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# Highly selective enrichment of phosphopeptides with high-index facets exposed octahedral tin dioxide nanoparticles for mass spectrometric analysis

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## ABSTRACT

High-index facets exposed octahedral tin dioxide (SnO<sub>2</sub>) nanoparticles were successfully synthesized and applied to selectively enrich phosphopeptides for mass spectrometric analysis. The high selectivity and capacity of the octahedral SnO<sub>2</sub> nanoparticles were demonstrated by effectively enriching phosphopeptides from digests of phosphoprotein ( $\alpha$ - or  $\beta$ -casein), protein mixtures of  $\beta$ -casein and bovine serum albumin, milk, and human serum samples. The unique octahedral SnO<sub>2</sub> with abundant unsaturated coordination Sn atoms exhibited enhanced affinity and selective coordination ability with phosphopeptides due to their high chemical activity. The strong affinity led to highly selective capture and enrichment of phosphopeptides for sensitive detection through the bidentate bonds formed between surface atoms and phosphate. The phosphopeptides could be detected in  $\beta$ -casein down to  $4 \times 10^{-9}$  M or in the mixture of  $\beta$ -casein and BSA with a molar ratio of even 1:100. The performance in selective enrichment of phosphopeptides from drinking milk and human serum showed powerful evidence of high selectivity and efficiency in identifying the low-abundant phosphopeptides from complicated biological samples. This work provided a way to improve the physical and chemical properties of materials by tailoring their exposed facets for selective enrichment of phosphopeptides.

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## 1. Introduction

Protein phosphorylation, one of the most important protein post-translational modifications, plays the key roles in regulating complex biological processes, such as cellular growth, division, and signaling transduction. Thus, the identification and localization of phosphorylation sites in proteins are of important significance for understanding biochemical pathways and disease states [1–4]. Mass spectrometry (MS) has emerged as the most powerful technique for examining the phosphorylation process due to its ultrahigh sensitivity, wide dynamic range and superior speed in analyzing complex samples. Unfortunately, owing to the low abundance of phosphorylated peptides, the substoichiometry of phosphorylation and the signal suppression of non-phosphorylated peptides, phosphorylation analysis still remains a great challenge for comprehensive understanding of protein phosphorylation [5,6]. Therefore, selective capture and enrichment of phosphorylated peptides from the complex mixtures prior to the interrogation of phosphorylation by MS has become an interesting research topic in bioanalysis.

A variety of enrichment strategies such as immobilized metal ion affinity chromatography (IMAC) [7–11], immunoprecipitation [12,13], and metal oxide affinity chromatography (MOAC) [14–17] have been developed to selectively capture and enrich the targeted phosphopeptides. Although IMAC is the most widely used technology, MOAC has shown higher capacity for selective enrichment of phosphopeptides and phosphoproteins [18]. Recently, various metal oxides such as titanium dioxide (TiO<sub>2</sub>) [19–21], zirconium dioxide [18], alumina [22], tin dioxide (SnO<sub>2</sub>) [23–28] and some composites [29–32] have been demonstrated to be specific in the trapping of phosphoproteins/phosphopeptides due to their specific and reversible interaction with phosphate groups through Lewis acid–base and bidentate bonds [14]. Especially, SnO<sub>2</sub> has been identified as an affinity probe for phosphopeptide enrichment and shown more excellent selectivity and less nonspecific binding than TiO<sub>2</sub> [23].

As an affinity probe, SnO<sub>2</sub> has been used for preparation of gas sensors [33,34], photocatalysis [35] and fabrication of lithium rechargeable batteries [36]. Recently, some surface scientists have demonstrated that the performance of SnO<sub>2</sub> in gas sensing and catalysis is greatly affected by the surface structure. In principle, phosphopeptides enrichment by metal-oxide like SnO<sub>2</sub> occurring on the surface metal-oxide, considering the fact that high-energy

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or high-index facets with abundant unsaturated coordination atoms usually exhibit high surface activity [37–43], the morphological control of metal oxides may provide a way to further tailor the physical and chemical properties of nanomaterials. Thus it is possible to use high-index facets tin oxide nanoparticles for efficiently improving sensitivity and selectivity of phosphopeptides enrichment.

This work investigated the high-index facets of octahedral  $\text{SnO}_2$  nanoparticles and observed the strong affinity of the nanoparticles to phosphopeptides due to the formation of bidentate bonds between high-density surface Sn atoms and phosphate [14]. The high surface activity made the nanoparticles become a novel metal oxide material for selective enrichment of phosphopeptides. By coupling with the enrichment process, a mass spectrometric method was thus proposed for simultaneous detection of three phosphopeptides in the tryptic digest of  $\beta$ -casein down to  $4 \times 10^{-9}$  M. The performance of octahedral  $\text{SnO}_2$  nanoparticles in selective enrichment of low abundance phosphopeptides from the tryptic digests of drinking milk and human serum clearly demonstrated its great capability in phosphoproteome analysis for real biological samples. This is the first example using the high-index facets exposed metal oxide materials for phosphopeptide capture, and provides the promising application in proteomics.

## 2. Experimental

### 2.1. Materials and reagents

Tin (IV) chloride pentahydrate ( $\text{SnCl}_4 \cdot 5 \text{H}_2\text{O}$ ), trifluoroacetic acid (TFA,  $\geq 90\%$ ), ammonium bicarbonate, DL-dithiothreitol (DTT), iodoacetamide (IAA), phosphoric acid ( $\geq 85\%$ ),  $\beta$ -casein (Mw  $\sim 24$  kDa), bovine serum albumin (BSA, Mw  $\sim 66$  kDa),  $\alpha$ -casein (Mw  $\sim 24$  kDa) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was obtained from Promega (Madison, USA). Acetonitrile (ACN) was obtained from Merck (Darmstadt, Germany). Hydrochloric acid (36.0–38.0%), polyvinylpyrrolidone (PVP, K-30) and ethanol ( $\geq 99.7\%$ ) were purchased from Nanjing Chemical Reagent CO., Ltd. (China). All these reagents were used as received without further purification. Drinking milk was purchased from a local grocery store. The clinical serum samples were from Jiangsu Institute of Cancer Prevention and Cure. All aqueous solutions were prepared using ultra-pure water ( $\geq 18 \text{ M}\Omega$ , Milli-Q, Millipore).

### 2.2. Synthesis of octahedral tin dioxide nanoparticles

Octahedral tin dioxide nanoparticles with high-index {211} facets were synthesized through a hydrothermal route at  $200^\circ\text{C}$  for 12 h according to previous report with some modifications [44]. About 1.2 mL of concentrated hydrochloric acid (mass fraction 36.5–38%) was added to 12 mL ethanol/distilled water (1/1, v/v) under intense ultrasonic treatment. About 0.5 g of  $\text{SnCl}_4 \cdot 5 \text{H}_2\text{O}$  was added into the mixture, sonicated for 15 min at a frequency of 40 kHz, and stirred for 60 min. Then 0.315 g PVP was added, sonicated for 15 min, and stirred for 60 min. The obtained solution was transferred to a Teflon-lined stainless steel autoclave and kept at  $200^\circ\text{C}$  for 12 h. The product was further underwent centrifugation and washed with deionized water and ethanol several times, and then dried in air at  $80^\circ\text{C}$  for 24 h.

### 2.3. Characterization of octahedral tin dioxide nanoparticles

Scanning electron microscopic images were obtained with a Hitachi S-3000 N scanning electron microscope at an acceleration voltage of 10 kV. The transmission electron microscopic (TEM)

images were gained on a JEM-2100 TEM (JEOL, Japan). Powder X-ray diffraction patterns were recorded on Rigaku Dmax 2200 X-ray diffractometer with Cu  $K\alpha$  radiation ( $\lambda = 1.5416 \text{ \AA}$ ). The dynamic light-scattering measurement was carried out using a Brookhaven BI-9000AT instrument (Brookhaven Instruments Corporation).

### 2.4. Digestion of proteins

About 1 mg of the desired protein ( $\beta$ -casein or  $\alpha$ -casein) was dissolved in 1 mL of  $\text{NH}_4\text{HCO}_3$  solution (25 mM,  $\text{pH} \sim 8$ ), and digested with trypsin at an enzyme-to-protein ratio of 1:40 (w/w) for 16 h at  $37^\circ\text{C}$ . The digestion of BSA was performed by adding  $20 \mu\text{L}$  of 100 mM DTT in  $100 \mu\text{L}$  of  $4 \text{ mg mL}^{-1}$  BSA in 25 mM  $\text{NH}_4\text{HCO}_3$  solution ( $\text{pH} \sim 8$ ) to incubate at  $100^\circ\text{C}$  for 10 min, followed with cooling, addition of  $20 \mu\text{L}$  of 1.0 M IAA, incubation in the dark for 45 min at room temperature, and addition of  $20 \mu\text{L}$  of DTT to react with the excessive IAA at room temperature for 1 h, and finally 10-fold diluting the mixture with 25 mM  $\text{NH}_4\text{HCO}_3$  and adding trypsin at an enzyme-to-protein ratio of 1:40 (w/w) to incubate for 16 h at  $37^\circ\text{C}$ . For real samples,  $30 \mu\text{L}$  of drinking milk was added in  $900 \mu\text{L}$  of  $\text{NH}_4\text{HCO}_3$  solution (25 mM,  $\text{pH} \sim 8$ ) and centrifuged at 16,000 rpm for 15 min to obtain the supernatant for tryptic digestion. After the supernatant was denatured at  $100^\circ\text{C}$  for 5 min,  $30 \mu\text{g}$  of trypsin was added in the solution to digest at  $37^\circ\text{C}$  for 16 h, which was then diluted 25 times with 0.5% TFA in 50% ACN (v/v). All the tryptic peptide mixtures were stored at  $-20^\circ\text{C}$  until use.

### 2.5. Selective enrichment of phosphopeptides

After the digestion product was further diluted with 0.5% TFA in 50% ACN (v/v),  $50 \mu\text{L}$  of octahedral  $\text{SnO}_2$  ( $6 \text{ mg mL}^{-1}$ ) was added into  $200 \mu\text{L}$  of the diluted peptide mixture (Fig. 1). The mixture was then vibrated at  $25^\circ\text{C}$  for 30 min and centrifuged to obtain the precipitates, which was washed thrice with 0.1% TFA in 60% ACN (v/v). The human serum sample could be directly used for phosphopeptide capture by mixing  $30 \mu\text{L}$  sample with  $120 \mu\text{L}$  of 0.5% TFA in 50% ACN (v/v) and then treating the mixture with octahedral  $\text{SnO}_2$  ( $6 \text{ mg mL}^{-1}$ ). After the trapped phosphopeptide was eluted using  $5 \mu\text{L}$  10%  $\text{NH}_3 \cdot \text{H}_2\text{O}$  under sonication for 10 min, the eluate was analyzed by MALDI-TOF MS.

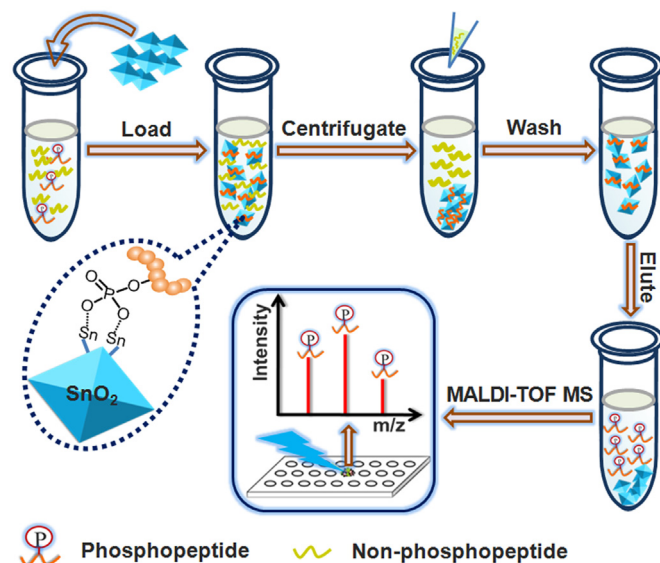


Fig. 1. Schematic illustration of selective enrichment of phosphopeptides using octahedral  $\text{SnO}_2$  for mass spectrometric analysis.

## 2.6. MALDI-TOF MS analysis

After 0.5  $\mu\text{L}$  of the eluate was deposited on a MALDI plate, 0.5  $\mu\text{L}$  of 2, 5-DHB (20 mg  $\text{mL}^{-1}$ ) in 50% ACN (v/v) containing 1%  $\text{H}_3\text{PO}_4$  was introduced as a matrix to perform the MALDI-TOF MS analysis in a positive ion mode on a 4800 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.

## 3. Results and discussion

### 3.1. Preparation and characterization of octahedral $\text{SnO}_2$ nanoparticles

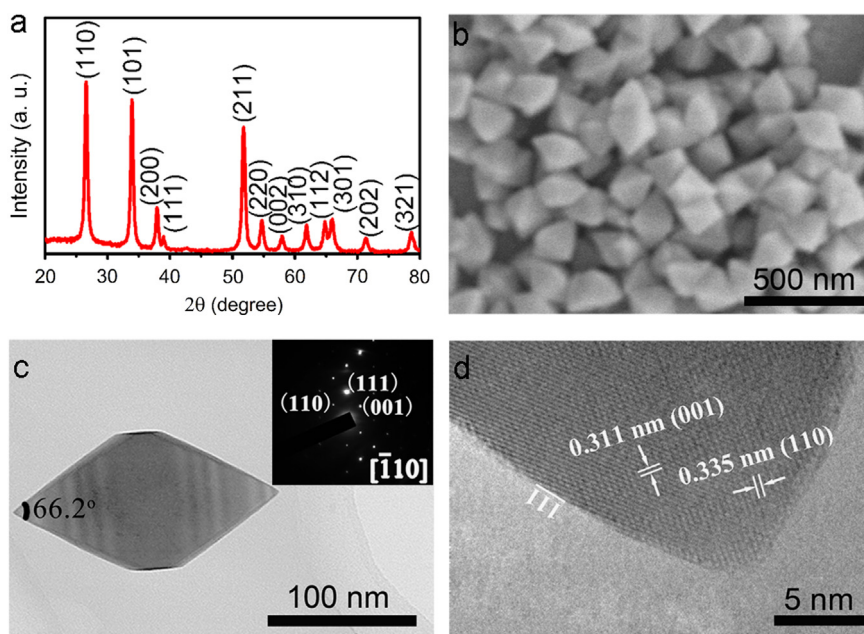
The crystalline structure of the as-prepared product was confirmed by X-ray diffraction (XRD). All the peaks in the XRD pattern could be indexed to the rutile phase of bulk  $\text{SnO}_2$  with cell constants of  $a=4.738$  and  $c=3.187$  Å (JCPDS No. 41-1445) (Fig. 2a). The scanning electron microscopic (SEM) image of the  $\text{SnO}_2$  nanoparticles showed a well-proportioned octahedral structure with a length of approximately 190 nm (Fig. 2b). Dynamic light scattering measurement also showed the hydrodynamic diameter of the  $\text{SnO}_2$  nanoparticles to be about 200 nm with a narrow size distribution (Fig. S1, Supplementary material). More-detailed structural information of an individual octahedral  $\text{SnO}_2$  particle was provided by transmission electron microscopy (TEM) (Fig. 2c). The corresponding selected-area electron diffraction (SAED) pattern could be indexed to the  $[\bar{1}10]$  zone axis of single-crystal tetragonal  $\text{SnO}_2$  (inset in Fig. 2c). The apex angle between the two side surfaces was about  $66.2^\circ$ . These structural features agree well with the model of octahedral  $\text{SnO}_2$  enclosed by  $\{221\}$  facets projected along the  $[\bar{1}10]$  direction [44]. The (221) surface can be described as combination of (111) terraces and (110) steps. Such a stepped surface composed of (111) terraces and (110) steps was directly captured in high-resolution TEM (Fig. 2d). These results clearly confirmed that the high-index facets  $\{221\}$  exposed octahedral  $\text{SnO}_2$  nanoparticles were successfully obtained. And the synthesized material has abundant unsaturated coordination atoms, which usually exhibits high surface activity.

### 3.2. Selective enrichment of phosphopeptides from tryptic digestion of $\beta$ -casein

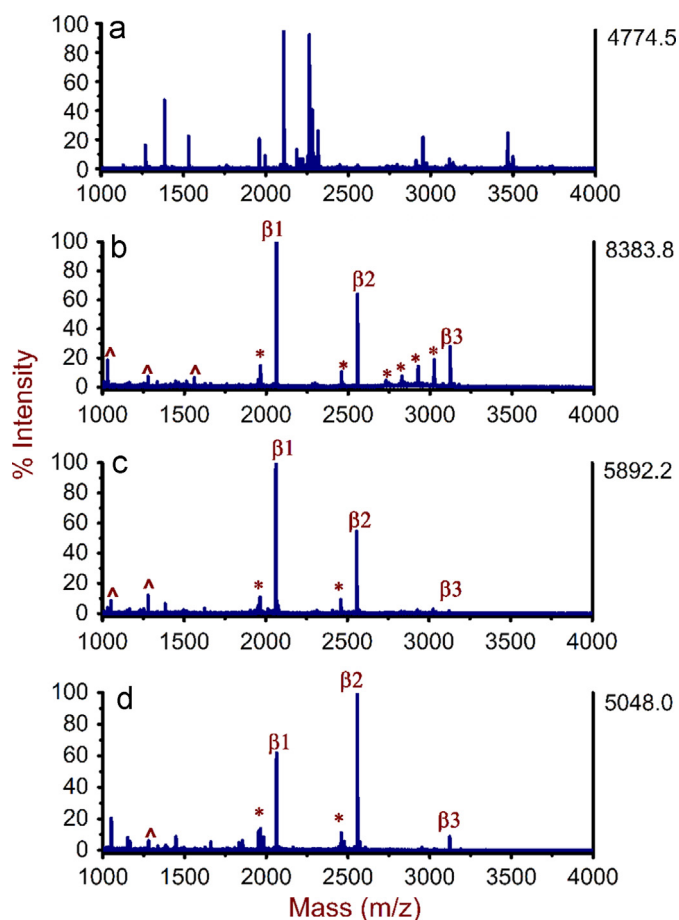
In light of the unique characters of the synthesized octahedral  $\text{SnO}_2$ , its selective enrichment capacity toward phosphopeptides in the tryptic digest of bovine  $\beta$ -casein was firstly examined by MALDI-TOF MS. The direct analysis of  $\beta$ -casein digest without enrichment did not show the MS signals of the phosphopeptides due to the overwhelming of many dominant peaks of non-phosphopeptides in the sample (Fig. 3a). After selective enrichment by octahedral  $\text{SnO}_2$ , the signals of  $\beta 1$ ,  $\beta 2$  and  $\beta 3$  phosphopeptides could be observed (Fig. 3b). Moreover these peaks dominated the spectrum, indicating a good enrichment efficiency of the octahedral  $\text{SnO}_2$ . This result demonstrated the selectivity of octahedral  $\text{SnO}_2$  nanoparticles toward phosphopeptides. The details of peptides detected in the MALDI-TOF spectra were summarized in Table S1 (Supplementary material).

To demonstrate the superiority of the high-index facets exposed octahedral  $\text{SnO}_2$  for enrichment of phosphopeptides,  $\text{SnO}_2$  nanosphere (Fig. S2, Supplementary material) and commercial  $\text{TiO}_2$  were used for comparison. As shown in Fig. 3b–d, the mass spectrum using octahedral  $\text{SnO}_2$  for enrichment exhibited three phosphopeptides with higher intensity than  $\text{SnO}_2$  nanosphere and commercial  $\text{TiO}_2$ , indicating stronger capture ability. This was possibly due to the distinguished feature of octahedral  $\text{SnO}_2$  with high densities of atom steps, ledges, kinks, and dangling bonds that usually exhibit high chemical-reaction activity. The  $\beta 3$  phosphopeptide exhibited more metastable losses of phosphoric acid (Fig. 3b) due to the high chemical-reaction activity of octahedral  $\text{SnO}_2$  [37].

The detection limit of  $\beta$ -casein digest with enrichment by octahedral  $\text{SnO}_2$  nanoparticles was also compared with that by  $\text{SnO}_2$  nanosphere. In the digest of  $4 \times 10^{-9}$  M  $\beta$ -casein, the ion signals of  $\beta 1$ ,  $\beta 2$  and  $\beta 3$  phosphopeptides could still be detected after enrichment with octahedral  $\text{SnO}_2$  nanoparticles (Fig. 4), while the MALDI mass spectrum after enrichment with  $\text{SnO}_2$  nanosphere could not give clear signal of  $\beta 3$  phosphopeptide at the concentration of even  $2 \times 10^{-7}$  M, and the intensities for  $\beta 1$  and  $\beta 2$  at  $4 \times 10^{-9}$  M were lower than three times ratio of signal-to-noise (Fig. 5). The detection limit was comparable with



**Fig. 2.** (a) XRD pattern and (b) SEM image of octahedral  $\text{SnO}_2$ , (c) TEM image of single octahedral  $\text{SnO}_2$  nanoparticle viewed along the  $[110]$  direction (inset: the corresponding SAED pattern), and (d) high-resolution TEM image taken from the top apex of the octahedron.

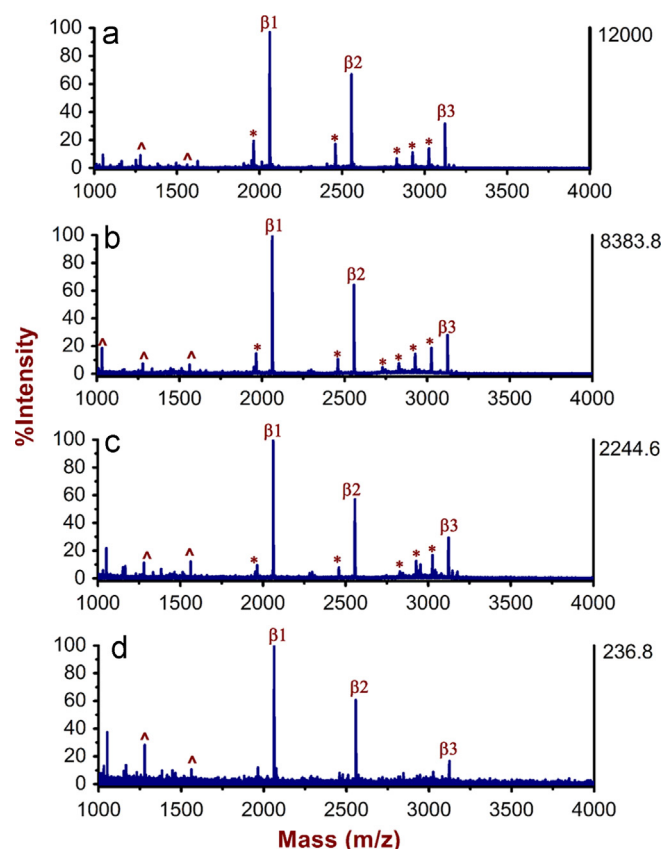


**Fig. 3.** MALDI mass spectra of tryptic digest of  $4 \times 10^{-8}$  M  $\beta$ -casein without (a) and with enrichment by octahedral  $\text{SnO}_2$  nanoparticles (b),  $\text{SnO}_2$  nanosphere (c) and commercial  $\text{TiO}_2$  (d) (\* metastable losses of phosphoric acid, ^ doubly charged phosphopeptide).

the previously reported  $\text{Fe}_3\text{O}_4@\text{C}/\text{SnO}_2$  core-shell microspheres ( $2 \times 10^{-9}$  M) [24] and  $\alpha\text{-Fe}_2\text{O}_3@\text{SnO}_2$  core-shell nanotubes ( $4 \times 10^{-9}$  M) [25] for  $\beta$ -casein digest enrichment. The good sensitivity could be attributed to the abundant unsaturated coordination Sn atoms that exhibited enhanced affinity and selective coordination ability to phosphopeptides. The high detection sensitivity further suggested that the octahedral  $\text{SnO}_2$  nanoparticles are promising nanomaterial for enrichment of phosphopeptides.

### 3.3. Highly specific enrichment of phosphorylated peptides from complex peptide mixtures

To further evaluate the enrichment specificity, a relatively complex sample, peptide mixture originating from a tryptic digest of  $\beta$ -casein and bovine serum albumin at molar ratio of 1:10 and 1:100, was used for test. Different from the direct analysis (Fig. 6a), the enrichment by octahedral  $\text{SnO}_2$  excluded the interference with three phosphopeptides from the highly contaminated mixture, even at the BSA concentration 100 times higher than  $\beta$ -casein (Figs. 6b and 6c). The selectivity was much better than the previously reported  $\text{Fe}_3\text{O}_4@\text{C}/\text{SnO}_2$  core-shell microspheres (1:50) [24] and  $\alpha\text{-Fe}_2\text{O}_3@\text{SnO}_2$  core-shell nanotubes (1:50) [25]. Furthermore, the high-concentration salt and denaturants such as ammonium bicarbonate, dithiothreitol and iodoacetamide used in the process of BSA digestion did not affect the specific capture of phosphopeptides. These results demonstrated the reliable



**Fig. 4.** MALDI mass spectra of tryptic digests of (a)  $2 \times 10^{-7}$ , (b)  $4 \times 10^{-8}$ , (c)  $2 \times 10^{-8}$  and (d)  $4 \times 10^{-9}$  M  $\alpha$ -casein after enriched by octahedral  $\text{SnO}_2$  nanoparticles (\* metastable losses of phosphoric acid; ^ doubly charged phosphopeptide).

performance of the octahedral  $\text{SnO}_2$  nanoparticles in the selective capture of phosphopeptides from the complex peptide mixture.

### 3.4. Selective enrichment of phosphopeptides from tryptic digestion of $\alpha$ -casein

The selective capture ability of octahedral  $\text{SnO}_2$  nanoparticles could be used for enrichment of more phosphopeptides. For example, in the absence of enrichment only two weak phosphopeptide peaks were observed for the tryptic digest of  $\alpha$ -casein that contains more phosphopeptides, while the mass spectrum after enrichment showed 18 phosphopeptides with considerable intensities (Fig. 7). The detectable number exceeded those with  $\text{Zr}^{4+}$ -IMAC (14) [9] and  $\text{Ti}^{4+}$ -phosphate functionalized cellulose (14) [10] for  $\alpha$ -casein digest enrichment. These results clearly indicated the high selectivity of the octahedral  $\text{SnO}_2$  nanoparticles. The details of phosphopeptides detected in  $\alpha$ -casein were listed in Table S2 (Supplementary material).

### 3.5. Highly specific enrichment of phosphopeptides from milk and human serum

Nonfat bovine milk contains two main phosphorylated proteins,  $\alpha$ -casein and  $\beta$ -casein, which have been widely used as standard proteins for enrichment of phosphopeptides. The capability of octahedral  $\text{SnO}_2$  to selectively trap phosphopeptides was thus evaluated using drinking milk as real samples. Fig. 8a displays the direct analysis of the tryptic digests of milk, in which non-phosphopeptides dominated the spectrum and only two weak peaks were generated from phosphopeptides. However, the mass

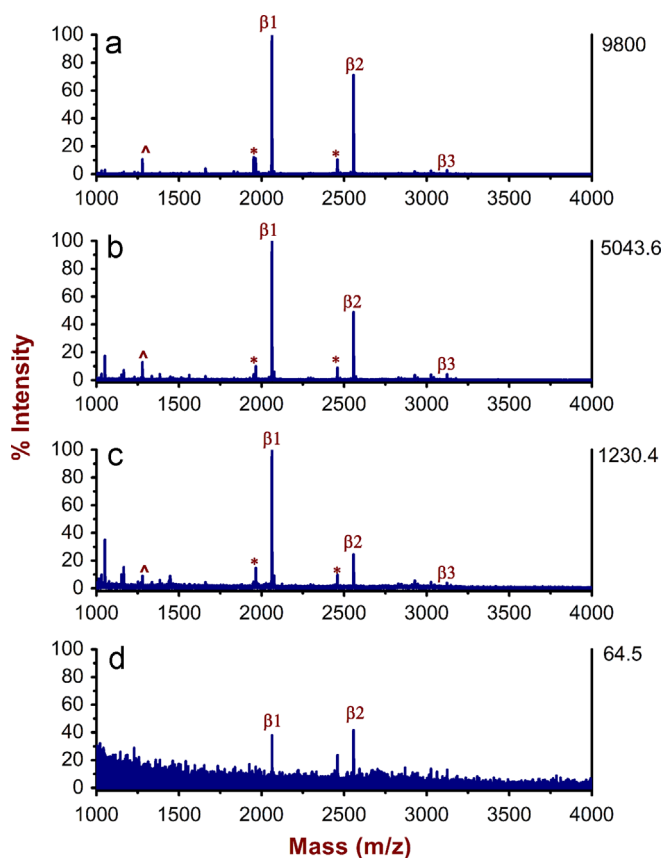


Fig. 5. MALDI mass spectra of tryptic digests of (A)  $2 \times 10^{-7}$ , (B)  $4 \times 10^{-8}$ , (C)  $2 \times 10^{-8}$  and (D)  $4 \times 10^{-9}$  M  $\beta$ -casein after enrichment by  $\text{SnO}_2$  nanosphere (\* metastable losses of phosphoric acid; ^ doubly charged phosphopeptide).

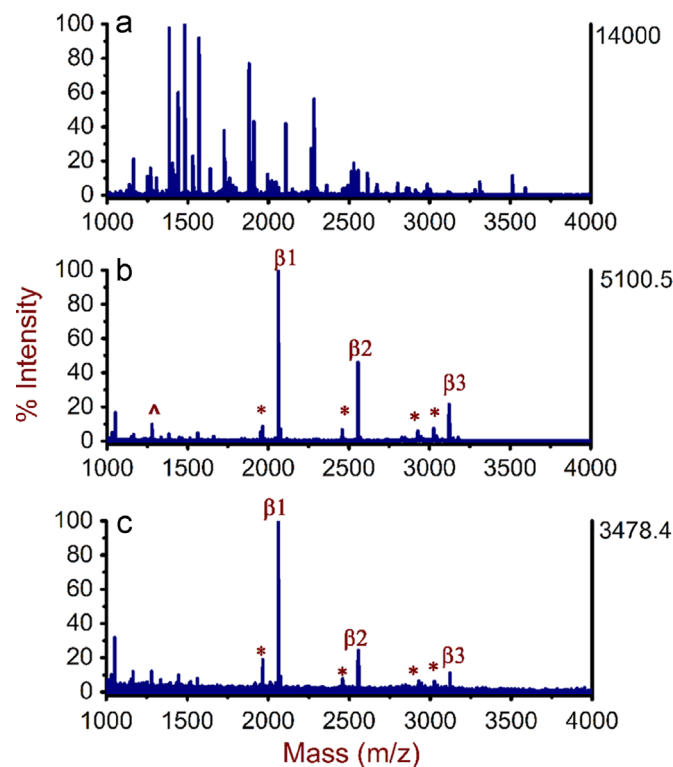


Fig. 6. MALDI mass spectra of tryptic digest of a mixture of  $\beta$ -casein/BSA at molar ratios of 1:10 for direct analysis (a) and with enrichment by octahedral  $\text{SnO}_2$  (b), and (c) 1:100 with enrichment by octahedral  $\text{SnO}_2$  (\* metastable losses of phosphoric acid, ^ doubly charged phosphopeptide).

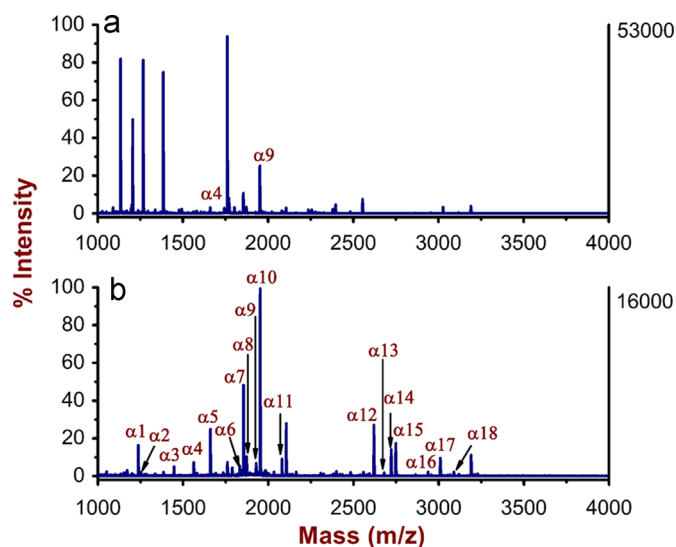


Fig. 7. MALDI mass spectra of tryptic digest of  $\alpha$ -casein (a) without and (b) with enrichment by octahedral  $\text{SnO}_2$  nanoparticles.

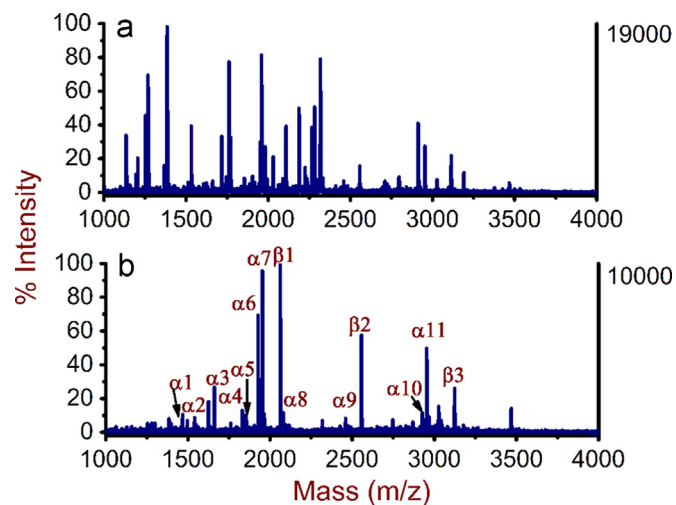


Fig. 8. MALDI mass spectra of tryptic digest of nonfat milk (a) without and (b) with enrichment by octahedral  $\text{SnO}_2$ .

spectrum of tryptic digest of nonfat milk was dominated by 14 phosphorylated peptide peaks after enrichment by octahedral  $\text{SnO}_2$  (Fig. 8b, Table S3 in Supplementary material). It was much better than that with  $\text{SnO}_2$  nanoparticles (10) and comparable with  $\text{SnO}_2$ - $\text{ZnSn}(\text{OH})_6$  composite (15) [28]. The detectable number also exceeded the number using  $\text{Ce}^{4+}$ -chelated magnetic silica microspheres (10) [11]. Thus the octahedral  $\text{SnO}_2$  nanoparticles possessed high selectivity and effectiveness in enrichment of phosphopeptides from real biological samples.

The human serum sample was also used for testing the specificity in enrichment of endogenous low-abundant phosphopeptides. The direct analysis of human serum is not shown here due to the presence of extremely high concentrations of salts that led to significant suppression of ionization in MALDI. Human serum sample without digestion showed four peaks of phosphopeptides derived from phosphorylated fibrinopeptide A [45], and some phosphopeptides showed the metastable losses of phosphoric acid (Fig. 9 and Table S4, Supplementary material). These results support the capability of octahedral  $\text{SnO}_2$  to selectively isolate and enrich phosphopeptides from the extremely complicated biological system.

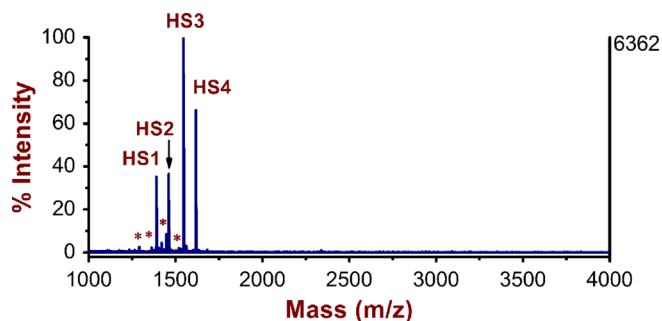


Fig. 9. MALDI mass spectrum of human serum sample after enriched by octahedral  $\text{SnO}_2$  nanoparticles (\* metastable losses of phosphoric acids).

#### 4. Conclusions

Octahedral  $\text{SnO}_2$  nanoparticles with exposed high-energy {221} facets were firstly applied in the selective capture and enrichment of phosphopeptides. The octahedral  $\text{SnO}_2$  showed strong affinity to phosphopeptides by forming the bidentate bonds between high-density surface atoms and phosphate, and thus can achieve the selective enrichment of phosphopeptides. The octahedral  $\text{SnO}_2$  shows excellent performance for selective enrichment of phosphopeptides from real complex samples. The advantages of this material for enrichment include its high adsorption capacity, high selectivity, easy separation from solution, and high sensitivity for mass spectroscopic detection. All the tests indicated that the octahedral  $\text{SnO}_2$  can act as an ideal adsorbent for phosphopeptides. This work provides a way to improve the physical and chemical properties of materials for selective enrichment of phosphopeptides, and extends the application of metal oxides with high-energy facets in molecular identification, especially in the MS-based phosphoproteomic study.

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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.2013.11.049>.

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