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Polydopamine-coated eppendorf tubes for Ti^{4+} immobilization for selective enrichment of phosphopeptides

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ABSTRACT

Mass spectrometric technique has emerged as a preferred technique in the analysis of protein phosphorylation. Owing to the low stoichiometry of phosphopeptides and the signal suppression effect by non-phosphopeptides, there is a demand for efficient enrichment of phosphopeptides. The selective enrichment of phosphopeptides in modified eppendorf tubes prior to mass spectrometry analysis, which can minimize sample loss as well as nonspecific interferences effectively, has become a hot topic in current proteomics field. In our work, an easy-to-use phosphopeptide-selective eppendorf tube was initially prepared, with its inner surface being modified with a Ti^{4+}-immobilized polydopamine (PDA) layer. The unique Ti^{4+}-immobilized PDA-modified eppendorf tubes (EP tube@PDA-Ti^{4+}) are investigated for its application in selective enrichment of phosphopeptides from complex biological samples. Due to the high Ti^{4+} loading amount on the surface of PDA, the EP tube@PDA-Ti^{4+} exhibits remarkable phosphopeptide enrichment ability in protein digests and human serum, which presents a powerful evidence for its high selectivity in detecting the low-abundance phosphopeptides from complex biological samples.

1. Introduction

Protein phosphorylation is a common post-translational modification (PTM) in mammalian systems, which can broaden biological functionality of proteins. Protein phosphorylation affects around 30% of a proteome and plays an important role in major regulatory machinery for many complex biological processes, such as intracellular signaling transduction and cellular growth and division [1–3]. However, owing to the complexity of the phosphoproteome, the low stoichiometry of phosphopeptides, the signal suppression effect by non-phosphorylated peptides in protein digests, and the dynamic nature of signaling networks during mass spectrometry analysis, phosphopeptides may be difficult to be detected by mass spectrometry [4]. Therefore, more and more techniques have been developed to selectively enrich phosphopeptides from highly complex mixtures in order to increase detection sensitivity for efficient phosphopeptides identifications, such as immobilized metal ion affinity chromatography (IMAC), metal oxide affinity chromatography (MOAC), strong cation exchange chromatography, mesoporous nanostructure materials, chemical-modification strategies and immunoprecipitation [5–9].

Among these techniques, IMAC and MOAC are the most effective and convenient and have been widely applied in the selective enrichment of phosphopeptides. For example, Lu et al. reported synthesis of Fe_3O_4@mesoporous TiO_2 microspheres for selective enrichment of phosphopeptides for phosphoproteomics analysis [10] and modified graphene with TiO_2 for specific capture of phosphopeptides [11]. Yan et al. reported functionalized carbon nanotubes with titania nanoparticles for selective enrichment of phosphopeptides for mass spectrometry analysis [12]. Xu et al. reported synthesis of magnetic microspheres with immobilized metal ions for selective enrichment and analysis of phosphopeptides [13].

Recently, it has been proved that dopamine (DOPA), a biomolecule with catechol and amine functional groups found in high concentration in the adhesive protein Mefp-5 (Mytilus edulis foot protein 5) secreted from mussels, has several advantages, such as excellent environmental stability, good biocompatibility and splendid hydrophilicity. DOPA can generate into a thin adherent polydopamine (PDA) films on a variety of substrates via the oxidative self-polymerization of the PODA in a basic solution [14]. In addition, the catechol groups in PDA coating are capable of strong metal ions coordination [15,16], which can facilitate to in situ deposit metal ions on the surfaces of PDA at mild conditions. More recently, in our group, PDA has successfully been developed as a novel chelating ligand for metal ions to achieve enrichment of phosphopeptides [17,18].

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In order to reduce sample loss due to adsorption on container walls and lead to high-throughput analyses, IMAC and MOAC have been developed to on-plate selective enrichment and pipette-tips selective enrichment in the recent years. For example, Lu et al. modified the MALDI target plate with alumina hollow spheres proposed for pre-treatment of phosphopeptides in biological samples [19]. Hsieh et al. developed a titanium dioxide nanoparticle pipette-tip for selective enrichment of phosphorylated peptides [20]. However, to our knowledge, there is no eppendorf tubes (EP tubes) strategy using IMAC for the selective enrichment of phosphopeptides. EP tubes, which are used to store up liquid, are commonly requisites in laboratory. It can eliminate carry-over and minimize the sorbent consumption to develop IMAC to EP tubes selective enrichment since there is no need for magnetic separation or high-speed centrifugation. Therefore, developing EP tube enrichment technique for phosphopeptides is very interesting and important.

In this study, an easy-to-use phosphopeptide-selective EP tube based on dopamine chemistry was initially prepared, with its inner surface being modified by a Ti^{4+}-immobilized PDA layer. The performance of the as-prepared EP tube@PDA-Ti^{4+} was investigated for its application in selective enrichment of phosphopeptides from complex biological samples. Due to the high Ti^{4+} loading amount on the surface of polydopamine, the EP tube@PDA-Ti^{4+} exhibits remarkable ability of enrichment for phosphopeptides in the presence of numerous nonphosphopeptides in protein digests and human serum. The excellent hydrophilic property, selectivity, sensitivity, detection limit, reusability and stability were also proven.

2. Experimental

2.1. Chemicals and reagents

β-casein, bovine serum albumin, L-1-tosylamido-2-phenylethyl-chloromethyl ketone (TPCK) treated trypsin (from bovine pancreas), ammonium bicarbonate (NH_4HCO_3), trifluoroacetic acid (TFA), 3-(trihydroxysilyl)propyl methylphosphate, 2,5-dihydroxybenzoic acid (DHB) and Tris were purchased from Sigma Chemical (St. Louis, MO). Dopamine hydrochloride was purchased from Alfa Aesar Johnson Malthey Company. $Ti(SO_4)_2$ was purchased from Sinopharm Chemical Regents Co. Ltd (Shanghai, China). Acetonitrile was purchased from Shanghai Lingfeng Chemical Reagents Co. Ltd (Shanghai, China). Eppendorf tubes were purchased from Axygen Inc. All aqueous solutions were prepared using Milli-Q water purified by Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Modification of the inner surface of eppendorf tubes

The synthetic protocol for preparation of EP tube@PDA-Ti^{4+} is presented in Fig. 1a. Firstly, Tris (12.1 mg) was dissolved in 10 mL distilled water by sonication to form 10 mM Tris buffer. 200 μL solution of PDA (2 mg/mL, dissolved in 10 mM Tris buffer) was added into eppendorf tube (500 μL). The tube was exposed to light and capped and left at room temperature for 24 h to produce a PDA coating on the inner surfaces of eppendorf tube. The obtained PDA-coated eppendorf tubes were washed with Milli-Q water (400 μL) three times by sonication for 1 min and dried in vacuum at 50 °C over night (0.085 mbar).

Secondly, 200 μL solution of $Ti(SO_4)_2$ (100 mM, aqueous solution) was added into the eppendorf tubes modified by PDA, and left at room temperature for 2 h to immobilize Ti^{4+} cation. The resultant eppendorf tubes were washed with Milli-Q water (400 μL) three times by sonication for 1 min and dried in vacuum at 50 °C over night (0.085 mbar).

2.3. Characterization

A Philips XL30 electron microscope (The Netherlands) was employed to record scanning electronic microscope (SEM) images of materials operating at 20 kV. A LabRam-1B Raman spectrometer was employed to record the Raman spectra with a laser at an excitation wavelength of 632.8 nm at room temperature.

2.4. Sample preparation

The protein (bovine serum albumin or bovine β-casein) was dissolved in 25 mM NH_4HCO_3 buffer (pH 8.3) containing proteomic-grade trypsin (2%, w/w) at 37 °C with overnight shaking. The digested products were diluted to various concentrations and stored below 0 °C. Human serum was diluted to ten times with 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) aqueous solution (v/v).

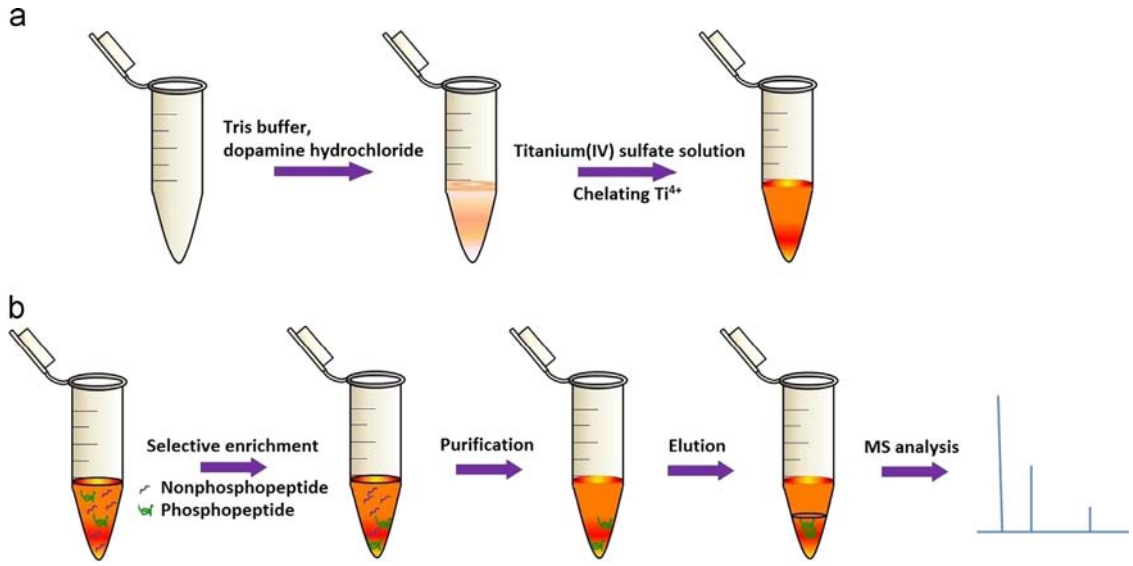

Fig. 1. (a) The synthetic procedure for Ti^{4+}-immobilized PDA-modified eppendorf tubes. (b) The procedure of phosphopeptides enrichment by utilizing Ti^{4+}-immobilized PDA-modified eppendorf tubes as adsorbent.

2.5. Phophopeptide enrichment

As shown in Fig. 1b, the enrichment of phosphopeptide from tryptic digestion of standard proteins was performed by using as synthesized Ti^{4+}-immobilized PDA-modified eppendorf tubes. The eppendorf tubes were washed with 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) water solution three times. Then, 200 μL of a 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) water solution containing 1 μL phosphopeptide digest (1 mg/mL–1 μg/mL) was added into tubes and shaken at 37 °C for 30 min. The phosphopeptide-loaded tubes were washed with 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) water solution (400 μL) three times by shaking for 1 min to remove nonspecific adsorption resulting from nonphosphopeptides. Subsequently, the phosphopeptides were eluted by an aqueous solution of NH_4OH (10 μL, 0.4 M) under shaking at 37 °C for 10 min and the eluate was analyzed by MALDI-TOF MS.

The enrichment of phosphopeptide from complex sample (the molar ratio of β-casein and BSA was 1:500) was performed by using as synthesized Ti^{4+}-immobilized PDA-modified eppendorf tubes. The eppendorf tubes were washed with 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) water solution three times. Then, 200 μL of a 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) water solution containing 1 μL tryptic digests of β-casein and BSA mixture (the molar ratio of β-casein and BSA was 1:500) was added into tubes and vibrated at 37 °C for 30 min. The phosphopeptide-loaded tubes were washed with a 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) water solution three times to remove nonspecific adsorption resulting from nonphosphopeptides. Subsequently, the phosphopeptides were eluted by an aqueous solution of NH_4OH (10 μL, 0.4 M) and the eluate was analyzed by MALDI-TOF MS.

The enrichment of phosphopeptides from human serum was performed by using as synthesized Ti^{4+}-immobilized PDA-modified eppendorf tubes. The eppendorf tubes were washed with 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) water solution three times. Then, 200 μL of a 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) water solution containing 2 μL human serum was added into tubes and vibrated at 37 °C for 30 min. The phosphopeptide-loaded tubes were washed with a 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) water solution three times to remove nonspecific adsorption resulting from nonphosphopeptides. Subsequently, the phosphopeptides were eluted by an aqueous solution of NH_4OH (10 μL, 0.4 M) and the eluate was analyzed by MALDI-TOF MS.

2.6. MALDI-TOF MS analysis

The above eluate of phosphopeptides was deposited on the MALDI target (Applied Biosystems, MDS SCIEX, Foster City, CA, USA) by the "dried-droplet" technique. 1 μL of eluate was pipetted onto the MALDI plate and left in the air at room temperature for evaporation of the solvent. Then, 1 μL of DHB aqueous solution (20 mg/mL, 50% acetonitrile and 1% H_3PO_4) was introduced as a matrix and left in the air at room temperature to form a thin matrix layer for further analysis by MALDI-TOF-MS. A Proteomic Analyzer (mode 5800, Applied Biosystems, Framingham, MA, USA) was used for MALDI analysis with the Nd: YAG laser at 366 nm, a repetition rate of 200 Hz and an acceleration voltage of 20 kV. Mass spectra were acquired in the positive ion reflection mode within a scan range of 1000–3500 *m/z*.

3. Results and discussion

It has been proven that DOPA can be coated on almost various substrates surfaces irrespective of substrate shape and type, including plastics, under solvent-free and non-toxic conditions. Moreover, the catechol groups in PDA coating can facilitate to in situ deposit metal ions at mild conditions. Owing to above advantages of PDA, we developed EP tube@PDA-Ti^{4+}, which can eliminate carry-over and minimize the sorbent consumption compared with other microsphere materials for selective enrichment of phosphopeptides since there is no need for magnetic separation or high-speed centrifugation. In this work, at first, we prepared EP tube@PDA-Ti^{4+}, and then evaluated its performance for phosphopeptide enrichment.

3.1. Preparation and characterization of EP tube@PDA-Ti^{4+}

As shown in Fig. 1a, dopamine hydrochloride could be oxidative self-polymerized in an alkaline solution (10 mM Tris buffer, pH 8.5) to produce a PDA coating on the inner surface of eppendorf tube. It can be observed that the color of solution changes to brown during the self-polymerization of dopamine (Fig. 2a). The catechol groups in PDA coating are capable of strong metal ions coordination, which can facilitate to in situ deposition of Ti^{4+} on the surfaces of PDA by simple addition of titanium (IV) sulfate precursor into PDA-modified eppendorf tube at mild conditions.

The as-prepared EP tube@PDA-Ti^{4+} was characterized by different techniques, including scanning electron microscopy (SEM), Energy dispersive X-ray spectroscopy (EDX) and Raman spectroscopy. The morphologies of PDA-modified eppendorf tubes were investigated by SEM characterization. As shown in Fig. 3a, before modification, the pristine eppendorf tube exhibits smooth and clean inner surface. After modified with PDA (Fig. 3c), the inner surface is covered with a robust PDA layer, which consists of small particles and a uniform thin film. According to the SEM

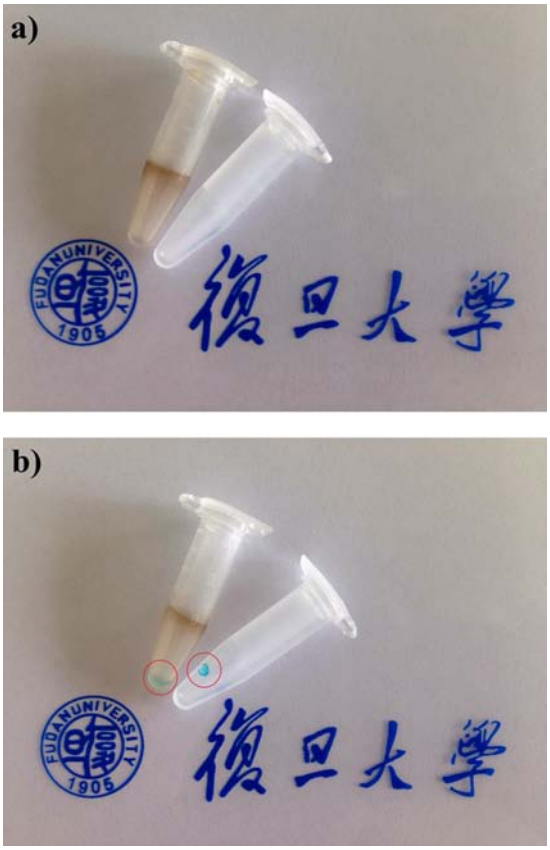

Fig. 2. (a) The digital photo of eppendorf tube before modification with PDA (right) and after modification with PDA (left); (b) the hydrophilic property of EP tube@PDA-Ti^{4+} (left) and the bare pristine eppendorf tube (right).

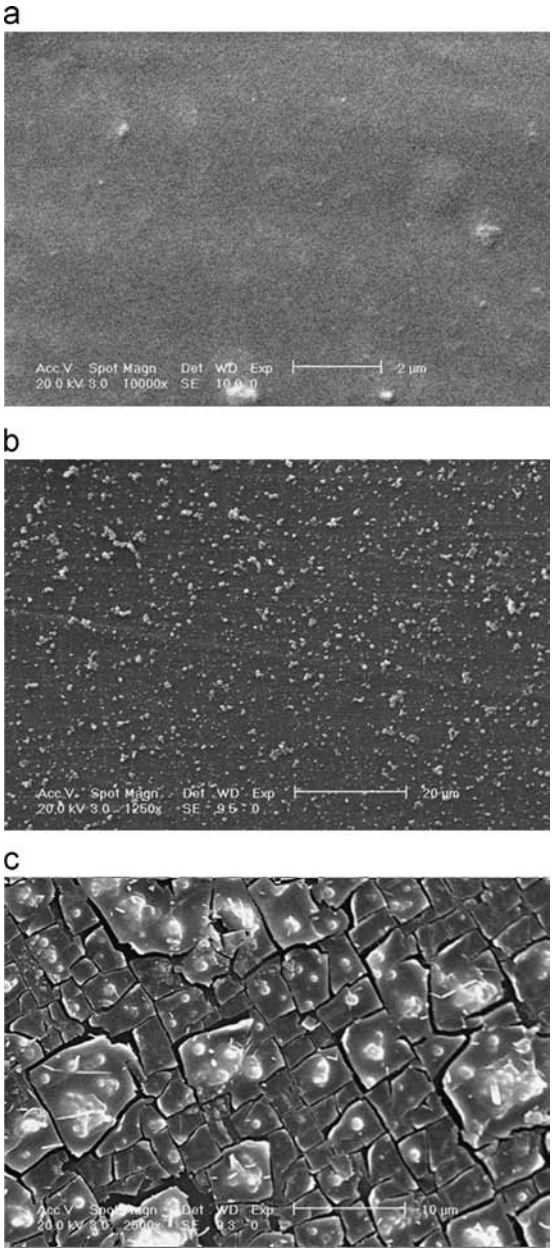

Fig. 3. SEM images of inner surface of (a) the bare pristine eppendorf tube, (b) the PDA-modified eppendorf tube (after 10 h-modificaiton), and (c) the PDA-modified eppendorf tube (after 20 h-modificaiton).

image, it could also be observed that at the first period of the polymerization (Fig. 3b), dopamine monomer has a tendency to form free PDA particles at high dopamine concentration [21]. Along with the reaction time, the PDA particles grow up into PDA thin films (Fig. 3c) which can undergo a variety of follow-up reactions to create different types of functional surfaces.

The immobilization of Ti^{4+} was characterized by energy dispersive X-ray spectroscopy (EDX), which is employed to identify the composition of materials. As shown in Fig. 4, the presence of C, N, O and Ti elements confirms the successful immobilization of Ti^{4+} on the PDA coating, which is formed by chelation between the catechol groups in PDA coating and Ti^{4+} in the addition of titanium (IV) sulfate precursor. The Raman spectra of PDA-modified eppendorf tubes are shown in Fig. 5. Before being coated with PDA (the blue line), the pristine eppendorf tube has no obvious characteristic peaks in the Raman spectrum. After being coated with PDA (the red line), two strong characteristic peaks at

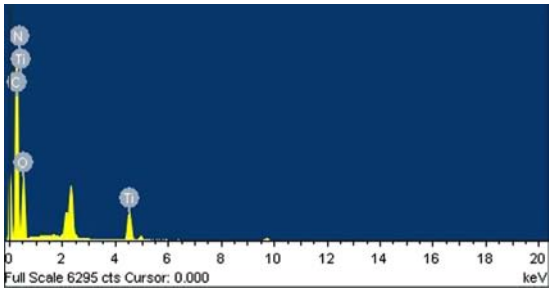

Fig. 4. The energy dispersive X-ray (EDX) spectrum data of EP tube@PDA-Ti^{4+}.

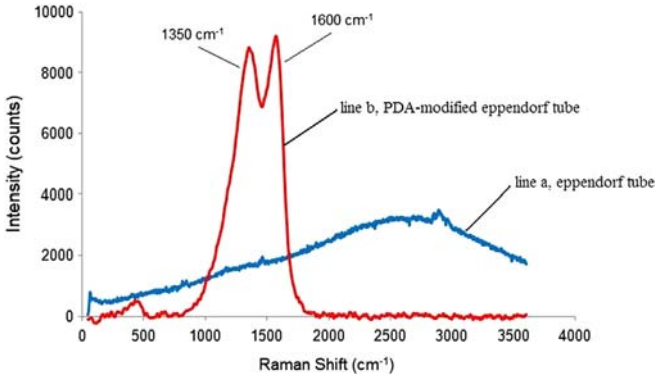

Fig. 5. The Raman spectra of the bare pristine eppendorf tube (line a) and PDA-modified eppendorf tube (line b).

around 1354 and 1572 cm^{-1} attributed to stretching and deformation of catechols confirm that dopamine has been polymerized on the inner surface of eppendorf tube successfully [21].

The wettability and hydrophilic property of EP tube@PDA-Ti^{4+} were investigated and the digital photos are shown in Fig. 2b. A drop of colored water was pipetted onto the inner surface of the bare pristine eppendorf tube and EP tube@PDA-Ti^{4+}, respectively. As shown in Fig. 2b, the drop of colored water in the bare pristine eppendorf tube agglomerates and presents a bead of colored water. On the contrary, the drop of colored water in the EP tube@PDA-Ti^{4+} spreads along the inner surface [22]. The evidence noticeably reveals that the modified inner surface turns to hydrophilic and thus improves its wettability and hydrophilic property, which could be attributed to the abundant polar catechol moieties and amino groups in PDA coating.

3.2. Evaluation of the performance of EP tube@PDA-Ti^{4+} for phosphopeptides enrichment

Tryptic digestion of β-casein was employed to evaluate the applicability of EP tube@PDA-Ti^{4+} in selective enrichment of phosphopeptides. β-casein is a common phosphoprotein containing five phosphorylation sites, and its tryptic digest is expected containing three phosphopeptides with molecular masses of 2061, 2556 and 3122 Da, respectively. However, some other phosphorylated fragments with miss cleavages are usually detected after enrichment. In our team, Yan et al. developed Ti^{4+}-immobilized Fe_3O_4@polydopamine core–shell microspheres for highly selective enrichment of phosphopeptides [17]. In her work, she chose an aqueous solution containing 50% of acetonitrile and 0.1% of trifluoroacetic acid (TFA) (pH 2.0) as loading and washing solution to minimize the undesired nonphosphopeptides and an aqueous solution containing 40% of NH_4OH was used as the elution solution, which achieved good results. In this study, we modified eppendorf tubes with polydopamine and immobilized Ti^{4+} on it

to enrich phosphopeptides, whose principle was similar with Yan's work. Because carboxyl groups in the peptides will be protonated at pH 2.0, which discriminates phosphate group from acidic residues on the modified inner surface. On the other hand, phosphopeptides adsorbed onto the modified inner surface can be released with a high pH buffer. Therefore, we continued to use an aqueous solution containing 50% of acetonitrile and 0.1% of trifluoroacetic acid (TFA) (pH 2.0) as loading and washing solution and an aqueous solution containing 40% of NH_4OH as the elution solution to enrich phosphopeptides. For comparison, a direct analysis of 40 pmol β-casein digests performed by MALDI analysis presents the result in Fig. S1a, in which nonphosphopeptide signals dominate the obtained spectrum due to the low concentration of phosphopeptides and the suppression of abundant nonphosphopeptides, directly resulting in nearly no presence of phosphopeptide signals. After enrichment with EP tube@PDA-Ti^{4+}, the signals of nonphosphopeptide apparently decrease and 10 peaks which could be assigned to phosphopeptides are detected and dominate the spectrum with high signal/noise ratio (S/N) in Fig. S1b (marked with "*"). The results indicate that the as-prepared EP tube@PDA-Ti^{4+} can selectively capture phosphopeptides from β-casein digests.

To further evaluate the specificity of the modified eppendorf tubes, a more complex peptide mixture of β-casein and BSA digests at a molar ratio of 1:500 constructing a mimic biological sample was employed. The concentration of β-casein digests is 40 pmol. As shown in Fig. 6, owing to the interference of high-abundance nonphosphopeptides, no phosphopeptides are detected in the mass spectrum before enrichment. However, after enrichment by EP tube@PDA-Ti^{4+}, almost no nonphosphopeptides are captured and 10 expected phosphopeptides with strong intensities, clear background and high S/N ratio are detected successfully.

The high sensitivity of Ti^{4+}-immobilized PDA-modified eppendorf tubes in enrichment of phosphopeptides was investigated from β-casein digests with ultra-low concentration. As shown in Fig. S2, when the concentration of β-casein digests is as low as 40 fmol, the ion signals from 6 phosphopeptides can still be observed with the modified eppendorf tubes. The results show the high detection sensitivity of the as-prepared eppendorf tube due to the hydrophilic property, the large amounts of immobilized titanium (IV) ions and the strong chelation of PDA with Ti^{4+}.

The reusability and stability of the modified eppendorf tubes were also tested in this work. The regenerated tubes, which were washed with the buffer solution, were reused to consecutively capture β-casein digests for five times. As shown in Fig. S3, the result of enrichment in the fifth time was almost the same as it in the first time, which demonstrated the modified eppendorf tubes can be reused at least five times with the same effects. To examine the stability of the modified tubes, the as-prepared EP tube@PDA-Ti^{4+} was stored at room temperature for one month, and the obtained result of enrichment was similar to that obtained by freshly made modified eppendorf tubes (Fig. S4). The loading and washing solution was of pH 2.0 and the elution solution was of pH 10.0. Therefore the as-prepared EP tube@PDA-Ti^{4+} kept stable during pH 2.0–10.0.

Encouraged by the advantages of EP tube@PDA-Ti^{4+} in the enrichment of phosphopeptides, the novel eppendorf tubes were further applied to analyze phosphopeptides of human serum to confirm the ability of enrichment for the real biosamples. The endogenous phosphopeptides contained in human serum can be used as biomarkers for therapeutic and diagnostic methods [23,24]. However, the detection of target phosphopeptides will be seriously interfered owing to the inorganic salts, abundant proteins and a large amount of nonphosphopeptides existing in the serum. Therefore, it is essential to develop techniques to capture phosphopeptides from human serum. As shown in Fig. S5a, almost no peptides are detected before enrichment due to the suppression by other contaminants in serum. However, 4 phosphopeptides are clearly observed with high S/N ratio at a mass range of 1000–3500 Da (Fig. S5b) after enrichment by modified eppendorf tubes. The above results clearly suggest that the modified eppendorf tubes hold great potential for the highly selective trapping of phosphopeptides from real biosamples.

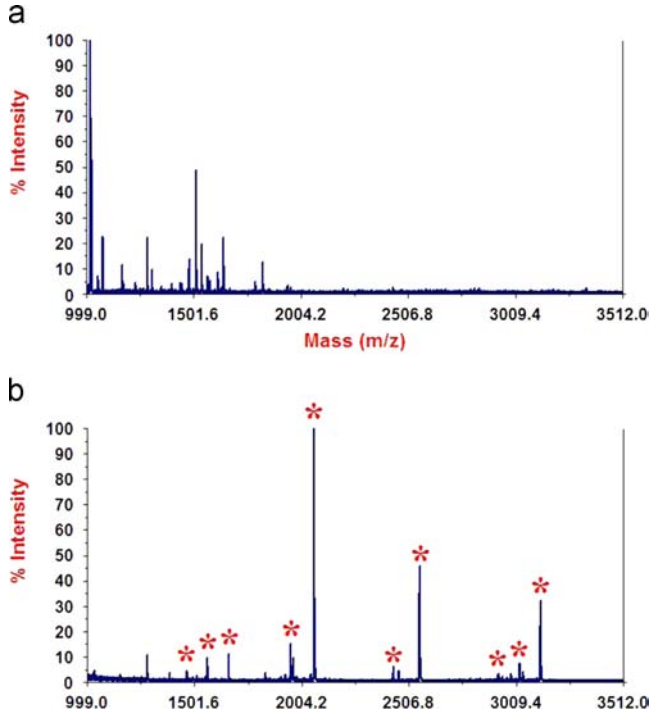

Fig. 6. The mass spectra of phosphopeptides derived from a peptide mixture of β-casein and BSA at a molar ratio of 1:500: (a) before and (b) after enrichment by EP tube@PDA-Ti^{4+}. The peaks of phosphopeptides are marked with "*".

4. Conclusion

In summary, a novel and easy-to use PDA-modified eppendorf tube immobilized with Ti^{4+} was prepared by using dopamine chemistry and chelation/coordination chemistry, and was successfully applied in the enrichment of phosphopeptides with MALDI analysis. The as-prepared material shows excellent hydrophilic property, selectivity, sensitivity, detection limit, reusability and stability. The modified eppendorf tubes can also be extended to selective enrichment of phosphopeptides from biosamples. Moreover, the enrichment process by EP tube@PDA-Ti^{4+} is quite convenient with no need for magnetic separation or high-speed centrifugation, which can eliminate carry-over and minimize the sorbent consumption. All of these strong points are expected to open up a promising enrichment strategy in enrichment of low-abundance phosphopeptides efficiently and sensitively in biosample.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.03.054.

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