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Facile synthesis of titania nanoparticles coated carbon nanotubes for selective enrichment of phosphopeptides for mass spectrometry analysis

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ABSTRACT

In this work, titania nanoparticles coated carbon nanotubes (denoted as CNTs/TiO_2 composites) were synthesized through a facile but effective solvothermal reaction using titanium isopropoxide as the titania source, isopropyl alcohol as the solvent and as the basic catalyst in the presence of hydrophilic carbon nanotubes. Characterizations using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) indicate that the CNTs/TiO_2 composites consist of CNT core and a rough outer layer formed by titania nanoparticles (5–10 nm). Measurements using wide angle X-ray diffraction (WAXRD), zeta potential and N_2 sorption reveal that the titania shell is formed by anatase titania nanoparticles, and the composites have a high specific surface area of about 104 m^2/g. By using their high surface area and affinity to phosphopeptides, the CNTs/TiO_2 composites were applied to selectively enrich phosphopeptides for mass spectrometry analysis. The high selectivity and capacity of the CNTs/TiO_2 composites have been demonstrated by effective enrichment of phosphopeptides from digests of phosphoprotein, protein mixtures of β-casein and bovine serum albumin, human serum and rat brain samples. These results foresee a promising application of the novel CNTs/TiO_2 composites in the selective enrichment of phosphopeptides.

1. Introduction

Protein phosphorylation is one of the most important and ubiquitous post-translational modifications (PTMs). It is a key regulator of almost all aspects of cellular processes in both prokaryotes and eukaryotes [1,2]. Mass spectrometry (MS) is a powerful technique for determining the phosphorylation profiles of proteins in phosphoproteome research because of its high sensitivity, high-throughput, and simplicity in identification of phosphorylation sites and quantification of changes in phosphorylation states [3–5]. However, phosphopeptides can be suppressed by non-phosphopeptides in MS detection due to their low abundance and low ionization efficiency [6]. To solve these problems, various affinity materials and affinity enrichment techniques have recently been introduced for the enrichment of phosphopeptides, such as immobilized metal ion affinity chromatography (IMAC), metal oxides affinity chromatography (MOAC) and metal oxide mesoporous materials etc. [7–14]. Generally, the basic principle for these selective enrichment techniques can be viewed as a Lewis acid–Lewis base interaction. The phosphate groups of phosphopeptides act as bidentate ligands (Lewis bases) which can effectively bind metal atoms (Lewis acids) upon contacting metal oxides or immobilized metal ions [15]. Following this principle, increasing efforts have been devoted to designing high performance nano-sorbents that possess high surface area, integrated functionalities, and novel nanostructures with an aim to achieve a highly selective, efficient, convenient and fast enrichment of phorsphopeptides from complex samples as well as an improved phosphopeptides recovery [14].

Since carbon nanotubes (CNTs) were first discovered in 1991 by Iijima [16], intensive studies of their properties have been carried out to explore their applications in various fields [17–21]. A great variety of CNTs-based hybrid nanomaterials have been reported, including gold nanoparticle-CNTs, magnetic nanoparticle-CNTs, and functional probe molecules-modified CNTs, which combine the fantastic physic-chemical properties of both carbon nanotubes and functional nanoparticles or molecules [22–26]. Moreover, carbon nanotubes (CNTs) were demonstrated as a unique support for nanomaterials. For instance, Chen et al. reported Pt nanoparticles-CNTs composites obtained by the microwave assisted polyol synthesis method, and successfully used the composites for methanol electrooxidation [27]. Peng et al. synthesized ceria supported on carbon nanotubes (CeO_2/CNTs) via a controlled sol–gel coating method. CeO_2 nanoparticles were found to uniformly deposit on the surface of CNTs without self-aggregation, which endowed the composites with high surface area for efficient removal of arsenate from water [28]. All these previous reports clearly indicate that CNTs are a useful support material for both in situ growth and post

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loading of functional nanoparticles due to their ability to prevent the macroscopic aggregation of nanoparticles, especially precious metal nanoparticles and active metal oxides. Therefore, it can be expected that, metal oxides nanoparticles that show affinity to phosphopeptide and can be coated on CNTs to form a CNTs-based nanocomposite have a promising application in phosphopeptide analysis.

Herein, we rationally designed and synthesized titania nanoparticles coated carbon nanotubes (CNTs/TiO_2 composites) through a facile solvothermal reaction for selective enrichment phosphopeptides for mass spectrometry analysis. The obtained CNTs/TiO_2 composite has an affinity metal oxide shell constructed by titania nanoparticles which can selectively enrich phosphopeptides. SEM, TEM and zeta potential observations reveal that the obtained CNTs/TiO_2 composites maintained the tubular structure but with rough surface morphology, and the walls of CNTs were fully coated by titania nanoparticles of 5–10 nm. WAXRD and N_2 sorption characterizations indicate that the titania nanoparticles have an anatase crystalline phase, and the surface area of the CNTs/TiO_2 composites is 104 m^2/g as a result of the close packing of nanoparticles on CNTs. Enrichment study shows that the CNTs/TiO_2 composites possess high performances in selective enrichment of phosphopeptides from complex peptide mixtures, its detection is 2×10^{-10} M, which suggests that the CNTs/TiO_2 composites are promising nanomaterials for specific enrichment of phosphopeptides.

2. Experimental

2.1. Chemicals

Bovine β-casein, bovine serum albumin (BSA), L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK) treated trypsin (from bovine pancreas), 2, 5-dihydroxybenzoic acid (DHB) and ammonium bicarbonate (NH_4HCO_3) were purchased from Sigma Chemical (St. Louis, MO). Acetonitrile was purchased from Merck (Darmstadt, Germany). All aqueous solutions were prepared using Milli-Q water by Milli-Q system (Millipore, Bedford, MA). Carbon nanotubes (multi-walled) with diameter of 20–40 nm were purchased from Nanoport Company (Shenzhen, China). Titanium(IV) isopropoxide (95%) and diethylamine (99%) were purchased from Alfa Aesar.

2.2. Synthesis of CNTs/TiO_2 composites

The pristine CNTs were firstly activated by treating in a concentrated nitric acid (65 wt%) at 100 °C under magnetically stirring for 12 h. The black dispersion was then diluted with water, and the CNTs were collected and washed with deionized water by filtration until the pH value of the filtrate reaches neutral. The acid-treated CNTs were dried in vacuum at 60 °C overnight for further use. The CNTs/TiO_2 composites were synthesized according to previous report with some modification [29]. Briefly, the activated CNT powder (30 mg) was dispersed in isopropyl alcohol (IPA, 50 mL) under sonication for 0.5 h. Then, 0.02 mL of diethylamine was added into the obtained stable dispersion followed by stirring for 5 min. Afterwards, 1.5 mL of titanium isopropoxide was added. After further stirring for 5 min, the solution was transferred into a Teflon-lined stainless-steel autoclave (100 mL capacity) for heating at 200 °C for 24 h in an oven. After reaction, the autoclave was taken out and left to cool down to room temperature. The product was collected by centrifugation and washed thoroughly with ethanol, and finally dried at 60 °C for 8 h. The dried sample was annealed at 400 °C in N_2 for 2 h with a heating rate of 1 °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 JEOL 2011 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). Nitrogen sorption isotherms were measured at 77 K with a Micromeritcs Tristar 3000 analyzer (USA). Before measurements, the samples were degassed in a vacuum at 200 °C for at least 6 h. The Brunauer-Emmett-Teller (BET) method was utilized to calculate the specific surface areas (S_{BET}) using adsorption data in a relative pressure range from 0.05 to 0.35.

2.4. Sample preparation

Bovine serum albumin was reduced with dithiothreitol [DTT] and carboxamidomethylated with iodoacetamide. Bovine serum albumin and bovine 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 with 50% acetonitrile and 0.1% trifluoroacetic acid [TFA] aqueous solution (v/v).

2.5. Preparation of the lysate of rat brain

Rats were sacrificed and the brains were quickly removed and placed in ice-cold homogenization buffer consisting of 7 M urea, 2 M thiourea and a mixture of protease inhibitor (1 mM phenylmethanesulfonylfluoride) and phosphatase inhibitors (0.2 mM Na_3VO_4, 1 mM NaF). After mincing with scissors and washing to remove blood, the brains were homogenized in a Pottter-Elvejhem homogenizer with a Teflon piston, using 5 mL of the homogenization buffer per 1 g of tissue. The suspension was homogenized for approximately 2 min, vortexed at 0 °C for 30 min, and centrifuged at 22,000*g* for 1.5 h. The supernatant contained the total brain proteins. Appropriate volumes of protein sample were precipitated as above, lyophilized to dryness, and redissolved in reducing solution (6 M guanidine hydrochloride, 100 mM NH_4HCO_3, pH 8.3) with the protein concentration adjusted to 2 mg/mL. Then, 200 mg of this protein sample (100 mL volume) were mixed with 10 mL of 0.5 M DTT. The mixture was incubated at 37 °C for 1 h, and then 20 mL of 0.5 M 2-iodoacetamide were added and incubated for an additional 30 min at 37 °C in the dark. The protein mixtures were exchanged into 50 mM NH_4HCO_3 buffer, pH 8.5, and incubated with trypsin (40:1) at 37 °C overnight.

2.6. Enrichment of phosphopeptides from tryptic digestion of standard proteins

In a typical process, a suspension of CNTs/TiO_2 composites (10 μL, 2 mg/mL) was added to 200 μL of a peptide mixture originating from tryptic digestion. The mixture was then vibrated at 25 °C for 30 min. The phosphopeptide-loaded CNTs/TiO_2 composites were collected by centrifugation and washed with 200 μL of a 50% acetonitrile and 0.1% TFA water solution three times. Subsequently, an aqueous solution of NH_4OH (5 μL, 0.4 M) was added to elute the captured phosphopeptides and the eluate was analyzed by MALDI–TOF MS.

2.7. Enrichment of phosphopeptides from tryptic digestion of rat brain

The tryptic digests of rat brain were lyophilized and then dissolved in loading buffer. CNTs/TiO_2 composites (2.0 mg) were added into 400 μL of diluted rat brain digests. Then the mixed solutions were vibrated at 25 °C for 30 min. After that, the phosphopeptides adsorbed CNTs/TiO_2 composites were collected and washed with 50% (v/v) acetonitrile and 0.1% (v/v) TFA aqueous solution for three times. Then the CNTs/TiO_2 was eluted with 5 μL of 0.4 M ammonia aqueous solution for 30 min. The eluate was lyophilized and then dissolved in 35 μL loading phase. Finally, the solution was submitted for LC–ESI-MS analysis.

2.8. MALDI-TOF MS analysis

The above phosphopeptides eluted from the CNTs/TiO_2 composites were deposited on the MALDI target using the dried droplet method. 0.5 μL of washing buffer 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 experiments were performed in positive ion mode on a 5800 Proteomics Analyzer (Applied Biosystems, USA) with the Nd-YAG laser at 355 nm, a repetition rate of 200 Hz and an acceleration voltage of 20 kV.

2.9. Nano-LC-ESI-MS/MS analysis and database searching

The peptide solution eluted from CNTs/TiO_2 composites were dried thoroughly by lyophilization and then redissolved with 5% ACN aqueous solution containing 0.1% formic acid, separated by nano-LC and analyzed by on-line electrospray tandem mass spectrometry. The experiments were performed on a Nano Aquity UPLC system (Waters Corporation, Milford, USA) connected to an LTQ Orbitrap XL mass spectrometer (Thermo Electron Corp, Bremen, Germany) equipped with an online nanoelectrospray ion source (Michrom Bioresources, Auburn, USA). The separation of the peptides was performed in a Symmetry® C18, 5 μm, 180 μm id × 2 cm trap-column and a BEH300 C18, 1.7 μm, 75 μm id × 15 cm reverse phase column (Waters Corporation, Milford, USA).

The peptide solution was injected into the trap-column at a flow rate of 15 μL/min and subsequently eluted with a three-step linear gradient. Starting from 5% B to 45% B in 43 min (mobile phase A: 5% ACN aqueous solution with 0.1% formic acid; mobile phase B: 95% ACN aqueous solution with 0.1% formic acid), increased to 80% B in 3 min. After holding on 80% B for 1 min, it decreased to the initial condition of 5% B in 1 min. Finally, the column was re-equilibrated at 5% B for 12 min. The column flow rate was maintained at 300 nL/min and column temperature was maintained at 35 °C. The electrospray voltage of 1.4 kV versus the inlet of the mass spectrometer was used. LTQ Orbitrap XL mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra with two microscans (m/z 400–1800) were acquired in the Orbitrap with a mass resolution of 60,000 at m/z 400, followed by 10 sequential LTQ-MS/MS scans. Dynamic exclusion was used with two repeat counts, 10-s repeat duration, and 60-s exclusion duration. For MS/MS, precursor ions were activated using 25% normalized collision energy at the default activation q of 0.25.

The obtained LTQ Orbitrap XL MS/MS data were identified by using SEQUEST [v.28 (revision 12)], Thermo Electron Corp. against the mouse International Protein Index (IPI) database. To reduce false positive identification results, a decoy database containing the reverse sequences was introduced into the database. The searching parameters were set up as follows: enzyme; the variable modification was phosphorylation (S, T, Y) and oxidation of methionine; the peptide mass tolerance was 100 ppm, and the MS/MS tolerance was 1 Da. And then the obtained searching results were filtrated with following parameters: $\Delta Cn > 0.1$; the minimum Xcorr of 2.00, and 2.50 corresponding to 2+, and 3+ charge states, respectively. Based on the above parameters, false positive rate of less than 5% was calculated to remove low-probability peptides, demonstrating the reliability of the identified results in this investigation.

3. Results and discussion

3.1. Synthesis of CNTs/TiO_2 composites

The activation treatment of pristine CNTs with oxidative nitric acid is necessary, because it can generate enormous hydrophilic groups like hydroxyl and carboxyl groups on the walls of CNTs, which can improve their dispersibility and ensure a successful coating with titania in a polar solvent. The CNTs/TiO_2 composites were easily synthesized via a solvothermal reaction. In this process, the titanium isopropoxide precursor underwent a quick hydrolysis and condensation catalyzed by diethylamine and was finally converted into nanosized titania that further deposits on CNTs to decrease the interface surface energy. Notably, diethylamine acted as not only the basic catalyst, but also the chelating agent because it can chelate titanium atoms. It helps to prevent the growth of nanosized titania into large particles. As shown in the SEM image, numerous tiny TiO_2 nanoparticles were found to deposit on individual CNTs, resulting in CNTs/TiO_2 composites with rough surface (Fig. 1a). No large particles were observed,

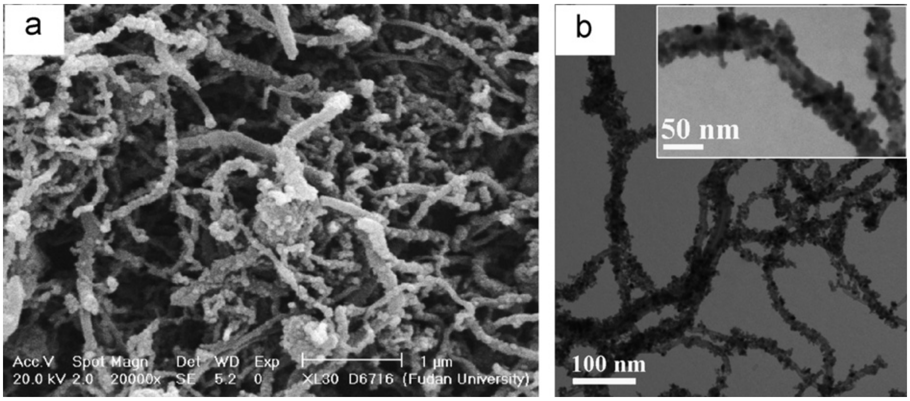

Fig. 1. (a) SEM and (b) TEM images of TiO_2 nanoparticles coated carbon nanotubes. The inset in (b) is the enlarged image showing the complete encapsulation of CNTs by $Ti0_2$ nanoparticles.

indicating that the growth of nanosized titania was effectively suppressed. TEM image shows that each CNT was homogeneously coated by a layer of TiO_2 nanoparticles, and the nanoparticles are 5–10 nm in diameter estimated from TEM image (Fig. 1b, inset). Additionally, the CNTs/TiO_2 composites are well dispersed without significant aggregation, which is beneficial to their application for enrichment.

Wide-angle X-ray diffraction patterns of the CNTs/TiO_2 composites (Fig. 2) display typical diffraction peaks of titania, which can be indexed to anatase TiO_2 (JCPDS card no. 21-1272 [30]. The broad diffraction peaks imply that the titania phase has a very small particle size. Additionally, two diffraction peaks at 26.1 and 43.5°, assignable to the diffractions of 004 and 100 planes, respectively, were also observed. It suggests that the structure of CNTs was well retained during the whole synthesis process. These results are in good agreement with the SEM and TEM observations. The N_2 adsorption–desorption measurement at 77 K indicates that the CNTs/TiO_2 composites have a high specific surface area of 104 m^2/g. The high surface and rough surface morphology are beneficial to the enrichment applications.

The zeta potential measurement was carried out for two materials in water. As shown in Fig. 3, the zeta potential of CNTs was -19.4 mV, after surface modification, CNTs/TiO_2 has increased zeta potential of 2.52 mV, which can be explained by that CNTs was modified by TiO_2. Only one narrow peak was observed for CNTs/TiO_2 material, whereas the peak at -19.4 mV for CNTs cannot be observed after surface modification. The above zeta potential measurements have displayed that the TiO_2 have been successfully functionalized onto the surface CNTs for CNTs/TiO_2, in agreement with nitrogen sorption, SEM, TEM and XRD results. The electrostatic force between the material and PO_4^{3-} of phosphopeptides contributes to the enrichment of phosphopeptides.

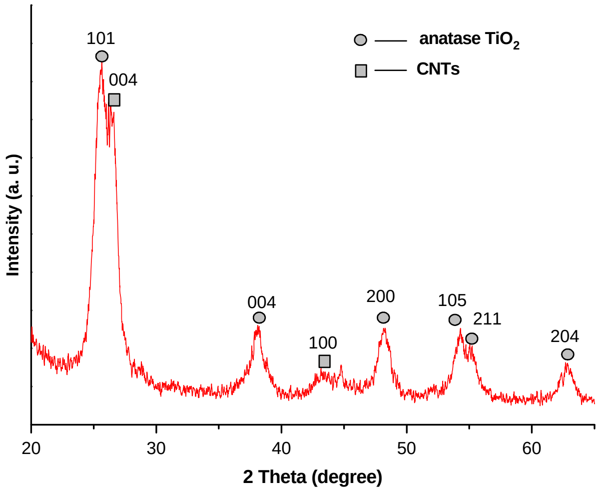

Fig. 2. Wide angle XRD patterns of TiO_2 nanoparticles coated carbon nanotubes.

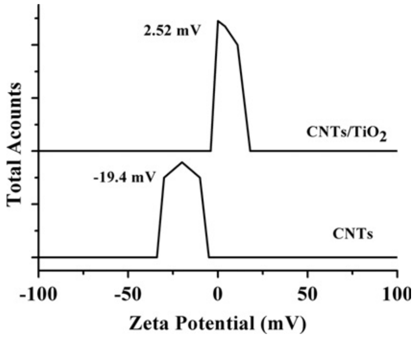

Fig. 3. The zeta potential distributions of CNTs and TiO_2 nanoparticles coated carbon nanotubes.

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

Bovine β-casein was used to evaluate the performance of the CNTs/TiO_2 composites for selective enrichment of phospho peptides. A tryptic digest from 1×10^{-6} M of β-casein was incubated in the presence of the nanotube. After separation of the composites from solution and thorough washing, 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, with the result presented in Fig. 4a. Only some nonphosphopeptides can be observed and no phosphopeptide peaks. The same amount of tryptic digest was also pretreated with the CNTs/TiO_2 composites using the same enrichment procedure, with the corresponding MALDI-TOF MS spectrum illustrated in Fig. 4b. The signals for the phosphopeptides (marked with a asterisk) significantly increased and dominated the spectrum and no nonphosphorylated peptides were observed, indicating a high enrichment efficiency of the CNTs/TiO_2 composites.

To further evaluate the sensitivity and capacity of the CNTs/TiO_2 composites for phosphopeptides enrichment, tryptic β-casein digests at different concentrations with a fixed volume (200 μL) were enriched using the CNTs/TiO_2 composites and then analyzed by MALDI-TOF MS. The mass spectra were shown in Fig. 5. It can be seen that when the concentration of β-casein digest was 2×10^{-10} M, after enrichment by CNTs/TiO_2, the ion signals from the phosphopeptides could still be detected, which indicated the high detection sensitivity of the CNTs/TiO_2 composites. The results suggest that the CNTs/TiO_2 composites we report here are promising materials for the enrichment of phosphopeptides.

To evaluate the specificity of enrichment of phosphopeptides with the CNTs/TiO_2 composites, a relative complex sample, BSA-derived tryptic peptides were added to the tryptic digest of β-casein at different molar ratios (β-casein:BSA=1:10 and 1:50). Fig. 6 represented the mass spectra of the mixtures before and after enrichment by CNTs/TiO_2. With the molar ratio increased

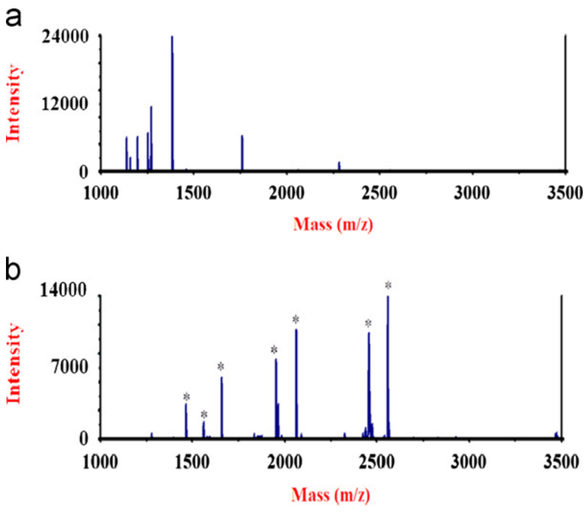

Fig. 4. MALDI mass spectrum of peptides derived from β-casein (a) before enrichment and (b) enriched by TiO_2 nanoparticles coated carbon nanotubes, where the * indicates the phosphopeptides.

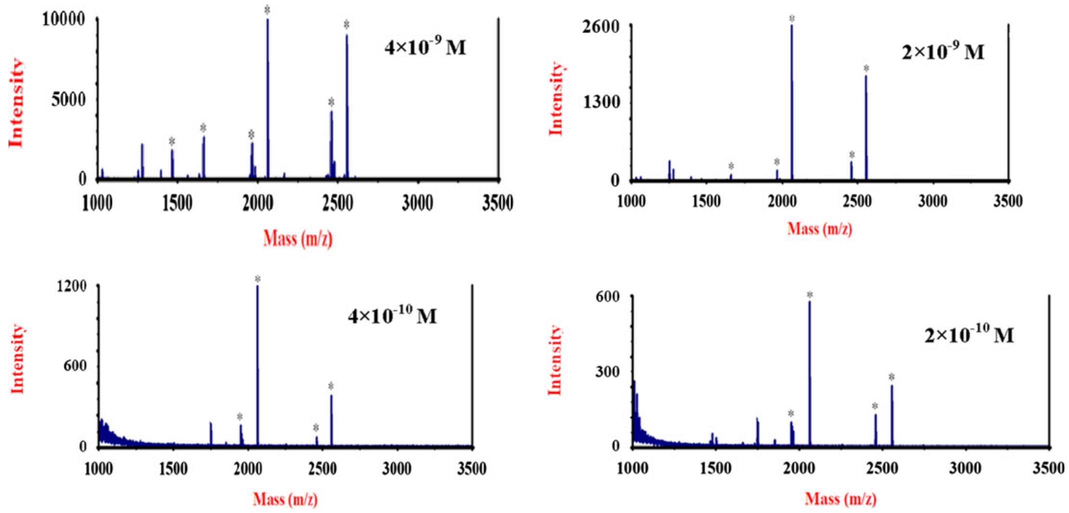

Fig. 5. MALDI mass spectra of phosphopeptides enriched from β-casein with different concentrations using TiO_2 nanoparticles coated carbon nanotubes, where the * indicates the phosphopeptides.

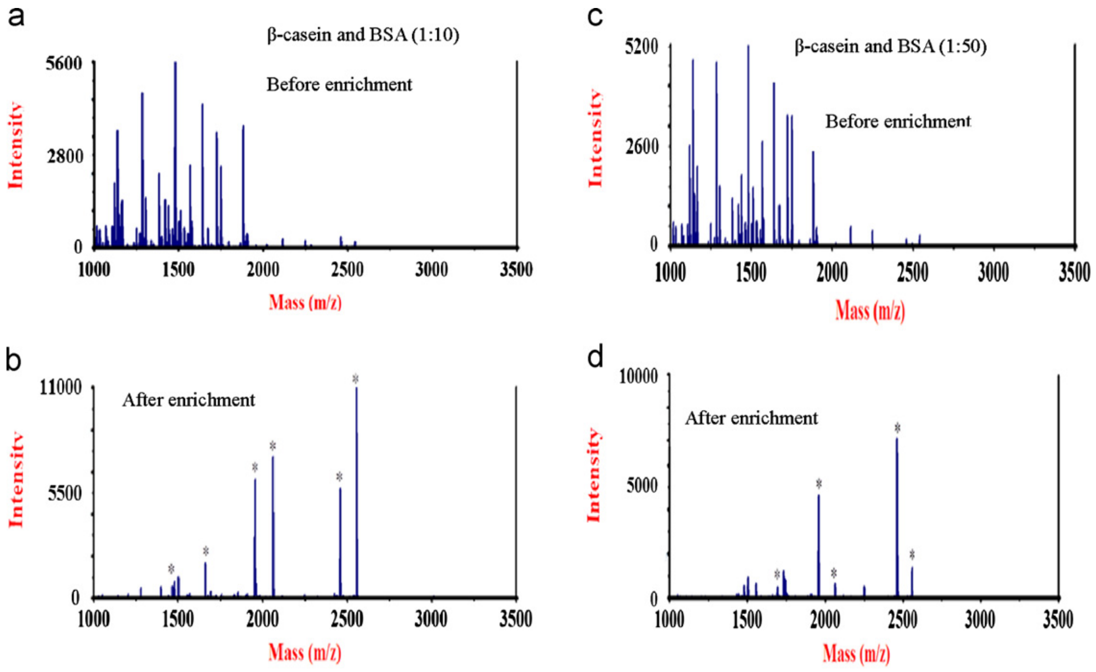

Fig. 6. MALDI-TOF mass spectra of mixtures containing the tryptic digest of β-casein and nonphosphorylated (BSA) protein tryptic digests at varying ratios of (a, b) 1:10 and (c, d) 1:50 enriched with TiO_2 nanoparticles coated carbon nanotubes, where the * indicates the phosphopeptides.

from 1:10 to 1:50, the peak intensity remained at a stable level for CNTs/TiO_2. This indicated that the CNTs/TiO_2 composites had a promising efficacy in separating phosphopeptides from a complex samples with different amounts of proteins.

3.3. Selective enrichment of phosphopeptides from tryptic digestion of human serum and rat brain

We have used human serum which contains phosphopeptides to examine the effectiveness and selectivity of the CNTs/TiO_2 composites in the enrichment of phosphopeptides from a complex sample. As shown in Fig. 7, the peak of phosphopeptides can be clearly observed after enrichment, compared with that no phosphopeptides were detected before enrichment and the peaks of nonphosphopeptides dominated the spectra. The result suggested that CNTs/TiO_2 composites were capable of selectively trapping phosphopeptides from naturally obtained complex sample.

To further evaluate the performance of CNTs/TiO_2 composites for phosphopeptide enrichment, the microspheres were employed to enrich phosphopeptides from tryptic digest of rat brain. 2 mg CNTs/TiO_2 composites were used to enrich the phosphopeptides derived from 100 mg of rat brain protein digest. The peptides eluted from CNTs/TiO_2 composites were analyzed by LC–ESI–MS. The acquired MS/MS spectra were searched separately by the Sequest program as Section 2 described. The searching results were filtered with Xcorr values obtained by statistical calculation of reverse database searching results. For $p < 0.05$, the Xcorr > 2.0, 2.9, and 3.73 for singly, doubly, and triply charged ions, respectively and DCn > 0.1 were used to filter the MS/MS database searching results. In total, 240 phosphorylation sites—211 on serine (87.92%), 27 on threonine (11.25%) and 2 on tyrosine (0.83%)—were identified. The detailed information of the phosphopeptides enriched from tryptic digests of rat brain by using CNTs/TiO_2 composites were listed in the ESI (see Supplementary Table S1†).

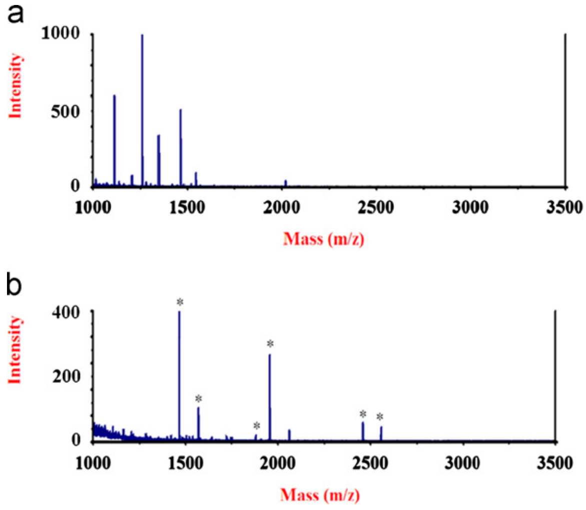

Fig. 7. MALDI mass spectrum of peptides derived from human serum (a) without enrichment and (b) enriched by TiO_2 nanoparticles coated carbon nanotubes, where the * indicates the phosphopeptides.

4. Conclusions

In this work, a titania nanoparticles coated carbon nanotubes composite has been synthesized through a facile but effective solvothermal reaction. The obtained CNTs/TiO_2 composites consist of CNT core and a rough outer layer formed by anatase titania nanoparticles (5–10 nm). The composites have a high specific surface area of about 104 m^2/g. As a result of their high surface area and affinity to phosphopeptides, the CNTs/TiO_2 composites were successfully applied to selectively enrich phosphopeptides for mass spectrometry analysis. The high selectivity and capacity of the CNTs/TiO_2 composites have been demonstrated by effectively enriching phosphopeptides from digests of phosphoprotein, protein mixtures of β-casein and bovine serum albumin, human serum and rat brain samples. It is expected that the novel CNTs/TiO_2 composites could be widely used as novel absorbents for selective enrichment and analysis of phosphopeptides from various complex samples by MALDI-TOF MS.

Acknowledgments

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Appendix A. Supplementary materials

Supplementary materials associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.12.026.

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