALDI mass spectrum of the tryptic digest product of α-casein (10^{-5} M, 0.3 μL) prior to enrichment. The peaks marked with Arabic numbers correspond to the amino acid sequences for each peptide displayed in Table 1. Only the peaks labeled with 13, 15, and 16 at m/z 1660.57, 1927.66, and 1951.83, respectively, are phosphopeptides. However, after enrichment by using the magnetic particles coated with alumina from of the tryptic digest product of α-casein (10^{-6} M), 10 phosphopeptide residue ions generated from α-casein appear in the MALDI mass spectrum with quite good intensities (Figure 1b and Table 2). There are no ion peaks from non-phosphorylated peptide residues appearing in the mass spectrum. The results indicate that the selectivity of the magnetic particles coated with alumina is quite good. As the concentration of the sample is lowered down to 5×10^{-10} M, there are still several phosphorylated peptides marked with the numbers 1, 2, 3, 4, 5, 6, 7, and 9 appearing in the mass spectrum (Figure 1c) after

Table 1. Peptide Ion Peaks Observed in the Direct MALDI Mass Spectrum of Tryptic Digest of α-Casein

no.	observed $[M + H]^+$	calculated $[M + H]^+$	sequences[a]
1	824.40	824.43	YPELFR (S1/146−151)
2	831.40	831.38	EDVPSER (S1/84−90)
3	910.48	910.47	EGIHAQQK (S1/125−132)
4	946.51	946.52	EKVNELSK (S1/35−42)
5	958.46	958.47	FPQYLQY (S2/92−98)
6	971.54	971.50	FYPELFR (S1/145−151)
7	979.62	979.56	FALPQYLK (S2/174−181)
8	1107.51	1107.51	QLDAYPSGAW (S1/155−164)
9	1195.69	1195.68	NAVPITPTLNR (S2/115−125)
10	1267.70	1267.70	YLGYLEQLLR (S1/91−100)
11	1337.67	1337.68	HIQKEDVPSER (S1/80−90)
12	1384.73	1384.73	FFVAPFPEVFGK (S1/23−34)
13	1660.57	1660.79	VPQLEIVPN*s*AEER (S1/106−119)
14	1759.80	1759.95	HQGLPQEVLNENLLR (S1/8−22)
15	1927.66	1927.69	DIG*s*E*s*TEDQAMEDIK (S1/43−58)
16	1951.83	1951.95	YKVPQLEIVPN*s*AEER (S1/104−119)
17	2104.90	2104.97	TDAPSFSDIPNPIGSENSEK (S1/174−193)

[a] "*s*" stands for phosphorylated serine.

Table 2. Peptide Ion Peaks Observed in the MALDI Mass Spectrum of Tryptic Digest of α-Casein after Enrichment by Magnetic Particles Coated with Alumina

no.	observed $[M + H]^+$	calculated $[M + H]^+$	sequences[a]
1	1237.33	1237.47	TVDME*s*TEVF (S2/138−147)
2	1660.73	1660.79	VPQLEIVPN*s*AEER (S1/106−119)
3	1927.69	1927.73	DIG*s*E*s*TEDQAMEDIK (S1/43−58)
4	1951.93	1951.95	YKVPQLEIVPN*s*AEER (S1/104−119)
5	2618.87	2618.90	NTMEHV*sss*EESII*s*QETYK (S2/2−21)
6	2703.62	2703.50	LRLKKYKVPQLEIVPN*s*AEERL (S1/99−120)
7	2720.94	2720.91	QMEAE*s*I*sss*EEIVPN*s*VEQK (S1/59−79)
8	2935.29	2935.16	EKVNEL*s*KDIG*s*E*s*TEDQAMEDIK (S1/35−58)
9	3008.15	3008.03	NANEEEYSIG*sss*EE*s*AEVATEEVK (S2/46−70)
10	3087.37	3087.33	*s*T*s*EENSKKTVDME*s*TEVFTKKTL (S2/129−153)

[a] "*s*" stands for phosphorylated serine.

enrichment by the particles. The trapping capacity estimated by absorption spectroscopy was 60 μg of phosphopeptides trapped by 1 mg of our magnetic particles.

Selectivity of these particles for phosphopeptides was further demonstrated with a tryptic digest of the mixture of α-casein (composed of S1 and S2 units) and β-casein, at a low concentration (5×10^{-8} M for each protein). Figure 2a presents the direct MALDI mass spectrum of the tryptic digest product of the mixture of α-casein and β-casein prior to enrichment. There are only two non-phosphorylated peptide ions at m/z 1337.67 (#80−90, HIQKEDVPSER) and 1759.81 (#8−22, HQGLPQEVLNENLLR) derived from α-S1-casein revealing in the mass spectrum. After using the affinity particles to enrich phosphopeptides, phosphopeptide residue ions start to appear in the MALDI mass spectrum (Figure 2b and Table 3). The peaks marked with the numbers 1, 2, 9, and 12 are derived from α-S2-casein, whereas the peaks marked with the numbers 3, 4, 5, 10, and 11 are derived from α-S1-casein. The remaining peaks, marked with the numbers 6, 7, and 13, are derived from β-casein. The corresponding peptide sequences of these ions are listed in Table 3. All of the ions revealed in the mass spectrum are phosphopeptide residues. Although the concentration of the sample solution is very low, the alumina-coated magnetic particles still have excellent trapping capacity specific for phosphopeptides.

To create an even more complicated sample, we mixed the tryptic digest products of several proteins, including non-phosphorylated proteins (cytochrome C and bovine serum albumin (BSA)) and phosphoproteins (α-casein and β-casein). Figure 3a displays the direct MALDI mass spectrum of the

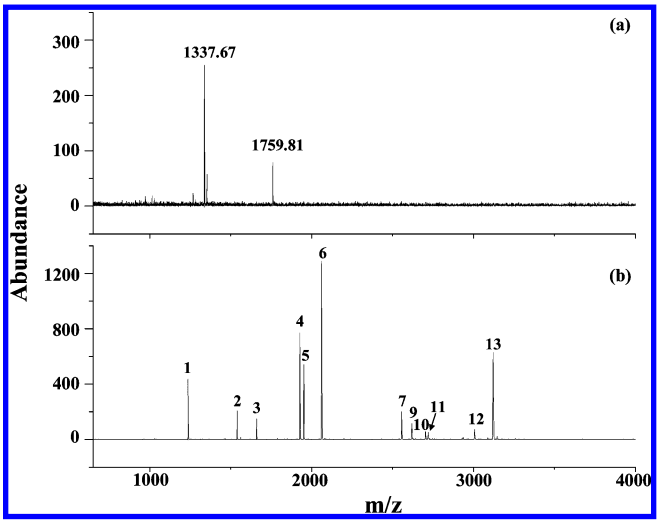


Figure 2. MALDI mass spectra of the tryptic digest product of the mixture (50 μL) of α-casein and β-casein (5×10^{-8} M for each protein) (a) prior to enrichment and (b) after enrichment by the magnetic particles coated with alumina.

tryptic digest product (0.3 μL) of cytochrome C (2.5×10^{-6} M), BSA (2.5×10^{-6} M), α-casein (5×10^{-8} M), and β-casein (5×10^{-8} M). At such a low concentration, only two weak phosphopeptide residue ions (nos. 1 and 7) are observed in the MALDI mass spectrum prior to enrichment. The remaining peaks are non-phosphorylated peptide residues derived from these proteins. However, after enrichment, 12 phosphopeptide

Table 3. Peptide Ion Peaks Observed in the MALDI Mass Spectrum of Tryptic Digest of Phosphoproteins (α- and β-Caseins) after Enrichment by Magnetic Particles Coated with Alumina

no.	observed $[M + H]^+$	calculated $[M + H]^+$	sequences[a]
1	1237.36	1237.47	TVDME*s*TEVF (α-S2/138−147)
2	1539.44	1539.60	EQL*s*T*s*EENSKK (α-S2/126−137)
3	1660.75	1660.79	VPQLEIVPN*s*AEER (α-S1/106−119)
4	1927.49	1927.69	DIG*s*E*s*TEDQAMEDIK (α-S1/43−58)
5	1951.85	1951.95	YKVPQLEIVPN*s*AEER (α-S1/104−119)
6	2061.86	2061.83	FQ*s*EEQQQTEDELQDK (β-33−48)
7	2556.11	2556.09	FQ*s*EEQQQTEDELQDKIHPF (β-33−52)
8	2588.07	2588.16	NAVPITPTLNREQL*s*T*s*EENSK (α-S2/115−136)
9	2618.92	2618.90	NTMEHV*sss*EESII*s*QETYK (α-S2/2−21)
10	2703.67	2703.50	LRLKKYKVPQLEIVPN*s*AEERL (α-S1/99−120)
11	2720.92	2720.91	QMEAE*s*I*sss*EEIVPN*s*VEQK (α-S1/59−79)
12	3008.13	3008.03	NANEEEYSIG*sss*EE*s*AEVATEEVK (α-S2/46−70)
13	3122.01	3122.27	RELEELNVPGEIVE*s*L*sss*EESITR (β-1−25)

[a] "*s*" stands for phosphorylated serine.

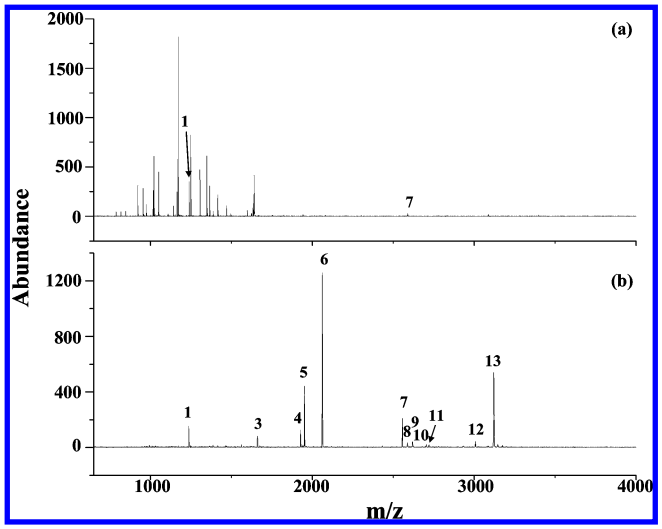


Figure 3. MALDI mass spectra of the tryptic digest product of the mixture (50 μL) of cytochrome C (2.5×10^{-6} M), BSA (2.5×10^{-6} M), α-casein (5×10^{-8} M), and β-casein (5×10^{-8} M) (a) prior to enrichment and (b) after enrichment by the magnetic particles coated with alumina.

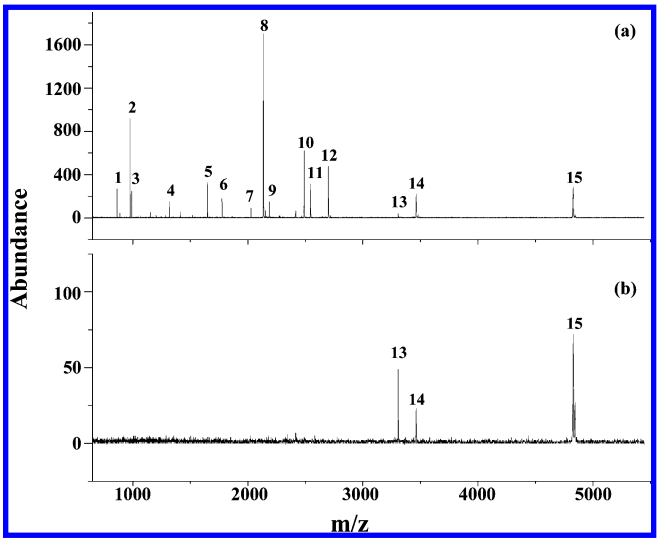


Figure 4. MALDI mass spectra of the tryptic digest product of PPI 1 (a) prior to enrichment (10^{-5} M) and (b) after enrichment (5×10^{-10} M, 50 μL) by the magnetic particles coated with alumina.

residue ion peaks appear in the mass spectrum. Their corresponding sequences are listed in Table 3. Among these peaks, five phosphopeptides marked with numbers 3, 4, 5, 10, and 11 are derived from α-S1-casein, and four phosphopeptides marked with numbers 1, 8, 9, and 12 are derived from α-S2-casein, whereas three phosphopeptides marked with numbers 6, 7, and 13 are derived from β-casein. Although there are more than 10 phosphopeptides in the same sample solution and the concentration of phosphorylated peptides are much lower than non-phosphorylated peptide residues derived from cytochrome C and BSA, there is no serious suppression effect between them during trapping and analysis, meaning that all the phosphopeptide resides can be trapped simultaneously with no interference. Furthermore, there are no non-phosphopeptides appearing in the mass spectrum. The results indicate that the alumina-coated magnetic particles have a quite good selectivity for phosphopeptides. The trapping selectivity is much better than that of titania-coated magnetic particles, which were previously demonstrated by our research group. In the previous study,[16] we demonstrated that when using the titania-coated magnetic particles as the affinity probes to selectively enrich their target peptides from a tryptic digest of the mixture of cytochrome C and β-casein, several non-phosphopeptides containing multi-acidic amino acids also appeared in the mass spectrum after enrichment by the titania-coated magnetic particles. The comparison implies that the alumina-coated magnetic particles have better trapping selectivity for phosphopeptides than the titania-coated magnetic particles.

Both caseins (α and β) are phosphorylated on serine sites, so we investigated a protein phosphorylated on threonine, human protein phosphatase inhibitor 1 (PPI 1), which contains one phosphorylated threonine. Figure 4a displays the direct MALDI mass spectrum of tryptic digest of PPI 1 (10^{-5} M) prior to enrichment. The corresponding sequences for all the peaks in the mass spectrum are listed in Supporting Information. The peaks marked with the numbers 13, 14, and 15 are the phosphopeptide residues. The peak marked with the number 13 is barely seen. However, after enrichment, the ion intensities of the phosphorylated peptides are greatly improved although the concentration is quite low (5×10^{-10} M, 50 μL). Furthermore, non-phosphorylated peptide residue ions do not appear in the mass spectrum. The results demonstrate that magnetic particles coated with alumina are effective trapping probes toward threonine phosphorylated peptides.

Additionally, we also investigated the possibility by using our particles to selectively trap phosphoproteins directly from a

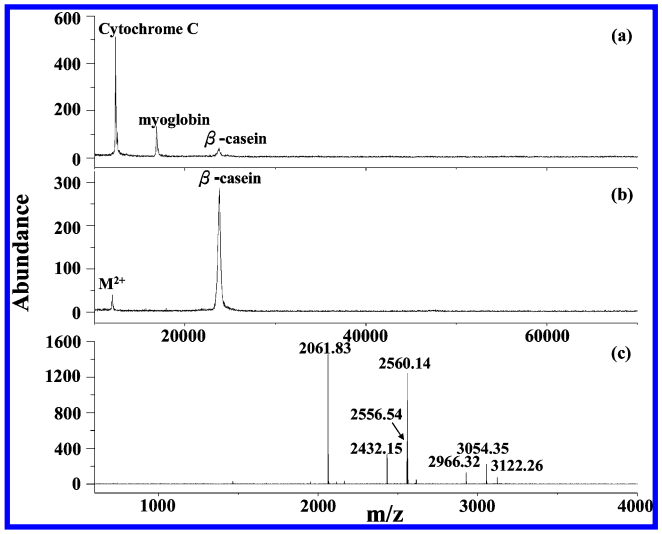


Figure 5. MALDI mass spectra of a protein mixture containing β-casein, cytochrome C, myoglobin, and BSA (2×10^{-6} M for each protein): (a) before enrichment and (b) after enrichment by the magnetic particles coated with alumina. The particles conjugated with target proteins were carried out on-particle tryptic digestion. (c) MALDI mass spectrum of the sample obtained using the particles remaining in the digest solution, the same sample that use for obtaining Figure 5b, to selectively trap target peptides from the same solution after acidification by 0.15% TFA.

protein mixture. Figure 5a presents the MALDI mass spectrum of a protein mixture containing β-casein, cytochrome C, myoglobin, and BSA (2×10^{-6} M for each protein). The molecular ion peak of cytochrome C dominates the mass spectrum, and myoglobin molecular ion peak also appears in the mass spectrum. A weak peak of β-casein reveals at m/z about 23500, while no BSA molecular ion peak is found in the same mass spectrum. Figure 5b display the MALDI mass spectrum of the protein mixture obtained using the particles coated with alumina to selectively trap their target proteins. The molecular ion peak of β-casein appearing at m/z about 23500 dominates the mass spectrum, while its doubly charged ion also appears at m/z about 11700 (M^{2+}). Furthermore, no cytochrome C and myoglobin ion peaks are observed in the mass spectrum after enrichment. For further characterization, the proteins trapped on the particles were carried out on-particle trypsin digestion at 38 °C for 20 min. Figure 5c presents the MALDI mass spectrum of the sample obtained using the particles remaining in the digest solution as that used in Figure 5b to selectively trap phosphopeptides from the same solution after acidification by 0.15% TFA. The peaks at m/z 2061.83 (#33−48), 2432.15 (#30−48), 2556.54 (#33−52), 2560.14 (#29−48), 2966.32 (#2−25), 3054.35 (#29−52), and 3122.26 (#1−25) are derived from β-casein. No non-phosphorylated peptide residues appear in the mass spectrum after enrichment. The results indicate that the magnetic particles coated with alumina also have the capacity to selectively enrich either phosphoproteins or phosphopeptides from a complex mixture. Furthermore, it only took 20 min to carry out on-particle tryptic digestion. The phosphorylated peptides could be readily enriched using the same particles remaining in the digest solution followed by characterization using MALDI MS.

To demonstrate the feasibility of this approach for a real sample, we employed our particles to selectively trap phosphoproteins from a milk sample, which commonly contains

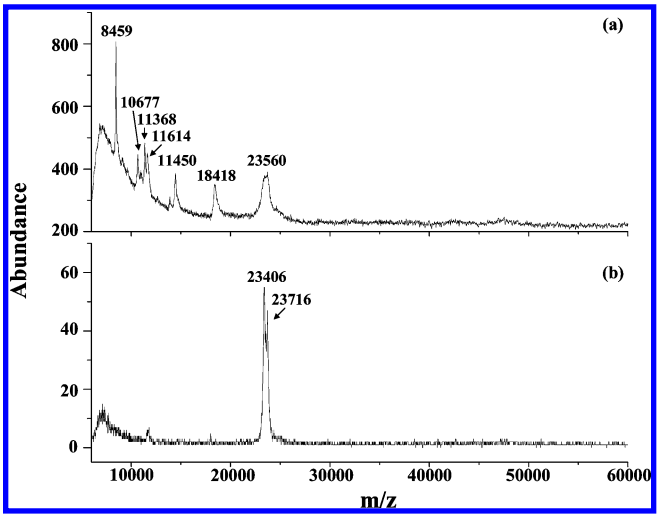


Figure 6. (a) Direct MALDI mass spectrum of a nonfat milk sample. (b) MALDI mass spectrum of the sample obtained using the magnetic particles coated with alumina to selectively enrich target proteins from the milk sample.

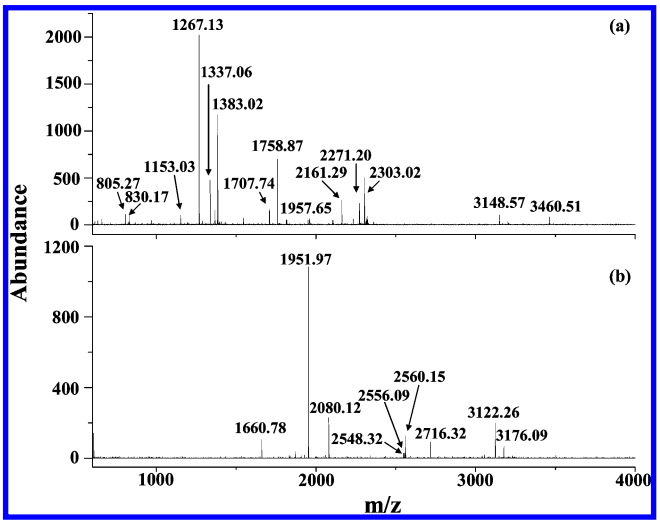


Figure 7. (a) MALDI mass spectrum of on-particle tryptic digest product of the sample, same as that used for obtaining Figure 6b. (b) MALDI mass spectrum of the sample obtained using the particles remaining in the digest solution, the same sample used for obtaining Figure 7a, to selectively trap target peptides from the same solution after acidification by 0.15% TFA.

abundant proteins including phosphoproteins, i.e., α- and β-caseins. Figure 6a presents the direct MALDI mass spectrum of a milk sample. There are several peaks appearing at $m/z >$ 8000. Figure 6b present the MALDI mass spectrum obtained using our particles to trap target species from the milk sample. Two unresolved peaks appearing at m/z about 23500 dominate the mass spectrum. The values of m/z resemble to those of α- and β-caseins. We confirmed the results by carrying out on-particle tryptic digestion by adding trypsin solution to the particle-target species conjugate. Figure 7a presents the MALDI mass spectrum of the on-particle digest product. There are many peaks appearing at m/z 800∼3500. Figure 7b displays the MALDI mass spectrum of the sample obtained using the particles remaining in the digest solution, the same as that used for obtaining Figure 7a, to selectively target phosphopeptides from the same solution after acidification by 0.15% TFA. The peaks at m/z 1660.78 (#106−119), 1951.97 (#104−119), 2080.12

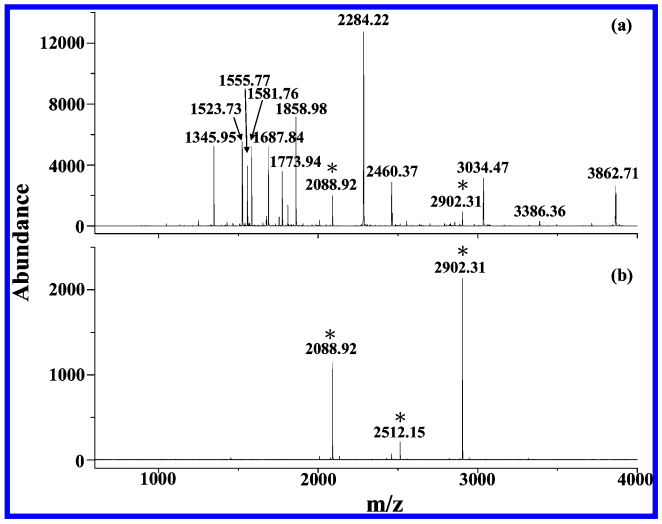


Figure 8. (a) Direct MALDI mass spectrum of the tryptic digest of egg white. (b) MALDI mass spectrum of the sample obtained using the magnetic particles coated with alumina to selectively enrich target species from the tryptic digest of egg white.

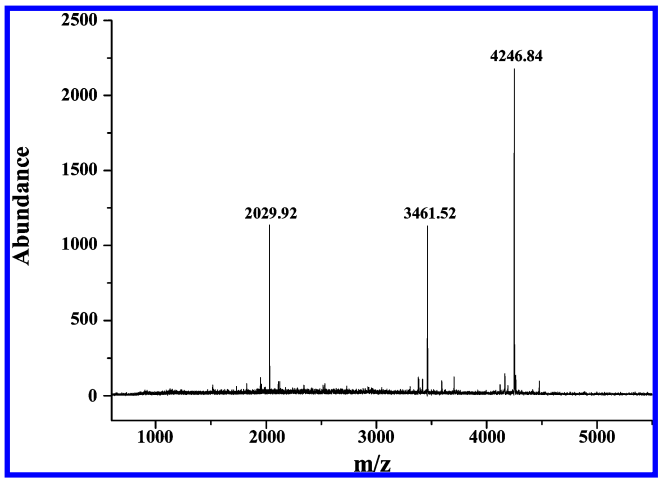


Figure 9. MALDI mass spectrum of the sample obtained using the magnetic particles coated with alumina to selectively enrich target peptides from the tryptic digest of HEp-2 cell lysate infected by *S. pyogenes*.

(#103−124), and 2548.32 (#104−124) are derived from α-casein (S1), whereas the peak at *m*/*z* 2716.32 (#115−137) is derived from α-casein (S2). Additionally, the peaks at *m*/*z* 2556.09 (#33−52), 2560.15 (#29−48), and 3122.26 (#1−25) correspond to the phosphopeptide residues of β-casein. All the peaks in the mass spectrum are identified as the phosphopeptide residues from either α-casein or β-casein. Without enrichment, no phosphopeptide peaks are observed in the MALDI mass spectra. The results demonstrate again that the magnetic particles coated with alumina are very effective in selectively trapping phosphopeptides from a very complex sample.

Additionally, we employed egg white, which contains abundant proteins including a phosphoprotein, i.e., ovalbumin, as the real sample to demonstrate the capacity of the affinity probes in selectively enriching phosphorylated species from this complex sample. Figure 8a presents the direct MALDI mass spectrum of the tryptic digest of egg white prior to enrichment. Many ions appear in the mass spectrum, while only the peaks marked with asterisks at *m*/*z* 2088.92 and 2902.31 are phosphorylated peptides generated from ovalbumin. The detailed sequences are listed in the Supporting Information. Figure 8b presents the MALDI mass spectrum obtained using our affinity probes to selectively enrich their target species from tryptic digest of egg white. Three noticeable peaks at *m*/*z* 2088.92, 2512.15, and 2902.31 (see Supporting Information) corresponding to the phosphorylated peptide residues generated from ovalbumin appear in the mass spectrum. The results indicate that the magnetic particles coated with alumina have a high selectivity for phosphopeptides from tryptic digest of egg white.

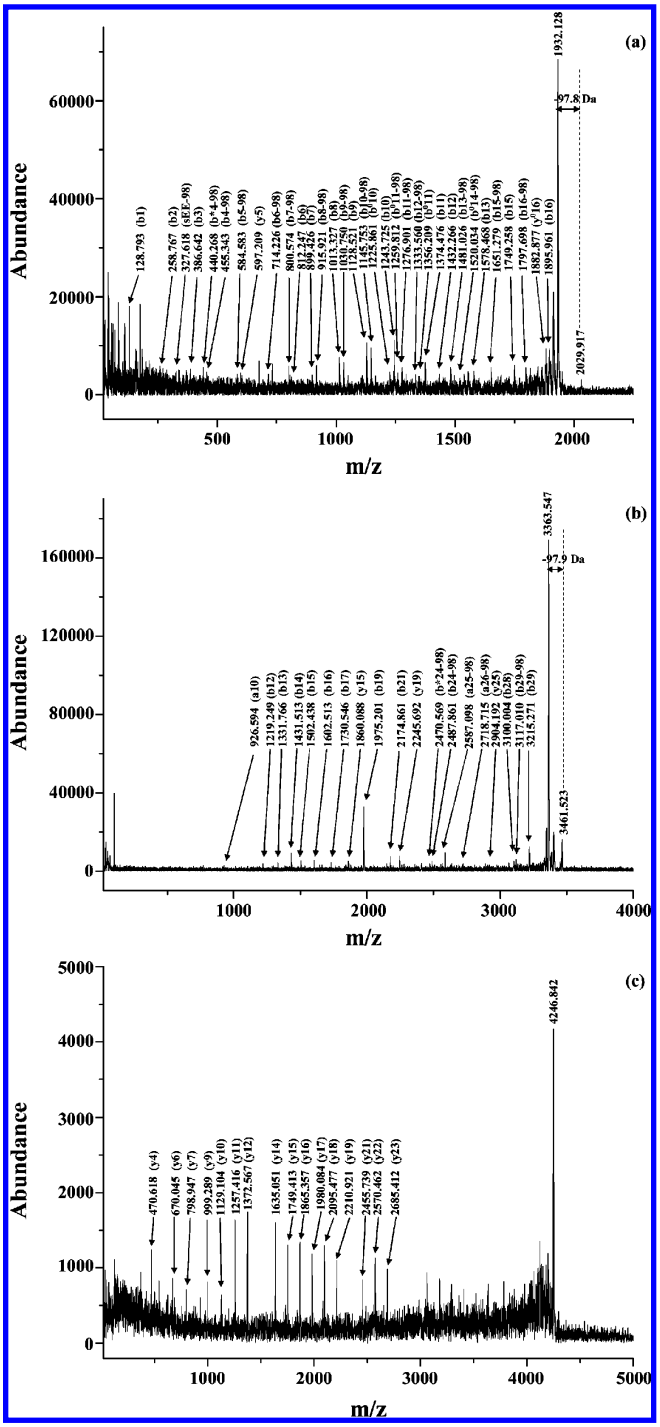


Figure 10. MALDI TOF−TOF mass spectra of the peaks at (a) *m*/*z* 2029.92, (b) *m*/*z* 3461.52, and (c) *m*/*z* 4246.84 (the same sample as that used in Figure 9).

Table 4. Peptide Ion Peaks Observed in the MALDI Mass Spectrum of Tryptic Digest of a HEp-2 Cell Lysate after Enrichment by Magnetic Particles Coated with Alumina

observed	Mr (expt)	Mr (calc)	delta	miss	score	expect	rank	sequences
2029.92	2028.91	2028.72	0.19	1	**93**	3.9e-08	1	KEE*s*EESDDDMGFGLFD*[a,b]
3461.52	3460.52	3460.41	0.10	0	**58**	1.1e-04	1	CGSGPVHISGQHLVAVE EDAE*s*EDEEEEDVK**[a,c]
4246.84	4245.83	4245.54	0.29	2	**114**	2.2e-10	1	LAADEDDDDDDEEDDD EDDDDDDFDDEEAEEKAPVKK**[c]

[a] "*s*" stands for phosphorylated serine. [b] "*" stands for 60S acidic ribosomal protein P1 (P05386), MASVSELACI YSALILHDDE VTVTEDKINA LIKAAGVNVE PFWPGLFAKA LANVNIGSLI CNVGAGGPAP AAGAAPAGGP APSTAAAPAE EKKVEAK**KEE SEESDDDMGF GLFD** [c] "**" stands for nucleophosmin (nucleolar phosphoprotein B23) (P06748), MEDSMDMDMS PLRPQNYLFG CELKADKDYH FKVDNDENEH QLSLRTVSLG AGAKDELHIV EAEAMNYEGS PIKVTLATLK MSVQPTVSLG GFEITPPVVL RLK **CGSGPVH ISGQHLVAVE EDAESEDEEE EDVK** LLSISG KRSAPGGGSK VPQKKVK**LAA DEDDDDDDEE DDDEDDDDDD FDDEEAEEKA PVKK**SIRDTP AKNAQKSNQN GKDSKPSSTP RSKGQESFKK QEKTPKTPKG PSSVEDIKAK MQASIEKGGS LPKVEAKFIN YVKNCFRMTD QEAIQDL-WQW RKSL

To enrich phosphorylated proteins and peptides directly from cell lysate samples remains as a common interest for the researchers involved in phosphoproteomics. Thus, we also investigated the feasibility of employing our affinity probes for selective enrichment of phosphopeptides from a cell lysate. The details for preparation of the cell lysate are stated in Experimental. Figure 9 presents the MALDI mass spectrum obtained using our affinity probes to selectively enrich target species from the tryptic digest of a HEp-2 cell lysate. Three remarkable peaks at m/z 2029.92, 3461.52, and 4246.84 appear in the mass spectra. To confirm the identities of these peaks, MALDI-TOF−TOF mass spectrometry was employed for characterization. Figure 10 a−c presents the MS/MS spectra for the peaks at m/z 2029.92, 3461.52, and 4246.84, respectively. These MS/MS ions were subjected into SwissProt protein database for matching the possible proteins. The MS/MS result of the peak at m/z 2029.92 is matched to a peptide residue derived from 60S acidic ribosomal protein P1 (P05386), whereas the MS/MS results of the peaks at m/z 3461.52 and 4226.84 are matched to two peptide residues from nucleolar phosphoprotein B23 (P06748). Table 4 lists the details of the matched sequences. The results indicate that the peaks at m/z 2029.92 and 3461.52 are phosphopeptide residues derived from 60S acidic ribosomal protein P1 and nucleolar phosphoprotein B23, respectively. Although the peak at m/z 4246.84 is not a phosphopeptide, the matched peptide sequence composed of 37 amino acids contains 26 acidic amino acids. Thus, it is not surprising to observe that these peptide residues in the cell lysate can be selectively enriched by our affinity probes. Nucelrolar phosphoprotein B23 has been known to be abundant in tumor cells.[25] On the basis of these results, it seems that theses two proteins are abundant in HEp-2 cells after bacterial infection, although further studies are required to clarify the details. Nevertheless, these results also support that the magnetic particles coated with alumina can be directly employed to enrich phosphopeptides from cell lysates without requiring any purification steps. To the best of our knowledge, we are the first to use affinity probe-based method combined with MALDI MS to characterize the phosphopeptides directly from a tryptic digest of a complex cell lysate without using any extra separation treatment or immunoprecipitation steps.

In this study, we always acidified the samples by TFA solution (0.15%) before enrichment by the affinity probes to prevent the binding from non-phosphopeptides containing acidic amino acids. Acidification for high concentrations of samples may result in precipitation of proteins. Although we did not observe any precipitation in our samples presented in this study owing to low concentrations of samples were used for extraction, it might be a problem for real sample containing high concentrations of proteins. When encountering this problem, a series of dilution can be performed before carrying out extraction to avoid the precipitation problem. Because we have demonstrated that this approach is quite sensitive, appropriate sample dilutions will not cause difficulties in detection. Alternatively, it might be possible to raise the extraction solution up to pH 6 as that Weckwerth and coworkers[21] have used for extraction experiments to prevent the problem from protein precipitation of high concentrations of sample solutions.

Conclusions

We present an approach to enrichment of phosphorylated peptide residues in complex samples using the magnetic particles coated with alumina. Enrichment by this method is the quickest ever reported. In only 30 s, phosphopeptides sufficient for characterization by MALDI-MS can be enriched from very low concentrations of sample solutions. The time required for performing the whole process is within 5 min, including the time needed for phosphopeptide enrichment and MALDI MS analysis. A small volume (50 μL) and a low concentration (5×10^{-10} M) of phosphopeptide sample can be dramatically enriched and characterized using this approach. The approach provides high trapping-capacity with specificity and sensitivity for the enrichment of phosphorylated peptides from complex sample solutions. Additionally, we also demonstrated that our approach can be practically employed to selectively enrich phosphoproteins from complex samples followed by on particle tryptic digestion. The same particles remaining in the digest solution were then used to selectively enrich phosphopeptides from the same solution. We also demonstrated that the magnetic particles coated with alumina-based enrichment method combined with MALDI MS/MS analysis can be used to quickly enrich and characterize the identities of phosphoproteins directly from the tryptic digest of a cell lysate without extra purification steps or tedious treatment. All the results indicate this affinity probe-based enrichment approach has quite high selectivity and sensitivity for phosphorylated species. The results obtained using the magnetic particles coated with alumina as the affinity probes for phosphorylated species were superior to those obtained from any existing affinity particles, such as the titania-coated magnetic particles, in terms of selectivity and specificity. Thus, employing this approach to diverse cell lyastes should be very suitable.

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Supporting Information Available: The TEM image of magnetic particles coated with alumina and the direct laser desorption/ionization mass spectrum of the magnetic particles are displayed in Supplementary Figures 1 and 2, respectively. The corresponding sequences of the peaks appearing in Figures 4 and 8 are listed in Supplementary Tables 1 and 2, respectively. These materials are available free of charge via the Internet at http://pubs.acs.org.

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