Analytica Chimica Acta 900 (2015) 46–55

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Facile synthesis of gallium ions immobilized and adenosine functionalized magnetic nanoparticles with high selectivity for multi-phosphopeptides

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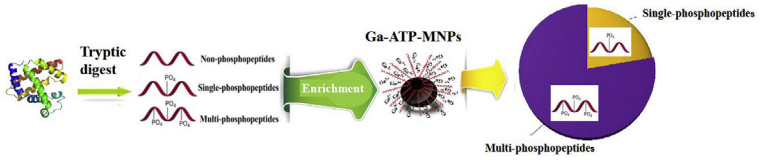
HIGHLIGHTS

- Ga^{3+}-ATP-MNPs have distinct selectivity for multi-phosphopeptides.
- Ga^{3+}-ATP-MNPs provide sensitivity for multi-phosphopeptides at 30 amol.
- Ga^{3+}-ATP-MNPs show superior efficiency for multi-phosphopeptides isolation.
- Ga^{3+}-ATP-MNPs show great promise for the large-scale phosphoproteome study.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 11 May 2015
Received in revised form
16 October 2015
Accepted 20 October 2015
Available online 24 October 2015

Keywords:
Multi-phosphopeptide
Adenosine triphosphate
Immobilized metal ion affinity chromatography
Magnetic nanoparticles
Gallium ion

ABSTRACT

Despite recent advances in phosphoproteome research, detection and characterization of multi-phosphopeptides have remained a challenge. Here we present a novel IMAC strategy for effective extracting multi-phosphopeptides from complex samples, through Ga^{3+} chelation to the adenosine triphosphate (ATP)-functionalized magnetic nanoparticles (Ga^{3+}-ATP-MNPs). The high specificity of Ga^{3+}-ATP-MNPs was demonstrated by efficient enriching multi-phosphopeptides from the digest mixture of β-casein and BSA with molar ratio as low as 1:5000. Ga^{3+}-ATP-MNPs were also successfully applied for the phosphoproteome analysis of rat liver mitochondria, resulting in the identification of 193 phosphopeptides with 331 phosphorylation sites from 158 phosphoproteins. In other words, 54.4% of the phosphopeptides trapped by Ga^{3+}-ATP-MNPs were observed with more than one phosphorylated sites, resulting in significant improvement on the identification of peptides with multi-phosphorylated sites. The high specificity of Ga^{3+}-ATP-MNPs towards multi-phosphopeptides may be due to the synergistic effect of the strong hydrophilic surface functionalized by ATP and the proper chelating strength provided by Ga^{3+}. Moreover, the unique magnetic core of Ga^{3+}-ATP-MNPs also facilitates the isolation process and on-plate enrichment for direct MALDI MS analysis with limit of detection as low as 30 amol. This new affinity-based protocol is expected to provide a powerful approach for characterizing multiple phosphorylation sites on proteins in complex and dilute analytes, which may be explored as complementary technique for improving the coverage of phosphoproteome.

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http://dx.doi.org/10.1016/j.aca.2015.10.012
0003-2670/

1. Introduction

Phosphorylation, an important and ubiquitous protein modification, plays a key role in signal transduction, cell cycle, metabolisms and enzymatic regulation [1,2]. Characterization of phosphoproteins is vital for understanding these biological pathways. Mass spectrometry (MS) based techniques has emerged as a premier and powerful tool for phosphoproteomes analysis [3]. However, the low abundance, dynamic nature, and difficult ionization of phosphopeptides, make phosphoproteomes research a substantial challenge. Therefore, the enrichment and concentration of phosphopeptides is essential prior to MS analysis.

Numerous improvements have been developed to isolate phosphopeptides [4–6]. Among them, immobilized metal ion affinity chromatography (IMAC) [7–10] is one widely used approach, which relies on the specific affinity of metal ions for phosphate group, usually Ga^{3+} [10–12] and Fe^{3+} [13–16]. The metal ions are immobilized by the chelating compound, that is, in particular, iminodiacetic acid (IDA) [13,17] and nitrilotriacetic acid (NTA) [14,15]. To overcome the poor selectivity derived from carboxyl groups of IDA and NTA, phosphate group as the metal binding ligand [7,18–20] has been developed. Most recently, Adenosine triphosphate (ATP) [21] was smartly adopted as the functional group to synthesize a novel IMAC material (Ti^{4+}-ATP-MNPs), for the first time by which the phosphopeptides could be trapped from peptides mixture diluted by 5000-fold non-phosphopeptides, and as low as 3 amol phosphopeptides from β-casein digests could be detected by MALDI-TOF MS after on-target enrichment, enabling a great improvement on the selectivity and sensitivity.

Lately, multi-phosphopeptides have drawn more attention due to their importance in many biological processes and pathways, and extensive efforts have been made to facilitate the detection of multi-phosphopeptides [22–26]. However, rooting in their decreased retention on reversed-phase column material due to increased hydrophilicity and severe suppressed ionization efficiency by the coexistence of unphosphorylated and mono-phosphorylated peptides, multi-phosphopeptides are rather excluded from the enriched mixtures during normally MS analysis [27]. More research is urgently needed to explore new avenues that can separate multi-phosphopeptides from not only non-phosphorylated peptides, but also from monophosphopeptides.

Different metal ions have been reported to have distinctly different selectivity and enrichment ability towards multi- and monophosphopeptides. Such as Ti^{4+} ions [7,28,29], which showed preference for monophosphopeptides, while mixed Ni^{2+}/Zn^{2+} ions [30] and Nb^{5+} ions [29] were reported to have affinity towards peptides with higher levels of phosphorylation. Here, based on our previous work [15,21,31], gallium ions were employed to be immobilized on ATP functionalized magnetic nanoparticles (Ga^{3+}-ATP-MNPs). The Ga^{3+}-ATP-MNPs have a high hydrophilic surface rooting in the adenosine parts of ATP, providing a far more accessible surface for multi-phosphopeptides, compared with currently used materials. Moreover, the immobilized gallium ions provide suitable chelating strength for multi-phosphopeptides, which thus enable efficient recovery toward multiply phosphorylated peptides. The performance of synthesized Ga^{3+}-ATP-MNPs were evaluated with tryptic digests of standard protein, nonfat milk and rat liver mitochondria for the phosphoproteome analysis, and compared with Ti^{4+}-ATP-MNPs thoroughly. All the experimental results demonstrate that a highly efficient, sensitive and selective phosphopeptides isolation process can be achieved for single and, more importantly, multiple phosphopeptides with the method we report here.

2. Experimental section

2.1. Reagents and materials

Bovine serum albumin (BSA), β-Casein (≥90%), trypsin (bovine pancreas), trifluoroacetic acid (TFA), urea, 1,4-dithio-D,L-threitol (DTT), iodoacetamide (IAA), formic acid (FA), 2,5-dihydroxybenzoic acid (2,5-DHB), glutaraldehyde solution (50% in H_2O, m/m) were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade) was ordered from Merck (Darmstadt, Germany). POROS 20 MC beads were purchased from Applied Biosystems (Foster, CA, USA). TiO_2 beads (5 μm) was purchased from GL Sciences (Tokyo, Japan). Adenosine-5′-triphosphate disodium salt (ATP-Na_2) (≥99.9%) was obtained from AMRESO (Solon, OH, USA). $Ga(NO_3)_3$ and all other chemical reagents were analytical grade and used without further purification unless otherwise described. Water was purified by Milli-Q system (Millipore Inc. Milford, MA).

2.2. Apparatus

The scanning electron microscopy (SEM) images were obtained with JSM-6360 LV (JEOL, Tokyo, Japan). Transmission electron microscopy (TEM) was performed using an FEI Tecnai G^2 spirit microscope (FEI, Eindhoven, Holland). The immobilization of gallium ions on the surface of the magnetic nanoparticles was characterized by X-ray photoelectron spectroscopy (XPS) on a VG ESCALAB MKII spectrometer (Crawley, UK).

MALDI-TOF MS experiments were performed in reflector positive mode on Ultraflex III MALDI-TOF/TOF MS (Bruker Daltonics, Bremen, Germany) with a Smartbeam Nd-YAG laser at 355 nm, a repetition rate of 200 Hz, and an acceleration voltage of 20 kV.

2.3. Preparation of Ga^{3+}-ATP-MNPs

The Ga^{3+}-ATP-MNPs were synthesized according to our previous work [21], but with some modifications. Briefly, the magnetic nanoparticles were synthesized through solvothermal reaction using $FeCl_3 \cdot 6H_2O$ (1.0 g) as a single iron source and 1,6-hexadiamine (3.6 g) as the ligand. A transparent solution of 1,6-hexadiamine, anhydrous sodium (4.0 g) and $FeCl_3 \cdot 6H_2O$ in ethylene glycol (50 mL) was obtained by vigorously stirring. Then, the mixed reagents were heated at 200 °C for 6 h in a Teflon-lined stainless steel autoclave, and the obtained products were washed thoroughly with water and ethanol.

In the surface modification step, the amine group of the nanoparticles (0.1 g) was activated under gentle rotation by 5 mL of glutaraldehyde solution (20%, m/m) in citrate buffer (100 mM, pH 5.0) for 2 h. The product was washed with citrate buffer (1 mL) five times to remove the excess glutaraldehyde. Then, the magnetic nanoparticles were functionalized with ATP groups by 3 mL of ATP solution (0.2 g/mL) in citrate buffer (100 mM, pH 5.0) for 2 h and then washed with citrate buffer (1 mL) five times to remove the excess ATP. Finally, the gallium ions were immobilized by 10 mL of $Ga(NO_3)_3$ (100 mM, 0.1% (v/v) FA) for 2 h. The nanoparticles were washed thoroughly with 0.1% (v/v) FA and storage in 0.1% (v/v) FA. All of the modification processes were performed at room temperature.

2.4. Sample preparation

β-Casein (1 mg) was dissolved in 1 mL of ammonium bicarbonate (25 mM, pH 8.0) and digested at 37 °C for 12 h with a 1:50 enzyme-to-protein ratio (m/m). 1 mg BSA was dissolved and denatured in 0.1 mL of 8 M urea at 56 °C for 10 min, reduced with 20 μL of 100 mM DTT at 56 °C for 1 h, and alkylated with 20 μL of