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Facile preparation of graphene/TiO_2 magnetic composite for highly selective and sensitive enrichment of phosphopeptides

Yulu Liang [a], Xiwen He [a], Langxing Chen *[a] and, Yukui Zhang[a,b]

[a]*State Key Laboratory of Medical Chemical Biology, Research Center for Analytical Science, College of Chemistry, Nankai University, Tianjin 300071, China. Collaborative Innovation Center of Chemical Science and Engineering (Tianjin).*

E-mail: lxchen@nankai.edu.cn; Tel: 0086+0 22-23505091; Fax: 0086+0 22-23502458

[b]*Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116011, P. R. China.*

E-mail: ykzhang@dicp.ac.cn; Tel: 0086+411-84379560; Fax:0086+411-84379560

Experimentals

Materials

All reagents were at least of analytical grade and used without further purification. Graphene oxide (GO) was provided by Nanjing XFNANO Materials Tech. Co. Ltd, China. Trypsin, β-casein and bovine serum albumin (BSA; Mw 66kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroaceticacid (TFA), dopamine hydrochloride and 2,5-dihydroxybenzoic acid (DHB) were from Aladdin (Shanghai, China). Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium acetate, ethylene glycol (EG), ethylene diamine (EDA), titanium (IV) sulfate ($Ti(SO_4)_2$), ammonium bicarbonate (NH_4HCO_3), ammonium hydroxide ($NH_3 \cdot H_2O$), formic acid (FA) and anhydrous ethanol were all purchased from Tianjin Chemical Reagent Company (Tianjin, China). Highly purified water was prepared with a Milli-Q water purification system (Millipore, Milford, MA).

Synthesis of Magnetic Graphene Material (Graphene/Fe_3O_4)

The preparation of graphene/Fe_3O_4 composite was carried out by a simple hydrothermal reaction. Typically, 50mg GO was dissolved into 25 mL ethylene glycol (EG), followed by the addition of 0.5 g $FeCl_3 \cdot 6H_2O$, 1.5g NaAc (as a protective agent) and 5 mL ethylene diamine (EDA) to form a homogeneous solution with the help of ultrasonic dispersion. The mixture was then transferred to a 100 mL Teflon-lined stainless steel autoclave for solvothermal reaction at 200℃ for 10 h. These magnetic black powders were washed several times with purified water and ethanol respectively. After that, the products were collected with the help of a magnet, then dried at 40℃ under vacuum and denoted as graphene/Fe_3O_4.

Synthesis of Magnetic Graphene/Fe_3O_4/TiO_2 Composite

Firstly, 60 mg of graphene/Fe_3O_4 and 30 mg of dopamine hydrochloride were added into 150 mL of 10 mM Tris buffer solution (pH 8.5) and dispersed by sonication for 10 min in an ice bath. The reaction mixture was stirred vigorously at 60℃ for 24 h. The hydrophilic polydopamine (PDA) coating was formed by self-polymerization of the dopamine in a mild basic condition. The PDA-coated graphene/Fe_3O_4 was washed by purified water, separated using a magnet, and then dried under vacuum. The PDA-coated graphene/Fe_3O_4 (50 mg) was

incubated in $Ti(SO_4)_2$ solution (100 mM) at room temperature for 2 hours under gentle agitation. Then, TiO_2 nanoparticles were prepared via the hydrolysis of $Ti(SO_4)_2$ by adjusting pH of solution with ammonium hydroxide. The obtained graphene/Fe_3O_4/TiO_2 composite was collected by magnetic separation and washed thoroughly with 0.1% (v/v) formic acid (FA) to remove residual titanium ions. The graphene/Fe_3O_4/TiO_2 composite was dispersed in 3mL 0.1% (v/v) FA (about 10mg/mL) before use.

Preparation of Protein Digests

Phosphoprotein β-casein (1 mg) was dissolved in 1 mL of 50 mM ammonium bicarbonate buffer. β-Casein and trypsin were mixed at a weight ratio of 50:1, and enzymatic digestion was carried out at 37℃ for 18 h. BSA (1 mg) was dissolved in 0.1mL of 50 mM ammonium bicarbonate buffer containing 8 M urea and incubated at 56℃ for 15 min. After adding 200μL of 100 mM dithiothreitol (DTT), the mixture was incubated at 56℃ for 1h. Then, 200μL of 200 mM iodoacetamide (IAA) was added and the mixture was incubated at 37℃ for 30 min in the dark. The obtained solution was diluted to 1mL with 50 mM ammonium bicarbonate and digested at 37℃ for 18 h with BSA to trypsin ratio of 50:1 (w/w). Semicomplex samples were mixtures originating from tryptic digestion of β-casein and BSA at molar ratio of 1:100 and 1:500 by keeping β-casein concentration at 20 fmol.

The protein concentration of nonfat milk was measured as 20 mg/mL by Bradford assay. Nonfat milk (50 μL) was first diluted by ten times with 25 mM ammonium bicarbonate to 250μL, and also denatured with urea (0.1 mL, 8 M) at 56℃ for 20 min. Then, the mixture was reduced with DTT (20μL 100 mM) at 56℃ for 1 h, and alkylated with IAA (20μL 200 mM) in the dark for 30 min at 37℃. Finally, proteins were diluted to 1 mL with 25 mM ammonium bicarbonate, and incubated at 37℃ for 16 h with the protein/enzyme ratio of 50:1 m/m. All the peptide solutions were acidified with 0.1% TFA to pH<3 and stored at -20℃ before use.

Selective Enrichment of Phosphopeptides Using Graphene/Fe_3O_4/TiO_2 Composite

In a typical process , graphene/Fe_3O_4/TiO_2 composite (10mg/mL, 2μL) was first washed with 50 μL loading buffer (50% ACN containing 1% TFA) and suspended in 200 μL of the peptide mixture originating from tryptic digestion product, and then the mixture was vibrated

at room temperature for 30 min. After incubation, phosphopeptide-loaded graphene/Fe_3O_4/TiO_2 composite was separated from the mixed solutions by applying an external magnet. After washing two times with loading buffer (50 μL) to remove the non-specifically adsorbed peptides, the trapped phosphopeptides were eluted with 50μL of 5% ammonia aqueous solution under vibration for 15 min. The supernatant was collected, lyophilized in DHB solution (25mg/mL, containing 50%ACN and 1%H_3PO_4) for MALDI-TOF-MS analysis. For the tryptic digestion of the nonfat milk as real sample, the similar enrichment method was carried out.

Characterization and Peptide Analysis

The morphology and structure of the resulting magnetic composites were evaluated using a Tecnai G2 T2 S-TWIN transmission electron microscope (TEM). Samples for TEM were prepared by placing a drop of dilute nanoparticles of solution in the ethanol solvent on a copper grid. The X-ray photoelectron spectra were obtained using a Shimadzu (Japan) Kratos AXIS Ultra DLD X-ray photoelectron spectrometer (XPS) with an Mg Kα node (15 kV, 400 W) at a take off angle of 45°. The source X-rays were not filtered and the instrument was calibrated against the C1s band at 284.6 eV. The infrared spectra were recorded on a Nicolet AVATAR-360 Fourier transform infrared (FT-IR) spectrometer. After vacuum drying, the samples were thoroughly mixed with KBr (the weight ratio of sample/KBr was 1%) in a mortar, and then the fine powder was pressed into a pellet. Then the FT-IR spectrum was recorded. The identification of the crystalline phase was performed on a Rigaku D/max/2500v/pc (Japan) X-ray diffractometer with a Cu Ka source. The 2θ angles probed were from 3° to 80° at a rate of 4°min^{-1}. The magnetic properties were analyzed with a vibrating sample magnetometer (VSM) (LDJ 9600-1, USA).

All mass spectra were measured in a Bruker AutoflexIII MALDI-TOF mass spectrometer (Bruker Daltonics Bremen, Germany) with a smartbeam laser at 337 nm. The acceleration voltage is 19 kV. Each typical spectrum was summed with 1000 laser shots. The matrix solution was prepared by dissolving 25 mg DHB into 1 mL of 50% ACN solution (containing 1% H_3PO_4). MALDI-TOF mass spectrometer was corrected using the external standard method, where the standard peptides (0.5μL) and matrix solution (0.5μL) deposited on the

target was running in advance. The phosphopeptides solution (0.5μL) and matrix solution (0.5μL) were deposited sequently on the target for MALDI-TOF measurements.

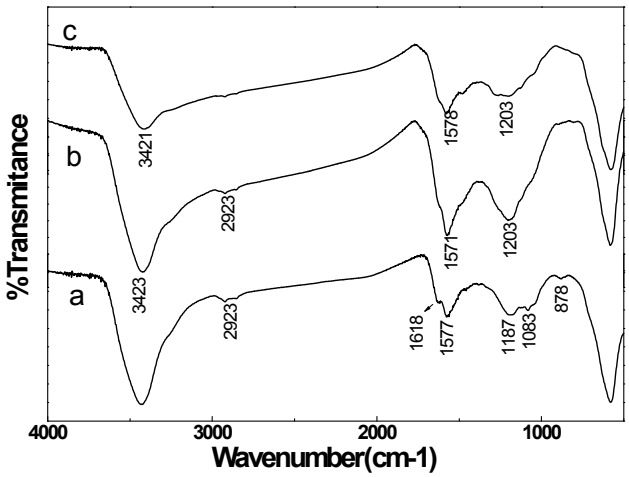

Fig.S1 The FTIR spectra of graphene/Fe_3O_4 (a), PDA-coated graphene/Fe_3O_4 (b) and graphene/Fe_3O_4/TiO_2 (c) magnetic composites.

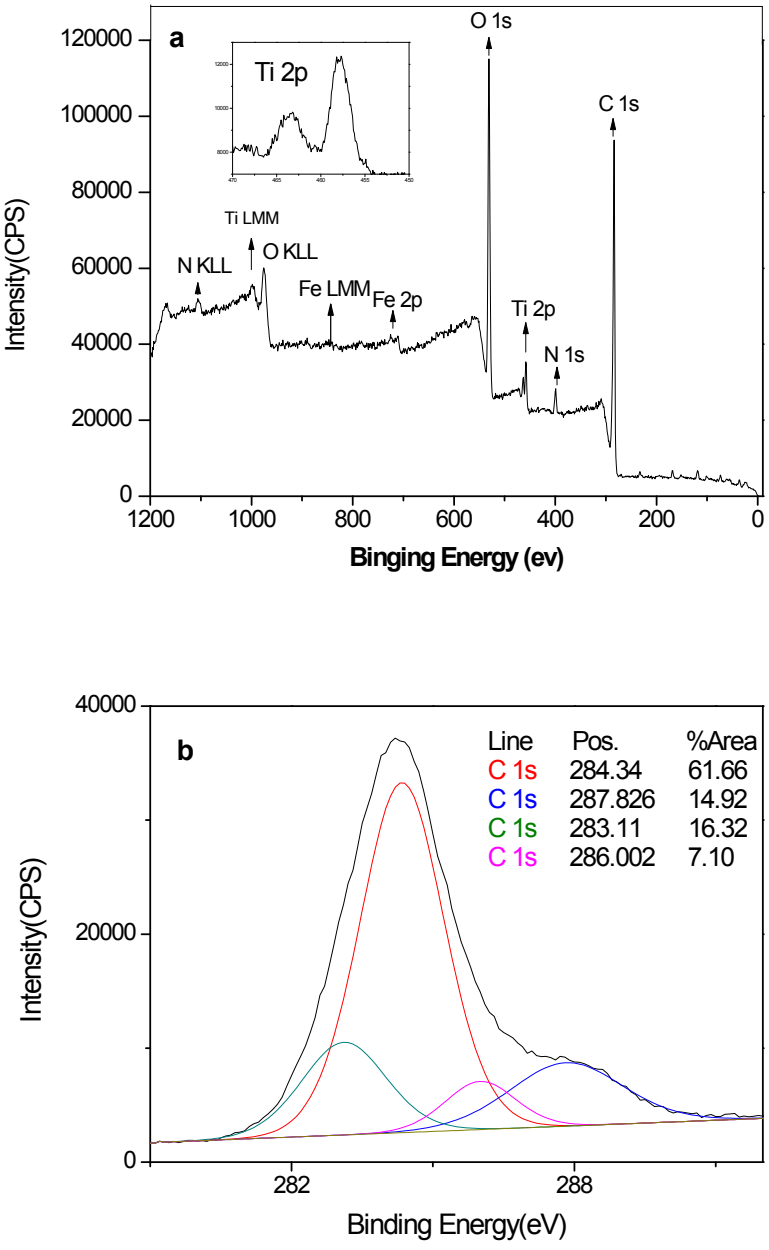

Fig.S2 XPS wide-scan spectra (a) and C1s core-level spectra (b) of graphene/Fe_3O_4/TiO_2 composite.

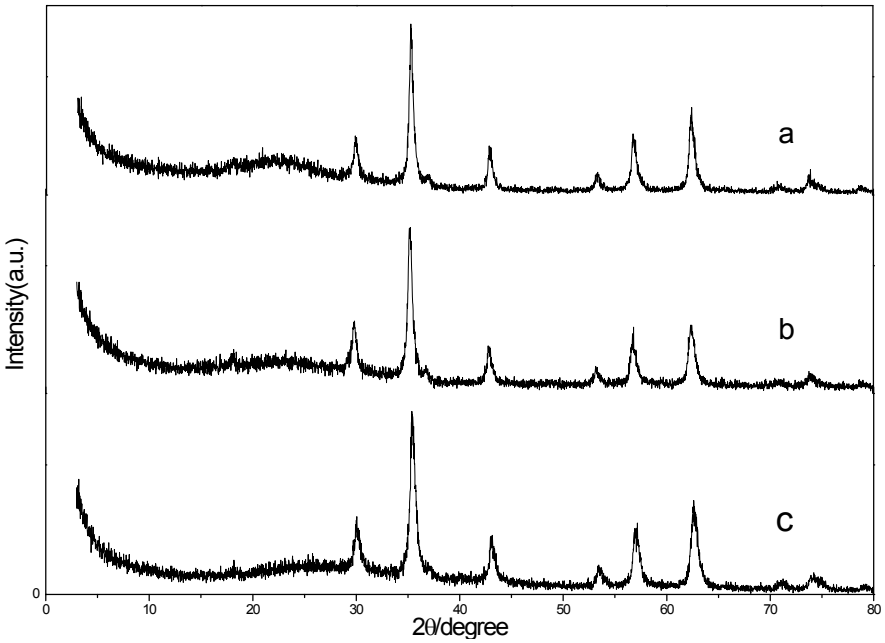

Fig.S3 XRD patterns of graphene/Fe_3O_4 (a), PDA-coated graphene/Fe_3O_4 (b) and graphene/Fe_3O_4/TiO_2 (c) magnetic composites.

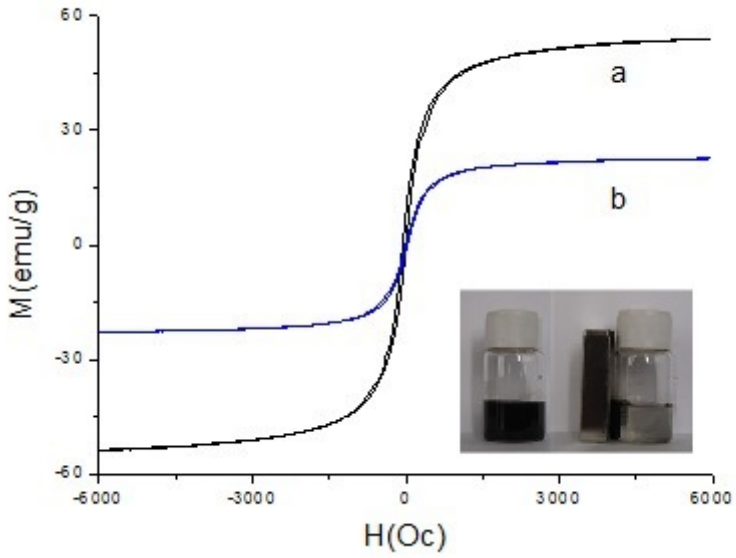

Fig.S4 The magnetic hysteresis loop of graphene/Fe_3O_4 (a), and graphene/Fe_3O_4/TiO_2 (b) magnetic composites.

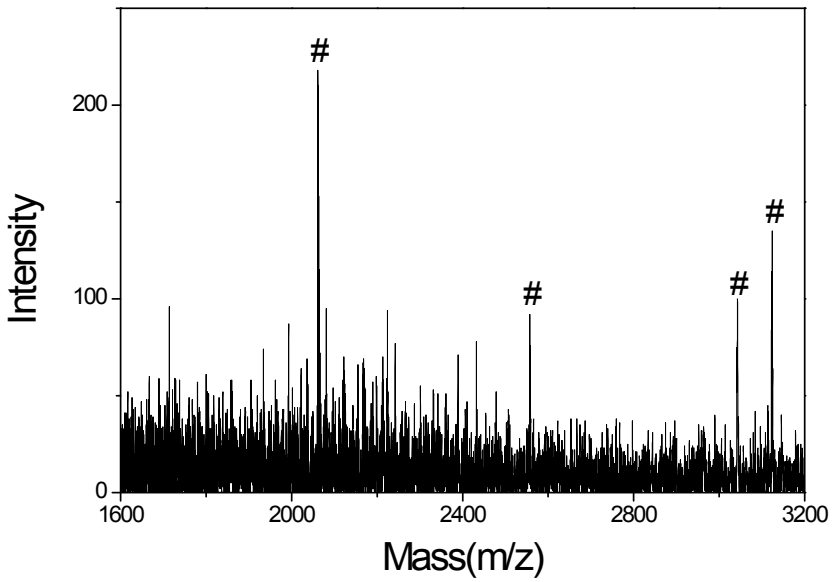

Fig.S5 MALDI-MS spectra obtained from the tryptic digest of β-casein with 0.2 amol after enrichment by graphene/Fe_3O_4/TiO_2 composite.

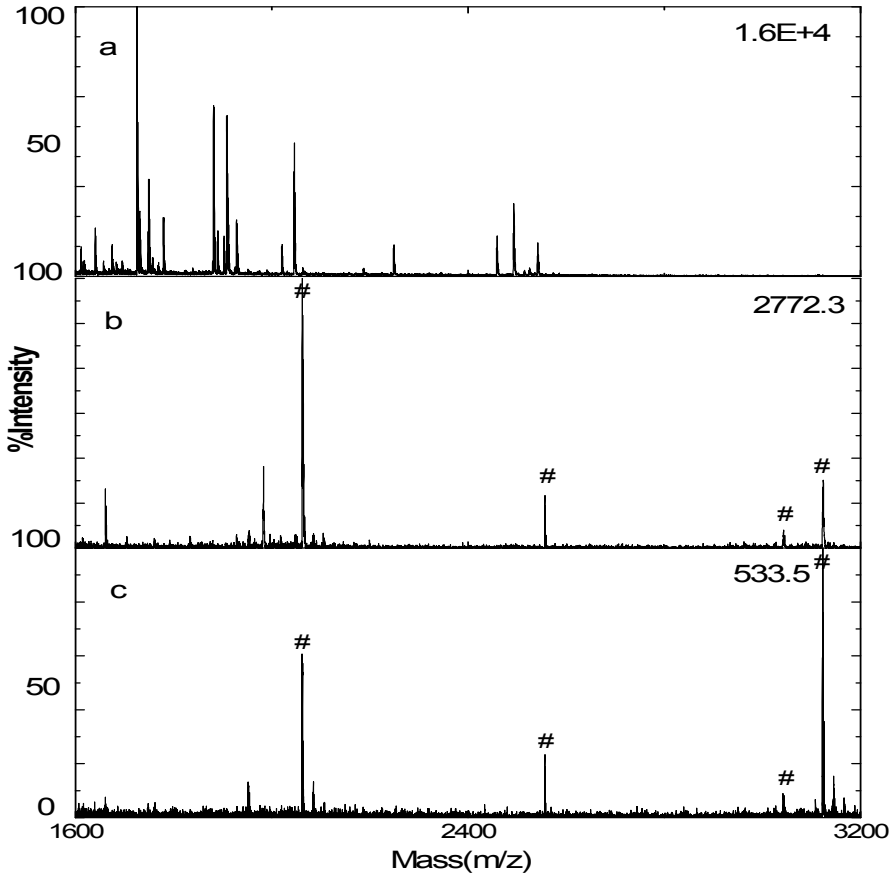

Fig.S6 MALDI-TOF mass spectra of the tryptic digest mixture of β-casein and BSA with different molar ratio. Direct analysis of the molar ratio of 1:100 (a), after enrichment by graphene/Fe_3O_4/TiO_2 composite with molar ratio of 1:100 (b), and 1:500 (c) respectively. The amount of β-casein was kept as 20 fmol, and the peaks of phosphopeptides are marked with #. The data in parentheses are signal to noise ratio (S/N) of the corresponding peaks.

Table S1. Detailed information of observed phosphopeptides from tryptic digest mixture of β-casein

$[M+H]^+$	Peptide residues	amino acid sequence	Phosphorylation site
2061.8	48-63	FQSpEEQQQTEDELQDK	1
2432.0	45-63	IEKFQSpEEQQQTEDELQDK	1
2556.8	44-63	KIEKFQSpEEQQQTEDELQDK	1
3042.2	16–40	RELEELNVPGEIVESLSpSpSpEESITR	3
3122.1	16–40	RELEELNVPGEIVESpLSpSpSpEESITR	4

Table S2. Detailed information of observed phosphopeptides from tryptic digest mixture of the nonfat milk

$[M+H]^+$	Peptide identity	amino acid sequence	Phosphorylation site
1660.65	α-S1-casein: 121–134	VPQLEIVPNSAEER	130
1927.58	α-S1-casein: 58–73	DIGSESTEDQAMEDIK	61,63
1951.84	α-S1-casein: 119–134	YKVPQLEIVPNSAEER	130
2061.71	β-casein: 48–63	FQSEEQQQTEDELQDK	50
2619.05	α-S2-casein: 17–36	NTMEHVSSSEESIISQETYK	23,24,25,31
2678.33	α-S1-casein: 52–73	VNELSKDIGSESTEDQAMED IK	56,61,63
2703.22	α-S1-casein: 114–135	LRLKkYKVPQLEIVPNSAEER	130
2720.41	α-S1-casein: 74–94	QMEAESISSSEEIVPNSVEQ K	79,81,82,83,90
2965.52	β-casein: 17–40	ELEELNVPGEIVESLSSSEE SITR	30,32,33,34
3042.43 3122.23	β-casein: 16–40	ELEELNVPGEIVESLSSSEE SITRINK	30,32,33,34