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Magnetic graphitic carbon nitride anion exchanger for specific enrichment of phosphopeptides

Gang-Tian Zhu [a,b], Xiao-Mei He [a], Xi Chen [c], Dilshad Hussain [a,d], Jun Ding [a], Yu-Qi Feng [a,*]

[a] Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), Department of Chemistry, Wuhan University, Wuhan 430072, PR China
[b] Key Laboratory of Tectonics and Petroleum Resources (Ministry of Education), China University of Geosciences, Wuhan 430075, PR China
[c] Wuhan Institute of Biotechnology, Wuhan 430072, PR China
[d] Division of Analytical Chemistry, Institute of Chemical Sciences, Bahauddin Zakariya University, Multan 60800, Pakistan

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ABSTRACT

Anion-exchange chromatography (AEX) is one of the chromatography-based methods effectively being used for phosphopeptide enrichment. However, the development of AEX materials with high specificity toward phosphopeptides is still less explored as compared to immobilized metal affinity chromatography (IMAC) or metal oxide affinity chromatography (MOAC). In this work, magnetic graphitic carbon nitride (MCN) was successfully prepared and introduced as a promising AEX candidate for phosphopeptide enrichment. Due to the extremely abundant content of nitrogen with basic functionality on the surface, this material kept excellent retention for phosphopeptides at pH as low as 1.8. Benefiting from the large binding capacity at such low pH, MCN showed remarkable specificity to capture phosphopeptides from tryptic digests of standard protein mixtures as well as nonfat milk and human serum. In addition, MCN was also applied to selective enrichment of phosphopeptides from the tryptic digests of rat brain lysate and 2576 unique phosphopeptides were successfully identified.

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1. Introduction

Phosphorylation is one of the most prominent post-translational modifications of proteins, which is involved in many biological processes including cell proliferation, differentiation, development and apoptosis [1–5]. Mass spectrometry (MS), including matrix-assisted laser desorption/ionization (MALDI) MS or electrospray ionization (ESI) MS, has been a central tool in shotgun based phosphoproteome analysis [6–9]. Phosphopeptide enrichment is a critical step to effectively decrease the complexity of proteome samples prior to MS analysis [10,11]. Until now, many approaches have been developed to enrich phosphopeptides, such as antibody-based method [12,13], chemical derivatization method [14,15] and affinity chromatography-based method [16,17]. The chromatography-based method mainly includes immobilized metal affinity chromatography (IMAC) [18–20], metal oxide affinity chromatography (MOAC) [21–23], cation-exchange chromatography [24] and anion-exchange chromatography (AEX) [25,26]. In most cases, single method cannot achieve desired goals due to the complexity of proteome sample and the wide range of physical and chemical properties of different phosphopeptides. In addition, each affinity sorbent has its own bias on subset of phosphopeptides [27]. It means that combination of multiple methods can effectively increase the phosphoproteome coverage and development of new types of sorbents may produce complementary or even better results to those of the existing methods.

Since phosphopeptides contain extra negative charge, AEX has been previously used to fractionate or enrich phosphopeptides because of the stronger retention of phosphopeptides compared to non-phosphopeptides [28,29]. Several reported AEX materials are also available as commercial AEX columns [30–32]. To improve the specificity, IMAC or MOAC is often needed to further purify the fractions eluted from AEX column [33,34]. Dong et al. prepared an organic-silica hybrid AEX monolithic capillary to enrich phosphopeptides from protein digests [28] where pH of loading solution was kept around 8 and the elution was carried out by 5% formic acid. Similarly, Atakay et al. investigated amine-functionalized silica gel for the enrichment of phosphopeptides [35]. The pH of loading solution and eluting solution was set at 4 and 1, respectively. Both of the two AEX materials showed unsatisfactory specificity for phosphopeptides.

* Corresponding author. Fax: +86 27 68755595.
E-mail address: yqfeng@whu.edu.cn (Y.-Q. Feng).

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The pH of solution is the key factor in specific enrichment of phosphopeptides by AEX. At a pH greater than 5, phosphopeptides are well-retained on AEX sorbent, while acidic non-phosphopeptides can be also adsorbed onto AEX. At a pH lower than 2, carboxyl groups are mostly uncharged and acidic non-phosphopeptides are hardly adsorbed on AEX. However, phosphate residues have just a single negative charge at such low pH, so the retention of phosphopeptides on AEX would weaken [29]. Therefore, development of AEX material with high specificity for phosphopeptides is still a challenge and it is desirable to introduce new AEX materials that can keep sustainable retention for phosphopeptides at low pH.

Graphitic carbon nitride (g-C_3N_4) is an analog of graphite consisting of C, N and some impurity H [36–38]. It is widely accepted that g-C_3N_4 is composed of tri-s-triazine rings crosslinked by trigonal nitrogen atoms which has unique electronic property and basic surface [39,40]. In addition, g-C_3N_4 possesses high thermal stability and is insoluble in water or organic solvent [41]. As a result, g-C_3N_4 has been developed as a hot material in many applications especially catalysis [42]. Indeed, it has been used as a solid base catalyst for a variety of reactions [41,43,44]. Besides, Lin et al. utilized g-C_3N_4 as a matrix for negative ion MALDI-MS, suggesting that g-C_3N_4 can act as a solid base and promote the charging process in the negative ion mode [45]. These previous reports inspired us to explore g-C_3N_4 as a potential adsorbent for selective capture of phosphopeptides.

Here, for the first time, g-C_3N_4 was developed as an AEX material for efficient enrichment of phosphopeptides. The mechanism of phosphopeptide enrichment with g-C_3N_4 was investigated and compared with amine-functionalized silica. Additionally, to simplify and expedite the enrichment procedure, we endowed the g-C_3N_4 sorbent with magnetic property. In previous work, magnetic g-C_3N_4 material, e.g., g-C_3N_4–Fe_3O_4, was prepared by physical blending of Fe_3O_4 and g-C_3N_4 [46] or chemical deposition of Fe_3O_4 nanoparticles onto g-C_3N_4 [47–49]. Whereas, the bare Fe_3O_4 on the surface of g-C_3N_4 makes it unstable in acidic solution, and can lead to nonspecific adsorption. In this article, we designed a facile method to prepare magnetic g-C_3N_4 by in-situ thermal polycondensation, in which g-C_3N_4 was immobilized on Fe_3O_4@SiO_2 particles. The magnetic g-C_3N_4 sorbent (MCN) was stable in acidic solution and was able to minimize the nonspecific adsorption. To investigate the performance of the resulting MCN, it was applied to enrich phosphopeptides from tryptic digests of standard protein mixtures, nonfat milk and human serum. Furthermore, MCN was applied to selective enrichment of phosphopeptides from tryptic digests of rat brain lysate.

2. Experimental

2.1. Chemicals and materials

Melamine, ferric trichloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium acetate (NaAc), ethylene glycol (EG), ethylene diamine, ethanol (EtOH) and ammonia hydrate ($NH_3 \cdot H_2O$, 25 wt.% in H_2O) were supplied by Shanghai General Chemical Reagent Factory (Shanghai, China). Tetraethyl orthosilicate (TEOS) was obtained from Chemical Plant of Wuhan University (Wuhan, China). HPLC grade acetonitrile (ACN) was obtained from Fisher Scientific (Pittsburgh, USA). Amine-functionalized silica was purchased from Weltech (Wuhan, China). Commercial TiO_2 (T104936) was purchased from Aladdin Chemical Reagent (Shanghai, China). Trifluoroacetic acid (TFA), 2,5-dihydroxybenzoic acid (2,5-DHB), bovine β-casein and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, USA). Sequencing grade trypsin was obtained from Promega (Madison, WI, USA). Purified water was obtained with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Nonfat milk was purchased from a local supermarket. Human serum sample was obtained from Wuhan Zhongnan Hospital according to their standard clinical procedures and stored at −70 °C until use.

2.2. Preparation of Fe_3O_4@SiO_2

Magnetic core–shell material (Fe_3O_4@SiO_2) was synthesized by a two step process, according to our previous works. Initially, Fe_3O_4 microspheres were prepared by solvothermal reaction [50] and silica shells were coated onto Fe_3O_4 microspheres through Stöber method with some modifications [51].

2.3. Preparation of pure g-C_3N_4 and MCN

Melamine was chosen as the precursor for synthesis of g-C_3N_4. Pure g-C_3N_4 was prepared by direct heating of melamine at 550 °C for 4 h under inert atmosphere [52]. MCN was synthesized by an in-situ chemical vapor deposition method. Typically, 1 g of melamine and 0.5 g of Fe_3O_4@SiO_2 were added to a mortar and grounded into homogeneous fine powder, and the resulting powder was transferred into a crucible and heated at 550 °C for 4 h under inert atmosphere to obtain MCN. Notably, the crucible was filled up to obtain homogeneous deposition.

2.4. Characterization of the prepared materials

Transmission electron microscopy (TEM) images were obtained from JEM-2100F transmission electron microscope (JEOL, Tokyo, Japan). Thermo-gravimetric analysis (TGA) was performed on NETZSCH STA449C thermal analyzer (Bavaria, Germany) under air flowing. The powder X-ray diffraction (XRD) measurements were recorded on a D/MAX-RB X-ray powder diffractometer (RIGAKU, Tokyo, Japan) using Cu Kα radiation (λ = 1.5406 Å) with scattering angles (2θ) of 1–6°. Fourier transform infrared spectrum (FT-IR) was performed with a Thermo Nicolet 670 FT-IR instrument (Boston, MA, USA).

2.5. Preparation of samples

Bovine β-casein was originally prepared into stock solutions of 1 mg/mL. Proteins were digested with trypsin using an enzyme to substrate ratio of 1:50 (w/w) in 100 mM Tris–HCl (pH 8.5) and the digestion was performed at 37 °C for 16 h.

Similarly, BSA (1 mg) was dissolved in 100 μL of denaturing buffer solution (8 M urea in 100 mM Tris–HCl, pH 8.5). The protein solution was mixed with 5 μL of 100 mM tri(2-chloroethyl)phosphate (TCEP) and incubated for 20 min at room temperature to reduce protein disulfide bonding. Iodoacetamide (IAA) (3 μL of 500 mM stock) was added to the solution and incubated for an additional 30 min at 25 °C in dark. The reduced and alkylated protein mixture was diluted with 300 μL of 100 mM Tris–HCl (pH 8.5). Then, 9 μL of 100 mM $CaCl_2$ was added to the above solution and the mixture was digested with trypsin at an enzyme to substrate ratio of 1:50 (w/w) by incubating at 37 °C for 16 h. All the tryptic digests were lyophilized to dryness and stored at −80 °C for further use.

For in-solution digestion, nonfat milk (50 μL) was first denatured by the ammonium bicarbonate solution (50 mM, 250 μL) containing urea (8 M) and incubated at 37 °C for 30 min. Then, dithiothreitol (DTT) solution (200 mM, 25 μL) was added and incubated at 55 °C for 1 h. After cooling to room temperature, the IAA solution (200 mM, 50 μL) was added and the mixture was kept in the dark for 3 h. Finally, the resulting mixture was incubated with trypsin (2 mg/mL, 5 μL) at 37 °C for 16 h. The tryptic digests were stored at −80 °C until further use.

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