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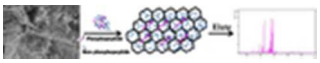

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Preparation of graphene-hafnium oxide composite for selective enrichment and analysis of phosphopeptides

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The reversible phosphorylation of proteins playes a crucial role in many regulatory processes. During the last decade, there has been considerable interest in the development of new methods for the identification of phosphorylation sites to allow for the comprehensive analysis of protein phosphorylation processes. However, the development of sensitive methods for the detection of trace quantities of phosphopeptides remains a significant challenge. In this study, we have prepared a novel graphene – hafnium oxide composite (GHOC) capable of enriching phosphopeptides and its application for the enrichment of phosphopeptides firstly prior to their analysis by matrix-assisted laser desorption/ionization mass spectrometry (MALDI - TOF/MS). The GHOC was prepared in a facile step using hydrothermal reaction. The surface morphology of the resultant materials was analyzed in its compound-bound form by TEM, SAED and XRD. According to theses results, the GHOC got an increased surface area result from the template of graphene and the modification of hafnium oxide which possess highly specificity toward phosphopeptides. Several complex samples (e.g. α- and β-casein, mixtures of β-casein and bovine serum albumin, and nonfat milk tryptic digest) were used to test the enrichment capability of the GHOC, and the results demonstrated that this material exhibited better selectivity towards mono- and multi-phosphorylated peptides comparing with the graphene-TiO_2 composite (GTOC) and graphene-ZrO_2 composite (GZOC). Furthermore, MALDI-TOF/MS experiments revealed no interference from nonphosphopeptides. The results demonstrate that our newly developed GHOC shows high specificity for the enrichment of phosphopeptides from biological samples and could therefore be applied in the field of phosphoproteomics.

Introduction

Graphene is a unique two-dimensional carbon nanostructure that is a single atom in thickness, with individual graphene sheets forming honeycomb network.[1] Graphene has attracted considerable interest in recent years because of its outstanding mechanical, catalytic, thermal, electrical, and optical properties, as well as theoretically high surface area of 2600 m^2 g^{-1}.[2] Based on the excellent properties of its large delocalized π-electron system, ultrahigh specific surface area and high loading capacity, graphene has been heralded as a promising material with a wide range of potential practical applications, notably the bioseparation.[3,4] However, graphene has a tendency to form hydrophobic interactions, which limits its application to biological samples and processes. Numerous oxidic derivatives of graphene have been developed in recent years via the incorporation of oxygen-containing functional groups (i.e., -O-, -COOH, and -OH), and these modifications have enhanced the hydrophilicity of graphene.[5,6] It was recently reported that graphene oxides decorated with metal oxide nanoparticles can be used to capture biomolecules from complex biological samples.[7-10]

The reversible phosphorylation of proteins is one of the most significant post-translational modification processes, and plays a crucial role in many regulatory mechanisms, including cellular growth, metabolism, differentiation, cell-cycle control, signal transduction, proliferation, and apoptosis.[11-13] Abnormal phosphorylation can be seen as the molecular signatures of organisms in a specific physiological state, and could provide valuable information for the early diagnosis of numerous diseases.[14,15] In particular, protein phosphorylation is related to the regulation of the biological pathways in tumor cells.[16] For these reason, there has been considerable interest in the identification of phosphorylation sites to allow for the comprehensive analysis of protein phosphorylation in the field of proteomics. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) techniques have been widely used to identify the phosphorylation sites of numerous proteins because of its high sensitivity and high-

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throughput comparing with other biochemical techniques.[17,18] However, the analysis of phosphopeptides by MALDI-MS remains particularly challenging because of the presence of abundant non-phosphopeptides, which can interfere with the analysis of trace phosphopeptides with low ionization efficiency.[19, 20]

Various methods have been developed to allow for the enrichment and concentration of the phosphoproteins or phosphopeptides in complex biological samples prior to their analysis by MS. The commonly used methods are mainly based on antibody-based affinity enrichment, immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC).[21-23] Among them, amphoteric metal oxides allowing for the reversible chemical adsorption of phosphate groups and a reduction in nonspecific binding, have made MOAC one of the most powerful enrichment methods.[24] Titanium dioxide,[25] zirconium oxide,[26] aluminum oxides,[27] and several other metal oxide particles[28,29] have been reported to possess specific enrichment capabilities towards phosphopeptides because of their strong Lewis acid- base properties. Furthermore, the results of several reports[30, 31] have shown that titania exhibits a strong affinity for multiphosphopeptides, whereas zirconia-supplemented titania exhibits a much stronger affinity towards monophosphopeptides. However, recent reports[32, 33] have indicated that the behavior of zirconia is similar to that of titania, exhibiting a low affinity for monophosphopeptides. Hafnium is the third member of subgroup IV in the periodic table and possesses similar characteristics as zirconium. Rivera and co-workers[29] have reported that hafnium dioxide could potentially be used as a supplement material to titania and zirconia for the enrichment of phosphopeptides.

The direct use of graphene or graphene oxide sheets as adsorbents can lead to their irreversible aggregation, which could ultimately lead to a reduction in their adsorption capacity and efficiency. For this reason, considerable research efforts have been directed towards the development of graphene- and graphene oxide-based templates that have been modified with metal oxides as promising candidates for bio-separation and enrichment applications.[34-37] In this study, we present, for the first time, a novel one-step strategy for the hydrothermal synthesis of graphene-hafnium oxide composite (GHOC), and the application of this material for the selective enrichment of phosphopeptides. The GHOC prepared in this study combines the advantages of the high surface area of graphene with the strong specificity of HfO_2 for phosphopeptides. It was envisaged that this material could be used for the isolation and enrichment of phosphopeptides with numerous phosphorylation sites. For the comparison, the corresponding graphene-TiO_2 composite (GTOC) and graphene-ZrO_2 composite (GZOC) were also prepared and evaluated the selectivity and specificity of these materials towards phosphopeptides by bovine α- and β-casein, peptide mixtures, and nonfat milk, to assess the potential application of GHOC as affinity materials in phosphoproteomic field.

Experimental

Reagents and materials

Graphene oxide was purchased from Nanjing XFNANO Materials Tech Co., Ltd. Hafnium(IV) chloride, . α-casein, β-casein, bovine serum albumin (BSA), trypsin (from bovine pancreas, TPCK treated), iodoacetamide (IAA), dithiothretol (DTT), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The zirconium(IV) isopropoxide isopropanol complex (99.9%) used in this study was purchased from HEOWNSz Biochemical Technology Co., Ltd. 2, 5-Dihydroxybenzoic acid (DHB) was purchased from Bruker. Acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). Tetrabutyl titanate, hydrochloric acid (HCl), ammonium bicarbonate (NH_4HCO_3), and phosphoric acid (H_3PO_4), ammonia solution ($NH_3 \cdot H_2O$), anhydrous ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd.

Preparation of graphene- hafnium oxide composites (GHOC)

The GHOC was synthesized using facile hydrothermal reaction. Briefly, graphene oxide (10 mg) was added to 1 M hydrochloric acid (40 mL), and the resultant mixture was agitated under ultrasonic irradiation for 30 min to form a homogeneous suspension. Hafnium(IV) chloride (1 mmol) was then added to the graphene suspension and the resultant mixture was mixed under ultrasonic irradiation for 30 min. The mixture was then transferred to a Teflon-lined stainless steel autoclave (100 mL) and heated at 200 °C for 12 h. The mixture was then cooled to room temperature and centrifugated to give the crude composite product as a solid, which was washed sequentially with ethanol and deionized water to remove any unreacted impurities. The composite material produced by the hydrothermal reaction was subsequently lyophilized under vacuum to give the desired GHOC as a powder.

Preparation of GTOC and GZOC

The synthesis of the GTOC/GZOC materials involved two steps, including sol-gel and hydrothermal reactions. First, graphene oxide (10 mg) was added to anhydrous ethanol (50 mL), and the resultant mixture was agitated under ultrasonication for 1 h to form a homogenous suspension. Tetrabutyl titanate/ zirconium(IV) isopropoxide (1 mmol) was then added to the graphene oxide suspension, and the resultant mixture was subjected to ultrasonication for 30 min. The mixture was then treated by adding the 5:1 (v/v) mixture of ethanol and water (60 mL) in dropwise manner (3 mL/min) under magnetic stirring. Upon completion of the addition, the resultant mixture was stirred at room temperature for 8 h. Finally, the product of the reaction was collected and sequentially washed several times with ethanol and water by centrifugation. The composite was

then redispersed in water (30 mL), and the resultant mixture was transferred to a 100-mL Teflon-lined autoclave and heated at 200 °C for 12 h. The mixture was then cooled to room temperature and centrifugated to give the product of the hydrothermal reaction. This material was lyophilized under vacuum to give the GTOC/GZOC material as a powder.

Characterization

Scanning electron microscopy (SEM) images were recorded on a JSM-6700 SEM instrument (JEOL, Japan). Transmission electron microscopy (TEM) and selected area electron diffraction (SAED) analyses were conducted on a JEM-2100 TEM instrument (JEOL) equipped with an energy-dispersive X-ray analysis system. Powder X-ray diffraction (XRD) patterns were obtained on a D8 Advance X-ray diffractometer (Bruker AXS, Germany) using Cu Ka monochromated radiation. Fourier transform infrared spectroscopy analyses were conducted from 400 to 4000 cm^{-1} using a Tensor 27 Fourier spectrometer (Bruker). particle

Tryptic digestion of standard proteins and milk

Tryptic digests of α-casein, β-casein and milk were used as samples to investigate the particularity affinity and enrichment efficiency of the materials for phosphopeptide enrichment. Briefly, bovine α- and β-casein and trypsin (1 mg) were separately dissolved in 1mL of NH_4HCO_3 buffer (50 mM, pH 8.1). The tryptic digests process was performed with a mass ratio of 1/40 (enzyme/ protein) for 16 h at 37 °C, and the resulting mixtures were separated in centrifuge tubes and stored at 20 °C for further use. To prepare more complex samples to evaluate the sensitivities of the graphene-based materials, we mixed different amount of the tryptic digests of BSA and β-casein. The BSA was initially denatured in urea (8 M) for 3 h at 37 °C, and the resultant mixture was reduced with DTT (10 mM) at 56 °C for 2 h, before being alkylated with IAA (20 mM) in the absence of light at room temperature for 30 min. The urea, DTT, and IAA were dissolved separately in ammonium bicarbonate (50 mM). The resultant BSA was diluted 10-fold with an ammonium bicarbonate buffer solution (50 mM, pH 8.1) to reduce the effect of urea towards trypsin. The mixture was then digested with trypsin for 16 h at 37 °C in a shaker, using a mass ratio of 1/ 40 (enzyme/ protein).

This process was also conducted in a similar manner to prepare a nonfat milk tryptic digest

Selective enrichment of phosphopeptides from the tryptic digests using the different graphene-based materials

Prior to the enrichment process, the tryptic digest peptide mixtures (100 μL) were diluted to a final volume of 1 mL using a loading buffer (50% ACN, 1% TFA) solution. The graphene-based materials (0.5 mg) were rinsed with the incubation buffer and then separated by centrifugation. After being rinsed, the product materials were mixed with the diluted tryptic digests, and the resultant mixture were incubated under vibration at 4 °C for 30 min. After incubation, the supernatant solution containing the non-phosphopeptides was removed by centrifugation, and the phosphopeptide-loaded materials were washed three times (4 °C, 30 min) with the incubation buffer (50% ACN, 1% TFA) solution to remove any unbound non-phosphopeptides. Finally, the phosphopeptides captured by the composite materials were eluted using anaqueous ammonia solution (200 mL, 15%). The elution procedures was maintained for 30 min, and repeated three times. The final eluent was collected for dialysis, which was performed using bag filters (MWCO 500).

MALDI-TOF/MS analysis

MALDI-TOF analyses were conducted on a Bruker UltrafleXtreme™ time-of-flight mass spectrometer (Bruker, Bremen, Germany) equipped with a delayed ion-extraction device and a 337 nm pulsed nitrogen laser. Each spectrum represents the sum total of 500 laser shots. The sample solutions were diluted to specific concentrations using a 0.1% (v/v) solution of TFA in water. The DHB matrix (2 μL, 20 mg mL^{-1}, 30% ACN, and 1% H_3PO_4) was mixed with the protein digest (2 μL), and small samples (1 μL) of the resultant mixtures were sequentially deposited onto the target for MALDI-TOF/MS analysis.

Results and discussion

Characterization of the graphene composites

The GHOC used to capture phosphopeptides was prepared according to a one-step hydrothermal reaction (Scheme 1). Graphene oxide has a negatively charged surface because of its many carboxylic groups, which can bind to hafnium oxide through electrostatic interaction. The morphological characteristics of the surfaces of graphene oxide and the GHOC were shown in Fig. 1. The surface of graphene oxide was found to be smooth with a neat edge when it was evaluated in the absence of any metal oxide modification (Fig. 1a). However, after being coated with hafnium oxide, the graphene oxide surface became rough because it was evenly covered with tiny nanoparticles with an average diameter of 100 nm (Fig. 1b). This result indicated that hafnium oxide had been successfully attached to the graphene oxide surface. TEM images of the GHOC were shown in Fig. 2a. These images showed that the two-dimensional graphene sheets behaved as a template, and they were well decorated by a large quantity of uniformly distributed HfO_2 particles. Notably, the graphene oxide sheet prevented the aggregation of the metal oxide particles, which contributed to the exposure of more activesites for the enrichment of phosphopeptides. The TEM images of GTOC (Fig. 2c) and GZOC (Fig. 2e) revealed that the graphene oxide had been modified in a homogenous manner with TiO_2 and ZrO_2 particles of 5 mm in diameter. Given that most of the phosphopeptides are around 0.75 nm in size, they could be readily adsorbed on these composite materials. The difference in the

morphological characteristics of the GHOC, GTOC and GZOC materials could be clearly seen in their TEM images. In addition, the GHOC was also analyzed by energy-dispersive X-ray analysis, which revealed the presence of elemental C, O, Hf, and Cu (Fig. S1). The result therefore provided further confirmation of the successful modification of HfO_2 onto graphene oxide.

Selected area electron diffraction (SAED) patterns of GHOC, GTOC, and GZOC were shown in Figs. 2b, 2d, and 2f. The diffraction rings of ($\bar{1}$11), (111), (020), (112), (220), and (221) correspond to monoclinic phase of hafnium oxide (JCDP card no. 06-0318) in the crystal plane (Fig. 2b). As shown in Fig. 3a, the obvious diffraction peaks at 2θ= 28.271, 31.573, 34.682, 45.082, 50.476, and 55.660 were similar to the diffraction rings of hafnium oxide in the monoclinic phase. Furthermore, the results obtained by XRD analysis were consistent with those obtained by SAED analysis, and therefore provided further confirmation of the incorporation of crystal HfO_2. The data reported in JCDP card no. 21-1272 revealed that the diameters of the (101), (004), (211), (204), (220), and (215) diffraction rings in the SAED patterns (Fig. 2d) were consistent with the diffraction peaks recorded by XRD (Fig. 3b). This result therefore confirmed the successful synthesis of anatase TiO_2. The results shown in Figs. 2f and 3c revealed that ZrO_2 (tetragonal phase) had been successfully deposited on the surface of the graphene oxide. For example, the SAED patterns of the GZOC material showed diffraction rings at (101), (110), (112), (211), (202), and (220), which were consistent with ZrO_2 (tetragonal phase). Furthermore, the XRD peaks of the GZOC material were consistent with those reported on JCDP card no. 79-1770 for ZrO_2.

Variations in graphene oxide groups following the hydrothermal treatment process were investigated by FTIR spectroscopy. As shown in Fig. 4b, the bands corresponding to the epoxide/ carboxy C-O vibrations (1056 cm^{-1},1368 cm^{-1}), and carboxy C=O adsorption (1731 cm^{-1}) in the FTIR spectrum of graphene oxide (Fig. 4a) almost completely disappeared after the hydrothermal reaction. This result indicated that the oxygen-containing functional groups in GHOC had been deoxygenized during the hydrothermal reaction. The FTIR spectrum of GHOC after the hydrothermal reaction revealed that it contained a peak corresponding to the skeletal vibrations of the C=C bonds (1622 cm^{-1}) belonging to the aromatic rings of graphene oxide. The FTIR spectrum of GHOC also contained peaks at 525, 634, and 769 cm^{-1}, which were attributed to HfO_2 (Fig. 4b). Taken together, these results confirmed that HfO_2 was successfully loaded onto the surface of the graphene using a facile one-step hydrothermal method. Furthermore, this process occurred with the concomitant deoxygenize of the oxygen-containing groups from the surface of the graphene oxide.

Application of the composites to the enrichment of phosphopeptides (α- and β-caseins)

A tryptic digest of bovine β-casein (with a trace of α-casein) was selected as a standard sample to assess the enrichment abilities of the GHOC, GTOC, and GZOC materials for phosphopeptides. The results for the direct analysis of the β-casein digests (5×10^{-7} M) were shown in Fig. 5a. These results revealed that only three weak signals were observed for the phosphopeptides, with the spectrum being dominated by signals corresponding to non-phosphopeptides. After the enrichment with the GHOC, we detected eight peaks corresponding to phosphopeptides by mass spectrometry (Fig. 5b). Most notably, there was almost no disturbance originating from nonphosphopeptides. TiO_2 and ZrO_2 have been widely used for the enrichment of phosphopeptides because of their special amphiprotic properties. With this in mind, we also synthesized the GTOC and GZOC materials and compared them with GHOC in terms of their ability to enrich the phosphopeptides from a β-casein digest. As shown in Fig. 5c, seven phosphopeptides were observed in terms of their enrichment by the GTOC. The ability of GZOC to capture phosphopeptides was weaker than those of GHOC and GTOC, with only six phosphopeptides being detected with some interference from the non-phosphopeptides (Fig. 5d). The results of these experiments were listed in Table S1, and showed that the GHOC material exhibited a higher level of selectivity for the enrichment phosphopeptides in the quantity and singal intensity than GTOC and GZOC, in particular, for the tetraphosphorylated peptides (β_5, m/z 3122.3) enrichment.

Bovine α-casein was also used to evaluate the enrichment efficiency and selectivity characteristics of GHOC because it contains more phosphorylation sites than β-casein. Only six phosphopeptide peaks could be directly detected in the α-casein digest without enrichment, with the mass spectrum of this digest being dominated by strong signals from the non-phosphopeptides (Fig. 6a). Following the use of GHOC, thirteen multiphosphopeptides and nine monophosphopeptides were detected in the α-casein digest without any obvious peaks corresponding to non-phosphopeptides. These results therefore indicated that the GHOC material could be used for the enrichment of phosphopeptides (Fig. 6b).

Evaluation of the sensitivity and selectivity characteristics of the different composite materials

The detection sensitivities of GHOC, GTOC, and GZOC for the enrichment of phosphopeptides were investigated using a tryptic digest of β-casein containing different molar ratios of BSA (e.g., 1:10, 1:100, 1:1000). As shown in Fig. 7a, only one phosphopeptide with a weak intensity (*m/z* 2061.8) was detected in the tryptic digest of a β-casein/ BSA mixture with a molar ratio of 1: 10. The intensities of the phosphopeptides were suppressed significantly by the high levels of non-phosphopeptides originating from the non-phosphorylated BSA. After an enrichment process using GHOC, eight phosphopeptides from α-, and β-casein respectively were observed with a clean background

(Fig. 7b). However, following the enrichment process with GTOC, we only observed seven phosphopeptides (Fig. S2a). Furthermore, GZOC gave a similar result to that of GTOC (Fig. S2b), although the baseline in the mass spectrum of GZOC was worse than that of GTOC. When the ratio of β-casein/BSA was adjusted to 1:100, we observed six phosphopeptides (Fig. 7c) following the GHOC enrichment process. The GTOC and GZOC materials lead to the detection of three phosphopeptides (α_3, β_1, β_4) and three phosphopeptides (α_6, β_1, β_4) under the same conditions, respectively (Fig. S2c and S2d). As the complexity of samples increased, so too did the level of interference in the backgrounds of the mass spectra resulting from the non-phosphopeptides in the BSA. Even when the molar ratio of β-casein to BSA was up to 1:1000, the results obtained using the GHOC enrichment process revealed that two signals corresponding to phosphopeptides (β1, β4) could be detected, despite the large number of peaks belonging to non-phosphopeptides in the background (Fig. 7d). In contrast, only one phosphopeptide (*m/z* 2061.8)was enriched by GTOC (Fig. S2e) and GZOC (Fig. S2f) under the same conditions. Compared with GTOC and GZOC, GHOC displayed better selectivity for phosphopeptides from the same complex BSA/phosphopeptide mixtures, suggesting that it has great potential as a material for the enrichment of phosphopeptides from complex biological samples, and captures more phosphopeptides from the lower concentration phosphopeptides samples.

Enrichment of phosphopeptides from milk tryptic digestion

Nonfat milk was used as a real sample to further demonstrate the practical application of the GHOC material for the selective enrichment of low-abundance phosphopeptides from biological samples. The results for the direct analysis of a nonfat milk digest by MALDI-TOF/MS were shown in Fig. 8a. This result showed that only four low-intensity MS signals were identified from the phosphopeptides in the milk together with high levels of interference from the abundant non-phosphopeptide present in the milk. However, 21 peaks corresponding to phosphopeptides (nine monophospho-peptides and twelve multiphosphopeptides) were distinctly detected with good resolution after the milk was enriched by GHOC (Fig. 8b). As shown in Fig. 8c, eight monophosphopeptides and nine multiphosphopeptides were captured by the GTOC material from nonfat milk. When the milk was enriched with the GZOC material, we observed 10 monophosphopeptides and nine multiphosphopeptides (Fig. 8d). Titania and zirconia have been widely used for capturing phosphopeptides because they exhibit high levels of selectivity for phosphopeptides. With this in mind, the GTOC and GZOC materials were selected for a comparison of their enrichment abilities with those of GHOC to evaluate the potential of GHOC as a material for the enrichment of phosphopeptides from complex samples. The results of a series of nonfat milk enrichment experiments revealed that GHOC was superior to GTOC and GZOC for the enrichment of monophosphopeptides and multiphosphopeptides. Detailed information pertaining to the different materials used to capture the phosphopeptides from the nonfat milk digest was shown in Table S1.

The standard phosphoprotein β-casein, the mixtures of non- phosphoprotein BSA and phosphoprotein β-casein, and non-fat milk real sample tryptic digestion were used to assess the ability of GHOC, all the data proved that it has stronger capability than GTOC and GZOC for capturing phosphopeptides. Titanium, zirconium and hafnium were in the same subgroup IV in the Periodic Table of the Elements with the same number of electrons in the outermost shell, so they have similar chemical properties and all of them exhibit strong specificity to phosphopeptides. However, with the increase of the radius of the electron orbit, the hafnium bounded outer electron capacity weaker than Titanium and zirconium which was the possible reasons that the GHOC possesses better ability in phosphopeptides enrichment.

Conclusions

In this study, we have prepared a novel GHOC using a facile one-step hydrothermal reaction and applied this material to the enrichment of phosphopeptides. The GHOC material combined the specific recognition properties of HfO_2 for phosphopeptides with the large specific surface area of graphene. This combination led to more activated sites being exposed to the sample matrix and led to an increase in the ability of this material to absorb phosphopeptides. The specificity and adsorption characteristics of GHOC, GTOC, and GZOC were evaluated for the enrichment of phosphopeptides from bovine β-casein, mixtures of β-casein and BSA, and nonfat milk tryptic digests. The specificity properties of GTOC and GZOC towards phosphopeptides were found to be inferior to those of GHOC, especially towards complex biological samples. These results therefore demonstrate that GHOC possesses high specificity for the enrichment of phosphopeptides from biological samples and great potential in terms of its application to phosphoproteomics.

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Notes and references

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38

Scheme 1 Schematic of the route used for the synthesis of GHOC and its subsequent use for the enrichment of phosphopeptides.

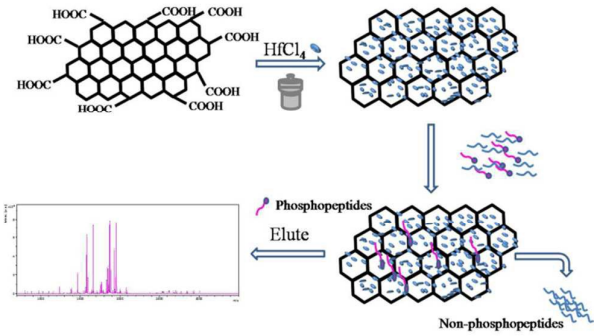


Fig. 1 SEM images of (a) graphene oxide, and (b) hafnium oxide embedded in graphene

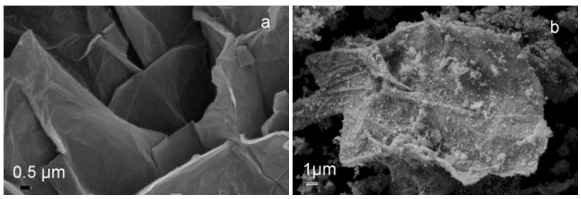
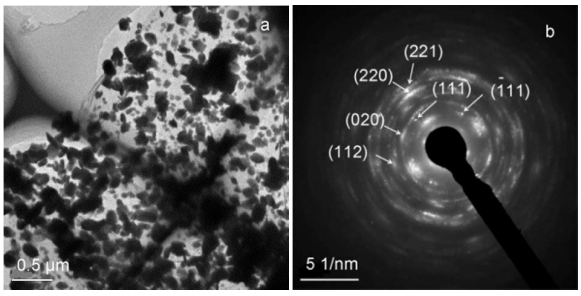


Fig. 2 TEM image of (a) GHOC, (c)GTOC, and (e) GZOC; selected area electron diffraction (SAED) patterns of (b) GHOC, (d)GTOC, and (f) GZOC.



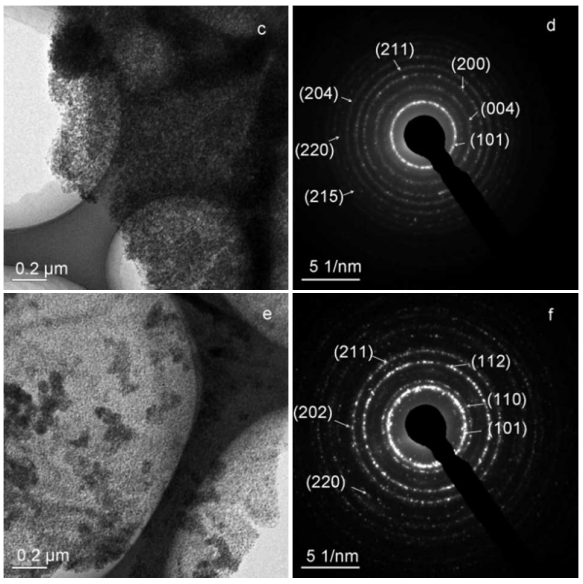


Fig. 3 X-ray diffraction (XRD) patterns of (a) GHOC, (b) GTOC, and (c) GZOC.

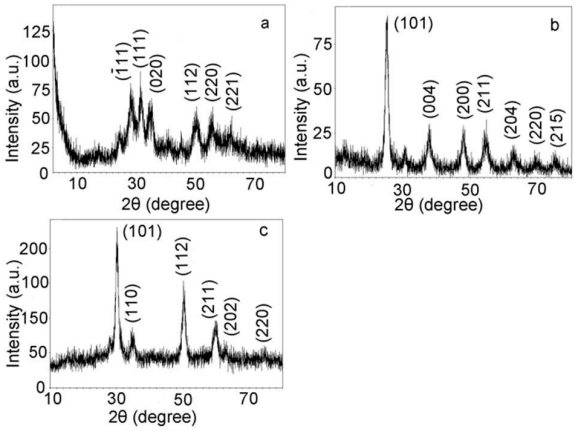


Fig. 4 Fourier transform infrared spectra of (a) graphene carboxyl, and (b) as-synthesized GHOC.

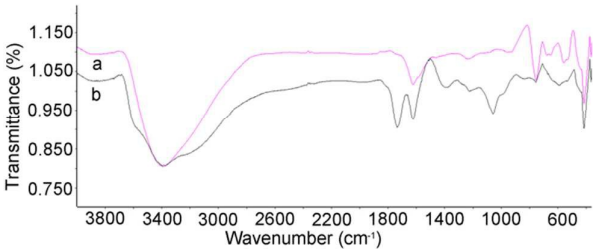


Fig. 5 MALDI-TOF mass spectra of the β-casein tryptic digest (a) without enrichment; and enriched by (b) GHOC, (c) GTOC, and (d) GZOC. The metastable losses of phosphoric acid have been labeled with an * symbol.

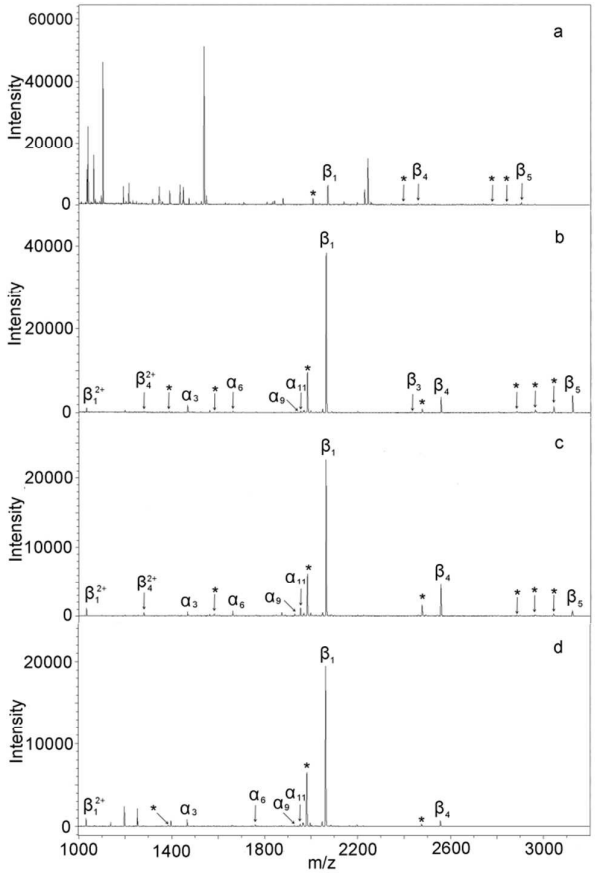


Fig. 6 MALDI-TOF mass spectra of the α-casein tryptic digest (a) without enrichment, and enriched by (b) GHOC. The metastable losses of phosphoric acid have been labeled with an * symbol.

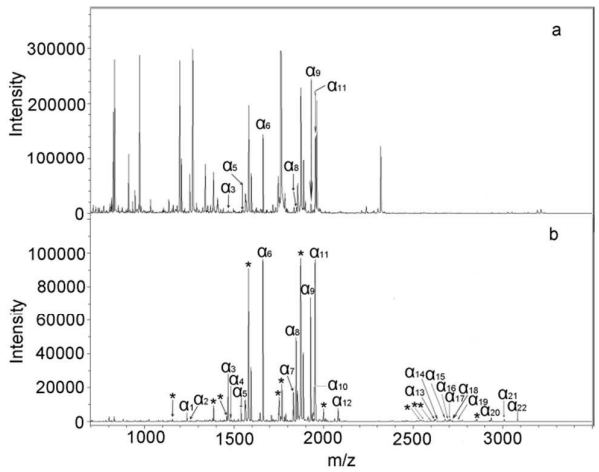


Fig. 7 MALDI-TOF mass spectra of the peptides from the mixtures of BSA and β-casein at different molar ratios. BSA and β-casein at amolar ratio of 1 : 10 (a)without enrichment; enriched with GHOC at molar ratios of (b) 1 : 10, (c)1 : 100, and (d)1 : 1000. The metastable losses of phosphoric acid have been labeled with an * symbol.

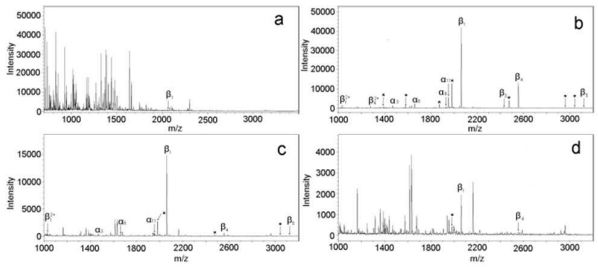


Fig. 8 MALDI-TOF mass spectra of the non-fat milk tryptic digests (a) without enrichment, enriched by (b) GHOC, (c) GTOC, and (d) GZOC. The metastable losses of phosphoric acid have been labeled with an * symbol.