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Preparation of a TiO_2 nanoparticle-deposited capillary column by liquid phase deposition and its application in phosphopeptide analysis

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ABSTRACT

Analysis of phosphopeptides from complex mixtures derived from proteolytic digestion of biological samples is a challenging yet highly important task. Since phosphopeptides are usually present in small amounts, enrichment is often necessary prior to their characterization by mass spectrometry. In this study, a thin layer of titanium dioxide (TiO_2) nanoparticles (NPs) was deposited onto the surface of capillary column by liquid phase deposition (LPD) technique and applied to selectively concentrate phosphopeptides from protein digest products. This is, to our knowledge, the first demonstration of using liquid phase deposition to construct in-tube solid phase microextraction devices for biological analysis. By coupling the device off-line or on-line with mass spectrometry analysis, experiments for systematic optimization of loading and washing conditions were carried out, and good trapping selectivity of TiO_2 NP-deposited capillary columns towards phosphopeptides was demonstrated.

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1. Introduction

Protein phosphorylation is an important protein post-translational modification (PTM) and plays a crucial role on various essential cell functions including signal transduction, metabolic maintenance and cell division [1–3]. In order to understand how these cellular processes are controlled by protein phosphorylation, it is essential to determine the sites of protein phosphorylation. Current mass spectrometry (MS)-based phosphorylation site analysis strategies [4–9], although successful in many cases, still possess difficulty. Due to the low abundance and low ionization efficiency nature of phosphopeptides, a phosphopeptide enrichment sample preparation process is a prerequisite for successful MS analysis. To be a good phosphopeptide enrichment technique, the method needs not only to be highly selective, but also to be applicable to samples with limited quantity. In other words, the method needs to be easy to perform, with high recovery rate, and cost effective.

Commonly used phosphopeptide enrichment methods can be separated into two main categories based on their enrichment principles: chemical derivatization-based methods and affinity purification-based methods [10–17]. Chemical derivatization methods generally involve multiple chemical reaction steps, and are difficult to apply to complex yet limited protein samples due to the concerns of sample loss [18–21]. Affinity-based enrichment methods include antibody-based affinity chromatography, strong cation and/or anion exchange chromatography, immobilized metal ion affinity chromatography (IMAC), metal oxide chromatography, etc. Affinity-based enrichment techniques are the most widely adopted methods at the moment.

In recent years, a titanium dioxide (TiO_2)-based method has been developed for enriching phosphopeptides from complex samples [22–27]. Analytes possessing phosphate functional groups can self-assemble onto the surface of TiO_2 particles and elution of the bound phosphopeptides can be achieved easily at an alkaline pH. Good compatibility to reversed-phase chromatography and less non-specific binding turn this method into a very interesting alternative to IMAC-based procedures. However, many challenges remain for on-line use of TiO_2-based materials with MS detection. Microsized TiO_2 particles (5-μm Titanspheres) are commonly used to pack capillary columns to realize on-line coupling with ESI-MS, but the packing and frit preparation are difficult and troublesome for many laboratories [23,27]. Nanosized TiO_2 particles were reported to have higher specific surface area and, hence, potentially higher trapping capacities toward phosphopeptides compared with microsized TiO_2 particles [28,29]. However, on-line application of TiO_2 nanoparticles with MS has not yet been realized due to the lack of appropriate frits to sustain these materials in chromatographic columns, and therefore limited their use in handling a relatively large amount of proteins. Furthermore, the

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reported preparation of nanoparticles is usually complex and time-consuming.

First reported by Kawahara et al. in 1980s [30,31], liquid phase deposition (LPD) method is a low-cost, environment-friendly process for thin film preparation. LPD refers to the formation of oxide thin films from an aqueous solution of a metal-fluoro complex which is slowly hydrolyzed by adding water, boric acid (H_3BO_3) or aluminum metal. The addition of water directly forces precipitation of the oxide, and boric acid (H_3BO_3) or aluminum metal acts as a fluoride scavenger, destabilizing the fluoro complex and promoting precipitation of the oxide. LPD was first developed for depositing SiO_2 thin films [32], and was later used to prepare other metal oxide films, such as TiO_2, tin oxide, zirconia or a variety of 3d transition metal oxides (V, Cr, Mn, Fe, Co, Ni, Cu, Zn, In, individually or combined) [33–38]. The chemical and physical properties of obtained metal oxide films can be tuned by several experimental parameters, such as pH and concentration for the precursor solution, deposition time and calcination temperature. LPD is very simple to perform and can be easily applied to various kinds of substrates with large surface area or complex morphology.

In the present work, we deposited a thin TiO_2 layer onto the inner surface of capillary column using the mixture of $(NH_4)_2TiF_6$ and boric acid by LPD method. The physical effects of coating cycles, capillary diameter and calcination on the nanostructure of obtained films were characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD) and X-ray photoelectron spectroscopy (XPS). This LPD-based approach is easier to perform and much more cost effective than the previously reported TiO_2 coating method based on sol–gel process [39–42]. After liquid deposition of TiO_2 films in capillaries, we examined the feasibility of using such capillaries as in-tube solid phase miroextraction (SPME) devices to selectively enrich phosphopeptides from proteolytic digest prior to MS detection. Using LPD technique for the construction of in-tube SPME devices that are compatible for on-line LC–ESI-MS usage, this should open up a new avenue for enriching and analyzing complex biological samples.

2. Experimental

2.1. Chemicals and materials

Fused-silica capillaries (365 μm o.d., 50 μm i.d. or 365 μm o.d., 75 μm i.d.) were purchased from Yongnian Optic Fiber Plant (Hebei, China). HPLC grade acetonitrile was obtained from Fisher Scientific (USA). Ammonium hexfluorotitanate ($(NH_4)_2TiF_6$), boric acid (H_3BO_3) and other chemicals were of analytical reagent grade and supplied by Shanghai General Chemical Reagent Factory (Shanghai, China). α-Casein, β-casein and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). A synthetic peptide from tryptic EGFR with phosphorylation site at amino acid residue Y1173 (GSTAENAEY(1173)LR, MW 1290), and the corresponding ^{15}N-labeled peptide (MW 1294) that incorporated ^{15}N-Gly, ^{15}N-Ala and ^{15}N-Leu were kindly provided by L.H. Ericsson (Seattle, Washington, USA) [47]. Trypsin was of sequencing grade and obtained from Promega (Madison, WI, USA). Purified water was obtained with a Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.2. Preparation and characterization of TiO_2 NP-deposited capillary columns

Fused-silica capillaries were activated by 1 mol/L NaOH and then 1 mol/L HCl. After rinsing with double distilled water, they were dried at 160 °C under N_2 flow for 10 h.

Mixture of equal volumes of 0.2 mol/L $(NH_4)_2TiF_6$ and 0.6 mol/L H_3BO_3 was stirred and used as the precursor solution. The activated capillary was filled with the precursor solution, sealed at both ends with silicone rubber and then incubated in a thermostat water bath controlled at 35 ± 1 °C for 16 h for LPD. After deposition, the capillary was washed with distilled water and dried at 120 °C for 4 h under constant N_2 flow. Fluorin residues in the TiO_2 film from the precursor solution were removed by washing the capillary with 0.1 mol/L NaOH and distilled water in sequence. The calcination of TiO_2 NP-deposited capillary column was performed by heating at a rate of 1 °C/min to 300 °C and holding for 2 h to age the TiO_2 film and increase cross-linking of the inorganic framework.

The morphology of the capillary inner surface was displayed by QUANTA-200 SEM (FEI, The Netherlands). In order to further characterize the TiO_2 film, LPD was also carried out on a quartz plate in a similar way to that performed in capillaries. The existence of TiO_2 film and its composition were determined by XSAM800 X-ray photoelectron spectroscopy (XPS, Kratos, UK), with Mg Kα radiation as the exciting source. The crystal structure of TiO_2 film was determined with a XRD-6000 X-ray diffractometer (Shimadzu, Japan) using Cu Kα radiation and a rotating anode operated at 40 kV and 30 mA.

2.3. Sample preparation

α-Casein and β-casein were originally made up into stock solutions of 1 mg/mL using distilled water. Proteins were digested using trypsin at an enzyme to substrate ratio of 1:50 (w/w) in 2 mol/L urea, 100 mmol/L Tris pH 8.5 overnight at 37 °C; the digest mixtures were then stored at −20 °C without further treatment.

BSA (4 mg) was dissolved in 1 mL denaturing buffer solution containing 8 mol/L urea in 50 mmol/L ammonium bicarbonate. The obtained protein solution was mixed with 20 μL of 50 mmol/L dithiothreitol (DTT) and incubated for 15 min at 65 °C to reduce protein disulfide bonding. 40 μL of 50 mmol/L iodoacetamide (IAA) was added, and the obtained solution was incubated for an additional 30 min at room temperature in dark. The above reduced and alkylated protein mixture was diluted 10-fold with 50 mmol/L ammonium bicarbonate and incubated overnight at 37 °C with trypsin at an enzyme to substrate ratio of 1:50 (w/w) to produce proteolytic digest mixture.

In order to control the pH value and ionic strength more precisely during the optimization of loading/washing/eluting conditions, we chose to use desalted tryptic digest mixture as experimental material. Desalting was achieved using C18 ZipTip pipette tips (Millipore, Billerica, MA) and the bound peptides were eluted by 50% acetonitrile/0.1% trifluoroacetic acid solution. Desalted digest mixtures were dried by speed-vac centrifugation, and were subsequently brought up in different concentrations of acetonitrile and acetic acid solution as specified in the text.

2.4. Enrichment of phosphorylated peptides using TiO_2 NP-deposited capillary columns

The extraction of tryptic digest was performed using a simple laboratory-made system [46], which is a combination of two microflow pumps (pump A and B), a six-port valve switching module (Unimicro Technologies, Shanghai, China) and a stainless steel sample loop (50 μL) (see Fig. 1). The peptides were first loaded onto the column in the solution of 0.1% (v/v) acetic acid/acetonitrile (1:9, v/v) at a flow rate of 20 μL/min by pump A. After washing with the same solution for 2.5 min at 20 μL/min, the six-port valve B was switched and the bound peptides were eluted with the mixture of 0.3% (v/v) ammonium hydroxide/acetonitrile (5:5, v/v) at 20 μL/min for 0.5 min by pump B.

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