

Video Article

Preparation of Highly Porous Coordination Polymer Coatings on Macroporous Polymer Monoliths for Enhanced Enrichment of Phosphopeptides

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Abstract

We describe a protocol for the preparation of hybrid materials based on highly porous coordination polymer coatings on the internal surface of macroporous polymer monoliths. The developed approach is based on the preparation of a macroporous polymer containing carboxylic acid functional groups and the subsequent step-by-step solution-based controlled growth of a layer of a porous coordination polymer on the surface of the pores of the polymer monolith. The prepared metal-organic polymer hybrid has a high specific micropore surface area. The amount of iron(III) sites is enhanced through metal-organic coordination on the surface of the pores of the functional polymer support. The increase of metal sites is related to the number of iterations of the coating process.

The developed preparation scheme is easily adapted to a capillary column format. The functional porous polymer is prepared as a self-contained single-block porous monolith within the capillary, yielding a flow-through separation device with excellent flow permeability and modest back-pressure. The metal-organic polymer hybrid column showed excellent performance for the enrichment of phosphopeptides from digested proteins and their subsequent detection using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The presented experimental protocol is highly versatile, and can be easily implemented to different organic polymer supports and coatings with a plethora of porous coordination polymers and metal-organic frameworks for multiple purification and/or separation applications.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52926/>

Introduction

Porous coordination polymers (PCPs) are coordination compounds based on metal centers linked by organic ligands with repeating coordination entities extending in 1, 2 or 3 dimensions that can be amorphous or crystalline¹⁻³. In recent years, this class of porous materials has attracted widespread attention due to their high porosity, wide chemical tunability, and their stability. PCPs have been explored for a range of applications including gas storage, gas separation, and catalysis³⁻⁶, and very recently, the first analytical applications of PCPs have been described⁷.

Because of their enhanced chemical functionality and high porosity PCPs have been targeted for their huge potential for the improvement of purification processes and chromatographic separations, and a number of reports concerning this topic have been published⁷⁻¹³. However, the performance of PCPs is not currently at an equivalent level with existing chromatographic materials likely due to fast diffusion through large interparticle voids in packed beds of these solids due to their typically irregularly shaped morphologies of their particles or crystals. This irregularly distributed packing leads to a lower than expected performance, as well as high column backpressures and undesirable peak shape morphologies^{14,15}.

In order to solve the problem of fast diffusion through the inter-particle voids and concomitantly enhance the performance of PCPs for analytical applications, the development of a hybrid material based on a macroporous polymer monolith¹⁶ that contains the PCP on the surface of the macropores would be desirable. Polymer monoliths are self-contained, single-piece materials that can sustain convective flow through their pores, which makes them one of the most efficient alternatives to bead packings and have been successfully commercialized by several companies^{17,18}. Porous polymer monoliths are usually based on the polymerization of a monomer and a crosslinker in the presence of porogens, which are typically binary mixtures of organic solvents. The obtained monolithic materials have a microglobular structure and a high porosity and flow permeability.

A simple approach to unify these materials to prepare a polymer monolith containing a PCP is based on the direct addition of as-synthesized PCPs in the polymerization mixture of the monolith. This approach resulted in PCPs mostly buried within a polymer scaffold, and not being active

for the further application of the final material^{14,15}. A different synthetic approach is clearly needed in order to, for example, develop uniform films of PCPs, or crystalline metal-organic frameworks (MOFs) where the majority of the pores contained within the crystal are accessible from the macropores of the polymer monolith.

Herein we report a simple protocol for the preparation of a metal-organic polymer hybrid material (MOPH) based on a macroporous polymer support with suitable functional groups for the attachment of PCPs, which can be easily implemented as a self-contained single-piece polymer monolith in a column format with optimum properties for flow-through applications. The polymer synthesis procedure is followed by a simple room temperature solution-based method to grow a PCP coating on the internal surface of the pores of the monolith^{19,20}. As the first example, we describe the preparation of an iron(III) benzenetricarboxylate (FeBTC) coordination polymer film within a macroporous poly(styrene-divinylbenzene-methacrylic acid) monolith. This method is effective for the preparation of bulk powders as well as capillary columns and the described protocol is readily implementable to other PCPs. As an example of the potential of MOPHs as functional materials for flow-through applications, we applied the developed FeBTC MOPH which contains a dense coating of Fe(III) centers to enrich phosphopeptides from digested protein mixtures exploiting the binding affinity of phosphopeptides to Fe(III). The developed protocol²¹ comprises three main parts: Preparation of the macroporous organic polymer monolith support; growth of the PCP coating on the surface of the pores of the monolith; application for the enrichment of phosphopeptides.

Protocol

NOTE: Before beginning, check all relevant material data sheets (MSDSs). Several of the chemicals used in the synthetic and application procedures are toxic. Please follow all appropriate safety practices and use adequate protective equipment (lab coat, full-length pants, closed-toe shoes, safety glasses, gloves). Please use all cryogenic personal protective equipment when handling liquid nitrogen for the nitrogen adsorption measurements (insulated gloves, face shield).

1. Porous Polymer Monolith Preparation in Bulk and Capillary Column Format

1. Bulk Polymer Monolith for Characterization

1. Purify styrene, divinylbenzene and methacrylic acid through a column of basic alumina, in order to remove the polymerization inhibitors. Place 10 g of basic alumina in a 25 ml disposable plastic syringe with a plug of glass wool fiber packed in the syringe tip. Percolate approximately 10 ml of the monomer through the column.
2. Load the monomers (50 mg styrene, 100 mg divinylbenzene and 50 mg methacrylic acid) and the pore forming agents (300 mg toluene and 300 mg isooctane) in a 1 ml glass vial. Add the initiator of the polymerization, 4 mg of 2,2'-azobisisobutyronitrile (AIBN, 1% with respect to monomers).
3. Homogenize by sonication for 10 min. Remove dissolved oxygen by bubbling nitrogen through the liquid for 10 min. Seal the vial cap with paraffin film and place it in a water bath at 60 °C for 6 hr to polymerize the mixture.
4. Cool to room temperature and break the vial carefully. Transfer the polymer monolith into a cellulose extraction thimble. Place the extraction thimble into a Soxhlet extraction chamber and assemble it to a round bottom flask that contains a volume of methanol, which is at least three times the volume of the extraction chamber. Assemble a condenser to the upper part of the extraction chamber. Perform Soxhlet extraction by boiling the methanol for 16 hr, ensuring the complete removal of the unreacted monomers and pore forming agents.
5. Dry overnight in a vacuum oven at 60 °C. Confirm the presence of carboxylic functional groups to attach the PCP by Fourier Transform Infrared Spectroscopy (FT-IR). Measure surface area by nitrogen adsorption porosimetry.

2. Functionalization of Silica Capillaries for the Preparation of Monolithic Columns

1. Cut 2 m of a polyimide-coated 100 µm i.d. fused silica capillary. Connect it to a 0.25-0.50 ml glass syringe and wash the capillary with acetone. Remove the acetone by rinsing the capillary with water.
2. In order to activate the internal silica coating of the capillary, use a syringe pump to flow a 0.2 M aqueous NaOH solution at 0.25 µl/min for 30 min. Rinse with water until the effluent is neutral.
3. Use pH paper strips to check effluent pH. In order to protonate the silanol groups of the capillary, pump a 0.2 M aqueous HCl solution through the capillary at 0.25 µl/min for 30 min. Rinse with water until the effluent is neutral. Rinse with ethanol.
4. Pump a 20% (w/w) ethanol solution of 3-(trimethoxysilyl)propyl methacrylate (pH 5 adjusted with acetic acid) at 0.25 µl/min for 1 hr. In this step, the silica capillary is functionalized with vinyl groups in order to attach the polymer monolith to the capillary inner surface.
5. Rinse with acetone, dry in a nitrogen stream and leave at room temperature overnight before use. Cut the capillary into shorter pieces of length 20 cm.

3. Preparation of Monolithic Capillary Columns

1. Prepare an identical polymerization mixture as for the bulk polymer monolith (section 1.1) in a 1 ml glass vial with a rubber septum. Add initiator 1% AIBN with respect to monomers. Homogenize by sonication for 10 min.
2. Purge the polymerization mixture with nitrogen by coupling a non-functionalized silica capillary to a nitrogen stream.
 1. Insert the nitrogen stream capillary through the rubber septum of the vial and immerse it into the polymerization mixture so that the nitrogen bubbles through the liquid. Leave the vial cap slightly loose to avoid overpressure. Purge for 10 min.
 2. Lift the nitrogen stream capillary from the polymerization mixture to the headspace of the vial, and close tightly the cap. Insert a functionalized capillary through the septum into the polymerization mixture. The excess of pressure generated into the capillary through the nitrogen injected into the headspace pumps the polymerization mixture through the functionalized capillary.
 3. Collect several drops of polymerization mixture from the effluent of the capillary to ensure that it is completely filled and close it with a rubber septum. Take the capillary out of the vial very carefully and close the inlet of the capillary with a rubber septum.

3. Polymerize the mixture contained in the capillary in a water bath at 60 °C for 6 hr. Cool at room temperature and cut a few millimeters of both ends of the capillary. Remove unreacted monomers and pore forming agents by flushing the column with acetonitrile using an HPLC pump at 3 µl/min for 30 min. Check backpressure of the capillary column.

2. Growth of the Iron-benzenetricarboxylate (FeBTC) PCP

1. Growth of the FeBTC MOPH on a Bulk Polymer Monolith for Characterization
 1. Grind the previously dried monolith using a mortar and pestle.
 2. Immerse 100 mg of the monolith powder in 5 ml of 2 mM FeCl₃·6H₂O in ethanol for 15 min. Vacuum filter using a nylon filter (0.22 µm) and wash the powder with ethanol. Immerse the monolith powder in 5 ml of 2 mM 1,3,5-benzenetricarboxylic acid (BTC) in ethanol for 15 min. Vacuum filter using a nylon filter (0.22 µm) and wash the powder with ethanol.
 3. Repeat step number 2 as desired. The growth of the final metal-organic coating will be defined by the number of applied cycles. Typically, between 10 and 30 cycles are performed. Confirm the presence of new pores by nitrogen adsorption porosimetry. Measure the amount of additional metal sites by thermogravimetric analysis (TGA).
2. Growth of the FeBTC MOPH on a capillary monolithic column for the enrichment of phosphopeptides
 1. Using a syringe pump. Flush the capillary monolith with 2 mM FeCl₃·6H₂O in ethanol for 15 min at 2 µl/min. Wash with ethanol for 15 min at 2 µl/min. Flush the capillary monolith with a 2 mM BTC in ethanol for 15 min at 2 µl/min. Wash with ethanol for 15 min at 2 µl/min.
 2. Repeat step 1 as desired. The growth of the final metal-organic coating will be defined by the number of cycles performed.

3. Protein Digestion and Enrichment of Phosphopeptides

1. Protein Digestion
 1. Dissolve 0.5 ml of non-fat milk in 1 ml of water and divide it into 200 µl fractions.
 2. For the protein digestion add 160 µl 1 M ammonium bicarbonate and 50 µl 45 mM dithiothreitol to each fraction, in order to cleave the disulfide bonds. Incubate at 50 °C in a thermomixer for 15 min.
 3. Add gradually 50 µl of an aqueous solution of iodoacetamide 100 mM, while the solution cooled down to room temperature. Iodoacetamide will prevent the formation of new disulfide bonds.
 4. Incubate in the dark for 15 min at room temperature. Add 1 ml deionized water. Add 2 µg trypsin and digest proteins in a thermomixer at 37 °C for 14 hr.
 5. Terminate digestion by acidification with 10 µl of 1% trifluoroacetic acid, and placing it in the thermomixer for 5 min at room temperature. Store the digested proteins at -20 °C.
2. Enrichment of phosphopeptides using a capillary MOPH column.
 1. Flush the column with 100 µl of a 4:1 mixture of acetonitrile containing a 0.1% trifluoroacetic acid for 10 min at a flow rate of 1 µl/min. Pump the protein digestion through the column at 2 µl/min for 30 min.
 2. Wash out the non-phosphorylated peptides again with a 4:1 mixture of acetonitrile containing a 0.1% trifluoroacetic acid for 10 min at a flow rate of 1 µl/min. Wash with water for 10 min at a flow rate of 1 µl/min.
 3. Elute phosphopeptides using a 250 mM pH 7 phosphate buffer solution pumped at 1 µl/min for 15 min. Collect the eluent in a vial and desalt the solution using a standard protocol¹⁹. Prepare a 2 mg/ml 2,5-dihydroxybenzoic acid to use it as the matrix for the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Drawn 2 µl of the 2,5-dihydroxybenzoic acid into the tip to elute the phosphopeptides and spot them directly on to the MALDI plate.
 4. Analyze the spots by MALDI-TOF-MS and regenerate the column by flushing thoroughly with water and then methanol.

Representative Results

A schematic illustration of the PCP growth on the pore surface of the organic polymer monolith is shown in **Figure 1**. In this figure, we illustrate the initial Fe(III) atoms retained on the pore surface of the original polymer monolith coordinated to carboxylic functional groups. Using the protocol described herein additional organic ligand and Fe(III) ions are added to the surface, shaping a porous coordination network within the polymer monolith. **Figure 1** also shows schematically the use of the prepared capillary MOPH column as flow-through support for the enrichment of phosphopeptides. Surface area and pore distribution measurements, a scanning electron microscopy image (SEM), FT-IR and TGA were collected for the prepared materials (**Figure 2**). These characterization experiments provided valuable information about the appearance of new pores after the growth of the FeBTC PCP (**Figure 2A**). The morphology of the material after modification with the FeBTC PCP is shown in **Figure 2B**. Based on crystallographic simulation, the thickness of the each individual MOF layer is estimated to be 3 and 5 Å, depending on the orientation of the growing crystal. FT-IR spectra demonstrate the presence of functional groups in the as-synthesized polymer monolith and its modified counterparts with different numbers of FeBTC cycles (**Figure 2C**). TGA shows the thermal stability and the increase of metal sites (**Figure 2D**) obtained after modification of the original polymer monolith. The residue at 600 °C is α-Fe₂O₃, as confirmed by powder X-ray diffraction. The presence of iron in the capillary column format is detected by energy dispersive X-ray spectroscopy²¹. **Figure 3** shows an example of a real sample application of the developed MOPH material for the enrichment of phosphopeptides from a digestion of nonfat milk.

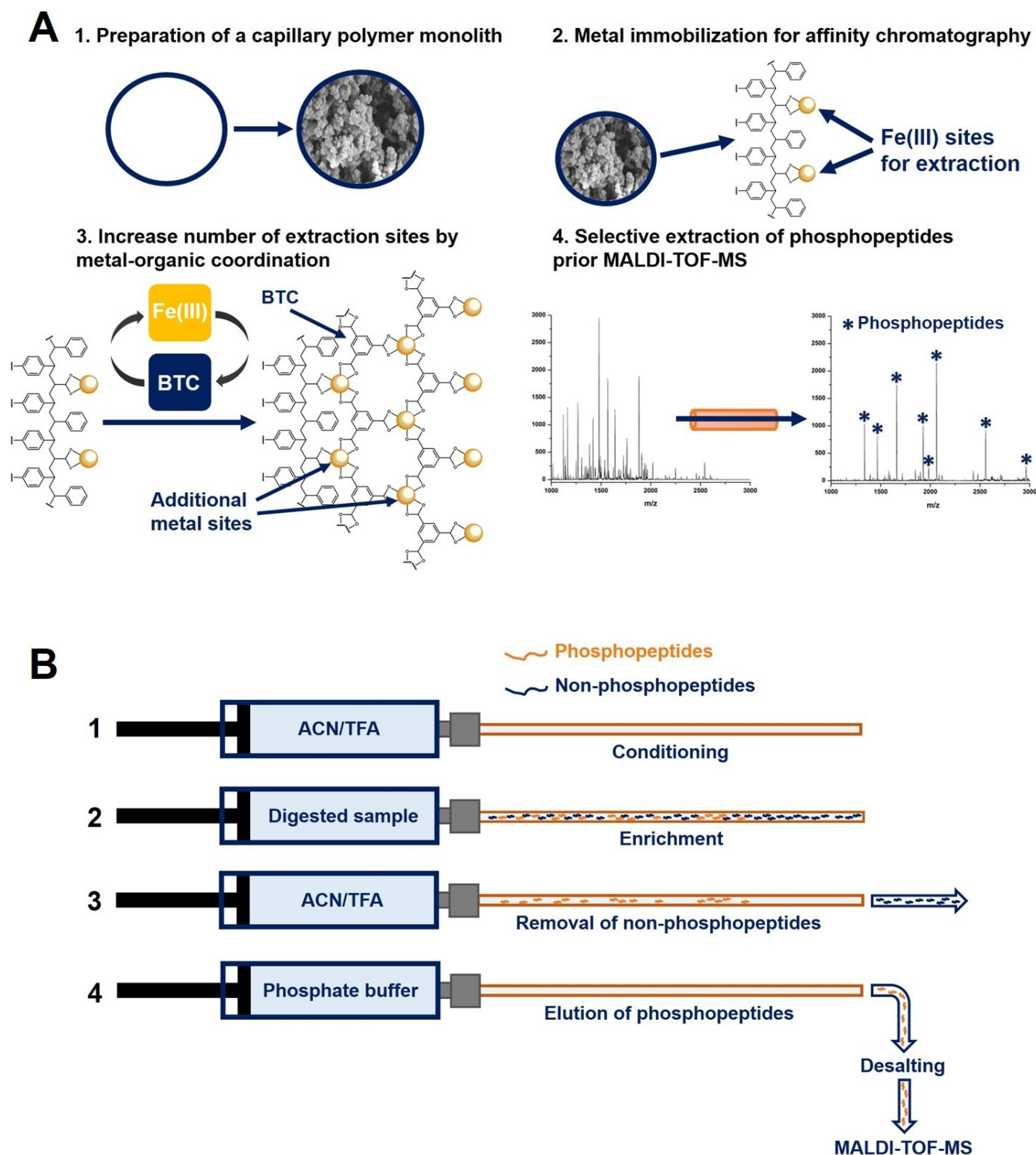


Figure 1: Scheme. (A) Illustration showing the principal steps for the preparation of a MOPH capillary column for the extraction of phosphopeptides. (B) Illustration of the procedure for the extraction of phosphopeptides using the prepared MOPH column.

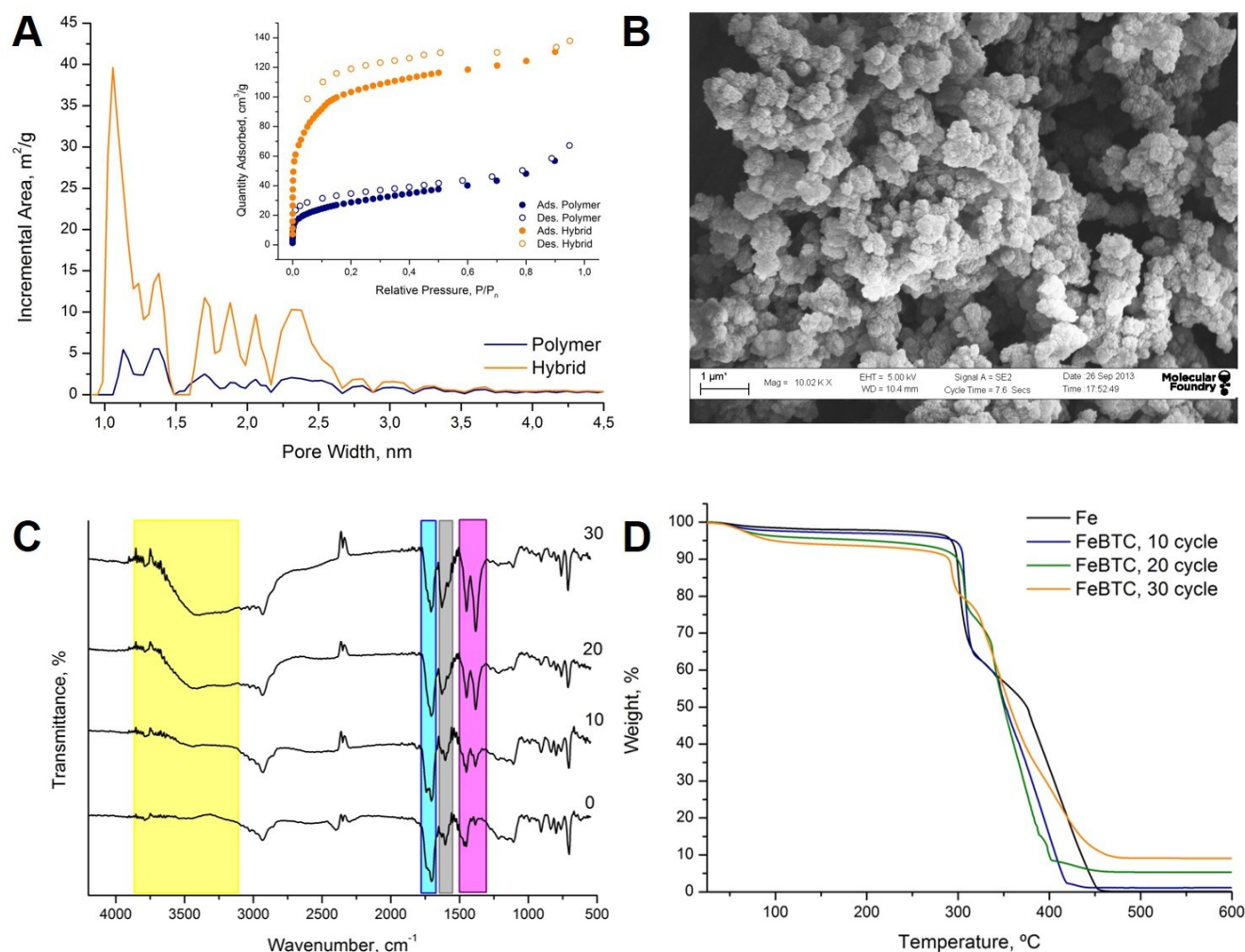


Figure 2: Bulk FeBTC MOPH characterization results. (A) Pore size distribution and nitrogen adsorption isotherms of the original organic polymer monolith and the MOPH after 30 coordination cycles. (B) SEM image of the MOPH after 30 coordination cycles. (C) FT-IR spectra of the original polymer monolith and the MOPH after 10, 20 and 30 coordination cycles. (D) TGA of the original polymer monolith after a single wash with the metallic precursor solution, and after 10, 20 and 30 coordination cycles. (Adapted from ref. 21 with permission from John Wiley & Sons.)

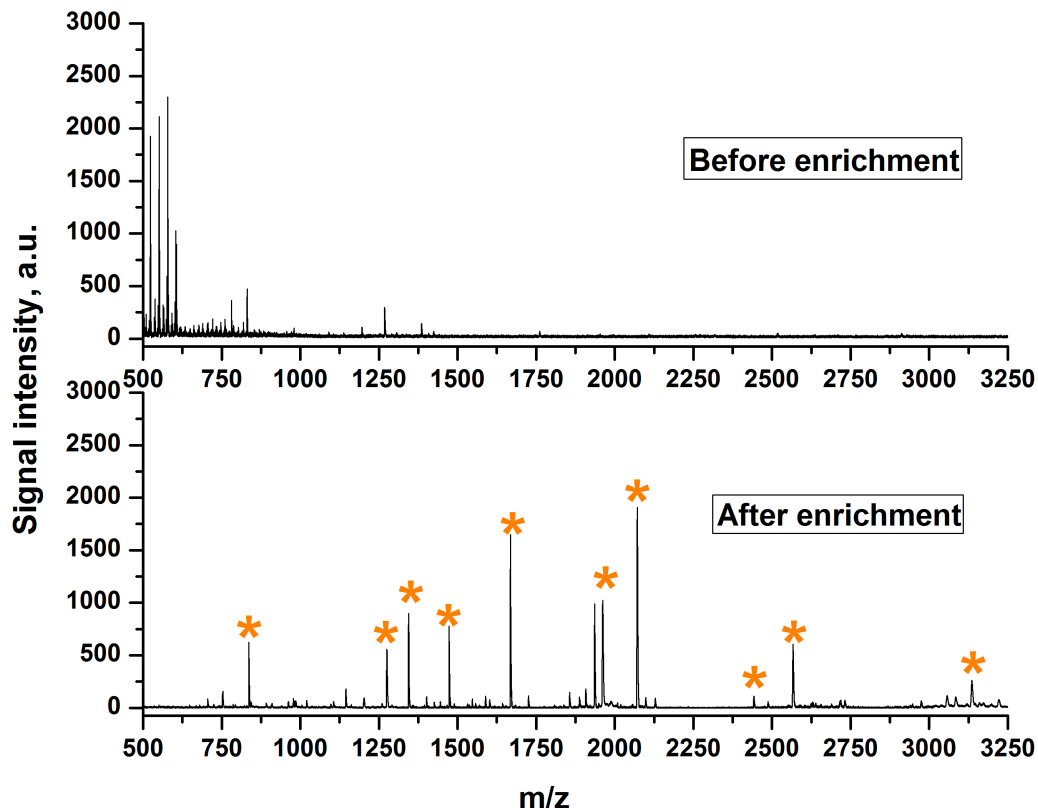


Figure 3: Enrichment of phosphopeptides from milk using a capillary FeBTC MOPH column. MALDI-TOF-MS spectra of a digested nonfat milk sample before and after enrichment using a MOPH capillary column after 10 FeBTC coordination cycles. MS peaks resulting from phosphopeptides are indicated with asterisks, while dephosphorylated fragments are indicated with hashes. Phosphopeptides were assigned using literature references²³⁻²⁷. (Reprinted from ref. 21 with permission from John Wiley & Sons.)

Discussion

The original polymer monolith contains carboxylic functional groups able to bind to metals. Coordinating the initial metal sites on the original material, we are able to grow a PCP coating (**Figure 1A**), incorporating a number of additional metal sites shaping a microporous network. This makes the presented MOPH materials attractive for extraction or purification procedures where metallic species are involved, such as the immobilized metal-ion affinity chromatography (IMAC) technique. The general procedure using a capillary column for the enrichment of phosphopeptides is shown in **Figure 1B**.

The preparation of the bulk powder monoliths enabled the characterization of the original monolithic material and its modified counterparts. We measured the N_2 uptake isotherms at 77 K (**Figure 2A**), which shows that after 30 PCP cycles the N_2 uptake at low P/P_0 largely increased, indicating the presence of new micropores in the material. The surface area of the original monolith increases nearly four times, from $106 \text{ m}^2/\text{g}$ to $389 \text{ m}^2/\text{g}$. Just performing a small number of cycles (10 PCP cycles) an increase of the porosity of the material to a surface area of $156 \text{ m}^2/\text{g}$ was measured. The preparation of porous materials using the detailed approach is not just limited to Fe-based PCPs. Substituting the Fe by Cu, just 10 cycles of the resultant CuBTC coating were required to increase the surface area of the MOPH from $106 \text{ m}^2/\text{g}$ to $219 \text{ m}^2/\text{g}$. The new pores present in the modified material have a diameter smaller than 3 nm as shown in the pore size distribution (**Figure 2A**). The distribution of the PCP coating on the surface of the polymer monolith was examined using SEM. **Figure 2B**, shows a monolith after 30 PCP cycles, which consists of a porous structure based on a microglobular network, thus retaining the initial morphology of the original polymer monolith. The large meso- and macropores remain intact after modification maintaining the excellent flow properties of the organic polymer monolith. Using FT-IR we confirmed the initial incorporation of carboxylic functional groups (band at $1,707 \text{ cm}^{-1}$) for the attachment of the FeBTC PCP, as well as monitor the growth of the coating by the increase of the bands $1,382$, $1,449$, $1,627$ and $3,400 \text{ cm}^{-1}$ (**Figure 2C**). Performing TGA we measured the increase of the amount of Fe(III) in the material (**Figure 2D**). Using powder X-ray diffraction we confirmed that the TGA residue at 600°C is $\alpha\text{-Fe}_2\text{O}_3$, and based on the mass of the residue, we calculate the mass % Fe on the original polymer monolith and the MOPHs. As an indicative example, the initial % Fe on the original monolith is 1.1%, and this value increased to a 10.5% after 30 PCP cycles.

The preparation of MOPHs is easily adaptable to a capillary column format for the development of flow through applications. In this case, the prepared MOPH containing a high abundance of Fe(III) sites on the surface of the pores makes it an excellent candidates for the IMAC enrichment of low abundant phosphopeptides. A gradual increase of the performance of the material is observed when the original support with immobilized Fe(III), is compared to an analogous support after 5 or 10 PCP cycles²¹. The critical step in the preparation of a MOPH capillary

column is to ensure that the number of cycles of the FeBTC coordination polymer is appropriate for the further application of the MOPH column. As an example, **Figure 3** shows the result obtained for the enrichment of phosphopeptides from digested commercial non-fat milk, using a MOPH capillary column. In this example, a MOPH column after 10 FeBTC cycles exhibited a remarkable selectivity for phosphopeptides. By direct analysis of the sample without enrichment, none of the low abundant phosphopeptides are detected. After enriching the same sample using the developed MOPH material, 12 different phosphopeptides are selectively extracted enabling their satisfactory detection. The capacity of a capillary column modified with 30 FeBTC cycles is 3.25 $\mu\text{mol ATP/ml}$, which is superior to commercially available iron affinity gels based on nitriloacetic acid²⁸. The developed Fe-based MOPH can be potentially implementable for the extraction of other organophosphates, such as organophosphorus pesticides and nerve agents. The selectivity of the MOPH towards enrichment of biomolecules can be tuned by selecting a metal with different binding properties for the preparation of the coordination polymer.

We have demonstrated a simple procedure for the growth of highly porous PCP coatings in a porous polymer monolith, which is the first example of a flow-through support containing a functional PCP uniformly coating the polymer macropores. The resulting MOPHs overcome the limitation of diffusional mass transport associated with flow through inter-particle voids, as well as penetration into the small pores of porous solids when packed in a column format or are embedded in porous polymers. We showed the utility of these materials for the enrichment of phosphopeptides by IMAC. The procedure reported here can be implemented using numerous PCPs and similar materials. The main limitation of the technique is the laborious manual preparation of the coating. However, current research by the authors is focused towards the automation of this methodology using computer-controlled flow techniques.

Disclosures

The authors have nothing to disclose.

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