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Development of Gd^{3+}-immobilized glutathione-coated magnetic nanoparticles for highly selective enrichment of phosphopeptides

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ABSTRACT

In this study, we designed a gadolinium-based immobilized metal ion affinity chromatography material for the selective enrichment of phosphopeptides. Gadolinium ion was immobilized on the surface of glutathione-coated magnetic nanoparticles through a facile and effective synthetic route. The adsorbent integrated the advantages of superparamagnetism of Fe_3O_4 core, good biological compatibility of glutathione, and strong interaction between gadolinium ion and phosphopeptides. It was employed to enrich phosphopeptides from standard protein digests coupled with MALDI-TOF MS. Results demonstrated that the adsorbent possessed high selectivity for phosphopeptides, good reusability and reproducibility. Moreover, the material provided selective enrichment of phosphopeptides from real samples including non-fat milk digests and human serum. The developed method exhibited high sensitivity (detection limit of 10 fmol), showing great potential in the detection of low-abundance phosphopeptides in biological samples.

1. Introduction

Protein phosphorylation is a reversible post translational modification, which plays vital roles in the regulation of many cellular processes such as cell proliferation, metabolism, differentiation, transcription, and signal transduction [1–3]. Nowadays, mass spectrometry (MS) based technology has become an important strategy for detecting phosphorylated proteins and phosphopeptides in phosphoproteome research [4,5]. However, the weak ionization efficiency and low abundance of phosphopeptides make MS a limited method for direct analysis [6,7]. Thus, it is indispensable to develop efficient enrichment methods to exclude interferences and improve the sensitivity of MS detection.

Therein, immobilized metal ion affinity chromatography (IMAC) based on chelation/coordination chemistry has been the most common method for enrichment of phosphopeptides. The selectivity of IMAC towards phosphopeptides is based on the affinity of phosphate groups to metal ions immobilized on supporting substrates [8–10]. Owing to the diverse empty orbitals and the positive charges of metal ions, various IMAC adsorbents exhibit biased affinities towards phosphopeptides [11,12]. Various metal ions, such as Fe^{3+}, Ga^{3+}, Ti^{4+}, Zr^{4+}, have been widely applied in IMAC [13–15]. Gadolinium (Gd), a rare earth element, has been employed for phosphopeptide capturing as well as labeling including GdF_3, Gd_2O_3, and $GdPO_4$ [16–18]. However, applications of Gd^{3+} ion based IMAC materials in phosphoproteomics have not been excavated.

The chelator also plays a crucial role in the enrichment of IMAC because it decides the amount of metal ions introduced and thus affects the binding specificity and capacity for phosphopeptides. Iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) are most commonly used chelators in the past years [19]. However, the loss of metal ions due to the weak interactions between IDA or NTA and metal ions always reduce the enrichment capacity of phosphopeptides [20,21]. Consequently, to develop novel chelators and design more stable IMAC materials for efficient enrichment of phosphopeptides are still urgently needed.

Glutathione (GSH), the most abundant cellular thiol and nucleophilic tripeptide, consists of various coordinating groups including carboxyl, amino, thiol, and amide [22]. GSH has a protective role against oxidative stress, and plays a crucial part in many detoxification procedures, e.g., help for heavy metal removal [23]. GSH can form a claw coordination structure with rare earth elements (such as La^{3+} and Gd^{3+}) through the carboxyl groups at both ends of GSH [24]. Considering the specificity of Gd^{3+} to phosphopeptides and the strong coordination interaction between GSH and Gd^{3+}, it is of significant interest to design Gd^{3+}-IMAC material with GSH as the chelator. As can be expected, such an IMAC material may be an attractive candidate for the enrichment of phosphopeptides.

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Herein, we synthesized Gd^{3+}-IMAC adsorbent and investigated its performance of phosphopeptides enrichment. The adsorbent was designed considering the following three reasons. (i) Magnetic nanoparticles were introduced because of their good biocompatibility and superparamagnetism, which allowed the enrichment process to be easily and rapidly performed with the help of an external magnetic field [25,26]. (ii) Gd^{3+} was chosen for phosphopeptide enrichment based on its affinity with the phosphate groups [16–18]. (iii) GSH had biofriendly nature and could chelate Gd^{3+} though carboxyl groups [23]. It was the first time that Gd^{3+} based IMAC material was employed for the capture of phosphopeptides with GSH as the chelator. The prepared material displayed high selectivity for phosphopeptides enrichment in the standard proteins and real biological samples. The new phosphopeptides enrichment strategy would have great potential for future phosphoproteome research.

2. Experimental section

2.1. Chemical and materials

Glutathione (GSH), gold chloride trihydrate ($HAuCl_4{\cdot}3H_2O$), and trifluoroacetic acid (TFA) were purchased from Aladdin Reagents (Shanghai, China). Iron trichloride ($FeCl_3{\cdot}6H_2O$), gadolinium chloride ($GdCl_3{\cdot}6H_2O$), sodium acetate (NaAc), ammonium hydroxide ($NH_3{\cdot}H_2O$), ammonium bicarbonate (NH_4HCO_3), acetonitrile (ACN), sodium borohydride ($NaBH_4$), ethylene glycol, and ethanol were purchased from Beijing Chemical Works (Beijing, China). L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin from bovine pancreas, β-casein from bovine milk, bovine serum albumin (BSA), and ovalbumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Non-fat milk was purchased from local supermarket. Human serum was obtained from China-Japan Hospital of Jilin University (Changchun, China) according to standard clinical procedures.

2.2. Synthesis of Fe_3O_4@Au-GSH-Gd^{3+}

Fe_3O_4 was prepared by a solvothermal reaction between $FeCl_3$ and ethylene glycol [27]. Briefly, 0.675 g $FeCl_3{\cdot}6H_2O$ was dissolved in 37.5 mL ethylene glycol and 1.80 g NaAc was added into the above solution. After stirring for 1 h, the formed homogeneous yellow solution was heated at 200 °C for 16 h in a Teflon-lined stainless steel autoclave, and then the obtained products were washed thoroughly with water and ethanol.

The procedure to prepare Fe_3O_4@Au-GSH was according to previously reported work [28,29]. Firstly, 60 mg Fe_3O_4 was dispersed in 168 mL water with sonication for 15 min. Next, 12 mL $HAuCl_4$ aqueous solution (6 mg mL^{-1}) was added into the above solution and the mixture was stirred at 0 °C for 1 h. Afterwards, 3.6 mL ice-cold $NaBH_4$ (0.2 mol mL^{-1}) was slowly added into the mixture. The reaction was carried out under stirring at 0 °C for 15 min to obtain Fe_3O_4@Au. Then, 10.56 mL GSH (0.1 mol mL^{-1}) was added into the solution under stirring at 25 °C for 24 h to obtain Fe_3O_4@Au-GSH. Finally, the products were dried under vacuum at 50 °C for 24 h.

3.717 g $GdCl_3{\cdot}6H_2O$ was dissolved in 75 mL water, and the pH was adjusted to be in the range of 5.0–5.5. Then, 100 mg Fe_3O_4@Au-GSH was added into the solution under stirring at 25 °C for 5 h. The products were separated with a magnet and rinsed with water for three times, and then vacuum-dried at 50 °C for 24 h to obtain Fe_3O_4@Au-GSH-Gd^{3+}.

2.3. Characterization

Scanning electron microscopic (SEM) and transmission electron microscopic (TEM) images were recorded on S4800 ESEM Hitachi microscope (Hitachi, Japan) and Hitachi H600 microscope (Hitachi, Japan), respectively. Dynamic laser scattering (DLS) size was measured on a Zetasizer Nano-ZS instrument (Malvern, UK). UV–Vis spectra were obtained on a UV-2450 spectrophotometer (Shimadzu, Japan). Fourier-transform infrared (FT-IR) spectra were carried out on a Thermo Nicolet 670 FT-IR instrument (Thermo Nicolet Corporation, USA) operated at the wavelength range of 4000–400 cm^{-1}. XPS data were obtained on an X-ray photoelectron spectrometer (XPS, Thermo Electron, USA). X-ray powder diffraction (XRD) measurement was performed to identify the phase on an X-ray diffractometer (R-AXIS RAPID-F Rigaku Corporation, Japan) with a Cu target ($\lambda = 1.5418$ Å) at a generator voltage of 40 kV and current of 20 mA. The basal spacing (d) of the adsorbent was calculated according to Bragg's equation ($n\lambda = 2d\sin\theta$). Vibrating sample magnetometry (VSM) was measured using a Superconducting Quantum Interference Device (SQUID XL-7, Quantum Design, USA). Thermogravimetric analysis/derivative thermogravimetry (TGA-DTG) analysis was performed on a Q500 thermal gravimetric analyzer (TA, USA).

2.4. Sample preparation

For protein digestion, β-casein, ovalbumin, and BSA were dissolved in NH_4HCO_3 buffer (50 mg mL^{-1}, pH 8.3) and treated with trypsin (1:50, *w/w*) at 37 °C for 16 h, respectively. All the resulted peptide mixtures were stored at −20 °C for further use.

Non-fat milk sample was treated according to our previous reported work [30]. Briefly, 30 μL non-fat milk was denatured using 1 mL NH_4HCO_3 buffer. The mixture was kept at 100 °C for 10 min and then treated with 40 μL trypsin (1 mg mL^{-1}) at 37 °C for 16 h. 50 μL human serum was mixed with 450 μL above buffer and incubated at 25 °C for 1 h. The supernatant was stored at −20 °C after centrifuged at 3000 rpm for 5 min.

2.5. Enrichment process

Typically, 0.5 mg Fe_3O_4@Au-GSH-Gd^{3+} was added into 100 μL loading buffer (50% ACN + 0.1% TFA) containing tryptic digests of peptides or human serum, and then the mixture was vibrated for 30 min and centrifuged to obtain the precipitates. After removing the supernatant, the precipitates were rinsed with 100 μL loading buffer three times. After that, the trapped phosphopeptides were eluted using 30 μL 10% $NH_3{\cdot}H_2O$ under sonication for 10 min and the eluent was analyzed by MALDI-TOF MS.

2.6. Optimization of enrichment conditions

Effects of loading buffer, eluent, adsorbent amount, and adsorption time on the enrichment efficiency were investigated. ACN solution (50%, *v/v*) containing TFA with various concentrations were employed as the loading buffer. The influence of TFA concentrations was studied in the range of 0.05–1%. The effect of $NH_3{\cdot}H_2O$ (eluent) concentrations on the enrichment ability was investigated ranged from 0.5% to 10%. In addition, the influence of adsorbent amount (0.1–0.7 mg) and adsorption time (10–40 min) on the enrichment efficiency was studied. All these optimization experiments were carried out with tryptic digests of β-casein (4 pmol).

2.7. MALDI-TOF MS analysis

A MALDI-TOF instrument (model 5800, Applied Biosystems, Foster City, CA) in the reflection positive-ion mode was used for MALDI-TOF MS detections. The DHB solution (25 mg mL^{-1}) was prepared in solutions containing 70% ACN, 28.9% water, 1% phosphoric acid, and 0.1% TFA. Typically, 0.5 μL eluent and 0.5 μL DHB were well mixed, and then loaded onto the sample plate for analysis. At least 1000 laser shots were accumulated with a laser pulse rate of 400 Hz in the MS mode with the m/z scan range of 1000–4000.