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Effective Enrichment and Mass Spectrometry Analysis of Phosphopeptides Using Mesoporous Metal Oxide Nanomaterials

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Abstract

Mass spectrometry (MS)-based phosphoproteomics remains challenging due to the low abundance of phosphoproteins and substoichiometric phosphorylation. This demands better methods to effectively enrich phosphoproteins/peptides prior to MS analysis. We have previously communicated the first use of mesoporous zirconium oxide (ZrO₂) nanomaterials for effective phosphopeptide enrichment. Here we present the full report including the synthesis, characterization, and application of mesoporous titanium dioxide (TiO₂), ZrO₂, and hafnium oxide (HfO₂) in phosphopeptide enrichment and MS analysis. Mesoporous ZrO₂ and HfO₂ are demonstrated to be superior to TiO₂ for phosphopeptide enrichment from a complex mixture with high specificity (>99%), which could almost be considered as “a purification”, mainly because of the extremely large active surface area of mesoporous nanomaterials. A single enrichment and Fourier transform MS analysis of phosphopeptides digested from a complex mixture containing 7% of α -casein identified 21 out of 22 phosphorylation sites for α -casein. Moreover, the mesoporous ZrO₂ and HfO₂ can be reused after a simple solution regeneration procedure with comparable enrichment performance to that of fresh materials. Mesoporous ZrO₂ and HfO₂ nanomaterials hold great promise for applications in MS-based phosphoproteomics.

INTRODUCTION

Protein phosphorylation is one of the most common and important post-translational modifications (PTMs). Approximately one-third of all proteins in a eukaryotic cell are phosphorylated at any one time on serine (~90%), threonine (~10%) and tyrosine (~<0.05%) residues.^{1–2} Phosphorylation plays a pivotal role in the regulation of many biological processes, such as cell growth, division, and signaling.^{2–3} Dysregulation of these phosphorylation-mediated signaling pathways has been found to be the underlying basis of many human diseases such as cancers and heart diseases.^{1,3–4} Mass spectrometry (MS) with the various tandem mass spectrometry techniques (MS/MS) has become the method of choice for the analysis of protein phosphorylation because of its sensitivity, speed, and simplicity in identification of phosphorylation sites and quantification of changes in phosphorylation states.

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Supporting Information Available:

Detailed discussion on the identification of phosphopeptides by MS/MS, optimization of enrichment solutions and procedures, final concentration, the quantity present in 6-protein mixture (Table S-1), and reproducibility of phosphopeptide enrichment (Table S-2) as well as supplemental figures (Figures S1–S6) as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

^{1,5–7} Since recently developed MS/MS techniques, electron capture dissociation (ECD)⁸ and electron transfer dissociation (ETD),⁹ preserve labile phosphorylation during the MS/MS process,^{8,10} they have been shown to be uniquely valuable for reliably mapping phosphorylation sites^{11–13} and determining phosphorylated positional isomers.¹⁴ However, MS analysis of phosphorylation on a proteome-wide scale remains a major challenge due to the low abundance of phosphoproteins and the low stoichiometry of phosphorylation as well as the highly temporal/dynamic nature of protein phosphorylation due to kinases/phosphatases functions.^{1–2,15–16} Therefore, isolation and enrichment of phosphoproteins/peptides are essential to facilitate the analysis of phosphorylation for MS-based phosphoproteomics.

Commonly used phosphopeptide enrichment methods^{1,5,17–18} can be grouped into chemical and affinity based methods.¹⁶ The former^{7,19–20} relies on specific chemical derivatization of phosphorylated amino acids, which could be compromised by side reactions, increased sample complexity, and potential loss of phosphate.⁵ The affinity based method is more routinely used due to its simplicity, of which the most well-known one is the immobilized metal affinity chromatography (IMAC)^{21–22} using surface-bound chelates of Ga(III), Fe(III), or other metals.^{5,16–18} However, IMAC-based enrichment methods suffer from the nonspecific binding of non-phosphorylated acidic peptides and the complexity of factors affecting the phosphopeptide binding and release which frequently result in low specificity and sensitivity for targeted phosphopeptides.^{17,23} Recently, metal oxide affinity chromatography (MOAC) methods¹⁷ have become more promising for phosphopeptide enrichment than IMAC since such oxides rely on specific and reversible chemisorption of phosphate groups on their amphoteric surface and have less non-specific binding (and thus higher specificity).²³ Microparticles of titanium dioxide (TiO₂),^{24–25} zirconium oxide (ZrO₂),²³ aluminum hydroxide [Al(OH)₃],²⁶ and other metal oxides,^{27–29} have been shown to selectively enrich phosphopeptides. Furthermore, nanoparticles of some of these metal oxides have been explored due to their potential higher capacities than the microparticles.^{18,30–31}

We have recently communicated the first use of mesoporous ZrO₂ nanomaterials for highly effective enrichment of phosphopeptides.³² Mesoporous materials^{33–40} are nanostructured materials with pore sizes typically between 2 – 50 nm. They have extremely large surface areas, well-ordered nanoscale porous structures, and flow-through capacity, are chemically stable and can be easily prepared at reasonable cost, therefore they have been utilized in many applications such as catalysis, separation, coating, electronics, and biology.^{39–40} As we preliminarily reported, their large surface areas combined with the many active surface sites on appropriate amphoteric metal oxides provide even higher loading capacity for binding phosphate groups than micro- and nanoparticles, and are ideal for enriching phosphopeptides for MS-based phosphoproteomics.³²

Herein we present a systematic investigation of the applications of mesoporous metal oxides of TiO₂, ZrO₂, and hafnium oxide (HfO₂) in phosphopeptide enrichment and MS analysis. We synthesized these mesoporous metal oxide nanomaterials using block copolymer template-directed sol-gel reactions, characterized their nanostructures, and investigated and optimized their performance in phosphopeptide enrichment. To our knowledge, this is the first reported use of mesoporous HfO₂ and TiO₂ for phosphopeptide enrichment. Although microparticles of TiO₂ and ZrO₂ have been frequently used for enrichment of phosphopeptides,^{1,5,17–18,23–25} the use of HfO₂ for phosphoenrichment has not been extensively explored except for a brief report on the use of microparticle HfO₂.²⁹ Hafnium is a heavier Group 4 metal element in the Periodic table of the elements which should have similar characteristics to those of zirconium. A comparison among these three mesoporous oxides has been made, which suggests that ZrO₂ and HfO₂ have higher specificity than TiO₂ for phosphopeptide enrichment. Methods for surface regeneration were also explored to demonstrate the reuse of these mesoporous materials.

MATERIAL AND METHODS

Reagents and Materials

Block copolymer $\text{HO}(\text{CH}_2\text{CH}_2\text{O})_{106}(\text{CH}_2\text{CH}(\text{CH}_3)\text{O})_{70}(\text{CH}_2\text{CH}_2\text{O})_{106}\text{H}$ (designated as EO106-PO70-EO106, or Pluronic® F127) was kindly provided as a gift from BASF (Florham Park, NJ). Anhydrous precursors, zirconium chloride (ZrCl_4), hafnium chloride (HfCl_4) was purchased from Alfa Aesar (Ward Hill, MA), and titanium chloride (TiCl_4) was purchased from Sigma Aldrich (Milwaukee, WI). All of the metal chlorides were stored in a glove box filled with inert atmosphere. Ethanol (200 proof) and HPLC grade water were purchased from Sigma Aldrich (St. Louis, MO). α -Casein from bovine milk, bovine serum albumin (BSA), porcine troponin from skeletal muscle, bovine ubiquitin, bovine ribonuclease B (RNase-B), and bovine β -lactoglobulin were purchased from Sigma (St. Louis, MO). Trypsin was a gift from Promega (Madison, WI). All proteins were used as received without further purification. Ammonium bicarbonate (NH_4HCO_3), trifluoroacetic acid (TFA), acetic acid, acetonitrile (ACN), 28% ammonium hydroxide (NH_4OH) and isopropanol were purchased from Fisher Scientific (Fair Lawn, NJ), phthalic acid from Acros Organics (Morris Plains, NJ) and used without further purification.

Synthesis of Mesoporous Metal Oxide Materials

Mesoporous ZrO_2 and HfO_2 were synthesized by adding Pluronic® F127 (0.396 g, 3.17×10^{-5} mol), absolute ethanol (2.35 mL for ZrO_2 and 3.17 mL for HfO_2) and either ZrCl_4 (0.634 g, 2.72×10^{-3} mol) or HfCl_4 (0.801 g, 2.50×10^{-3} mol) sequentially under vigorous stirring. After all precursors were dissolved, 14% NH_4OH (20 μL) was added to the sol and stirred for an additional 2 hrs. The precipitate that formed upon addition of NH_4OH dissolved within 2 hrs under stirring. The viscous sols were then poured into uncovered Petri dishes. For mesoporous TiO_2 , a previously reported procedure⁴¹ is followed. Briefly, Pluronic® F127 (0.312 g, 2.50×10^{-5} mol) was added to absolute ethanol (6.403 g) in a round bottom flask fitted with a septum. The flask was flushed with nitrogen for 5 mins, then the solution was heated to 30 °C with vigorous stirring before TiCl_4 (0.375 mL, 3.42×10^{-3} mol) was injected. After stirring for 1 hr, deionized water (0.60 mL) was injected into the sol in small aliquots, followed by an additional 2 hrs of stirring. The clear pale yellow sol was poured into uncovered Petri dishes, and allowed to rest at ambient conditions for 20 mins. Sols of all three metal oxides were aged for 3 days in an incubator at 40 °C with constant humidity maintained by a saturated aqueous KCl solution. The aged gels were subsequently transferred to alumina boats and calcined in air in a tube furnace. The furnace was ramped from room temperature to 360 °C over 6 hrs, and then held at 360 °C for 2 hrs, followed by a natural cool-down. Calcined samples, which are pale white or pale yellow powders, were ground with an agate mortar and pestle for structural characterization and phosphopeptide enrichment.

Characterization of Mesoporous Materials

Nanostructures of the synthesized mesoporous oxides were characterized with small angle x-ray scattering (SAXS) on a Rigaku SAXS (Rigaku, Texas, USA). The powders of mesoporous materials were placed in glass capillary tubes as the sample holder to carry out the SAXS analysis. Transmission electron microscopy (TEM) was taken with a Philips CM200UT TEM (Philips Electron Optics, Eindhoven, The Netherlands) with an accelerating voltage of 200 kV. TEM samples were prepared by grinding and suspending the synthesized products in ethanol and then dispersing them onto lacy carbon TEM grids.

Proteolytic Peptide Sample Preparation

The sample preparation procedures have been described in the supporting material of our previous communication.³² Briefly, trypsin digestion was performed with an enzyme-to-

protein ratio of 1:100 and incubated at 37 °C for 2 hrs. The 6-protein mixture was prepared using bovine serum albumin (BSA), α -casein, troponin, ubiquitin, RNase-B, and β -lactoglobulin. These proteins were combined just before digestion, desalted, reduced with dithiothreitol (85 mM) for 3 hrs and then alkylated with iodoacetic acid (90 mM) for 1 hr and finally digested by trypsin (1:50) overnight. For both pure α -casein and the 6-protein mixture, the resulting digest solution was quenched with 6 μ L of acetic acid, aliquoted, and stored at -20 °C. The peptide solutions were diluted 10 times with 20 mg/mL phthalic acid solution in 0.1% TFA in 50/50 water/ACN (pH 2.0) just before enrichment. This brought the final concentration of α -casein before enrichment to 4 pmol/ μ L for the pure α -casein. The final protein concentrations and quantities of the proteins present in the 6-protein mixture before enrichment are shown in Table S-1, with 0.8 pmol/ μ L for α -casein which is 7% of the total protein quantities (by weight).

Typical Phosphopeptide Enrichment Using Mesoporous TiO₂, HfO₂ and ZrO₂

In a 1.5 mL centrifuge tube, 2 mg of the calcined mesoporous metal oxide material was weighed out and pretreated with 200 μ L of a binding solution. In the optimized procedure, the binding solution consisted of a 20 mg/mL phthalic acid solution in 0.1% TFA in 50/50 H₂O/ACN (pH 2.0). The tubes were vortexed for 1 min, centrifuged for 1 min, and then the equilibrating solution was pipetted out and discarded. 100 μ L of peptide solutions digested from α -casein (4 pmol/ μ L) or the 6-protein mixture (Table S-1) in the binding solution were added to the mesoporous materials. The samples were mixed thoroughly for 5 mins and then centrifuged for 1 min before the supernatant was pipetted off. Then the metal oxide was rinsed twice with 1 mL of a 50 mM solution of NH₄HCO₃ in 50/50 H₂O/ACN mixture (pH 8.5) following the same procedure of vortexing, spinning down, and discarding the supernatants. Finally, the phosphopeptides were eluted from the mesoporous oxide powder with an aqueous solution of NH₄OH at pH 11.5 and the supernatant was collected. The eluted peptides were either directly used for negative ion mode MS analysis or dried down and reconstituted in a solution of 0.1 – 5% formic acid or acetic acid in 50:50 H₂O/ACN for positive ion mode MS analysis.

Regeneration of the mesoporous materials

The solution-based regeneration procedure was accomplished by combining all previously used materials into a single 1.5 mL centrifuge tube. The materials were washed with 1 mL of ACN and vortexed thoroughly for 5 minutes, centrifuged, and the supernatant was discarded. Then 1 mL of concentrated NH₄OH was added and vortexed for 5 mins, centrifuged, and the supernatant discarded. After the mesoporous materials were completely dried, they were ready to be reused for phosphopeptide enrichment.

Mass spectrometry analysis

Mass spectra were acquired on a standalone LTQ linear ion trap mass spectrometer and a 7 T linear trap/Fourier transform ion cyclotron resonance (FTICR) hybrid mass spectrometer (LTQ FT Ultra) (Thermo Scientific Inc., Bremen, Germany). Samples were introduced to the LTQ with an Eksigent nano 2D HPLC system (Eksigent Technologies, Dublin, CA). Reversed phase LC was run with a trap column (Agilent Zorbax 300SB C18) followed by a self packed C18 column (75 μ m $\times$ 150 mm Magic C18, 5 μ particle, 300 Å pore size) and a gradient of 0.1% formic acid in water and ACN at a flow rate of 300 nL/min for a one-dimensional LC separation. The phosphopeptides were detected in a neutral loss MS³ acquisition mode in which the mass spectrometer was set as a full scan MS followed by data dependent MS/MS. Subsequently MS³ spectrum was automatically triggered when the neutral loss of 98 Da for detection of phosphoric acid, H₃PO₄, (m/z of 98, 49, and 32.7 for 1+, 2+, 3+ charge states, respectively) and 80 Da for metaphosphoric acid (HPO₃) (m/z of 80, 40, and 26.7 for 1+, 2+, 3+ charge states, respectively). The data dependent MS/MS and MS³ spectra were searched against the

SwissProt non-redundant bovine and porcine protein database in Bioworks using SEQUEST algorithm considering variable phosphorylations of Ser, Thr, and Tyr residues.

For high resolution analysis, the samples were directly introduced to the LTQ FT mass spectrometer using an automated chip-based nanoESI source, the Triversa NanoMate (Advion BioSciences, Ithaca, NY) with a spray voltage of 1.2–1.6 kV versus the inlet of the mass spectrometer, resulting in a flow of 50 – 200 nL/min. Ion transmission into the linear trap and further to the FTICR cell was automatically optimized for maximum ion signal. The target values (the approximate number of accumulated ions) for a full MS scan linear trap (LT) scan, FTICR cell (FT) scan, MSⁿ linear trap scan and MSⁿ FTICR scan were 3×10^4 , 10^6 , 10^4 , and 5×10^5 , respectively. The resolving power of the FTICR mass analyzer was set at 100,000 m/ Δm 50% at m/z 400, resulting in an acquisition rate of one scan/s. Individual charge states of the protein molecular ions were first isolated and then dissociated by ECD using 5–6% “electron energy” and a 150–250 ms duration time with no delay. Typically, 100–300 transients were averaged per spectrum to ensure high quality ECD spectra (up to 1000 transients in some cases for the extremely low abundance precursor ions). For collisionally activated dissociation (CAD), precursor ions were activated using 10 – 35% normalized collision energy at the default activation q of 0.25 and dissociated in the linear ion trap followed by detection in FTICR cell. CAD spectra were typically averaged over 10–100 transients. All FTICR spectra were processed with Xtract Software (FT programs 2.0.1.0.6.1.4, Xcallibur 2.0.5, Thermo Scientific Inc., Bremen, Germany) using a signal-to-noise threshold of 1.5 and fit factor of 60% and validated manually. The resulting monoisotopic mass lists were further searched using in-house “ion-assignment” software.

RESULTS AND DISCUSSION

Templated Assembly and Synthesis of Mesoporous Metal Oxide Materials

Well-ordered 3-dimensional inorganic nanostructures with periodic porosity and high surface area are challenging to synthesize. Such mesoporous materials can be synthesized using ionic or block copolymer surfactants that form nanoscale micellar structures or lyotropic liquid-crystal phases to template the controlled hydrolysis and assembly of metal precursors through electrostatic, hydrogen bonding, or van der Waals forces (Scheme 1).^{33–34,37,39–40}

Mesoporous silica/silicate materials are well known and easy to synthesize and have found their way into many applications such as molecular sieves for proteins and drug delivery.^{42–45} Non-silicate mesoporous materials (such as those made of metal oxides) can be prepared using the more versatile non-ionic block copolymer surfactant templates and have found wider applications due to their diverse properties.^{36,39–40,46}

We synthesized mesoporous materials of metal oxides, specifically TiO₂, ZrO₂, and HfO₂, using commercially available amphiphilic triblock copolymer Pluronic® F127, modifying^{36,47} or following⁴¹ reported synthetic procedures. The F127 copolymers form ordered nanoscale micellar structures in alcohol solutions, which facilitates the controlled assembly of the hydrolyzed metal precursors in a so-called evaporation induced self assembly (EISA) process (Scheme 1). Mesoporous ZrO₂, and HfO₂,⁴⁸ unlike TiO₂, have not been frequently studied and reported. We found the metal chloride precursors work better than the alternative metal alkoxide precursors often used. In the mesoporous TiO₂ synthesis, TiCl₄ serves not only as the titanium source, but also generates HCl to catalyze the condensation reaction. Since TiCl₄ decomposes readily in air, reaction flasks are purged by inert gas to remove water and oxygen to prevent precursor decomposition during injection. The water (moisture) content needs to be carefully controlled during the sol-gel formation and aging stage of the synthesis. Too much moisture in the sol can lead to uncontrollable hydrolysis and precipitation of the oxide while insufficient moisture can lead to pore collapse during template removal. Control of the condensation reaction was found to be very useful for reproducible synthesis, which, in the

cases of mesoporous ZrO₂ and HfO₂, was accomplished by adding a minute amount of NH₄OH, which served to catalyze the condensation reactions. Fast evaporation of the solvent leaves pores disordered and kinetically trapped. Annealing in a moisture-rich atmosphere for long aging times (3 days) allows the gel to reach a thermodynamic equilibrium producing well-ordered mesoporous structures.

The ordered porous nanostructure of synthesized mesoporous TiO₂, ZrO₂, and HfO₂ were confirmed using transmission electron microscopy (TEM) and small angle x-ray scattering (SAXS) (Figure 1). TEM and SAXS are the most typical and conclusive structural characterization tools for mesoporous materials. TEM images clearly show the porous structures of these materials: clear pores in the head-on view in Figure 1a and the upper left domain of Figure 1e and side view of the pore channels in Figure 1c and the middle domain of Figure 1e. Pore size for a typical synthesis is 6.4 nm for TiO₂, 5.8 nm for ZrO₂ and 7.6 nm for HfO₂, as determined using many TEM images. SAXS allows the global determination of the long range order of a large (nanometer scale) “unit cell” of the mesoporous structures. The SAXS patterns (Figure 1b, 1d, 1f) are presented as scattering intensity vs. wave vector q . Wave vector q has the unit of Å⁻¹ and is related to the d spacing of the ordered structures by $d = 2\pi/q$. Therefore, by measuring the q values of the first peaks in SAXS patterns, we determined the periodicity to be 14.6 nm for TiO₂, 8.2 nm for ZrO₂, and 10.1 nm for HfO₂, which are in general agreement with the TEM evaluation. As we reported previously, Brunauer-Emmett-Teller (BET) analysis of N₂ absorption experiments revealed that the mesoporous ZrO₂ has a surface area of 72 m²/g,³² which is in good agreement with that previously reported for mesoporous ZrO₂ templated with block copolymer F127.⁴⁹ Mesoporous TiO₂ and HfO₂ templated with F127 should have comparable values. It is important to note that the variable and higher density of metal oxides such as ZrO₂ makes a direct comparison between ZrO₂ and silica difficult. However, Nawrocki and co-workers calculated that 30 m²/g of ZrO₂ is comparable to 90 – 120 m²/g SiO₂.⁵⁰ With this in mind our synthesized ZrO₂ has a comparable surface area of 216–288 m²/g to that of SiO₂. Note that even higher surface areas can be achieved for mesoporous materials by using surfactants with smaller molecular weights and dimensions. However, proteins or peptides and the aqueous solutions that contain them might not diffuse well into small pores and the adsorbed proteins/peptides may be more stable and protected from the external environment.⁴⁵ For maximum binding and recovery, it is likely advantageous to choose a large pore size to ensure the peptides have access to the entire pore surface and maximum interaction with the external environment.

Optimization of Enrichment Solutions and Procedures

We have sought to minimize non-specific binding by optimizing the buffers used in binding, washing, and eluting steps (Figure S-4). Group 4 transition metal oxides (TiO₂, ZrO₂, and HfO₂) are known to be very chemically stable and display amphoteric properties based on unsatisfied valences in both oxygen and zirconium/hafnium/titanium on their surface layer, which can preferentially (but reversibly) bind phosphate groups and other anionic species at low pH and release them at high pH.^{50–52} Here, the best results were achieved with the binding buffer of 20 mg/mL phthalic acid in 0.1% TFA in 50/50 water/ACN (pH 2.0), washing buffer of 50 mM NH₄HCO₃ in 50/50 water/ACN, and eluting buffer of NH₄OH (pH 11.5) (Figure S-4f).

Our data show that not only the pH value but also the additives to the binding, washing, and elution solutions such as phthalic acid are all critical to obtain the most selective (specific) enrichment of phosphorylated peptides, in agreement with observations made previously on metal oxide microparticles.^{23,25,53} The most phosphopeptides recovered are bound in a low pH solution with 20 mg/mL phthalic acid, which has been found to help decrease nonspecific binding through competition for binding sites and functions⁵³ similar to dihydroxybenzoic acid

(DHB), another commonly used additive to decrease nonspecific binding.^{25,54–55} Such ability to withstand the harsh solution conditions, including the use of organic acid at low pH to improve the phosphopeptide binding specificity, is one of the benefits of metal oxides.¹⁷

Phosphopeptide Enrichment Using Mesoporous Metal Oxide Nanomaterials

Using the optimized procedures, we first evaluate the abilities of mesoporous TiO₂, HfO₂ to ZrO₂ to enrich a mixture of peptides from tryptic digests of α -casein. HfO₂ is a material that is not commercially available as chromatographic media or enrichment kits but shows amphoteric property similar to that of TiO₂ and ZrO₂.⁵⁶ The enrichments using all three mesoporous metal oxides are quite effective as shown by the high resolution Fourier transform (FT) MS spectra in both positive ion mode (Figure 2) and negative ion mode (Figure 3) of the α -casein digest before and after the enrichment. Only 8 MS peaks corresponding to 6 phosphopeptides were detected before enrichment in both positive and negative ion mode MS spectra (Figure 2a, 3a); all of which are relatively low abundance peaks owing to ion suppression from abundant non-phosphopeptides. In contrast, after enrichment with mesoporous oxides, many more phosphopeptides were routinely detected in a single mass spectrum with much higher signal-to-noise ratios. Apparently, the effective enrichment by mesoporous metal oxide substantially enhanced the signal of phosphopeptides. For example, the insets in Figure 3 highlight a quintuply phosphorylated peptide, p14, which was completely suppressed by non-phosphopeptides without enrichment (Figure 3a) and was observed only after enrichment (Figure 3b, c, d) underscoring the effectiveness of this enrichment procedure. Overall, 22 and 26 multiply charged MS peaks corresponding to 18 and 19 phosphopeptides were detected after enrichment using mesoporous TiO₂, in positive (Figure 2b) and negative ion mode (Figure 3b) MS spectrum, respectively. 29 and 27 multiply-charged MS peaks corresponding to 20 and 18 phosphopeptides were detected after enrichment with mesoporous ZrO₂, in positive (Figure 2c) and negative ion mode (Figure 3c), respectively. 30 multiply charged MS peaks corresponding to 21 phosphopeptides were detected after enrichment with HfO₂ in both positive and negative ion mode (Figure 2d, 3d). The enrichment performance of mesoporous ZrO₂, and HfO₂ is comparable while TiO₂ seems somewhat less effective than the other two because there are still a number of non-phosphopeptides present in the MS spectra after TiO₂ enrichment whereas both ZrO₂ and HfO₂ enrichments generated much higher purity phosphopeptides as indicated in both positive and negative ion mode spectra.

Overall, the numbers of phosphopeptides detected in positive ion mode (Figure 2) are comparable to that observed in negative ion mode (Figure 3). However, in agreement with other studies,^{23,57} we found negative mode ionization provided much higher sensitivity and much better stability and reproducibility for phosphopeptides than positive mode ionization due to the acidity of the phosphates which could make phosphopeptides more difficult to protonate than nonphosphopeptides. Hence, consistent with previous studies,^{5,23,29,57–58} the MS of phosphopeptides was preferably performed in negative ion mode for the later enrichment experiments although the MS/MS experiments were primarily performed in the positive ion mode for phosphopeptide identification mainly due to its relatively higher dissociation efficiency (Supporting information, Figure S-1 and S-2).

To further evaluate the enrichment specificity, we tested the mesoporous TiO₂, ZrO₂, and HfO₂ using a more complicated peptide mixture digested from a 6-protein mixture containing a substantial fraction of 5 non-phosphorylated proteins (BSA, skeletal troponin, ubiquitin, RNase-B, and β -lactoglobulin) and a phosphorylated α -casein (7%, by weight of the total proteins) (Table S-1). Before enrichment many non-phosphopeptides in this mixture dominate the MS spectrum (Figure 4a) so that even the most abundant phosphopeptide, p3, is severely suppressed and hardly observable. After enrichment with mesoporous TiO₂, 22 multiply charged MS peaks corresponding to 16 phosphorylated peptides were identified (Figure 4b).

After enrichment with mesoporous ZrO₂, 28 multiply charged MS peaks corresponding to 19 phosphorylated peptides were identified (Figure 4c) whereas an enrichment by mesoporous HfO₂ resulted in 30 multiply charged MS peaks corresponding to 21 phosphorylated peptides (Figure 4d). Note all of the phosphopeptides and phosphorylation sites identified from the peptide mixture digested from pure α -casein were also recovered in enrichments using mesoporous ZrO₂ and HfO₂ from this much more complex peptide mixture.

The sequences of all the identified singly to quintuply phosphorylated peptides from the tryptic digest of α -casein and 6-protein mixture are tabulated in Table 1. Overall, we have identified 20 unique phosphorylation sites (out of a total of 21 potential phosphorylation sites as reported in the literature) for α -casein (s1 and s2 variants) (Figure S-3). A previously reported peptide at m/z 2702.8559 was found to match a different sequence that corresponds to a loss of ammonia from the N-terminal glutamine residue condensing to form pyroglutamate.

Comparison of Mesoporous TiO₂, ZrO₂, and HfO₂

Mesoporous HfO₂ and ZrO₂ materials show higher specificity than TiO₂ for enrichment of phosphopeptides in complex peptide mixtures since it is fairly consistently seen over many enrichment experiments that HfO₂ and ZrO₂ enrichments can remove nearly all of the non-phosphopeptides whereas a number of non-phosphopeptides are still present after the TiO₂ enrichment (Figures 4b, c, d). While the enrichment specificity using ZrO₂ and HfO₂ is fairly comparable, the number of phosphopeptides identified by mesoporous HfO₂ is slightly higher than that by ZrO₂ and TiO₂. Moreover, it is fairly consistently seen over many enrichment experiments that the number of identified phosphopeptides by mesoporous ZrO₂ is higher than that of TiO₂ in most cases (see some representative cases in Table S-2). Microparticles of ZrO₂ have previously been showed by Hakansson and co-workers to be more selectively for phosphopeptide enrichment than TiO₂.²³ Overall, our data suggest that mesoporous HfO₂ and ZrO₂ are superior to mesoporous TiO₂ for phosphopeptide enrichment from a complex mixture.

Under our optimized enrichment condition, mesoporous HfO₂ and TiO₂ appear to be somewhat more effective than ZrO₂ in enriching multiply phosphorylated peptides. For example, enrichment with mesoporous TiO₂ and HfO₂ yield higher intensity for multiply phosphorylated peptides and revealed two multi-phosphorylated peptides, p17 and p13, which were not observed for the mixtures enriched by mesoporous ZrO₂ (Figure 4b,c,d). This is consistent with the previous report by Hakansson and coworkers where microparticle TiO₂ showed higher selectivity for multiply-phosphorylated peptides than microparticle ZrO₂.²³ However, different from that report, we do not observe a significantly enhanced selectivity for monophosphorylated peptides using mesoporous ZrO₂ and HfO₂ in comparison to TiO₂. Instead, all mesoporous oxides appear to have similar affinity towards monophosphorylated peptides in general (although some minor differences were observed), which is in agreement with other reports.^{31,59} We speculate that different selectivity of singly- vs. multiply phosphorylated peptides with the same metal oxide observed by different groups⁵⁹ could possibly due to i) the forms of the different materials (e.g. micro, nano, mesoporous), ii) enrichment condition including different binding, washing, and eluting buffers (Figure S-4), and iii) MS ionization and tuning conditions. Taking into account all aspects of the enrichment performance, our data suggest the great potential of utilizing mesoporous HfO₂ for highly effective and specific enrichment of both singly- and multiply phosphorylated peptides, which will be nicely complementary to TiO₂ and ZrO₂.

The High Specificity of Mesoporous ZrO₂ and HfO₂ for Phosphoenrichment

As demonstrated, mesoporous HfO₂ and ZrO₂ nanomaterials offer extremely high specificity (>99%) for enrichment of phosphopeptides even from complex mixtures (Figures 3c,d; 4c,d). In both cases, nearly all of the non phosphopeptides were removed by the enrichment procedure

leaving all abundant peaks as phosphopeptides, which could almost be considered as a “purification”. We have previously reported in our communication that the mesoporous ZrO_2 demonstrated significantly higher specificity for phosphopeptide enrichment over the leading commercial products based on IMAC and ZrO_2 microparticles in a side-by-side comparison.³² We attribute this specificity to the extremely large surface areas of the mesoporous materials. For example, the mesoporous ZrO_2 can have a high surface areas of 72 m^2/g as revealed by Brunauer-Emmett-Teller (BET) surface area analysis.³² Such large surface areas, together with the many active surface sites, can offer even higher loading capacity for binding phosphate groups than micro- and nanoparticles.

The specificity of the enrichment significantly enhanced the signal-to-noise ratio of phosphopeptides and yielded highly abundant peaks for easy isolation of the peaks and further MS/MS fragmentation to render complete or nearly complete coverage for phosphopeptides sequencing and of phosphorylation site localization. In particular, ECD has relatively lower efficiency than CAD which especially requires higher signal-to-noise ratios for precursor ions thus demands efficient enrichment methods to facilitate its effective applications in phosphoproteomics.⁵⁷

Reuse of Mesoporous Metal Oxides

These thermally and chemically stable mesoporous materials were tested for their performance in repeated enrichments. We used a simple solution-based chemical regeneration of mesoporous materials by soaking them with ACN and concentrated NH_4OH even after exposure to phthalic acid. The regenerated ZrO_2 and HfO_2 mesoporous material were used to enrich a blank (the binding buffer only, no peptides) and peptide mixture digested from α -casein with trypsin, respectively (Figure 5). Only baseline noise peaks were observed in the negative ion mode ESI/FTMS spectra of the blank after enrichment with regenerated ZrO_2 and HfO_2 , respectively (Figure 5a, c). This indicated the nanomaterials have been regenerated effectively with no carryover of phosphopeptides from previous enrichment. Highly abundant phosphopeptides were detected after the enrichment of regenerated mesoporous materials with α -casein digest, using regenerated mesoporous ZrO_2 and HfO_2 , respectively (Figure 5b, d), demonstrating that mesoporous metal oxides are highly reusable. The first- versus the second-use of the same mesoporous materials appear to have similar efficacy for enriching phosphopeptides as the ESI/FTMS spectra of phosphopeptide with a fresh (first-use) and regenerated (second-use) mesoporous ZrO_2 and HfO_2 are highly comparable (Figure S-5 and S-6). Multiple experiments performed on different days using regenerated mesoporous nanomaterials showed highly consistent results. Such highly reusable materials not only cut down the material cost but also could contribute to the practice of a “green laboratory”.

CONCLUSION

We have investigated the synthesis and use of mesoporous metal oxide nanomaterials for enriching phosphopeptides from complex mixtures. The mesoporous ZrO_2 and HfO_2 nanomaterials show high specificity (>99%) for phosphopeptide enrichment which could almost be considered as “purifications” afforded by the extremely high surface areas. A single enrichment and FTMS analysis of phosphopeptides digested from a complex mixture containing 7% of α -casein identified 21 out of 22 phosphorylation sites for α -casein. Mesoporous TiO_2 can also enrich phosphopeptides but its specificity is inferior to that of ZrO_2 and HfO_2 especially in complex mixtures. Furthermore, mesoporous ZrO_2 and HfO_2 can be readily reused for more enrichment with high performance after a simple regeneration procedure. Hence, the large surface area, low cost, easy-to-synthesize, and reusable mesoporous ZrO_2 and HfO_2 materials have great potentials for phosphopeptide enrichment with extremely high specificity and address the current challenge in MS-based

phosphoproteomics. Further systematic investigation of mesoporous metal oxide nanomaterials for their practical applications in phosphoproteomic study of complex biological samples (i.e. tissue lysate) is currently in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structural characterization of the mesoporous metal oxide nanomaterials
TEM micrographs for mesoporous TiO₂ (a), ZrO₂ (c), and HfO₂ (e); and the SAXS diffraction patterns for mesoporous TiO₂ (b), ZrO₂ (d), and HfO₂ (f).

Figure 2. Mesoporous metal oxide for phosphopeptide enrichment from a tryptic α -casein digest
 Positive ion mode ESI/FTMS spectra of peptide mixtures before enrichment (a), and after enrichment with mesoporous TiO_2 (b), ZrO_2 (c), and HfO_2 (d). Circle, double circle, triangle, square, and star indicate singly, doubly, triply, quadruply and quintuply phosphorylated peptides, respectively. Phosphopeptides labeled with numbers are identified and shown in Table 1.

Figure 3. Comparison of mesoporous metal oxide for phosphopeptide enrichment from a tryptic α -casein digest

Negative ion mode ESI/FTMS spectra of peptide mixtures before enrichment (a), and after enrichment with mesoporous TiO₂ (b), ZrO₂ (c), and HfO₂ (d). Circle, double circle, triangle, square, and star indicate singly, doubly, triply, quadruply and quintuply phosphorylated peptides, respectively. Phosphopeptides labeled with numbers are identified and shown in Table 1. Insets are expanded MS spectra at m/z 1365–1369; a singly charged non-phosphopeptide at m/z 1366 in (a), and doubly charged quintuply phosphorylated peptide (QMEAES*IS*S*S*EEIVPNS*VEQK), p14, at m/z 1367 in (b, c, d).

Figure 4. Comparison of mesoporous metal oxide for phosphopeptide enrichment from a tryptic digest of the 6-protein mixture

Negative ion mode ESI/FTMS spectra of peptide mixtures before enrichment (a), and after enrichment with mesoporous TiO₂ (b), ZrO₂ (c) and HfO₂ (d). Circle, double circle, triangle, square, and star indicate singly, doubly, triply, quadruply and quintuply phosphorylated peptides, respectively. Phosphopeptides labeled with numbers are identified and shown in Table 1. Insets are expanded MS spectra at *m/z* 962–964; a singly charged non-phosphopeptide at *m/z* 962.47 in (a) and a doubly charged bisphosphopeptide (DIGS_pES_pTEDQAMEDIK) at *m/z* 962.33 in (b, c, d).

Figure 5. Reuse of mesoporous metal oxides

Negative ion mode ESI/FTMS spectra of blank, and peptide mixtures digested from α -casein with trypsin enriched with mesoporous ZrO_2 (a, b) and HfO_2 (c,d) materials that were regenerated with concentrated NH_4OH and ACN. Asterisks indicate identified phosphopeptides shown in Table 1. NL: normalized intensity level of the most abundant peak.

Scheme 1.
Mesoporous metal oxide materials synthesis scheme.

Table 1

List of phosphopeptide identified in the negative ion mode FTMS spectra of tryptic digests from α -casein and 6-protein mixture before enrichment (a), after enrichment with mesoporous TiO_2 (b), ZrO_2 (c), and HfO_2 (d), respectively.

Peptide #	Expt'l (m/z)	Charge State	Expt'l Mass	Calc'd Mass	Error (ppm)	Sequence Identified	Phosphorylation sites	Assignment
p ¹	828.8824	-2	1659.7759	1659.7869	-6.6	VPQLEIVPNS*AEER	1	α -S1[121-134] ^{b,c,d}
	1658.7689	-1	1659.7779	1659.7869	-5.4	VPQLEIVPNS*AEER	1	α -S1[121-134] ^{a,b,c,d}
p ²	922.3468	-2	1846.7159	1846.7179	-1.1	DIGS*ESTEDQAMEDIK	1	α -S1[58-73] ^{b,c,d}
p ³	962.3299	-2	1926.6759	1926.6842	-4.3	DIGS*ES*TEDQAMEDIK	2	α -S1[58-73] ^{a,b,c,d}
	1925.6644	-1	1926.6679	1926.6842	8.5	DIGS*ES*TEDQAMEDIK	2	α -S1[58-73] ^{a,b,c,d}
p ⁴	974.462	-2	1950.9359	1950.9451	-4.7	YKVPQLEIVPNS*AEER	1	α -S1[119-134] ^{a,b,c,d}
	1949.9218	-1	1950.9279	1950.9451	-8.8	YKVPQLEIVPNS*AEER	1	α -S1[119-134] ^{a,b,c,d}
p ⁵	1337.4917	-2	2676.9959	2677.0155	-7.3	VNELS*KDIGS*ES*TEDQAMEDIK	3	α -S1[52-73] ^{a,b,c,d}
p ⁶	899.9485	-3	2702.8673	2702.8626	1.8	pyroQMEAES*IS*S*EEIVPNS*VEQK	5	α -S1[114-135] ^{b,c,d}
	1350.423	-2	2702.8559	2702.8626	2.5	pyroQMEAES*IS*S*EEIVPNS*VEQK	5	α -S1[114-135] ^{a,b,c,d}
p ⁷	905.6242	-3	2719.8839	2719.9055	-7.9	QMEAES*IS*S*EEIVPNS*VEQK	5	α -S1[74-94] ^{b,c,d}
	1358.9367	-2	2719.8959	2719.9055	-3.5	QMEAES*IS*S*EEIVPNS*VEQK	5	α -S1[74-94] ^{a,b,c,d}
p ⁸	1466.06	-2	2934.1359	2934.1530	-5.8	EKVNLS*KDIGS*ES*TEDQAMEDIK	3	α -S1[50-73] ^{b,c,d}
p ⁹	704.2372	-2	1410.4959	1410.4952	0.47	EQLS*TS*EENSK	2	α -S2[141-151] ^{a,b,c,d}
	1409.4785	-1	1410.4879	1410.4952	-5.2	EQLS*TS*EENSK	2	α -S2[141-151] ^{b,c,d}
p ¹⁰	731.792	-2	1465.5959	1465.6047	-6.0	TVDMES*TEVFTK	1	α -S2[153-164] ^{a,b,c}
	1464.5881	-1	1465.5979	1465.6047	-4.7	TVDMES*TEVFTK	1	α -S2[153-164] ^{a,b,c}
p ¹¹	1307.9331	-2	2617.8801	2617.8879	-2.3	NTMEHV*S*S*EESIS*QETIK	4	α -S2[17-36] ^{c,d}
p ¹²	1001.3274	-3	3007.0139	3007.0221	-2.7	NANEEY SIGS*S*S*EES*AEVATEEVK	4	α -S2[61-85] ^{c,d}
	1502.4936	-2	3006.9959	3007.0221	-8.7	NANEEY SIGS*S*S*EES*AEVATEEVK	4	α -S2[61-85] ^{b,c,d}
p ¹³	1542.4767	-2	3086.9759	3086.9884	-4.1	NANEEY SIGS*S*S*EES*AEVATEEVK	5	α -S2[61-85] ^{b,d}
p ¹⁴	1366.9350	-2	2735.8847	2735.9004	-5.8	QMEAES*IS*S*EEIVPNS*VEQK	5	α -S1[74-94] ^{b,c,d}
p ¹⁵	914.32	-3	2745.9839	2745.9923	-3.1	NTMEHV*S*S*EESIS*QETIK	4	α -S2[17-37] ^{c,d}
	1371.9798	-2	2745.9742	2745.9559	6.6	NTMEHV*S*S*EESIS*QETIK	4	α -S2[17-37] ^{a,b,c,d}
p ¹⁶	970.3289	-2	1942.6724	1942.6791	-3.0	DIGS*ES*TEDQAMEDIK	2	α -S2[58-73] ^{b,c,d}
p ¹⁷	1294.9093	-2	2591.8332	2591.8332	8.7	QMEAES*IS*S*EEIVPNS*VEQK	5	α -S1[74-93] ^{b,d}

Peptide #	Expt'l (m/z)	Charge State	Expt'l Mass	Calc'd Mass	Error (ppm)	Sequence Identified	Phosphorylation sites	Assignment
p ₁₈	1318.9558	-2	2639.9262	2639.9392	-4.9	QMEAES*IS*S*EIVPNSVEQK ⁴	4	α -S1[74-94] ^{b,c,d}
p ₁₉	1331.9985	-2	2666.0116	2665.9896	8.2	NTMEHVS*S*EESIISQET ⁴ YKQ	3	α -S2[17-36] ^{b,c,d}
p ₂₀	1310.4443	-2	2622.9032	2622.8833	7.6	pyroQMEAES*IS*S*EIVPNSVEQK	4	α -S1[74-94] ^{b,c,d}
p ₂₁	768.2900	-2	1538.5945	1538.5902	2.8	EQLS*TS*EENSKK	2	α -S2[141-152] ^{b,d}
p ₂₂	795.8395	-2	1593.6935	1593.6997	3.9	TVDMES*TEVFTKK	1	α -S2[153-165] ^{b,c,d}

M – represents oxidized methionine