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# Facile synthesis of $Fe_3O_4$@PDA core-shell microspheres functionalized with various metal ions: A systematic comparison of commonly-used metal ions for IMAC enrichment

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ABSTRACT

Metal ions differed greatly in affinity towards phosphopeptides, and thus it is essential to systematically compare the phosphopeptides enrichment ability of different metal ions usually used in the IMAC techniques. In this work, for the first time, eight metal ions, including $Nb^{5+}$, $Ti^{4+}$, $Zr^{4+}$, $Ga^{3+}$, $Y^{3+}$, $In^{3+}$, $Ce^{4+}$, $Fe^{3+}$, were immobilized on the polydopamine (PDA)-coated $Fe_3O_4$ (denoted as $Fe_3O_4$@PDA-$M^{n+}$), and systematically compared by the real biosamples, in addition to standard phosphopeptides. $Fe_3O_4$ microspheres were synthesized via the solvothermal reaction, followed by self-polymerization of dopamine on the surface. Then through taking advantage of the hydroxyl and amino group of PDA, the eight metal ions were easily adhered to the surface of $Fe_3O_4$@PDA. After characterization, the resultant $Fe_3O_4$@PDA-$M^{n+}$ microspheres were applied to phosphopeptides enrichment based on the binding affinity between metal ions and phosphopeptides. According to the results, different metal ions presented diverse phosphopeptides enrichment efficiency in terms of selectivity, sensitivity and the enrichment ability from real complex samples, and $Fe_3O_4$@PDA-$Nb^{5+}$ and $Fe_3O_4$@PDA-$Ti^{4+}$ showed obvious advantages of the phosphopeptides enrichment effect after the comparison. This systematic comparison may provide certain reference for the use and development of IMAC materials in the future.

## 1. Introduction

Reversible phosphorylation of proteins is one of the most important post translational modifications (PTM) involved in regulation of cell division, growth, migration, differentiation, and intercellular [1,2], which is important for transmitting regulatory signals in all living cells [3], and its deregulation unsurprisingly could be implicated in cancer [4,5] or any other diseases. Therefore, characterization and quantification of protein phosphorylation status and changes may greatly improve the understanding of mechanisms of the diseases. In the past decades, mass spectrometry (MS) has become a prior method of choice for phosphoproteomics analysis [6,7]. However, considering the highly dynamic nature, low ionization efficiency and the signal suppression of non-phosphopeptides, it remains a major challenge to detect phosphorylated peptides by MS directly. Thus, specific and selective phosphopeptides enrichment strategies prior to MS analysis are both challenging and essential.

A variety of strategies have been developed for enrichment of phosphopeptides so far. These selective methods can be mainly classified into two categories: (1) immobilized metal ion affinity chromatography (IMAC), and (2) metal oxide affinity chromatography (MOAC). These two techniques for phosphopeptides enrichment are basically based on the affinity of metal ions/metal oxide with the phosphate group. For now, various metal ions have been used in IMAC materials, such as $Ti^{4+}$, $Zr^{4+}$, $Ga^{3+}$, $Fe^{3+}$ and so on [8–19]. Researches about different IMAC materials applied to phosphopeptides enrichment were also frequently reported in recent years. Generally, a part of the studies focus on exploring new metal ions [20–22], while some of others devoted continuously to the innovation of the structure or composition of the whole materials to improve the enrichment efficiency, involving different chelates, as well as various carriers [12,16,23–33].

To date, various metal ions have been applied in enrichment of phosphopeptides and gained distinctly different enrichment effects [9,10,34–37]. For example, Feng et al. compared $Zr^{4+}$-IMAC with $Fe^{3+}$-IMAC and the results indicated that $Zr^{4+}$-IMAC had much higher specificity and selectivity for phosphopeptides enrichment than $Fe^{3+}$-

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IMAC [37]. Also, $Ga^{3+}$ was applied to enrich phosphopeptides from peptide mixture and reported to have better performance of capturing phosphopeptides than the commonly used $Fe^{3+}$ ion in IMAC [9]. The group of Zou reported that $Ti^{4+}$-IMAC offered distinct advantages over $Fe^{3+}$-IMAC and $Zr^{4+}$-IMAC in terms of the selective enrichment and effective isolation of phosphopeptides from the same tryptic digest samples [10,35]. In addition, metal ions may exhibit different bias affinity towards monophosphorylated or multiply phosphorylated peptides due to different binding affinity and coordination geometries. Zhou et al. developed several strategies including $Ti^{4+}$-IMAC, $Fe^{3+}$-IMAC, $Zr^{4+}$-IMAC, $TiO_2$ and $ZrO_2$ for enrichment of phosphopeptides and found that $Zr^{4+}$ preferred to enrich multiphosphopeptides, while $Ti^{4+}$ tended to enrich monophosphopeptides [35]. In our previous work, $Nb^{5+}$ and $Ti^{4+}$ were investigated in phosphopeptides enrichment, and $Nb^{5+}$ was demonstrated to have better enrichment efficiency for multiphosphopeptides than $Ti^{4+}$ [21]. The above research results suggest that metal ions vary widely in affinity towards phosphopeptides and preferential isolation of multi- or mono-phosphopeptides. Though some reports have shown the existing significant different affinity for different metal ions towards phosphopeptides with the analysis of only two or three metal ions [9,10,34–37], few studies have been reported to be committed to systematically comparing the phosphopeptides enrichment ability of various metal ions usually used in the IMAC techniques. Therefore, a systematic comparison of commonly-used metal ions in IMAC enrichment were made for the first time in this study.

Polydopamine has many advantages of simple synthesis approach, high hydrophilicity and good biocompatibility, resulting in increased attention in recent years. As abundant catechol and amine groups in PDA layer could adhere to metal ions through secondary reaction, polydopamine has been utilized as chelating agents for phosphoproteomics research [20–22]. Previously, $Nb^{5+}$ and $Ti^{4+}$ have been successfully immobilized on PDA layer of $Fe_3O_4$@PDA composites in our group [21]. Herein, in addition to $Nb^{5+}$ and $Ti^{4+}$, other six metal ions which are mostly used in IMAC materials, including $Zr^{4+}$, $Ga^{3+}$, $Y^{3+}$, $In^{3+}$, $Ce^{4+}$, $Fe^{3+}$, were also immobilized on $Fe_3O_4$@PDA composites. Furthermore, we compared the phosphopeptides enrichment efficiency of these eight metal ions from the point of view of selectivity, sensitivity, as well as the real bio-samples, which may provide certain reference for the use and development of IMAC materials in the future.

## 2. Experimental

### 2.1. Materials and chemicals

Bovine β-casein, bovine serum albumin (BSA), trifluoroacetic acid (TFA), L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK) treated trypsin (from bovine pancreas), ammonium bicarbonate ($NH_4HCO_3$) and 2, 5-dihydroxybenzoic acid (DHB) were acquired from Sigma Chemical (St. Louis, MO). Acetonitrile and DTT were purchased from Merck (Darmstadt, Germany). Iodoacetaamide (IAA) was purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). Iron chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ethanol, ethylene glycol and sodium acetate were obtained from Shanghai Chemical Corporation. Dopamine hydrochloride was purchased from Aladdin Chemistry Co. Ltd. Niobium (V) oxalate hydrate, titanium sulfate, yttrium chloride, nitrate indium, gallium chloride, cerium sulfate and zirconium hydroxide were bought from Alfa Aesar. Nonfat milk was purchased from a local shop. Deionized water used in all experiments was purified using Milli-Q water by Milli-Q system (Millipore, Bedford, MA). All other chemicals and reagents were of the highest grade commercially available.

### 2.2. Synthesis of different metal ions of IMAC materials

Magnetic particles were synthesized through a solvothermal reaction via previous methods [33]. Briefly, 1.35 g of $FeCl_3{\cdot}6H_2O$ was dissolved in 75 mL of ethylene glycol, followed by adding 3.6 g of sodium acetate. The mixture kept stirring at room temperature to form a uniform solution and then shifted to a 200 mL Teflon-lined stainless-steel autoclave. The autoclave kept under 200 °C for 16 h. After cooling to room temperature, the produced magnetic particles were rinsed several times with deionized water and then dried in vacuum.

According to a reported procedure [16], modification of $Fe_3O_4$ magnetic particles with polydopamine was achieved as follows: 10 mg of the obtained magnetic particles was firstly dispersed in 10 mL of Tris buffer (10 mM, pH 8.5) with an addition of 20 mL ethanol. Subsequently, under continuous stirring, 15 mL aqueous solution containing 40 mg of dopamine hydrochloride was added into the resulting suspension, and the mixture kept stirring at room temperature for 12 h. The obtained product was cleaned with deionized water several times and dried in vacuum. For the preparation of $Fe_3O_4$@PDA-$M^{n+}$, 50 mg of the obtained polydopamine-coated magnetic particles were mixed under ultrasonication with 250 mL of 50 mM metallic salt solutions (Niobium (V) oxalate hydrate, $Ti(SO_4)_2$, $FeCl_3$, $In(NO_3)_3$, $YCl_3$, $ZrOCl_4$, $GaCl_3$, $Ce(SO_4)_2$), respectively. In order to make the metal ions immobilize on the PDA shell firmly, the mixed dispersion was stirred mechanically for 4 h. The $Fe_3O_4$@PDA-$M^{n+}$ was finally collected by a magnet, washed thoroughly with deionized water for 5 times and ethanol for 3 times, and then were dried in vacuum at 50 °C for 20 h.

### 2.3. Instruments

The morphological characteristics of $Fe_3O_4$@PDA-$M^{n+}$ were obtained by transmission electron microscope (TEM) and energy dispersive X-ray (EDX). TEM images were recorded on a JEOL 2011 microscope (Japan) at 200 kV, and EDX analysis were from a Philips XL30 electron microscope (Netherlands) at 20 kV.

### 2.4. Sample preparation

A certain amount of BSA was dissolved in $NH_4HCO_3$ buffer (25 mM, pH 8.3), followed by addition of DTT to reduce the disulfide bonds of proteins and IAA to alkylate the protein. Then, the obtained solution was diluted with $NH_4HCO_3$ buffer and treated with trypsin (2%, w/w) at 37 °C for 16 h for digestion. For bovine β-casein, $NH_4HCO_3$ buffer (25 mM) was added, and then digested with trypsin (2%, w/w) at 37 °C for 16 h. Nonfat milk was exchanged into $NH_4HCO_3$ (25 mM) and then centrifuged at 16,000 rpm for 15 min. The supernatant was collected, denatured at 100 °C for 15 min, and incubated with trypsin for 16 h at 37 °C to produce proteolytic digests for further phosphopeptides enrichment. The above obtained tryptic digests were preserved at −80 °C until further use.

### 2.5. Enrichment of phosphopeptides from tryptic digestion of standard proteins

The resultant $Fe_3O_4$@PDA-$M^{n+}$ was dispersed by sonication in deionized water at a concentration of 2 mg/mL, then, 10 μL of the suspension was mixed with 200 μL peptide mixture and vibrated at 25 °C for 30 min. Subsequently, the phosphopeptide-loaded $Fe_3O_4$@PDA-$M^{n+}$ microspheres were collected through magnetic separation and washed three times with 200 μL of the loading buffer (50% ACN containing 0.1% TFA) to remove the non-specifically adsorbed peptides. The captured phosphopeptides were eluted with $NH_3{\cdot}H_2O$ (5 μL, 0.4 M) at 25 °C for 10 min and the eluent was analyzed by MALDI-TOF MS.

### 2.6. Selective enrichment of phosphopeptides from tryptic digests of nonfat milk

$Fe_3O_4$@PDA-$M^{n+}$ composites (200 μg) were added into the mixture solution containing 200 μL loading buffer and 2 μL milk tryptic digest.