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Short communication

Preparation of on-plate immobilized metal ion affinity chromatography platform via dopamine chemistry for highly selective isolation of phosphopeptides with matrix assisted laser desorption/ionization mass spectrometry analysis

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ABSTRACT

In this study, a novel on-plate IMAC technique was developed for highly selective enrichment and isolation of phosphopeptides with high-throughput MALDI-TOF-MS analysis. At first, a MALDI plate was coated with polydopamine (PDA), and then Ti^{4+} was immobilized on the PDA-coated plate. The obtained IMAC plate was successfully applied to the highly selective enrichment and isolation of phosphopeptides in protein digests and human serum. Because of no loss of samples, the on-plate IMAC platform exhibits excellent selectivity and sensitivity in the selective enrichment and isolation of phosphopeptides, which provides a potential technique for high selectivity in the detection of low-abundance phosphopeptides in biological samples.

1. Introduction

Protein phosphorylation is one of the most essential and universal post translational modifications (PTMs) of proteins, which is involved in almost all aspects of cell life, such as cell growth, division, migration and differentiation [1–3]. With the intention of studying these bioprocesses, much effort has been devoted to develop the methods and techniques of systematic identifying and characterizing phosphoproteins. Mass spectrometry (MS) strategies, the workhorse for detection and characterization of phosphoproteins, have emerged as a powerful technique due to its high speed and high throughput features, such as matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis [4–6]. Unfortunately, the large amount of non-phosphopeptides contained in various biological samples suppresses the detection of phosphopeptides, which calls for the urgent demand of selective isolation of phosphopeptides from biological samples before MALDI-TOF-MS analysis.

Plenty of traditional off-target methods, such as immobilized metal ion affinity chromatography (IMAC), metal oxide affinity chromatography (MOAC) and functionalized nanoparticles, have been developed for selective enrichment and isolation of phosphopeptides before MALDI-TOF-MS analysis [7–11]. Zou et al. prepared Fe_3O_4@SiO_2@$(HA/CS)_{10}$-Ti^{4+} nano-materials for selective enrichment of phosphopeptides [12,13]. In our lab, Yan et al. developed polydopamine-coated grapheme with Ti^{4+} or TiO_2 immobilized as a platform for phosphoproteome analysis, which leads to excellent selectivity and sensitivity in the isolation of phosphopeptides for MALDI-MS analysis. However, the above methods mentioned will result in a series of problems, such as inevitable loss of samples, waste of materials and potential contaminants. In Tsai's work, a tip was packed with Ni-NTA silica resin and immobilized multimetal on it to enrich phosphopeptides, which could avoid the above problems mentioned. However, it was hard for it to realize high throughput analysis, and it also needed to elute during the process of phosphopeptides enrichment like other off-target methods [14]. To avoid these problems, on-target isolation prior to MALDI-TOF-MS analysis has been developed and attracted much attention recently, which involves in on-plate MOAC platform for the selective isolation of phosphopeptides. Lu et al. synthesized alumina hollow spheres for on-plate isolation of phosphopeptides [15]. Tan et al. functionalized a MALDI target plate with magnetic nanoparticles for phosphopeptides isolation [16]. Zeng et al. developed on-plate selective isolation of peptides/proteins for direct MALDI MS analysis [17]. Nonetheless, on-plate MOAC platform for selective isolation will

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lead to destruction of the ion source of mass spectrometry, owing to the access of those nanoparticles impacted by laser [18,19]. Therefore, it is crucial to develop novel on-plate isolation techniques for highly selective enrichment of phosphopeptides.

Many researches have proved that dopamine (DOPA) can self-polymerize on various substrates (such as metal) in mild condition [20–23]. Moreover, the metal ions such as Ti^{4+} can be directly immobilized on the surface of polydopamine (PDA) in mild conditions through the catechol groups in PDA coating [24,25] Based on these, in this study, we developed a novel on-plate IMAC platform, Ti^{4+}-immobilized MALDI plate by PDA layer, for isolation of bound phosphopeptides in direct MALDI-TOF MS analysis. At first, the MALDI plate was coated with PDA. And then Ti^{4+} was immobilized on the PDA layer. Our method avoided the problems of off-target methods and the MOAC platform. And it also realizes the high throughput analysis of phosphopeptides since the pretreatment is finished on the MALDI plate directly. Moreover, the prepared on-plate IMAC platform was applied to the highly selective enrichment and isolation of phosphopeptides without elution both in protein digests and human serum, which greatly shortened the enrichment time. Owing to no loss of samples, the Ti^{4+}-immobilized PDA-coated MALDI target plate (Ti^{4+}-PDA-plate) exhibits excellent selectivity and sensitivity in the isolation of phosphopeptides for MALDI-MS analysis.

2. Experimental

2.1. Chemicals and reagents

Dopamine hydrochloride was purchased from Alfa Aesar Johnson Malthey Company (Beijing, China). $Ti(SO_4)_2$ and NH_4OH were purchased from Sinopharm. Chemical Regents Co. Ltd. (Shanghai, China). The stainless-steel plate (384 Opti-TOF 123 mm × 81 mm SS) was purchased from AB Sciex (Massachusetts, USA). The standard peptide was purchased from China Peptides Co., Ltd. (Shanghai, China). Tris (hydroxymethyl) aminomethane (Tris), trifluoroacetic acid (TFA), β-casein, bovine serum albumin (BSA), trypsin (from bovine pancreas, TPCK treated), ammonium bicarbonate (NH_4HCO_3), and 2, 5-dihydroxybenzoic acid (DHB) were purchased from Sigma Chemical (St. Louis, MO). Acetonitrile was purchased from Shanghai Lingfeng Chemical Reagents Co. Ltd. (Shanghai, China). Distilled water was purified by a Milli-Q system (Milford, MA, USA). All other chemicals and reagents are of the highest grade and commercially available.

2.2. Preparation of IMAC plate

The synthetic strategy of IMAC plate is shown in Fig. 1. First, 400 mg of dopamine hydrochloride was dissolved in 200 mL of Tris buffer (10 mM, pH 8.5). The stainless-steel plate was washed with distilled water and ethanol several times and dried at room temperature. Then the clean target plate was immersed in the prepared solution of dopamine at room temperature for 24 h to form a PDA coating on the surface of the plate. The obtained PDA-coated plate was washed with distilled water for several times and immersed again in the aqueous solution of $Ti(SO_4)_2$ (100 mM) at room temperature for 2 h to immobilize Ti^{4+}. The product plate was washed with distilled water for several times and dried at room temperature.

2.3. Characterization

A Phillips XL30 electron microscope (Netherlands) was used to record scanning electronic microscope (SEM) images of materials which operated at 20 kV. An energy dispersive X-ray spectroscopy (EDX) was employed to identify the composition of materials.

2.4. Sample preparation

The protein (bovine serum albumin or bovine β-casein) was dissolved in NH_4HCO_3 buffer (25 mM, pH 8.3) and treated with proteomic-grade trypsin (2%, w/w) at 37 °C for 16 h. The digested products were stored below 0 °C. Human serum was centrifuged and the supernatant was stored below 0 °C.

2.5. On-plate selective enrichment and isolation of phosphopeptides

As shown in Fig. 1, the enrichment of phosphopeptides was performed by the modified MALDI plate. The IMAC plate was washed with 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) water solution three times. The digests of β-casein, the mixture digests of β-casein and BSA and human serum were diluted to various concentrations with 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) aqueous solution (v/v). Then 1 μL diluted digests were pipetted onto the modified plate and incubated for 30 min at room temperature and washed with 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) aqueous solution (v/v) several times to remove nonspecific adsorption. Finally, 1 μL of DHB aqueous solution (20 mg/mL, 50% acetonitrile and 1% H_3PO_4) was added at the spots as a MALDI matrix for further analysis by MALDI-TOF-MS.

2.6. MALDI-TOF MS analysis.

MALDI-TOF MS experiments were performed by a Proteomic Analyzer (mode 5800, AB Sciex, Framingham, MA, USA) with the Nd: YAG laser at 355 nm, a repetition rate of 200 Hz and an acceleration voltage of 20 kV in the positive ion reflection mode within a scan range of 1000–3500 *m/z*.

3. Results and discussion

Herein, a novel IMAC plate was prepared (Fig. 1). The synthetic IMAC plate was applied in the selective enrichment and isolation of phosphopeptides, which could minimize the loss of samples, the consumption of materials and reduce potential contaminants. Moreover, there was no need of elution, which resulted in the fast and high-throughput MALDI MS analysis after the phosphopeptides enrichment and isolation. The Ti^{4+} based IMAC plate was characterized by scanning electronmicroscopy (SEM) and energy dispersive X-ray spectroscopy (EDX).

3.1. Preparation and characterization of IMAC plate

Dopamine hydrochloride can self-polymerize oxidatively on the surface of various substrates in mild condition. The MALDI plate is silver metallic luster before modified with PDA. After the self-polymerization of DOPA, the color of MALDI plate changes to light brown, which indicates the successful polymerization of DOPA. The morphology of the PDA-modified plate was evaluated by SEM characterization. As shown in Fig. 2a, after immersing in DOPA solution for 10 h, DOPA monomer has a tendency to form free PDA (Fig. 2a) and we can also see that the MALDI plate exhibits the characteristic surface of metal defects. As time goes by, the free PDA particles grow up into PDA films [26]. In Fig. 2b, after modified with PDA for 24 h, the surface of MALDI target plate is firmly coated with a robust PDA layer. The thickness of PDA layer is determined by the concentration of DOPA solution and polymerization time. After modification of PDA, plenty of Ti^{4+} in the addition of titanium (IV) sulfate precursor was immobilized on the

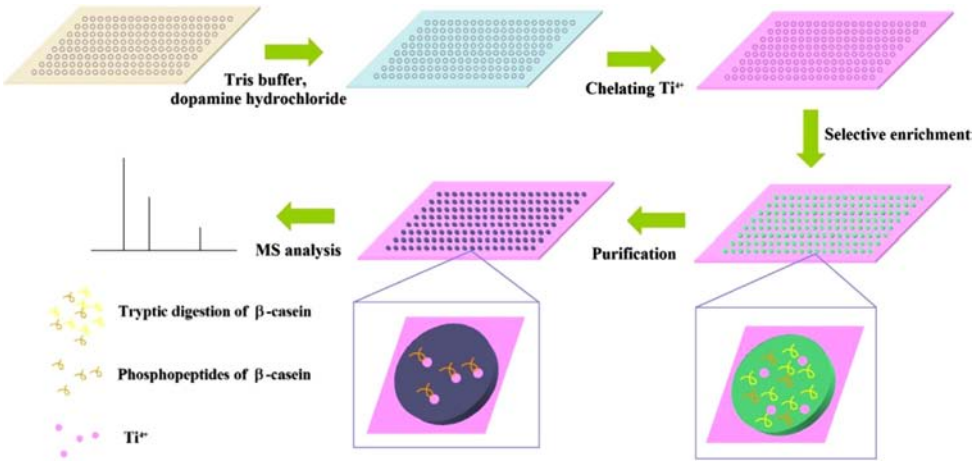

Fig. 1. The synthetic procedure for Ti^{4+}-PDA-plate and the procedure of phosphopeptides enrichment by utilizingTi^{4+}-PDA-plate as adsorbent.

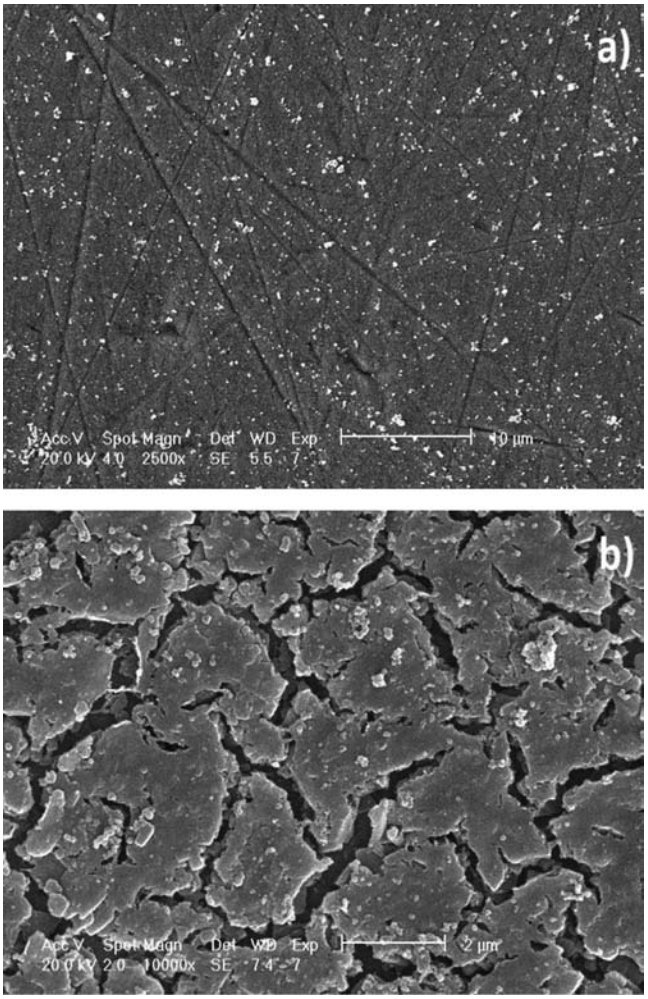

Fig. 2. SEM images of the PDA-modified plate. (a) After 10 h-modification and (b) after 24-h modification.

PDA layer. Ti^{4+} was characterized by EDX to confirm the successful immobilization [27]. As shown in Fig. S1, the detection of Ti element confirms the successful chelation of Ti^{4+} and catechol groups in PDA layer.

3.2. On-plate selective enrichment and isolation of phosphopeptides in tryptic digests of β-casein

Bovine β-casein, a standard phosphoprotein, which is commonly contaminated with a trace of α-casein, was employed to evaluate the performance of on-plate capture of Ti^{4+}-PDA-plate. As a control experiment, a tryptic digest of 0.8 pmol β-casein was deposited on the pristine MALDI target directly for comparison. As presented in Fig. S2a, direct analysis without enrichment leads to the detection of non-phosphopeptides, and only a few very weak peaks assigned to phosphopeptides can be observed, which illustrates that the detection of phosphopeptides is severely impacted by the suppression of non-phosphopeptides and the low concentration of phosphopeptides. After isolation with Ti^{4+}-PDA-plate, the target plate was analyzed by MALDI-TOF MS directly without the need of elution. As shown in Fig. S2b, 13 captured phosphopeptides dominate the spectrum and the signals of non-phosphopeptides apparently decrease. The peaks at *m*/*z* 2061, 2432, 2556 and 3122 are determined by phosphopeptides of β-casein digests. The peak at *m*/*z* 3122 corresponds to quadruple phosphopeptide of β-casein digests and the peaks at *m*/*z* 2061, 2432 and 2556 represent monophosphopeptides of β-casein digests. Table S1 lists the phosphopeptides detected in β-casein digests, including the mass of the phosphopeptides, their sequence and position of the phosphorylation. Furthermore, the double charge signal assigned to the one-half mass unit interval of the isotopic peaks at *m*/*z* 1031, 1562 and 1279, the dephosphorylated fragment of the phosphopeptides at *m*/*z* 1963, 2458 and 3025 through a mass shift of 98 Da, and three phosphopeptides derived from α-casein at *m*/*z* 1466, 1660 and 1952 are also observed successfully. The results indicate the specificity of the Ti^{4+}-PDA-plate for enrichment and isolation of phosphopeptides from β-casein digests.

In order to identify phosphopeptides, a MS/MS spectrum of the parent ion *m*/*z* 2061 signal is displayed in Fig. S3. The neutral loss of H_3PO_4 presented by the detected phosphopeptides in Fig. S3 provides a reliable proof to identify phosphopeptides. The peak at *m*/*z* 1964 accounts for the loss of H_3PO_4 (98 Da), which clearly reveals its monophosphorylation. It can also indicate that the phosphorylation site of the peak at *m*/*z* 2061 is at serine by 167 Da mass difference between fragment ions y13 (*m*/*z* 1619) and y14 (*m*/*z* 1786) corresponding to a phosphoserine residue. Therefore, the MS/MS spectrum in Fig. S3 explains the sequence and position of the phosphorylation.

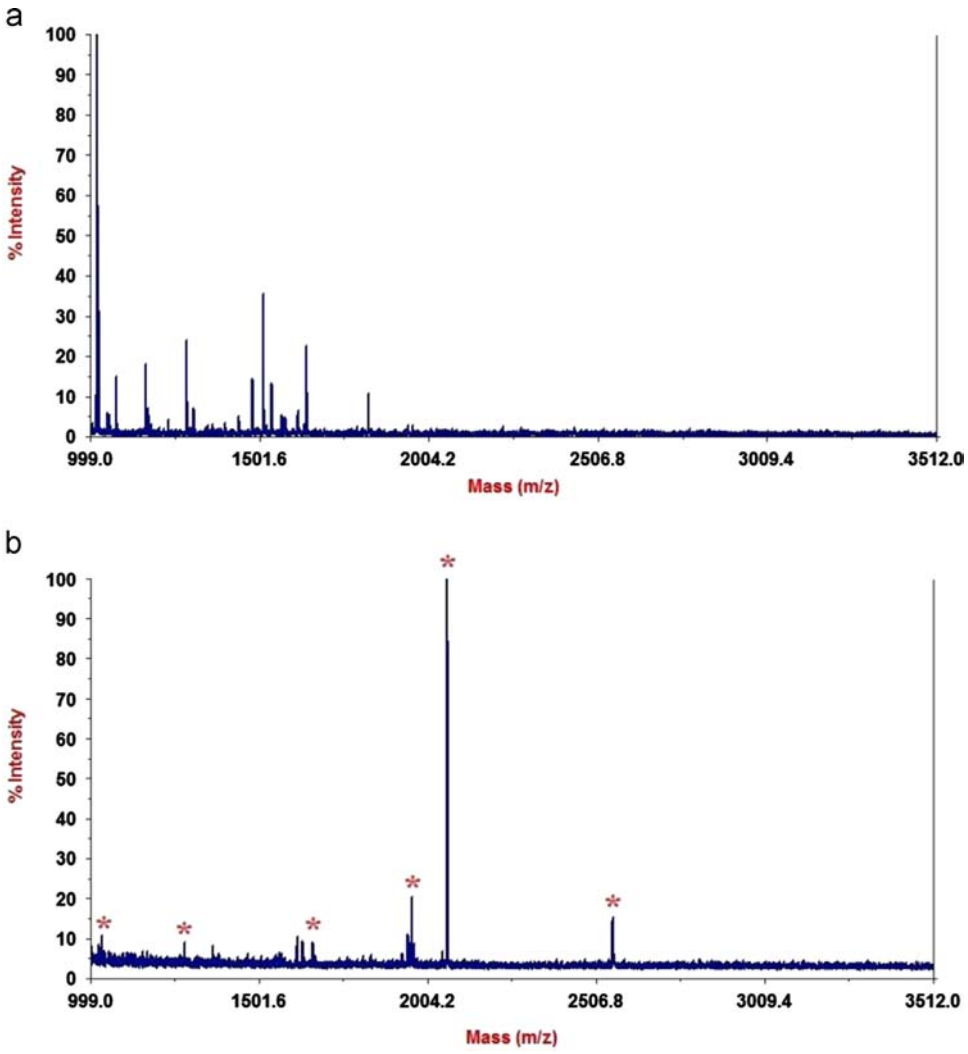

Fig. 3. The mass spectra of phosphopeptides derived from a peptide mixture of β-casein and BSA at a molar ratio of 1:200: (a) before and (b) after enrichment by Ti^{4+}-PDA-plate. The peaks of phosphopeptides are marked with "*".

3.3. The detection sensitivity and saturation point of IMAC plate.

To further test the detection sensitivity of Ti^{4+}-PDA-plate in enrichment and isolation of phosphopeptide, β-casein digests with ultra-low concentrations were employed. The captured phosphopeptides from the tryptic digests of β-casein at 80 fmol, 8 fmol and 0.8 fmol are displayed in Fig. S4. When the concentration is 0.8 fmol, there are still 6 peaks of phosphopeptides detected in the mass spectrum after isolation with Ti^{4+}-PDA-plate, which showed much higher detection sensitivity than that in Tan's work (10 fmol) [16]. The result shows that Ti^{4+}-PDA-plate can be introduced to detect β-casein digests with ultra-low concentration, which indicates the high detection sensitivity of the Ti^{4+}-PDA-plate.

The saturation point of Ti^{4+}-PDA-plate was also studied. The standard peptide (pSADGQHAGGLVK) at *m*/*z* 1219 with different concentrations was employed. As shown in Fig. S5a, the peak of phosphopeptide from the supernatant of the standard peptide with concentration at 1.25 μg/mL can be detected. When the concentration is as low as 312.5 ng/mL, a weak peak of phosphopeptide can be detected (Fig. S5b). The result indicates that the maximum amount of phosphopeptides that can be enriched is 110.524 $\mu g/m^2$, which may be related to the number of available sites and the stoichiometry of the binding.

3.4. On-plate selective enrichment and isolation of phosphopeptides in the mixture of tryptic digests of β-casein and BSA

The selective trapping ability of Ti^{4+}-PDA-plate toward phosphopeptides was investigated with a peptide mixture of tryptic digests including β-casein (0.8 pmol) and BSA at ratios of 1:200 (mol/mol). As a control experiment, a direct analysis of peptide mixture on the pristine MALDI target is displayed in Fig. 3a. Owing to the introduction of BSA, the signals corresponding to phosphopeptides are severely suppressed by non-phosphopeptides and no phosphopeptide signals can be detected before enrichment. While after the mixtures treated with Ti^{4+}-PDA-plate, 6 intensive peaks originated from phosphopeptides are easily captured with strong intensities, high S/N ratio and clear background (Fig. 3b). The results indicate that Ti^{4+}-PDA-plate has a high trapping ability toward phosphopeptides even with 200-fold dilution by peptides from non-phosphoproteins. Moreover, PDA based materials do not have serious nonspecific adsorption of target molecules.

3.5. The stability and reusability of the IMAC plate.

We also studied the stability of Ti^{4+}-PDA-plate by employing β-casein digests in this work. The modified plate was stored at room temperature for one month and employed to enrich phosphopeptides

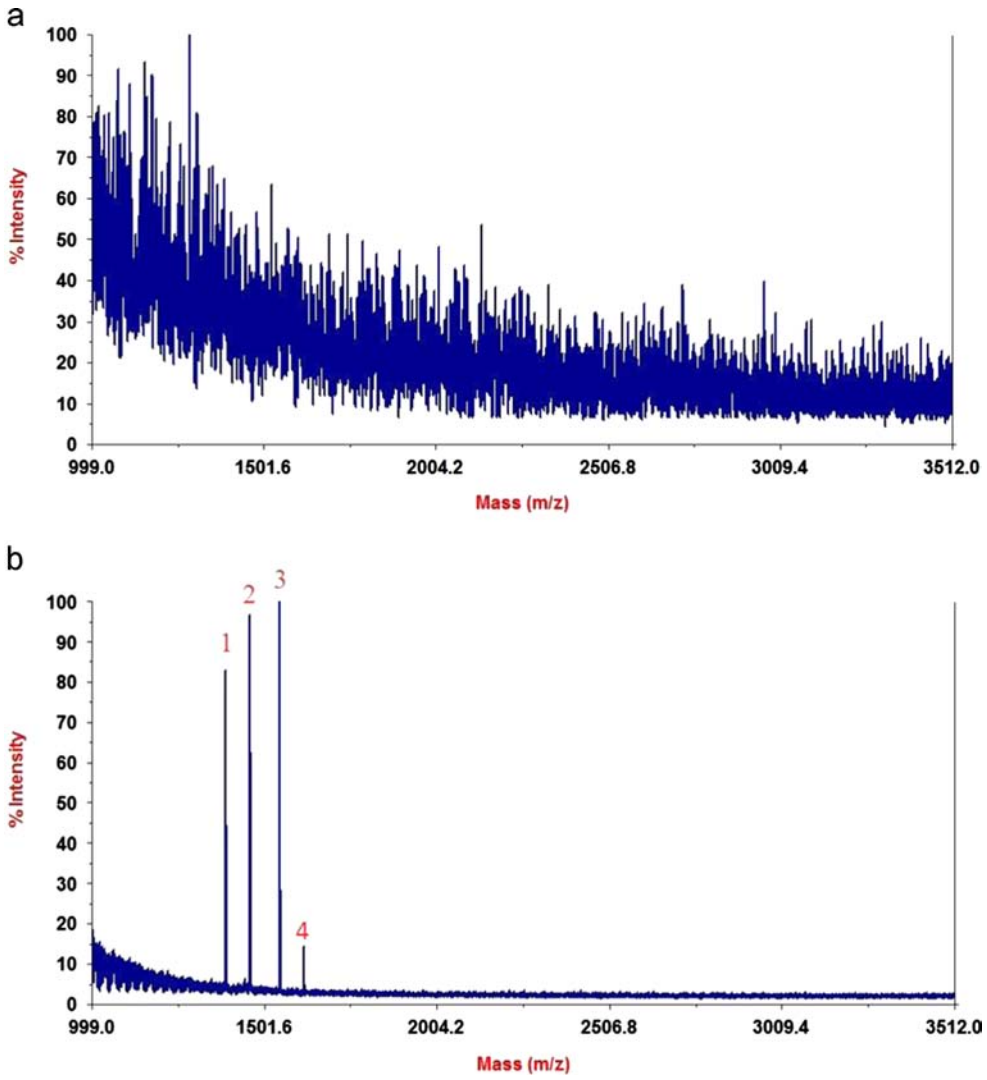

Fig. 4. The mass spectra of peptides derived from human serum (a) before enrichment and (b) after enrichment by Ti^{4+}-PDA-plate. The peaks of phosphopeptides are marked with numbers.

from β-casein digests. As presented in Fig. S6, the peak intensity of final spectrum was nearly the same as that in the spectrum obtained by freshly made modified plate, which proves the good stability of Ti^{4+}-PDA-plate. To evaluate the reusability of Ti^{4+}-PDA-plate, we employed a peptide mixture of standard peptide (pSADGQHAGGLVK) at *m*/*z* 1219 and BSA tryptic digests at ratios of 1:1 (mol/mol) in this work. The used Ti^{4+}-PDA-plate was regenerated and incubated with 0.4 M of NH_4OH for 20 min and rinsed with water for three times to remove the absorbed phosphopeptide. The regenerated spots were used to enrich the peptide mixture for five times repeatedly. We calculated the relative standard deviation of the peak intensities, which is 5.56% for the detection of the peptide mixture. It was also shown in Fig. S7 that similar spectra were obtained in the fifth time as the first one, which indicates the excellent reusability of Ti^{4+}-PDA-plate.

3.6. On-plate selective enrichment and isolation of phosphopeptides in human serum.

The as made Ti^{4+}-PDA-plate reveals excellent capacity of phosphopeptides enrichment. In order to demonstrate the feasibility of our method to complex samples, we carried out the on-plate enrichment of phosphopeptide from human serum, which is expected containing four endogenous phosphopeptides with molecular masses of 1389, 1460, 1545 and 1616 Da coming from fibrinogen α-chain [28–31]. Because a large amount of non-phosphopeptides, abundant proteins and inorganic salts existing in the serum increases the complexity of condition largely, the detection of endogenous phosphopeptides contained in human serum will be suppressed, which results in almost no peptides found in the spectrum before enrichment (Fig. 4a). However, after enrichment with Ti^{4+}-PDA-plate, 4 phosphopeptide peaks were detected clearly with high S/N ratio and strong intensities in Fig. 4b. The peaks at *m*/*z* 1389, 1460, 1545 and 1616 are assigned to endogenous monophosphopeptides coming from fibrinogen α-chain in human serum. Table S2 lists the phosphopeptides detected in human serum, including the mass of the phosphopeptides, their sequence and position of the phosphorylation. Cheng et al. prepared magnetic affinity microspheres with meso-/macroporous shells for selective enrichment and fast separation of phosphorylated biomolecules [29]. He also elevated the prepared microspheres in human serum for phosphopeptides enrichment, the results of which are similar with ours. However, our method realizes the high throughput analysis of phosphopeptides. So the results we obtained provide a satisfactory approach to enrich endogenous phosphopeptides in human serum.

4. Conclusion

In summary, we developed a new phosphopeptide-selective MALDI plate with coated PDA layer and immobilized Ti^{4+} in this work. The synthetic IMAC plate shows superb selectivity, sensitivity, and stability both in protein digests and complex biosamples. In addition, the modified plate strategy can minimize the loss of samples, the consumption of materials and reduce potential contaminants. Most importantly, during the process of enrichment and isolation, there is no need of elution, which results in the fast and high-throughput MALDI MS analysis. This method provides a promising method for highly selective enrichment and isolation of phosphopeptides.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.12.041.

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