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Selective enrichment of phosphopeptides by titania nanoparticles coated magnetic carbon nanotubes

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ABSTRACT

Selective enrichment of phosphoproteins or phosphopeptides from complex mixtures is essential for mass spectrometry (MS)-based phosphoproteomics. In this work, for the first time, titania nanoparticles coated magnetic carbon nanotubes (denoted as MagCNTs@TiO_2 composites) were synthesized through a facile but effective solvothermal reaction for selective enrichment of phosphopeptides. The MagCNTs@TiO_2 material demonstrated low limit of detection (20 fmol), along with an exceptional great specificity to capture phosphopeptides from a tryptic digest of the mixture of a nonphosphorylated protein BSA and a phosphorylated protein β-casein with molar ratios of BSA/β-casein up to 200:1. In addition, the high magnetic susceptibility allowed convenient separation of the target peptides by magnetic separation. Experimental results demonstrated that the MagCNTs@TiO_2 composites showed excellent potential for the selective enrichment of phosphopeptides for MS analysis.

1. Introduction

Magnetic microspheres have attracted wide interest because of their wide range of current and potential applications in the biomedical field [1–6]. They allow mechanical sorting, trafficking, and other forms of micro-manipulation to be easily performed in biological systems simply via the application of an external magnetic field [7,8]. Along this line, the application of magnetic microspheres to proteomics research has also attracted much attention [9,10]. As one of the most important and ubiquitous post-translational modifications (PTMs), protein phosphorylation is a key regulator of almost all aspects of cellular processes in both prokaryotes and eukaryotes [11,12]. Mass spectrometry (MS) has become a powerful technique for determining the phosphorylation profiles of proteins in phosphoproteome research due to its high sensitivity, high-throughput, and simplicity in identification of phosphorylation sites and quantification of changes in phosphorylation states [13,14]. However, the identification and characterization of phosphoproteins remains a challenge due to their low abundance and low ionization efficiency [15]. The selective enrichment of phosphoproteins or phosphopeptides from complex mixtures is therefore essential for MS-based phosphoproteomics. To date, various affinity materials and techniques have been introduced to specific capture phosphopeptides. Immobilized metal ion affinity chromatography (IMAC) is the most commonly used method to enrich phosphopeptides, which relies on the affinity of the phosphate groups to metal ions immobilized on a matrix. In recent years, metal oxide nanoparticles such as TiO_2, HfO_2, and ZrO_2 have been demonstrated to be more potential in phosphopeptides analysis than conventional IMAC because such oxides rely on specific and reversible chemisorption of phosphate groups on their amphoteric surface and have less non-specific binding [16–18]. In particular, recent advances in TiO_2 synthesis have led to the development of the current state of the art phosphate-adsorbing materials, which have a higher enrichment capacity and better selectivity than solid oxides [19,20].

Carbon nanotubes (CNTs) were first discovered in 1991 by Iijima [21], from then on, intensive studies have been carried out to explore their applications in various fields [22,23]. To date, many hybrid nanomaterials based on CNTs have been reported, which combine the fantastic physical–chemical properties of both carbon nanotubes and functional nanoparticles or molecules [24–26], such as magnetic nanoparticle–CNTs and gold nanoparticle–CNTs. These reports demonstrated that CNTs were useful support material because of their ability to prevent the macroscopic aggregation of nanoparticles. Therefore, it can be expected that, metal oxides nanoparticles that show affinity to phosphopeptide and can coated on CNTs to form CNTs-based composites may have potential performance on enrichment of phosphopeptide. However, when the phosphopeptide-bound materials are harvested using centrifugation, high-molecular-weight non-phosphopeptides are sedimented at high

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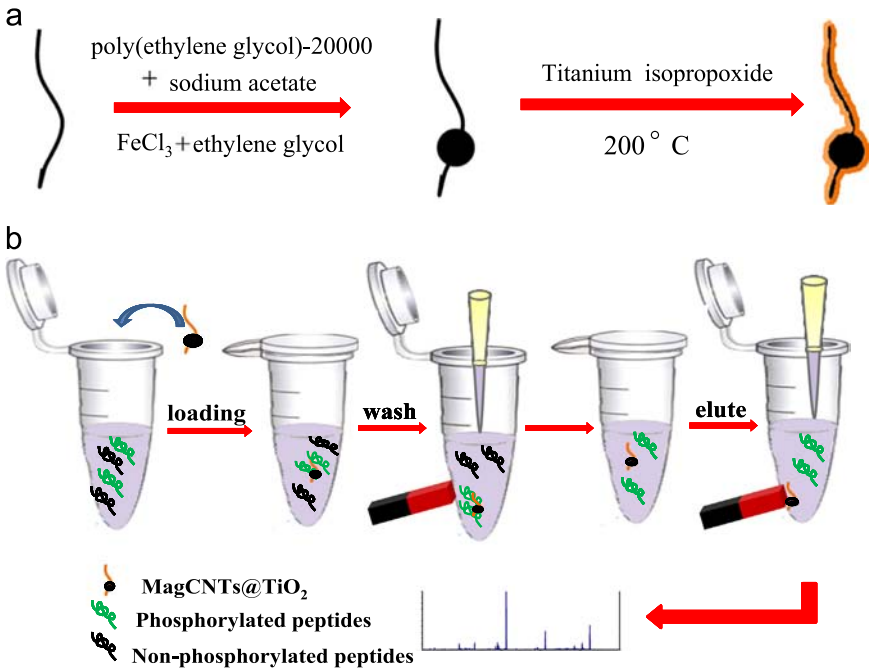

Scheme 1. Schematic illustration of (a) synthetic procedure for the fabrication of the MagCNTs@TiO_2 composites and (b) the selective process for the enrichment of phosphorylated peptides using MagCNTs@TiO_2 composites and magnetic separation..

rotation speeds. We therefore formulated a rational design to combine magnetic nanomaterials with porous TiO_2, with the aim of achieving the simple and efficient separation and enrichment of the phosphopeptides from complex samples.

Herein, we synthesized titania nanoparticles coated magnetic carbon nanotubes (MagCNTs@TiO_2 composites) through a facile solvothermal reaction, and applied them for highly selective enrichment of phosphopeptides. The synthesized procedure for the MagCNTs@TiO_2 composites is illustrated in Scheme 1a. To demonstrate the highly selective enrichment ability for phosphopeptides, MagCNTs@TiO_2 composites were applied to enrich the phophopeptides in different biological samples for mass spectrometry analysis (Scheme 1b).

2. Experimental

2.1. Materials and chemicals

Trifluoroacetic acid (TFA), β-casein, ammonium bicarbonate (NH_4HCO_3), bovine serum albumin, trypsin from bovine pancreas. 3-(trihydroxysilyl)propyl methylphosphate and 2,5-dihydroxybenzoic acid (DHB, Sigma). Acetonitrile was purchased from Merck (Darmstadt, Germany). Human serum was supplied by Zhongshan Hospital. All aqueous solutions were prepared using Milli-Q water by Milli-Q system (Millipore, Bedford, MA).

2.2. Synthesis of MagCNTs@TiO_2 composites

The magnetic CNTs were prepared via a modified hydrothermal method reported by Jia et al. [27]. Briefly, multiwalled CNTs (400 mg, diameter 20–40 nm, Shenzhen Nanotech Port Co., Ltd.) were dispersed into 50 mL concentrated nitric acid at 60 °C with magnetic stirring for 7 h. Then the pretreated CNTs (150 mg) and $FeCl_3 \cdot 6H_2O$ (810 mg) were dispersed into 40 mL ethylene glycol solution with trisodium citrate (0.15 g), sodium acetate (3.6 g) and poly(ethylene glycol)-20000 (1.0 g) by ultrasonication and stirring for 3 h. The mixture was sealed in the autoclave to be heated at 200 °C for 10 h. Finally the gained MagCNTs were washed and collected.

The MagCNTs@TiO_2 composites were synthesized according to previous report with some modification [28]. Briefly, the MagCNT powder (30 mg) was dispersed in isopropyl alcohol (50 mL) under sonication for 0.5 h. Then, 0.02 mL of diethylamine was added and stir for 5 min. Afterwards, 1.5 mL of titanium isopropoxide was added. The solution was transferred into a autoclave for heating at 200 °C for 24 h. The product was collected and washed thoroughly, and finally dried at 60 °C for 8 h. The dried sample was annealed at 400 °C in air for 2 h with a heating rate of 1.0 °C/min.

2.3. Characterization and measurements

Scanning electron microscopy (SEM) images were obtained on a Philips XL30 electron microscope (Netherlands) operating at 20 kV. Transmission electron microscopy (TEM) images were taken with a JEOL2011 microscope (Japan) operating at 200 kV. Wide-angle X-ray diffraction (WAXRD) patterns were recorded on a Bruker D4 X-ray diffractometer (Germany) with Ni-filtered Cu KR radiation (40 kV, 40 mA).

2.4. Sample preparation

Bovine serum albumin was reduced with dithiothreitol [DTT] and carboxamidomethylated with iodoacetamide. Then bovine serum albumin and β-casein were dissolved in 25 mM NH_4HCO_3 buffer at pH 8.3 and treated with trypsin (2% w/w) for 16 h at 37 °C, respectively. Human serum was diluted 10 times with 50% acetonitrile and 0.1% trifluoroacetic acid [TFA] aqueous solution (v/v).

2.5. Enrichment of phosphopeptides from tryptic digestion of standard proteins

MagCNTs@TiO_2 composites (200 μg) were added to 200 μL of a peptide mixture originating from tryptic digestion. The mixture was vibrated at room temperature for 30 min. After washed with 200 μL of

a 50% acetonitrile and 0.1% TFA water solution, an aqueous solution of NH_4OH (5 μL, 0.4 M) was added to elute the captured phosphopeptides.

2.6. Enrichment of phosphopeptides from human serum

MagCNTs@TiO_2 composites (200 μg) were added into 200 μL of a 50% acetonitrile and 0.1% TFA water solution which contain 2 μL human serum. The mixture was then vibrated at room temperature for 30 min. Then an aqueous solution of NH_4OH (5 μL, 0.4 M) was added to elute the captured phosphopeptides.

2.7. MALDI-TOF MS analysis

0.5 μL of elute was deposited on the plate and then another 0.5 μL of DHB aqueous solution (20 mg/mL, 50% acetonitrile and 1% H_3PO_4) was introduced as a matrix. MALDI-TOF MS

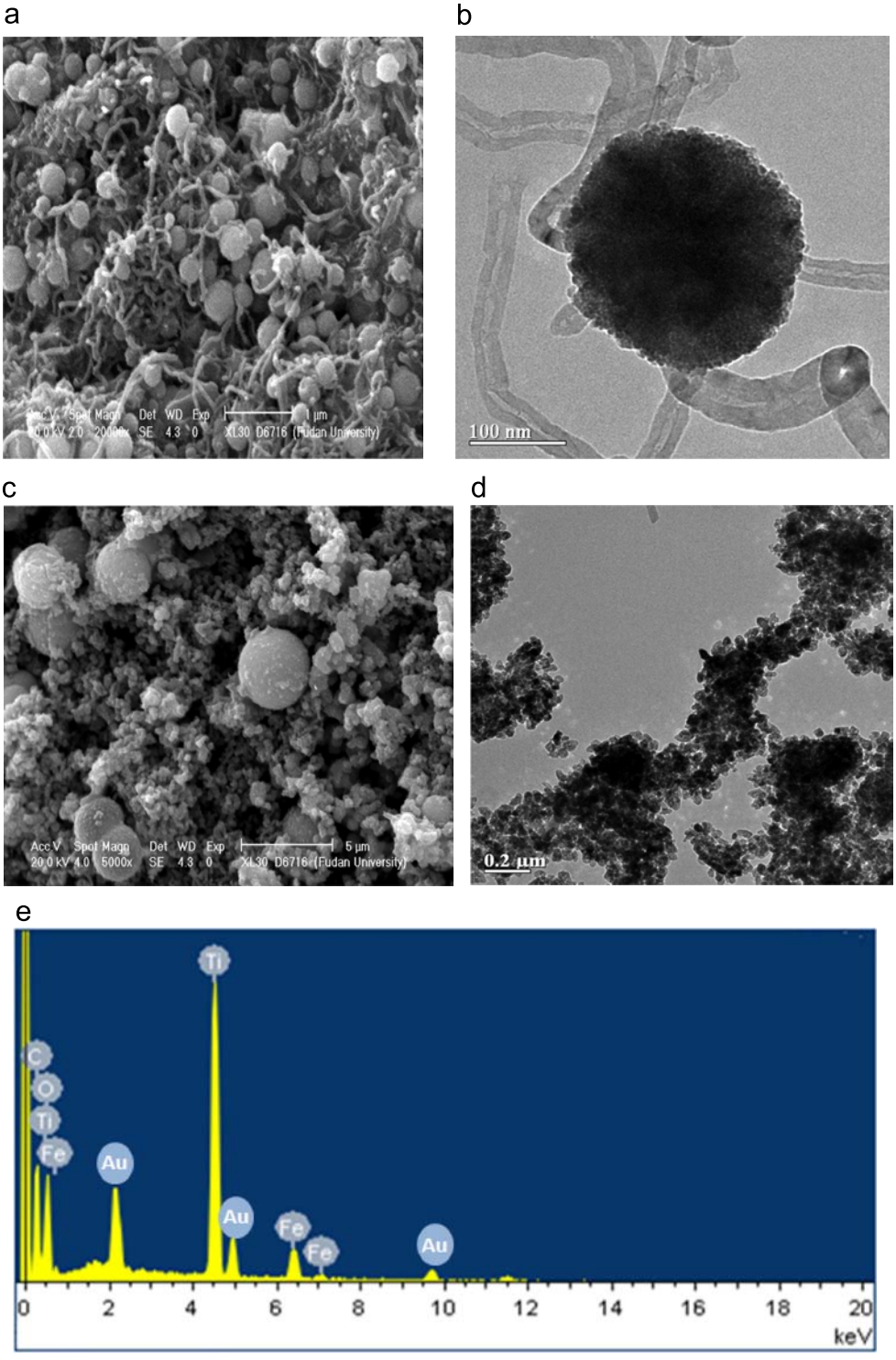

Fig. 1. SEM images of MagCNTs (a) and MagCNTs@TiO_2 composites (c); TEM images of MagCNTs (b) and MagCNTs@TiO_2 composites (d); and the energy dispersive X-ray (EDX) spectrum data of MagCNTs@TiO_2 composites (e).

experiments were performed in positive ion mode on a 5800 Proteomics Analyzer.

3. Results and discussion

3.1. Synthesis of MagCNTs@TiO_2 composites

The designed synthesis strategy of MagCNTs@TiO_2 composites are shown in Scheme 1. Briefly, first, the magnetic CNTs were prepared via a hydrothermal method, then TiO_2 nanoparticles were coated on MagCNTs to form MagCNTs@TiO_2 composites. The obtained MagCNTs@TiO_2 composites are hybrid material, it possesses the following merits. First, it has a porous affinity metal oxide shell constructed by titania nanoparticles which can selectively enrich phosphopeptides. Second, a high-magnetic-response MagCNTs core, allow separation to be simply and efficiently performed by using a magnet. The MagCNTs@TiO_2 composites with the above unique properties are anticipated to have excellent performance for the selective enrichment of phosphopeptides, which would be highly beneficial for mass spectrometric analysis.

The SEM and TEM images of MagCNTs (Fig. 1a and b) revealed that the CNTs were orderly decorated with the spherical particles with mean diameters of about 200 nm. Fig. 1c shows that numerous tiny TiO_2 nanoparticles were found to deposit on individual MagCNTs, resulting in MagCNTs@TiO_2 composites with rough surface. No large particles were observed, indicating that the growth of nanosized titania was effectively suppressed. TEM image showed that each MagCNTs were homogeneously coated by a layer of TiO_2 nanoparticles (Fig. 1d). Additionally, the MagCNTs@TiO_2 composites were well dispersed without significant aggregation, which was beneficial to their application for enrichment. The energy-dispersive X-ray analysis (EDX) (Fig. 1e) of the illuminating electron beams on the obtained MagCNTs@TiO_2 composites revealed the existence of Fe, C, Ti and O elements, further confirming the successful modification of MagCNTs with TiO_2.

Wide-angle X-ray diffraction patterns of the MagCNTs@TiO_2 composites (Fig. 2) displayed typical diffraction peaks of titania and Fe_3O_4, which could be indexed to anatase TiO_2 (JCPDS card no. 21-1272) and the phase of Fe_3O_4 (JCPDS file no. 190629). The broad diffraction peaks implied that the titania phase had a very small

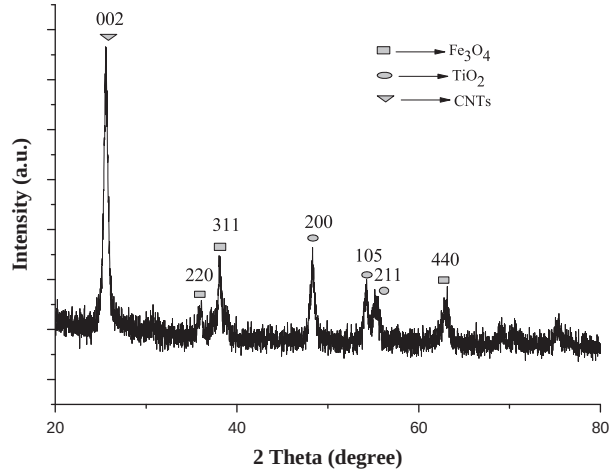

Fig. 2. Wide angle XRD patterns of MagCNTs@TiO_2 composites.

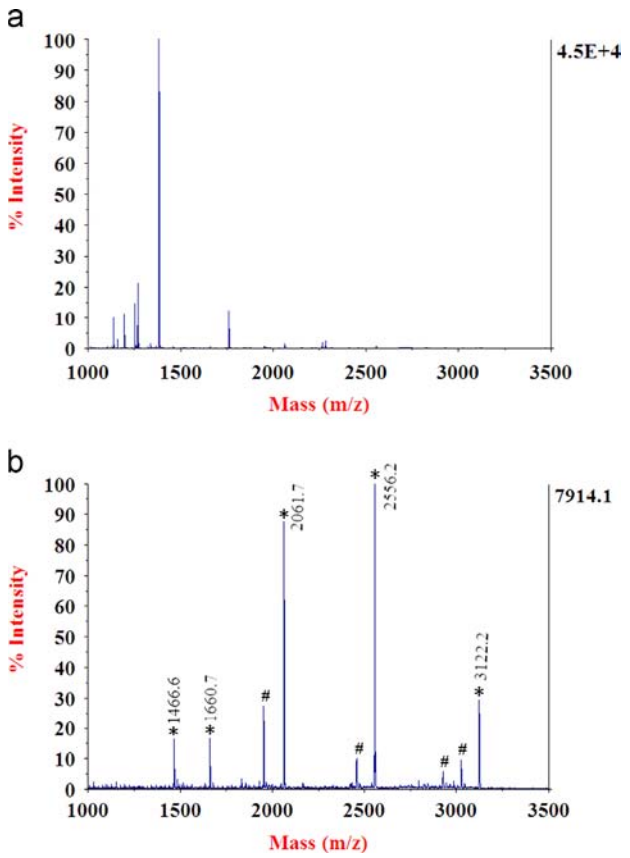

Fig. 3. MALDI mass spectrum of peptides derived from β-casein: (a) before enrichment and (b) enriched by MagCNTs@TiO_2 composites, where the * indicates the phosphopeptides and # indicates the metastable losses of phosphoric acid.

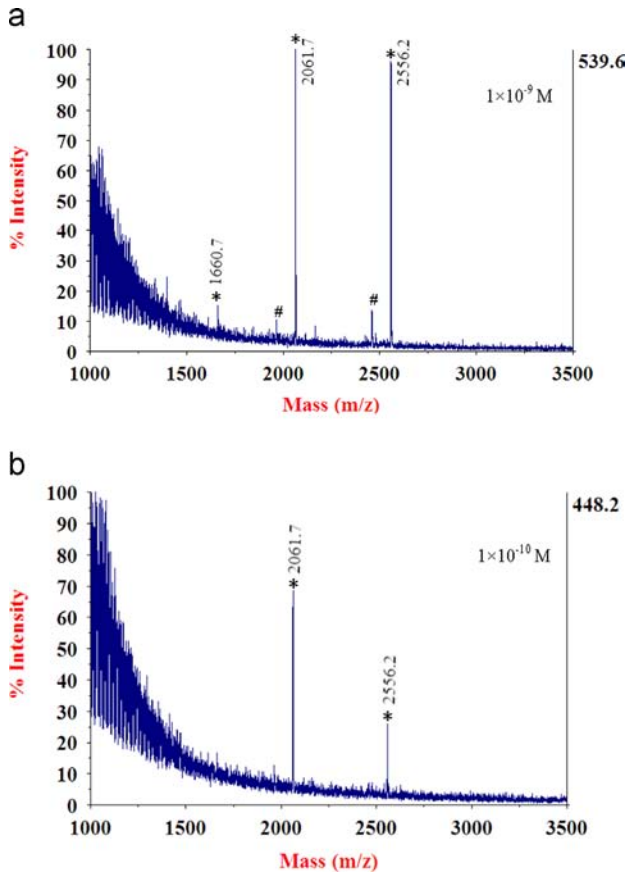

Fig. 4. MALDI mass spectra of phosphopeptides enriched from β-casein with different concentration using MagCNTs@TiO_2 composites, where the * indicates the phosphopeptides and # indicates the metastable losses of phosphoric acid.

particle size. Additionally, the peak at 26.1°, assignable to the diffractions of 002 plane, were also observed. It suggested that the structure of CNTs was well retained during the whole synthesis process. These results were in good agreement with the SEM, TEM and EDX observations.

3.2. Selective enrichment of phosphopeptides from tryptic digestion of standard proteins

We first investigated the enrichment capacity of MagCNTs@TiO_2 composites. A tryptic digest from 1×10^{-6} M of β-casein was incubated with the composites. After separation of the composites from solution, the trapped phosphopeptides were eluted by an aqueous solution of NH_4OH (0.4 M) for MALDI-TOF MS analysis. For comparison, direct analysis of the β-casein digest was also performed by MS analysis (Fig. 3a). Before enrichment, there were no phosphopeptide peaks and only some nonphosphopeptides could be observed. After pretreated with the MagCNTs@TiO_2 composites (Fig. 3b), the signals for the phosphopeptides (marked with asterisk) significantly increased and dominated the spectrum with clear background, the signals marked with a hash could be assigned to a dephosphorylated fragment of the phosphopeptide through loss of H_3PO_4. The observed phosphopeptides marked with numbers and the detailed information of the observed phosphopeptides are listed in Table S1. The selective enrichment of phosphopeptides of MagCNTs@TiO_2 composites was better than many previous reports [29,30], which is mainly because MagCNTs@TiO_2 was hybrid nanomaterial, indicating a high enrichment capacity of the MagCNTs@TiO_2 composites.

To study the detection limit of MagCNTs@TiO_2 composites used for enrichment of phosphopeptides, β-casein digest solutions with different concentrations were applied. The mass spectra are shown in Fig. 4. When the concentration of β-casein digest was 1×10^{-10} M (20 fmol), after enrichment by MagCNTs@TiO_2 composites, the ion signals from the phosphopeptides could still be detected, which indicated the high detection sensitivity of the material. The results suggested that MagCNTs@TiO_2 composites we report here were promising materials for the enrichment of phosphopeptides.

To investigate whether MagCNTs@TiO_2 composites can be recycled and reused for phosphopeptides enrichment, the materials were regenerated by washing with the buffer solution three times. The regenerated MagCNTs@TiO_2 composites were reused to

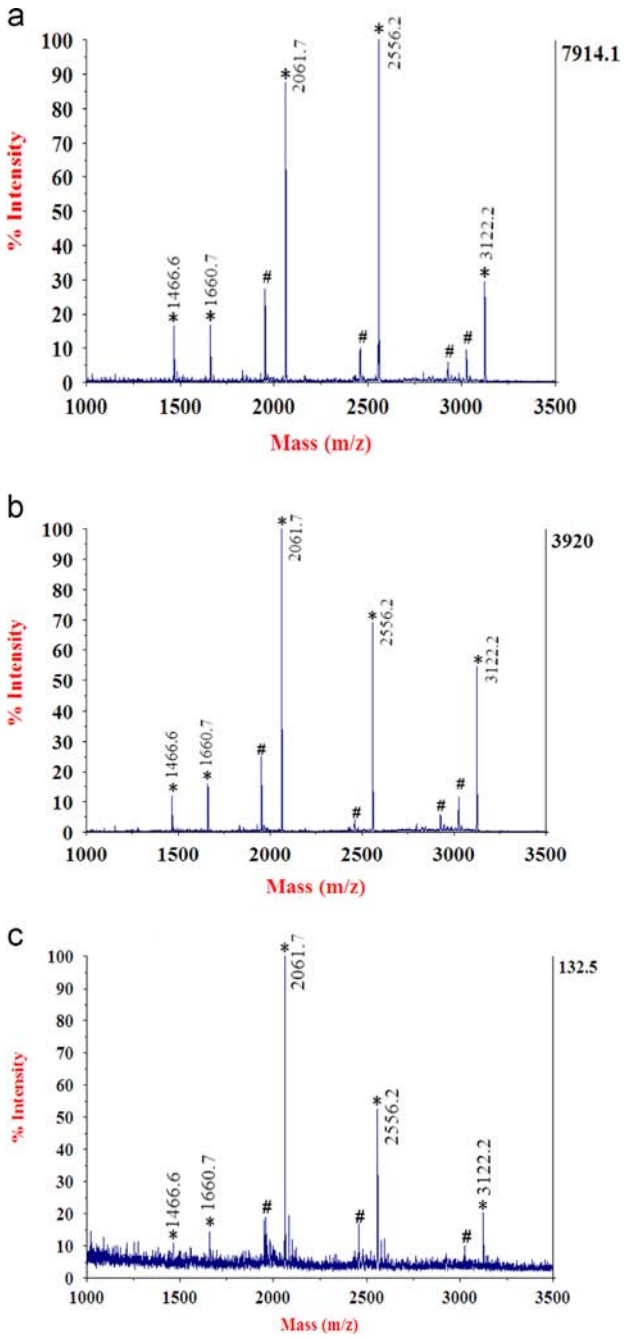

Fig. 5. MALDI mass spectrum of phosphopeptides enriched from β-casein using MagCNTs@TiO_2 composites: (a) for the first time; (b) for the fifth time and (c) for the tenth time.

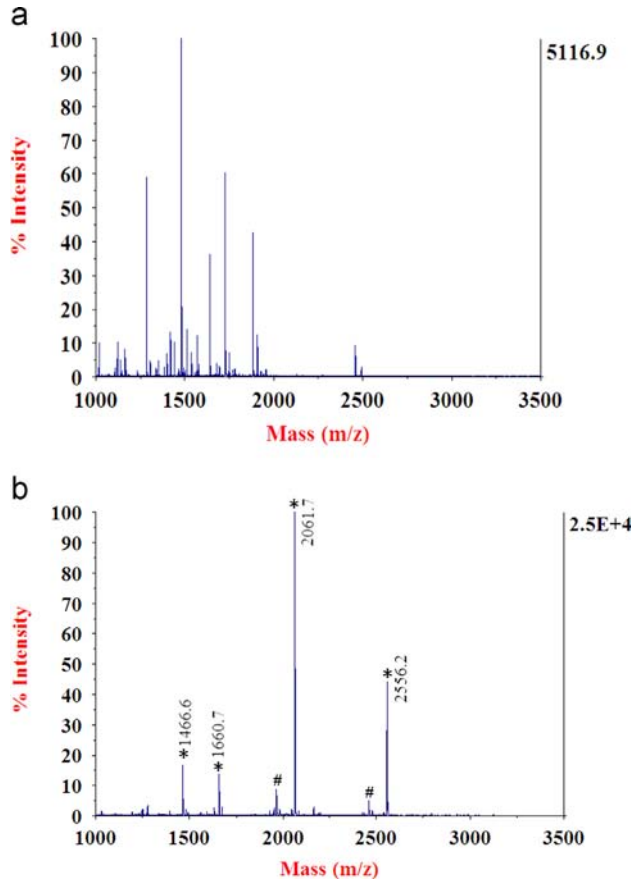

Fig. 6. MALDI mass spectrum of peptides derived from a peptide mixture of β-casein and BSA at a molar ratio of 1:200: (a) without enrichment and (b) enriched by MagCNTs@TiO_2 composites, where the * indicates the phosphopeptides and # indicates the metastable losses of phosphoric acid.

enrich phosphopeptides from β-casein digest 10 times. As shown in Fig. 5, the MS spectrum of phosphopeptides after enriching 10 times were similar to that for the first time, confirming that our new synthesized materials can be recycled and reused for phosphopeptides enrichment.

To further evaluate the ability of MagCNTs@TiO_2 composites to capture phosphopeptides in complex samples, MagCNTs@TiO_2 composites were applied to trap phosphopeptides in mixtures of a tryptic digest of β-casein and bovine serum albumin (BSA) (with a molar ratio of 1:200). As shown in Fig. 6a, before enrichment, no phosphopeptides were detected due to the presence of large amounts of non-phosphopeptides (from the BSA). After incubation with MagCNTs@TiO_2 composites, all six phosphopeptides could be easily detected with a very clean background (Fig. 6b). This indicated that the MagCNTs@TiO_2 composites had a promising efficacy in separating phosphopeptides from a complex samples with different amounts of proteins.

3.3. *Highly specific selective enrichment of phosphopeptides from human serum*

Human serum contain endogenous peptides, we have used the real sample of human serum which contains endogenous phosphopeptides to examine the effectiveness and selectivity of the MagCNTs@TiO_2 composites in the enrichment of phosphopeptides from a complex sample. As shown in Fig. 7, the peak of eight phosphopeptides could be clearly observed after enrichment, compared with that in which no phosphopeptides were detected before enrichment and the peaks of nonphosphopeptides dominated the spectra. To the best of our knowledge, the selectivity was much better than previous reports [31,32], which may be mainly because MagCNTs@TiO_2 was CNTs-based hybrid nanomaterial. The results suggested that MagCNTs@TiO_2 composites were capable of selective trapping phosphopeptides from naturally obtained complex sample.

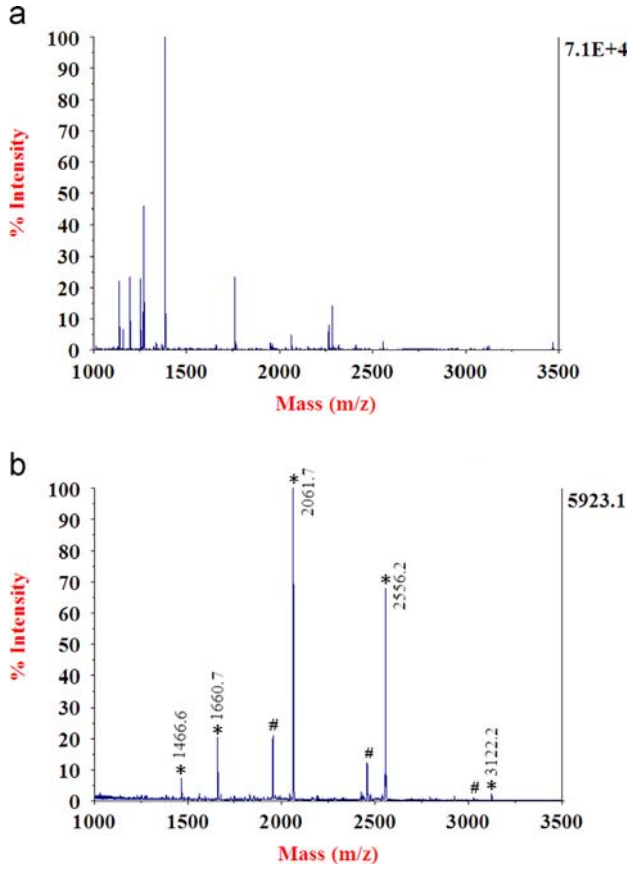

Fig. 7. MALDI mass spectrum of peptides derived from human serum (a) without enrichment and (b) enriched by MagCNTs@TiO_2 composites, where, the * indicates the phosphopeptides and # indicates the metastable losses of phosphoric acid.

4. Conclusions

In summary, we have presented a new facile, repeatable, and mass spectrometry-friendly synthetic route for the preparation of MagCNTs@TiO_2 composites with a MagCNT core and a rough outer layer formed by anatase tiania nanoparticles. As a result of selective affinity to phosphopeptides, the MagCNTs@TiO_2 composites were successfully applied to selectively enrich phosphopeptides for mass spectrometry analysis. The phosphopeptide enrichment experiments confirmed that the MagCNTs@TiO_2 composites had excellent selectivity for phosphopeptides. This showed that MagCNTs@TiO_2 composites had great potential for phosphoproteome research.

Acknowledgments

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.09.036.

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