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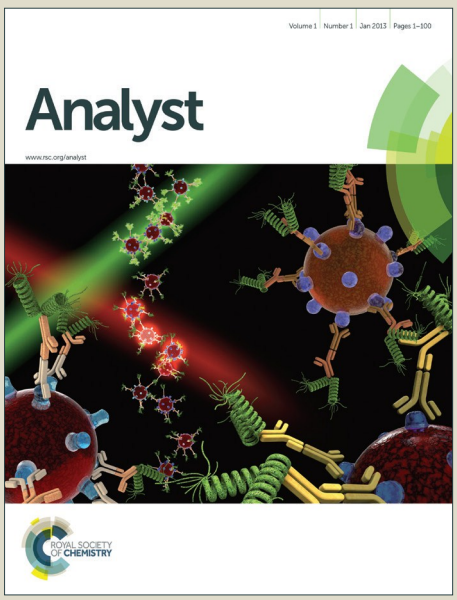

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Hydrophilic modification of titania nanomaterials as a biofunctional adsorbent for selective enrichment of phosphopeptides

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Abstract

TiO_2-based metal oxide affinity chromatography (MOAC) nanomaterials show high potential in the phosphoproteome mass-spectrometric (MS) analysis. However, a drawback of TiO_2 nanomaterials is poor water solubility,which will greatly reduce the enrichment efficiency of phosphopeptides and eventually limit its use in the phosphoproteome MS analysis. In this work, a hydrophilic TiO_2 hybrid material (denoted as $NH_2@TiO_2$) is successfully designed with 1,6-hexanediamine modified on the surface of nanoparticle TiO_2 and applied as a biofunctional adsorbent for selective enrichment of phosphopeptides. The novel TiO_2 hybrid material with high hydrophilicity and biocompatibility is charactered using scanning electron microscopy (SEM), the energy dispersive X-ray (EDX) spectrum and infrared (IR), and its performance in selective enrichment of phosphopeptides is evaluated with the standard protein digests, human serum and the tryptic digests of nonfat milk.

Key words: mass spectrometry, phosphopeptides, enrichment, TiO_2-based metal oxide affinity chromatography

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1.Introduction

As one of the most important and ubiquitous post-translational modifications (PTMs), protein phosphorylation plays the key roles in eukaryotic cells, such as cell division, growth and intercellular signaling transduction[1-3]. However, due to the low abundance of phosphopeptides and the suppression effect of nonphosphorylated peptides in protein digests, the detection of phosphopeptides may be still a major challenge by mass spectrometry (MS)[4,5] . Therefore, it is essential to selectively enrich phosphopeptides from complicated mixtures prior to MS analysis.

To solve these problems, various enrichment strategies such as immunoprecipitation, chemical-modification strategies, immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC) have been widely studied and used in the enhancement of phosphopeptides[6-9]. Among them, TiO_2-based MOAC nanomaterials have been regarded as one of the most promising materials for phosphopeptides enrichment, because TiO_2 could provide lots of interaction sites due to the large specific surface area, which leads to a higher enhancement capacity and better selectivity in the process of analysis of phosphopeptides. However, TiO_2 nanomaterials show strong hydrophobicity, which greatly reduces the enhancement efficiency, because phosphopeptides show more hydrophilicity than other peptides[10-12]. Thus, hydrophilicity surface of TiO_2 nanomaterials is crucial for the enhancement of phosphopeptides.

It has been proved that amino groups could be modified to the surface of nanomaterials to improve water solubility and biocompatibility due to the formation of hydrogen bonds between the amino groups and water. The good water solubility and biocompatibility could make nanomaterials more convenient for use in bioseparation and biomedical application. In addition, amino groups are easily protonated and carried a positive charge at strong acid solution(pH=2), because most primary amines were reported with a mean pK_a of 9. Protonated amino groups will provide a possible means of enriching phosphopeptides by interaction with the negatively charged phosphate groups on the phosphopeptides, which will be very beneficial for the improvement of the enhancement efficiency[13,14].

Herein, a hydrophilic TiO_2 hybrid material (denoted as NH_2@TiO_2) with 1,6-hexanediamine modified on the the surface of nanoparticle TiO_2 was sythesized and applied for selective

enrichment of phosphopeptides. TiO_2 nanomaterials are functionalized with amino groups, which makes $NH_2@TiO_2$ water soluble and able to offer enhanced affinity to phosphopeptides due to the interaction between the phosphate groups and amine groups. The nanohybrids $NH_2@TiO_2$ possess high hydrophilicity and biocompatibility, improved selectivity to phosphopeptides, large loading amounts of TiO_2 nanomaterials. The $NH_2@TiO_2$ with the above properties are anticipated to have excellent performance for the selective enrichment of phosphopeptides.

2. Experimental

2.1. Materials and chemicals

Trifluoroacetic acid (TFA), β-casein, bovine serum albumin (BSA), ammonium bicarbonate(NH_4HCO_3), DL-dithiothreitol(DTT), iodoacetamide (IAA) and 2,5-dihydroxybenzoic acid (2,5-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). Human serum was supplied by Jiangsu Province Hospital of TCM. Nonfat milk was purchased from a local supermarket. All aqueous solutions were prepared using Milli-Q water by Milli-Q purification system (Millipore, Milford, MA, USA).

2.2. Synthesis of $NH_2@TiO_2$

The hydrophilic TiO_2 hybrid material $NH_2@TiO_2$ was prepared by a sol–gel method with some modification[15]. A solution of hydrochloric acid (pH=2, 10 mL) and ethanol (20 mL) were dispersed into 20 mL of ethanol solution containing 4 mL of acetic acid and 4 mL of tetrabutyltitanate ($C_{16}H_{36}O_4Ti$) with magnetic stirring for 3 h to form a steady colloid solution. The colloid solution was first dried at 100 °C and then calcined in a muffle at 450 °C for 3 h to obtain TiO_2 nanoparticle. $NH_2@TiO_2$ nanohybrids were synthesized by stirring TiO_2 (0.1 g) and 1,6-hexanediamine (0.6 g) for 24 h in 30 mL of glycol at room temperature(RT). The obtained solution was further underwent centrifugation and washed with ethanol and deionized water several times, and then dried at 100 °C for 5 h.

2.3. Characterization of $NH_2@TiO_2$

A Nicolet NEXUS 670 Fourier transform infrared spectrometer was employed to record the Infrared spectra. A JSM-5610LV scanning electron microscope system was used to gain Scanning electron microscopy (SEM) images. A HORIBA EX-250 Energy-dispersive X-ray(EDX) spectrometer was used to obtain the chemical composition of the $NH_2@TiO_2$ nanohybrids. Thermogravimetric analysis (TGA) studies were carried out on a PerkinElmer Pyris Diamond (PerkinElmer, USA) from 30 °C to 700 °C with a heating rate of 10 °C/min under nitrogen.

2.4. Sample preparation

The protein (bovine β-casein or bovine serum albumin (BSA)) was dissolved in 25 mM ammonium bicarbonate buffer solution (pH=8.1) containing trypsin at the ratio of enzyme-to-protein of 1:40 (w/w) at 37 °C with overnight shaking. For the real sample, 50 μL of nonfat milk was diluted to 250 μL in 50 mM ammonium bicarbonate buffer solution. After centrifugation at 14000 rpm for 25 min, the supernatant was denatured at 100 ℃ for 5 min, and then incubated for 12 h at 37 ℃ with the addition of 10 μg of trypsin. Human serum was diluted to five times with 50% acetonitrile and 0.1% TFA aqueous solution (v/v).

2.5. Phosphopeptide enrichment

As shown in Fig. 1b, the peptide mixtures originating from tryptic digestions of standard proteins were firstly diluted by 50% acetonitrile and 0.1% TFA water solution (v/v). Then 0.3mg $NH_2@TiO_2$ was added into 100 μL of the diluted peptide mixture. The mixture was vibrated for 30 min and centrifuged to obtain the precipitates, which was rinsed with 50% acetonitrile and 0.1% TFA water solution (v/v) (100 μL) three times. Finally, the phosphopeptides trapped by $NH_2@TiO_2$ nanohybrids were eluted using 10 μL 10% $NH_3 \cdot H_2O$ under sonication for 10 min and the eluate was analyzed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). For phosphopeptide enrichment of peptide mixture digested from nonfat milk, the dilution, binding, washing and elution steps were similar to standard proteins digests.

The enhancement of phosphopeptides from human serum sample was performed by mixing 20 μL human serum sample with 80 μL of 50% acetonitrile and 0.1% TFA aqueous solution(v/v) and then treating the mixture with 0.3 mg $NH_2@TiO_2$ in the procedure elucidated above for tryptic digestions of standard proteins capture.

2.6. MALDI-TOF MS analysis

For MALDI-TOF MS, the above eluate of phosphopeptides (1μL) was deposited on a MALDI plate and dried in the air at room temperature, and then another 1μL of 2,5-DHB (20 mg/mL, dissolved in 50% ACN (v/v) containing 1% H_3PO_4) was introduced as a matrix. Analysis of phosphopeptides was performed with MALDI TOF/TOF MS (BRUKER Daltonics, Germany) in reflected positive-ion mode. The instrument has been equipped with a "Smartbeam™-II technology" laser. The following instrument parameters were used for the positive-ion reflection mode: number of shots, 200; acceleration potential, ±25 kV.

3. Results and discussion

3.1. Synthesis and Characterization of $NH_2@TiO_2$

The hydrophilic TiO_2 hybrid material $NH_2@TiO_2$ was prepared according to Fig. 1a. The TiO_2 nanoparticle was firstly synthesized by a sol-gel process, then the hydroxyls on the surface of TiO_2 (preferentially from the chemisorbed water[16]) reacted with the terminal primary amine on 1,6-hexanediamine by a condensation reaction to obtain the $NH_2@TiO_2$ nanohybrids. The $NH_2@TiO_2$ obtained by the proposed approach was characterized by scanning electron microscopy (SEM), Energy-dispersive X-ray(EDX), infrared (IR) and Thermogravimetric analysis (TGA).

To determine 1,6-hexanediamine was bonded on the surface of TiO_2, we performed IR analysis on $NH_2@TiO_2$ nanohybrids. As shown in Fig. S1(Supplementary material), in the IR spectrum of pure TiO_2, there two peaks at 3425 cm^{-1} and 1351 cm^{-1} were assigned to the stretching and bending vibration of surface OH groups[17]. Fig. 2 showed IR spectrum of the $NH_2@TiO_2$ nanohybrids. The strong absorption band at 530 cm^{-1} was attributed to vibration level of Ti–N bond. Another, some characteristic peaks around 1626,1090,1014 cm^{-1} matched well with that from free 1,6-hexadiamine, which indicated that the free -NH_2 group was retained on the $NH_2@TiO_2$ nanohybrids[18,19].

The composition of the $NH_2@TiO_2$ nanohybrids was fruther investigated by EDX spectrometry. As shown in Fig. 3, it was obviously observed that Ti, C, O and N elements existed in the $NH_2@TiO_2$ nanohybrids , fruther confirming the successful modification of TiO_2 by 1,6-hexanediamine.

The SEM images showed that TiO_2 nanoparticle exhibited a clean surface (Fig. 4a). After modified by 1,6-hexanediamine (Fig. 4b), the surface of TiO_2 was covered with a robust layer. The results indicated that 1,6-hexanediamine has been immobilized on the surface of TiO_2 nanoparticle with high surface coverage.

To test whether the $NH_2@TiO_2$ nanohybrids had a significant hydrophilic property, TiO_2 nanoparticle and $NH_2@TiO_2$ nanohybrids were all dispersed in water with the ultrasonication for 20min. After removing the ultrasonication, the water solutions of TiO_2 and $NH_2@TiO_2$ kept standing for differernt hours. As shown in Fig. 5, the TiO_2 nanoparticle almost completely aggregated to the bottom of the vial within 1 hour. However, the $NH_2@TiO_2$ nanohybrids has formed an uniform water solution and even remained for more than within 3 hours. The results

noticeably revealed that the $NH_2@TiO_2$ nanohybrids had a better water solubility, which was very beneficial for the selective enrichment of phosphopeptides[20,21].

TGA was used to estimate the content of 1,6-hexanediamine and evaluate the stability of $NH_2@TiO_2$. Fig. 6 presented the TGA curves of $NH_2@TiO_2$, it can be observed that the weight loss around 250 ℃ indicated that the content of 1,6-hexanediamine was as high as 27.5 wt% and $NH_2@TiO_2$ was very stable at room temperature.

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

To investigate the enrichment capacity of $NH_2@TiO_2$ nanohybrids toward phosphopeptides, a tryptic digest of β-casein (4×10^{-8} M) was firstly examined by MALDI-TOF MS. The direct analysis of β-casein digests without enhancement only observed two MS signals of phosphopeptides at low intensity due to the suppression of nonphosphopeptides in the sample (Fig.7a). After selective enrichment by $NH_2@TiO_2$, the signals for the phosphopeptides were detected and dominated the spectrum with clear background and high signal/noise ratio (S/N) in Fig.7b, indicating a high enrichment efficiency of $NH_2@TiO_2$ nanohybrids. The detailed information of the observed phosphopeptides was listed in Table S1(Supplementary material).

To demonstrate a better phosphopeptides enhancement capacity of the $NH_2@TiO_2$ nanohybrids, pure TiO_2 was used for comparison. After enrichment with pure TiO_2, only two phosphopeptides could be observed, and some non-phosphopeptides with strong signals also appeared in the mass spectrum(Fig.7c). The results indicated that the $NH_2@TiO_2$ nanohybrids showed a better performance for the enhancement of phosphopeptides.

To study the sensitivity of $NH_2@TiO_2$ used for enrichment of phosphopeptides, β-casein digest solutions with different concentrations were used as the test samples. It can be seen that when the concentration of β-casein digests was 5×10^{-11} M (5 fmol), the signals from phosphopeptides could still be easily detected by MALDI-TOF MS after enrichment by $NH_2@TiO_2$ (Figure 8). The results showed that $NH_2@TiO_2$ had high detection sensitivity for phosphopeptides.

The improved enhancement capacity and higher detection sensitivity might be attributed to high hydrophilicity and enhanced affinity to phosphopeptides due to the surface functionalization of TiO_2 nanomaterials with amino groups. The $NH_2@TiO_2$ nanohybrids with good water solubility could reduce the loss of phosphopeptides through the strong hydrophilic interaction between the amino groups on the surface of TiO_2 and phosphopeptides. On the other hand, the

amino groups on the surface of TiO_2 could provide an additional selectivity for the enhancement of phosphopeptides by the interactions between phosphate groups and amino groups[22,23].

To further evaluate the enrichment specificity of the NH_2@TiO_2 nanohybrids toward phosphopeptides, a more complex peptide mixture of β-casein and BSA at a molar ratio 1:1 and 1:10 was used as a mimic biological sample for test. Before enrichment, nonphosphopeptides peaks with high intensity dominated nearly the whole spectrum (Fig.9a). After enhancement with NH_2@TiO_2, the peaks from phosphopeptides were clearly detected with desired intensities(Fig.9b and Fig.9c). The results further demonstrated that the NH_2@TiO_2 nanohybrids had an excellent performance in selective capture of phosphopeptides from the complex peptide mixture.

3.3 Highly specific selective enrichment of phosphopeptides from human serum and nonfat milk

As a real biosample, the human serum sample without digestion contained four endogenous phosphopeptides derived from phosphorylated fibrinopeptide A[24, 25](The detailed information was listed Table S2, Supplementary material), which was also used for testing the specificity and selectivity of the NH_2@TiO_2 nanohybrids in the enrichment of phosphopeptides from a complex sample. The direct analysis of human serum by MALDI-TOF MS was still a great challenge due to the presence of inorganic salts, abundant proteins and a large amount of nonphosphopeptides that led to the suppression of signals from phosphopeptides[26, 27]. As shown in Fig. 10a, no phosphopeptides were detected before enrichment due to the suppression by other contaminants in serum. After enrichment with NH_2@TiO_2 nanohybrids, four phosphopeptides could be obviously identified and no non-phosphopeptides are observed (Fig. 10b). These results confirmed that the excellent performance of the NH_2@TiO_2 nanohybrids for the selective enrichment of phosphopeptides from real biological samples.

To further assess the feasibility in treating complex biosamples, we utilized the nanohybrids to enrich phosphopeptides from a tryptic digest of nonfat milk containg both the single-phosphopeptides and multi-phosphopeptides. Because of lots of non-phosphopeptides with strong signals, only four phosphopeptides with low signals could be observed(Fig. 10c). In contrast, 15 phosphopeptides with both mono- and multiphosphopeptides were identified after enrichment with NH_2@TiO_2 (Fig. 10d). The sequence information of the observed phosphopeptides was listed in Table S3 in supplementary material. These results further

demonstrated that the NH_2@TiO_2 nanohybrids were capable of selective capturing phosphopeptides from the complex biosamples.

Conclusions

A novel hybrid nanomaterial, the NH_2@TiO_2 nanoparticle was successfully synthesized by modifying 1,6-hexanediamine on the surface of nanoparticle TiO_2 for the enrichment of phosphopeptides with high specificity, extremely high detection sensitivity, enhanced affinity to phosphopeptides. The modification of 1,6-hexanediamine endowed the nanoparticle TiO_2 not only with excellent hydrophilicity and biocompatibility, but also higher affinity to phosphopeptides due to the interaction between the phosphate groups and amine groups, which would greatly improve the enrichment efficiency of phosphopeptides. This work was expected to provide an approach to prepare more efficient TiO_2-based MOAC nanomaterials for phosphoproteome research.

Acknowledgements

This work was supported by The Natural Science Foundation of Jiangsu Higher Education Institutions of China(NO. 15KJD150005). A project funded by the Priority Academic Program Development(PAPD) of Jiangsu Higher Education Institutions of China and NSFC for Talents Training in Basic Science (J1103507，J1210025).

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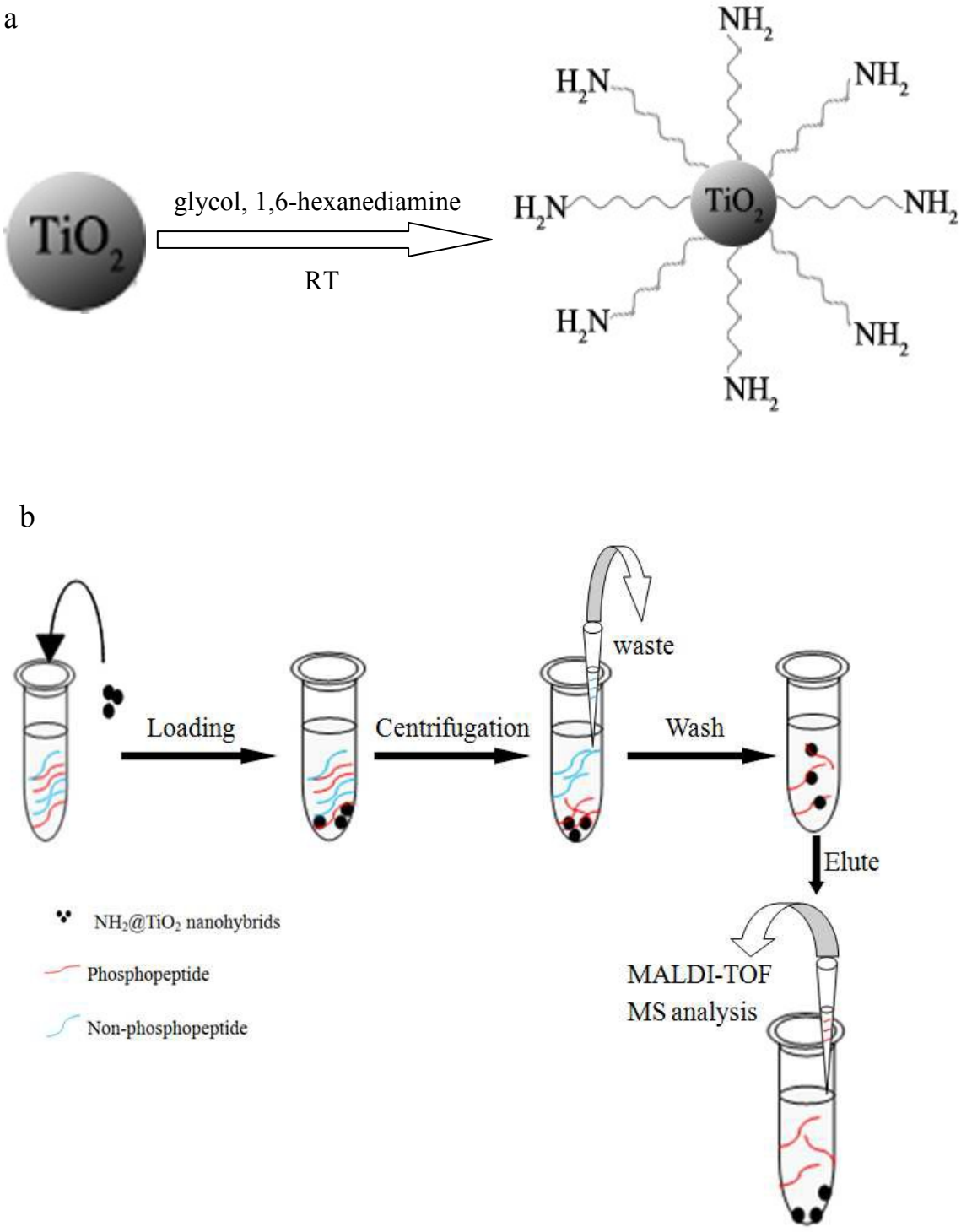


Fig. 1. Schematic illustration of (a) synthetic procedure of $NH_2@TiO_2$ and (b) the selective process for the enrichment of phosphopeptides using $NH_2@TiO_2$.

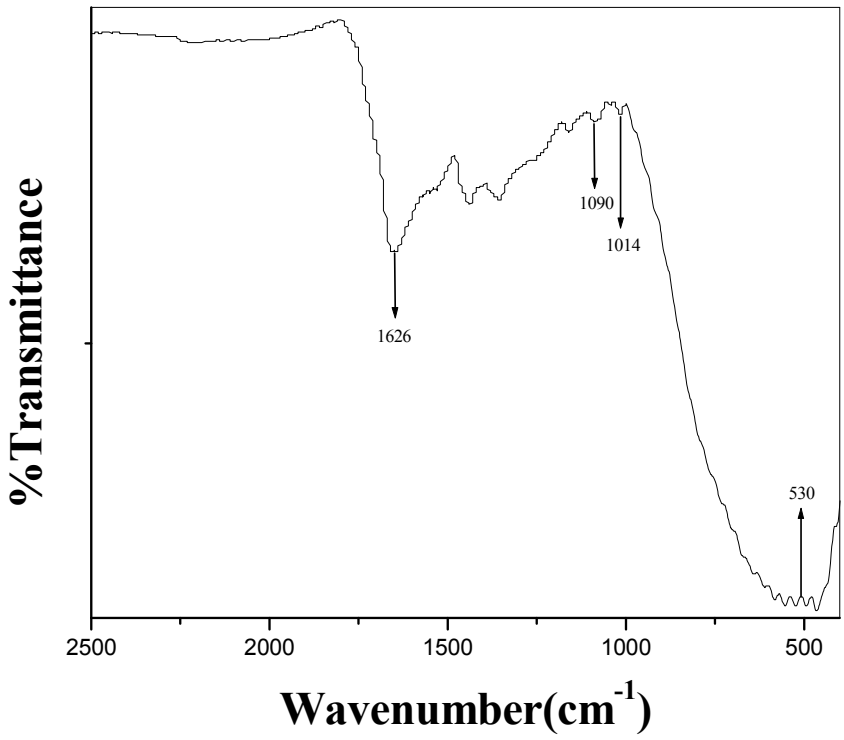


Fig. 2. FTIR spectra of NH_2@TiO_2.

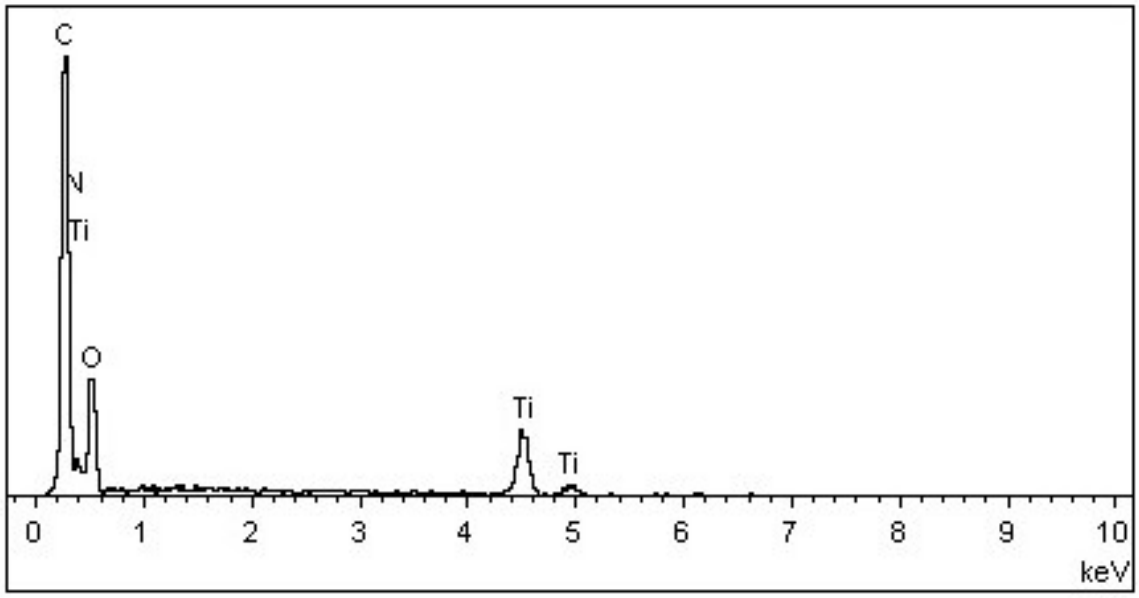


Fig. 3. The EDX spectrum of NH_2@TiO_2.

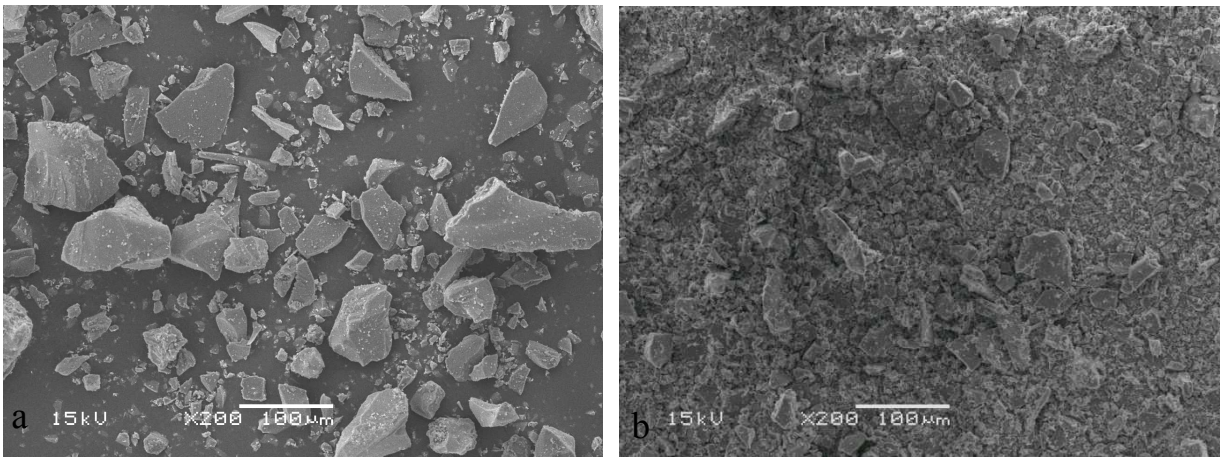


Fig. 4. SEM images of (a) TiO_2; (b) $NH_2@TiO_2$.

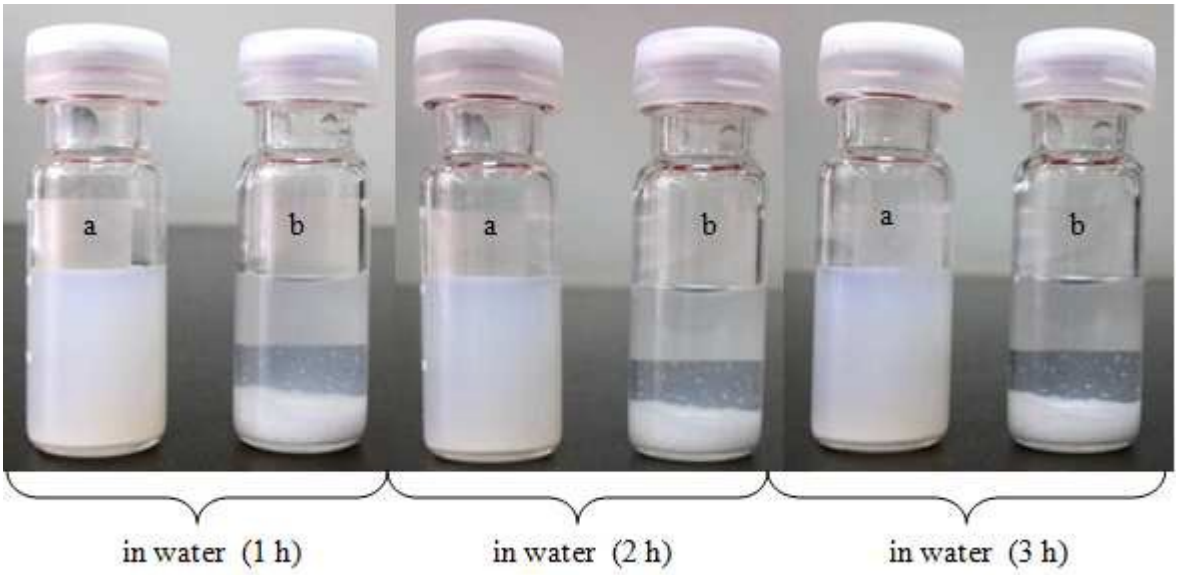


Fig. 5. The solubility in water of (a) NH_2@TiO_2; (b) TiO_2 for different hours.

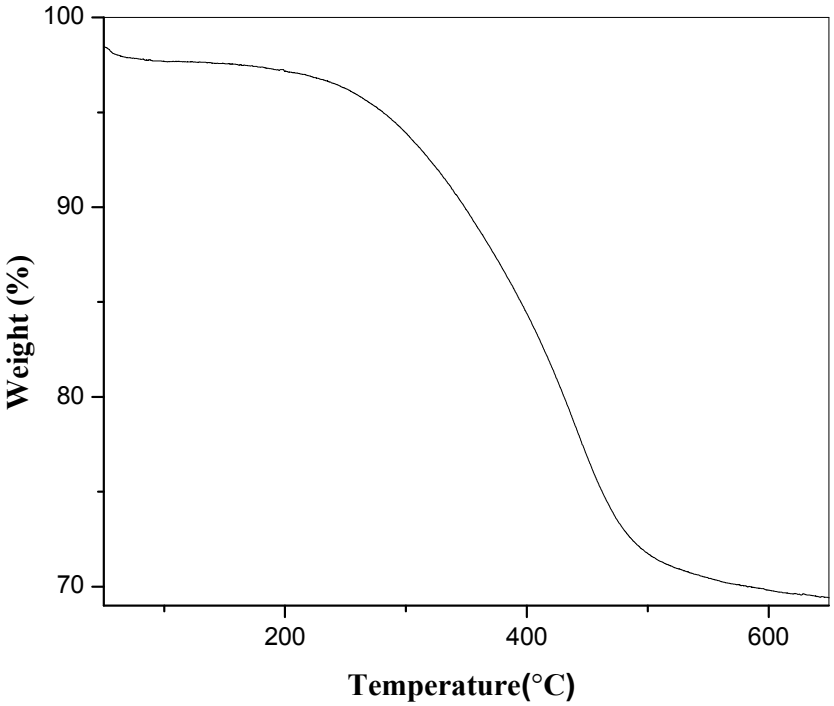
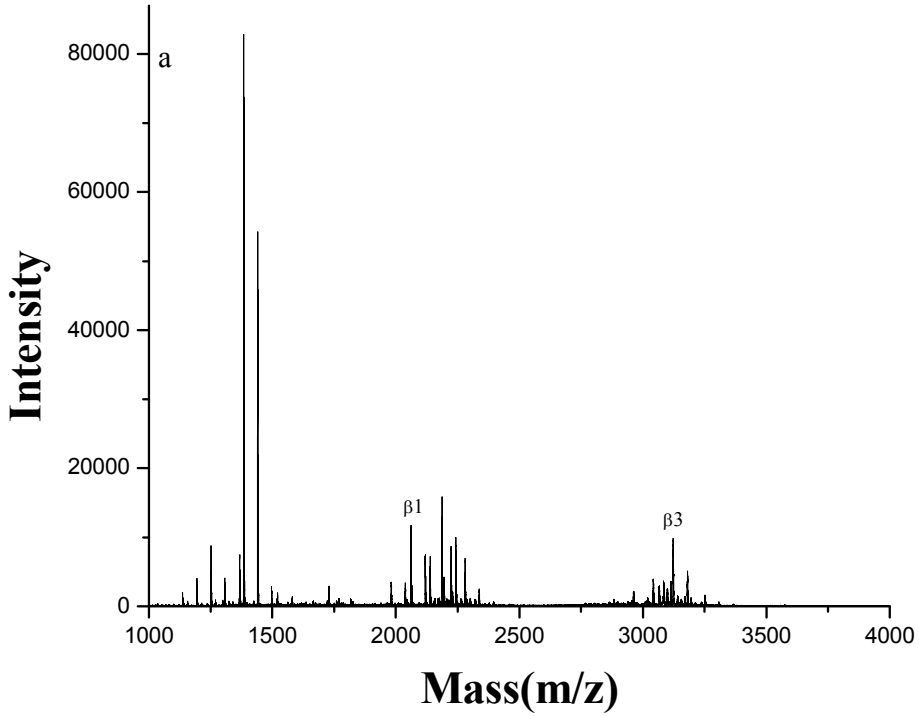
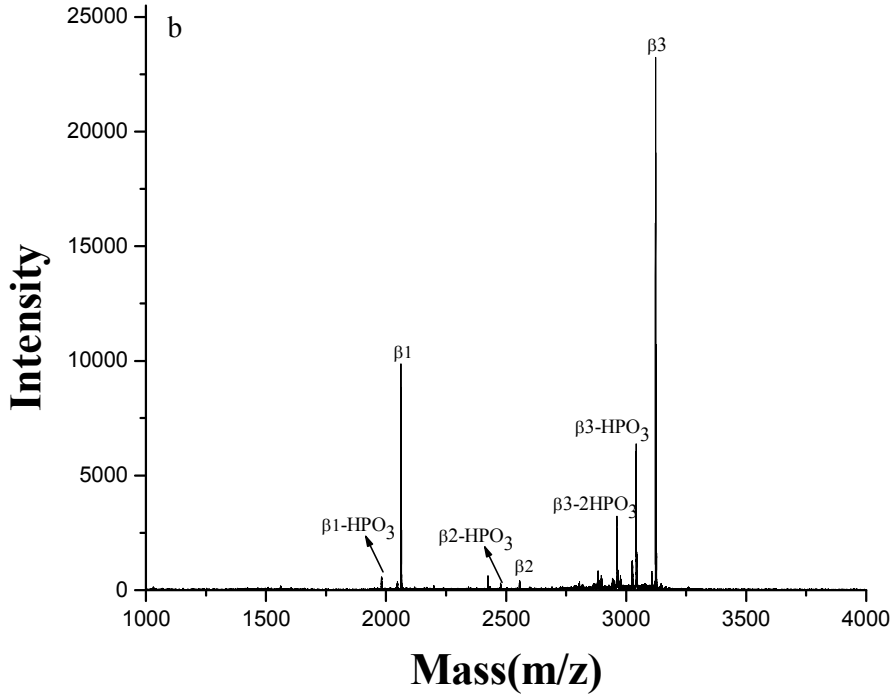


Fig. 6. TGA curves of $NH_2@TiO_2$





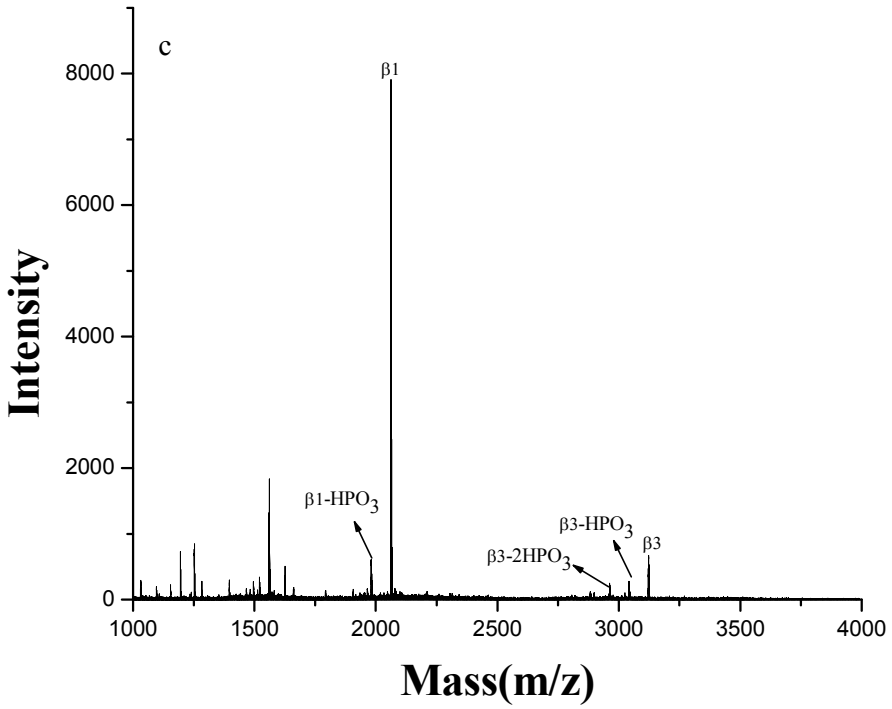
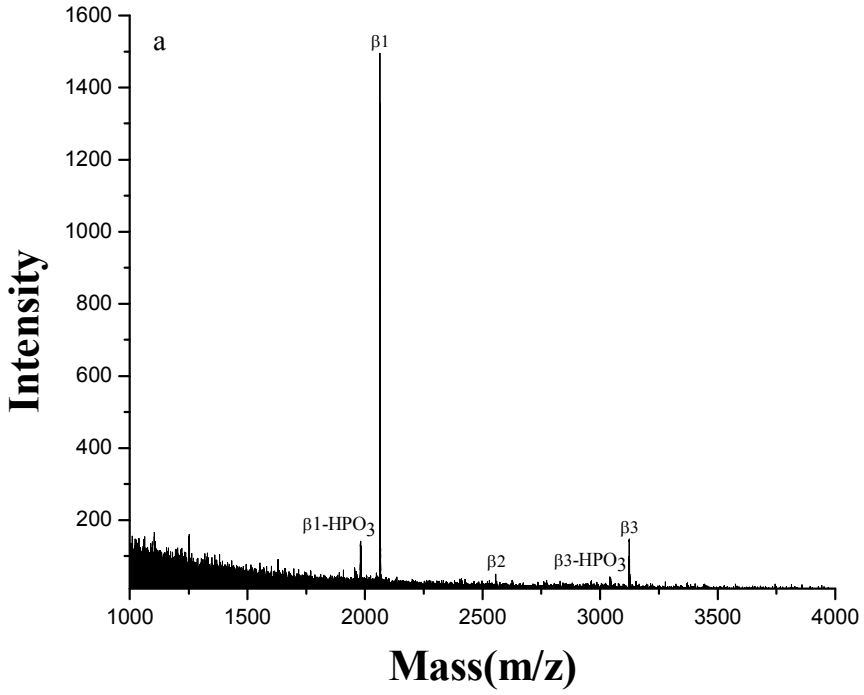


Fig. 7. MALDI-TOF mass spectrum of tryptic digests of 4×10^{-8}M β-casein : (a) before enhancement and after enriched (b) by $NH_2@TiO_2$ and (c) TiO_2.



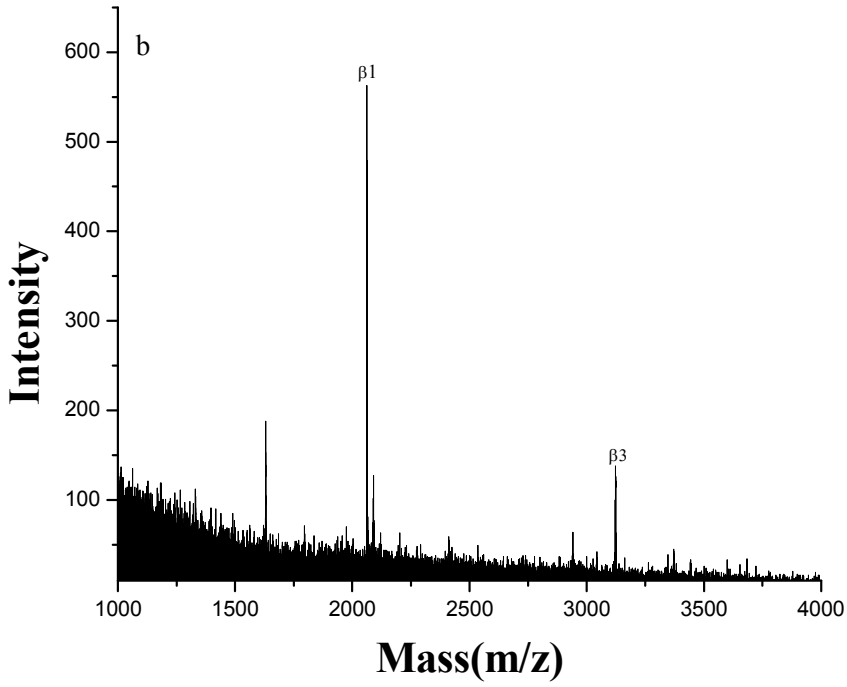
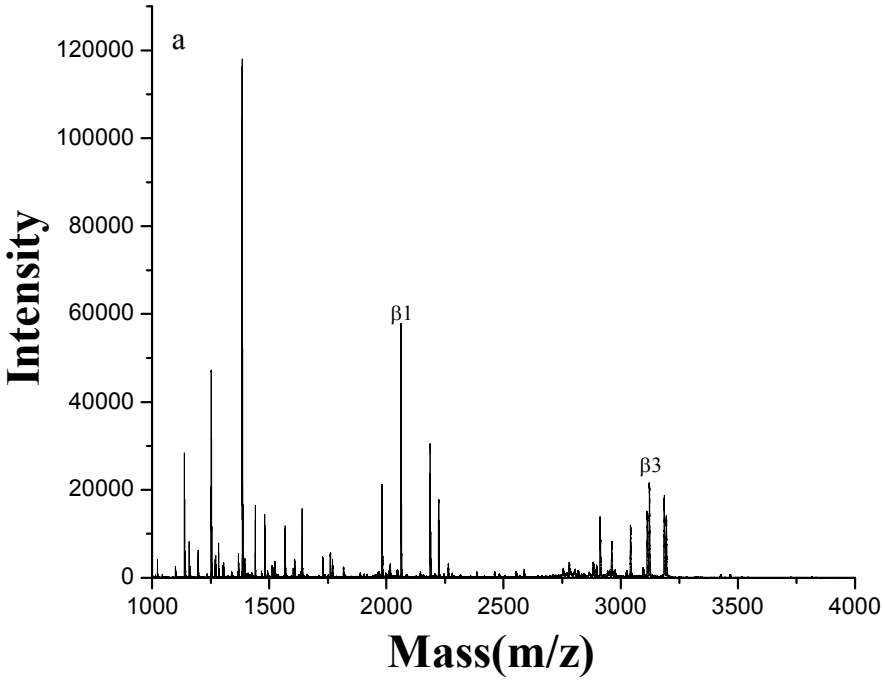
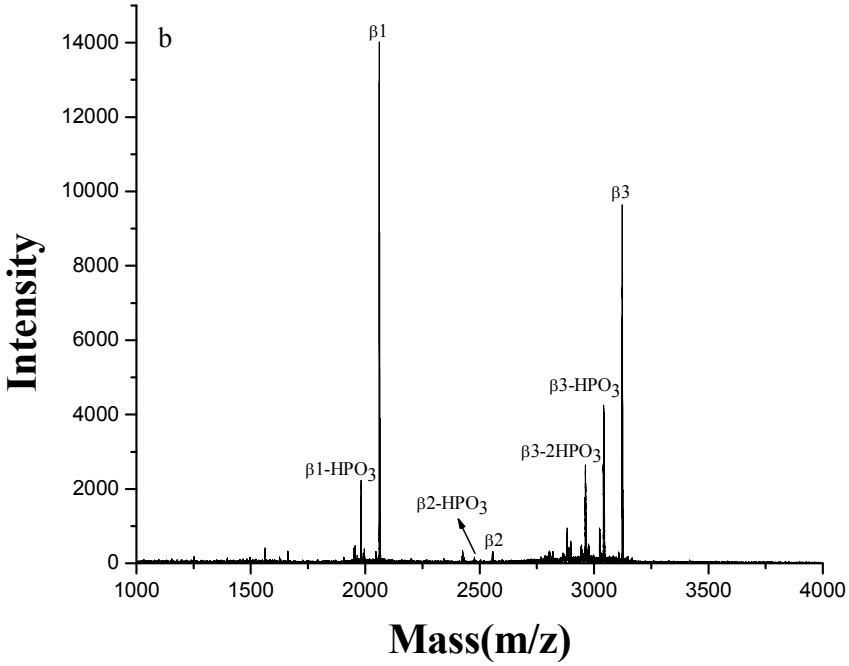


Fig. 8. MALDI-TOF mass spectra of tryptic digests of (a) 5×10^{-10}M (50 fmol) and (b) 5×10^{-11}M (5 fmol) β-casein after enrichment using $NH_2@TiO_2$.





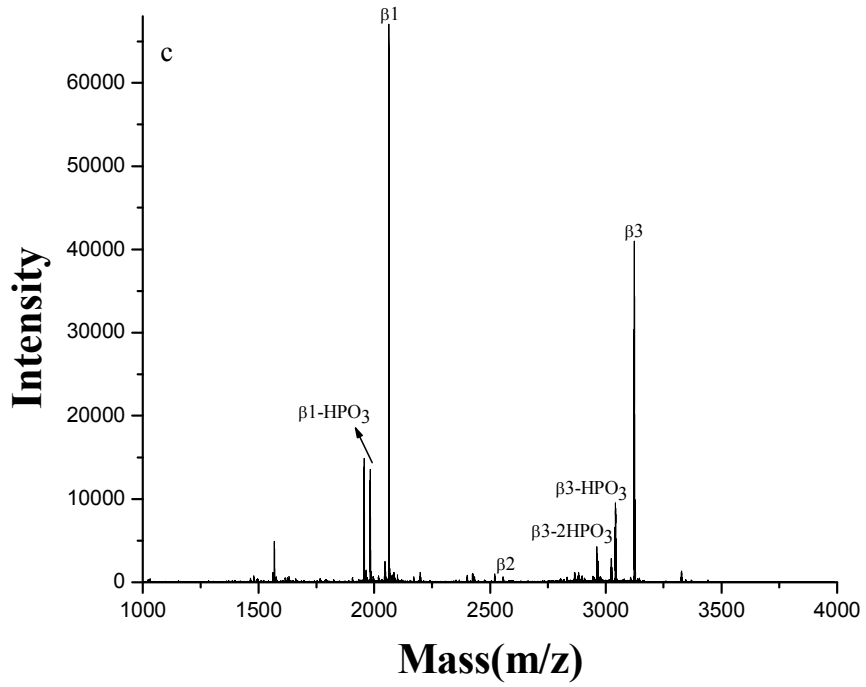
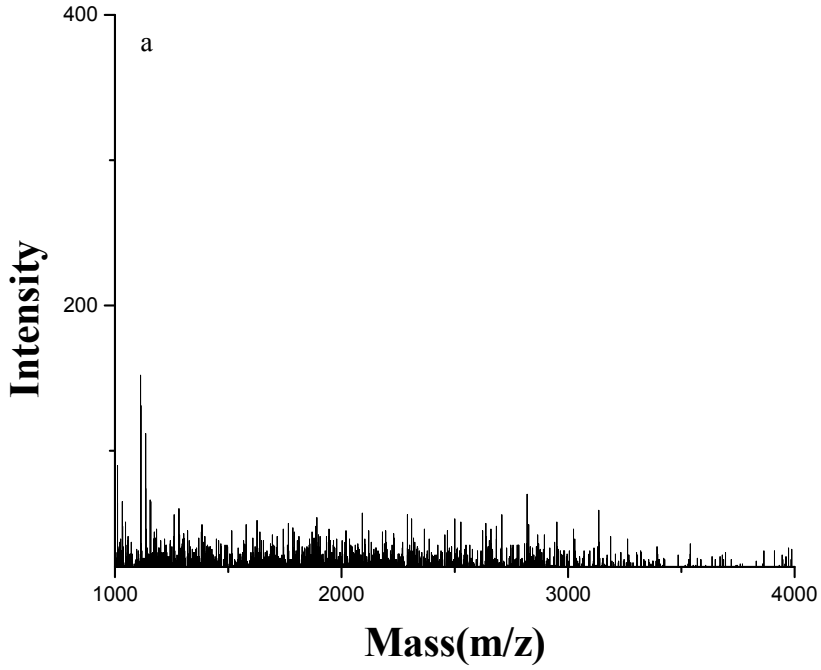
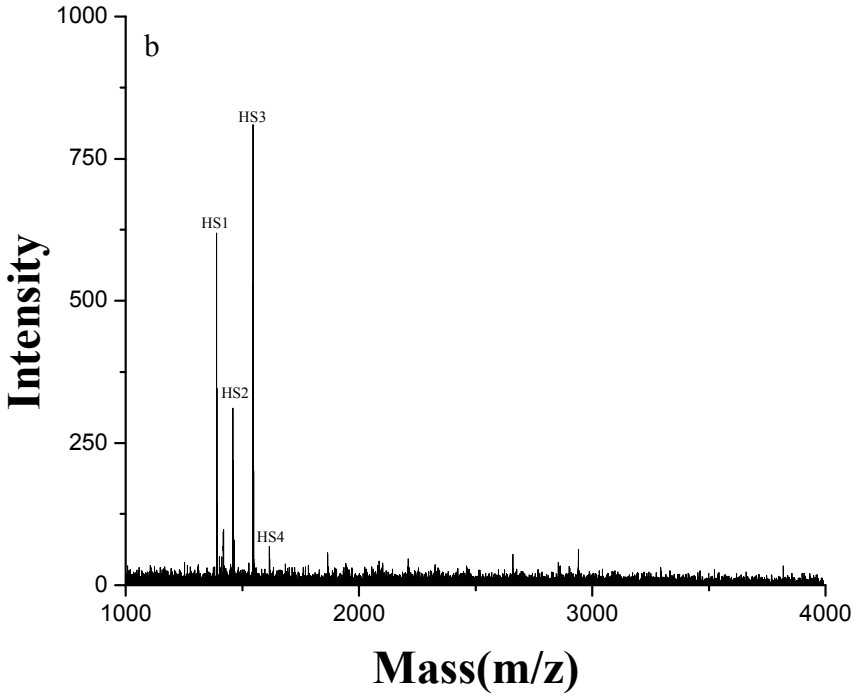
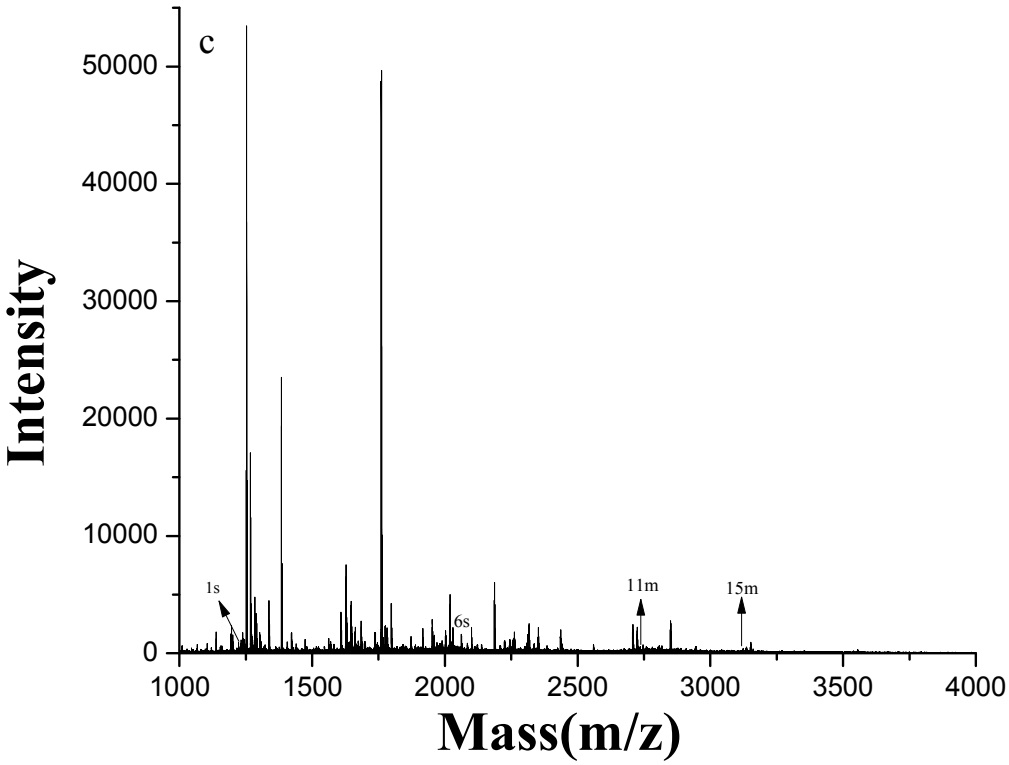


Fig. 9. MALDI-TOF mass spectra of tryptic digests of a mixture of β-casein and BSA : (a) direct analysisat at a molar ratio of 1:1; after enriched by $NH_2@TiO_2$ at molar ratios of (b) 1:1, (c) 1:10. The concentration of β-casein was 4×10^{-8}M







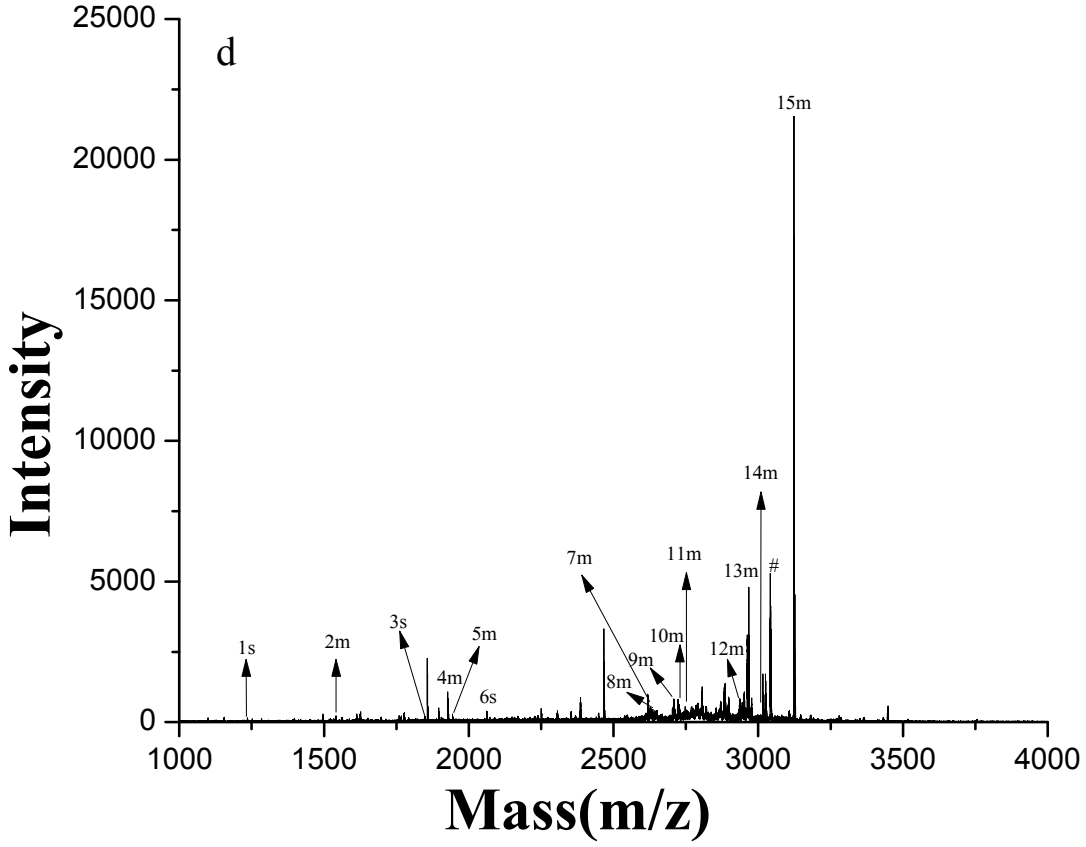


Fig. 10. MALDI-TOF mass spectra of human serum and tryptic digests of nonfat milk obtained by direct analysis (a and c) and enriched by NH_2@TiO_2 (b and d).