Journal of Chromatography A, xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Facile synthesis of titanium (IV) ion immobilized adenosine triphosphate functionalized silica nanoparticles for highly specific enrichment and analysis of intact phosphoproteins

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ARTICLE INFO

Article history:
Received 8 April 2018
Received in revised form 29 May 2018
Accepted 5 June 2018
Available online xxx

Keywords:
Ti^{4+}-ATP-NPs
β-Casein
Intact phosphoprotein
Enrichment
SDS-PAGE

ABSTRACT

Analysis of phosphoproteins always faces the challenge of low stoichiometry, which demands highly selective and efficient enrichment in the initial sample preparation. Here we report our synthesis of the novel titanium (IV) ion immobilized adenosine triphosphate functionalized silica nanoparticles (Ti^{4+}-ATP-NPs) for efficient enrichment of intact phosphoproteins. The average diameter of Ti^{4+}-ATP-NPs was about 128 nm with good dispersibility and the saturated adsorption capacity for β-casein was 1046.5 mg/g. In addition, Ti^{4+}-ATP-NPs exhibited high specificity and selectivity in enriching phosphoproteins from both standard protein mixtures and complex biological samples (non-fat milk, chicken egg white and mouse heart tissue extract) as demonstrated by SDS-PAGE.

1. Introduction

Reversible protein phosphorylation, one of the most important and ubiquitous protein post-translational modifications (PTMs), plays a crucial role in regulating various biological processes [1–3]. Approximately 30% of eukaryotic proteins have been reported to be phosphorylated [4], and the aberrant protein phosphorylation was related to many human diseases, such as heart disease [5,6], Parkinson's disease [7], Alzheimer's disease [8,9], etc. Phosphoproteomics has the potential to find diagnostic disease markers, unravel biological processes and find new strategies for the treatment of relevant diseases [10], Thus, it is of great significance to study protein phosphorylation; however, the direct analysis of protein phosphorylation in biological samples remains a great challenge due to low abundance of phosphoproteins, low stoichiometry of phosphorylation and the signal suppression of non-phosphoproteins. Hence, enrichment prior to analysis is prerequisite.

Many approaches for the enrichment of intact phosphoproteins have been reported during last decades, the common enrichment strategies mainly include immunoaffinity, chemical derivatization and affinity materials strategy [11–13]. Among them, affinity materials, especially immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC), have been most extensively studied due to their advantages of good enrichment performance, low-cost, operation simplicity, universality [14–17], etc.

As for the IMAC materials, the enrichment mechanism is that metal cations with unoccupied coordination orbitals could coordinate with the phosphoryl oxygen atoms in the phosphate groups at low pH. The enriched phosphoproteins on the surface of IMAC can be released by acid, base or competitive chelating reagents that contain phosphate ions [4,12]. Up to now, several IMAC materials have been successfully developed for the enrichment of intact phosphoproteins [18–20]. For example, the novel Zn^{2+}-immobilized superparamagnetic nanoparticles functionalized by multivalent ligand molecules were developed by Ge et al. and used for the phosphoprotein enrichment in the samples of protein mixtures (β-casein, pepsin and BSA), HEK 293 cell lysate and swine heart tissue extract [21,22]. Jia et al. synthesized the polydopamine assisted Ti^{4+}-decorated magnetic particles for phosphoprotein enrichment and applied the materials in non-fat milk system [23]. Wang et al. prepared the Ti^{4+}-immobilized monodisperse poly(ethylene glycol methacrylate phosphate) microgel for enriching phosphoproteins and the enrichment capability was tested in real complex sample (drinking milk) [24]. However, binding capacity, reproducibility and specificity are still far from fully satisfaction [25], and more

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https://doi.org/10.1016/j.chroma.2018.06.013
0021-9673/

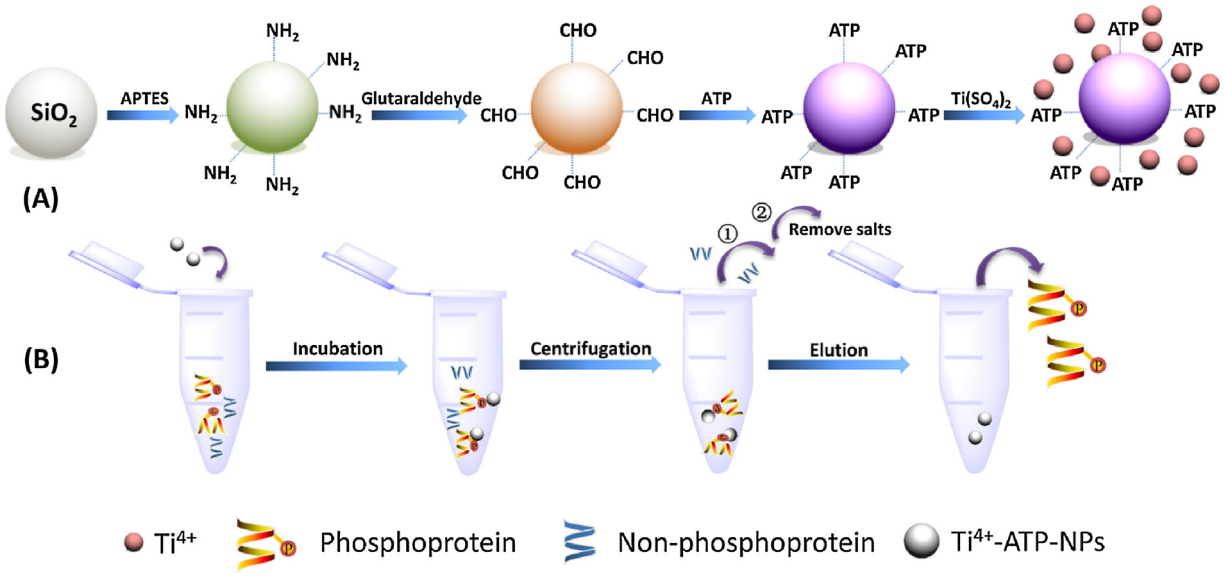

Fig. 1. Synthetic route of Ti^{4+}-ATP-NPs (A) and schematic procedure for the enrichment and analysis of phosphoproteins using Ti^{4+}-ATP-NPs.

research efforts are still urgently needed to improve efficiency and sensitivity.

In this article, a novel kind of titanium (IV) ion immobilized adenosine triphosphate functionalized silica nanoparticles (Ti^{4+}-ATP-NPs) was developed to enrich intact phosphoproteins and the adsorption isotherms of Ti^{4+}-ATP-NPs for the standard proteins (β-casein and BSA) was studied. Besides, excellent phosphoproteins enrichment performance was demonstrated by SDS-PAGE both in standard protein mixtures and complex biological samples (non-fat milk, chicken egg white and mouse heart tissue extract). The results showed that Ti^{4+}-ATP-NPs had a promising prospect for the analysis of the intact phosphoproteins.

2. Experimental

2.1. Materials and reagents

Acetonitrile (ACN) (CHROMASOLV gradient grade, 34851), trifluoroacetic acid (TFA, HPLC grade, 302031), ammonia hydroxide solution (320145, 28% v/v in H_2O), β-casein from bovine milk (C6905), bovine serum albumin (BSA, V900933), *N*,*N*'-methylenebisacrylamide (M7279), sodium orthovanadate (S6508) were purchased from Sigma Aldrich (St. Louis, MO, USA). PageRuler™ Prestained Protein Ladder (10 to 180 kDa, 26616) was purchased from Thermo Fisher Scientific (Wyman Street, Waltham, MA, USA). Sodium chloride (NaCl, 10019318) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Sodium fluoride (NaF, G82071B) was purchased from Tiatan (Shanghai, China). Citric acid (A501702), titanium(IV) sulfate (A502653), urea (A600148), tris (hydroxymethyl) aminomethane (Tris, A600194), sodium dodecyl sulfate (SDS, A600485), glycine (A610235), ammonium persulfate (APS, A600072), acrylamide (A601032), *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED, A610508), glycolic acid, 20 × PBS (phosphate buffered saline, SB0627), 2 × SDS PAGE sample loading buffer (C508321), BCA Protein Assay Kit (SK3021), Protein Stains H (C510041) and Protein Stains S (C500043) were purchased from Sangon Biotech (Shanghai, China). Tetraethoxysilane (TEOS, 83251B), (3-aminopropyl) triethoxysilane (APTES, 90056A), glutaraldehyde (50% in water, 14376C), adenosine 5-triphosphate disodium salt (112470A) and sodium citrate tribasic dehydrate (G70153B) were purchased from Adamas (Shanghai, China). Ultrapure water (18.2 MΩ cm) was produced with Millipore Simplicity® system (Billerica, MA, USA). Non-fat milk and fresh chicken eggs were purchased from local supermarket. All other reagents were of analytical grade or better and used without further purification.

2.2. Synthesis of Ti^{4+}-ATP-NPs

The synthetic route was demonstrated in Fig. 1A, the SiO_2 nanoparticles were prepared first and then functionalized with amino groups, aldehyde groups and ATPs step by step, at last the ATP-NPs were immobilized with titanium (IV) ions to synthesize the final product Ti^{4+}-ATP-NPs.

In details, silica (SiO_2) nanoparticles (NPs) were firstly synthesized according to the literature with minor modification [26]. In brief, 6 mL TEOS was added to the mixtures of 100 mL ethanol, 4 mL deionized water and 4 mL 28% ammonium hydroxide with vigorous stirring at 30 °C for 24 h. The resultant SiO_2 NPs were washed with ethanol and dried at 60 °C in a vacuum dryer.

Secondly, 100 mg SiO_2 NPs were dispersed in a mixture solution of 10 mL isopropanol and 0.2 mL APTES by ultrasonication and then transferred to the oven at 80 °C, the reaction was continued for 2 h to synthesize the amino-functionalized silica nanoparticles (denoted as SiO_2@APTES). The product was washed with ethanol and dried at 60 °C under vacuum for further use.

Thirdly, 10 mg SiO_2@APTES was dispersed in 0.5 mL glutaraldehyde solution (50% in water) and 0.5 mL 100 mM citrate buffer (pH = 5.0) mixtures, then 6 mg $NaBH_3CN$ was added, and reaction vessel was kept in the thermostat shaker at 37 °C for 4 h. Subsequently, wash the resultant thoroughly with citrate buffer to remove the excess glutaraldehyde.

The fourth step, SiO_2@APTES@glutaraldehyde was functionalized with ATP by adding 1 mL citrate buffer (100 mM, pH = 5.0) containing 200 mg ATP and 6 mg $NaBH_3CN$ for 2 h at 37 °C. Wash the SiO_2@APTES@glutaraldehyde@ATP with citrate buffer to remove the excessive ATP, this synthetic procedure was based on the research reported by Zhang et al [27,28].

At last, SiO_2@APTES@glutaraldehyde@ATP was incubated overnight in 20 mL 100 mM $Ti(SO_4)_2$ solution to immobilized Ti^{4+} cations. The obtained Ti^{4+}-ATP-NPs were washed with 0.1% TFA to remove the excess Ti^{4+} cations and stored in 0.1% TFA at 4 °C for further use.

Please cite this article in press as: H. Wang, Z. Tian, Facile synthesis of titanium (IV) ion immobilized adenosine triphosphate functionalized silica nanoparticles for highly specific enrichment and analysis of intact phosphoproteins, J. Chromatogr. A (2018), https://doi.org/10.1016/j.chroma.2018.06.013