Mesoporous metal oxide nanoparticles for selective enrichment of phosphopeptides from complex sample matrices†

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Selective enrichment of phosphopeptides is a key factor for successful detection of protein phosphorylations. Here, three types of mesoporous nanoparticles, ZnSn(OH)₆, P₀.₅–Ti–40 and P₀.₅–Ti–50, were evaluated for their efficiency in enriching phosphopeptides from β-casein and Arabidopsis thaliana leaf protein tryptic digests and compared with commercially available benchmarks. The three types of nanoparticles had different core metal ions and were synthesized under different reaction conditions, so they showed different characters in phosphopeptide enrichment. Various factors that may affect enrichment efficiency including the peptide to nanoparticle ratio and the composition and concentration of the loading buffer were optimized. Under optimal conditions, the three types of mesoporous nanoparticles were utilized to capture phosphopeptides from the Arabidopsis leaf protein digest. ZnSn(OH)₆ can enrich 3557 phosphopeptides with 3826 phosphosites from 1611 phosphoproteins in a 95 min nano-LC/MS run. For P₀.₅–Ti–40, 1474 phosphoproteins with 3850 phosphopeptides and 4589 phosphosites were identified. For P₀.₅–Ti–50, 1449 phosphoproteins, 3454 phosphopeptides and 3872 phosphosites were enriched. The three types of mesoporous metal oxide nanoparticles showed excellent enrichment efficiency towards phosphopeptides compared with commercially available benchmarks (TiO₂ beads and PolyMAC-Ti) and provided a low cost and efficient alternative for future protein phosphorylation studies.

1. Introduction

Protein phosphorylation is one of the most important protein post-translational modifications (PTM). Various biological functions are related to and regulated by protein phosphorylation, including cell growth, proliferation, metabolism, signal transduction and apoptosis. It is estimated that more than 30% of the proteins are phosphorylated at any time in a living cell.†

With the development of mass spectrometers of higher resolution, mass accuracy and sensitivity, in-depth coverage of phosphoproteomes is now possible.‡ But there still are challenges in phosphopeptide detection, due to low stoichiometry, poor ionization efficiency, rapid degradation of phosphopeptides and interference from non-phosphopeptides.§ So, it is crucial to enrich phosphopeptides prior to LC-MS analysis.

Various strategies have been developed for phosphopeptide enrichment, including immobilized metal ion affinity chromatography (IMAC), metal oxide affinity chromatography (MOAC), strong cation exchange chromatography (SCX), hydrophilic interaction liquid chromatography (HILIC) and electrostatic repulsion hydrophilic interaction chromatography (ERILIC). Among them, MOAC is the most extensively used method. The mechanism of MOAC enrichment is based on Lewis acid and alkali theory; under acidic conditions, metal oxides act as Lewis acids and capture Lewis basic phosphopeptides, and the phosphopeptides can be released under basic conditions. Various metal oxides such as TiO₂, ZrO₂, Al₂O₃ and SnO₂ have been developed for phosphopeptide enrichment. They showed different affinity properties for phosphopeptides. TiO₂ is the most commonly used material due to its high enrichment efficiency and specificity. When enriching phosphopeptides with MOAC, acidic amino acids can also interact with the metal ion and result in non-specific enrichment. Low pH can suppress ionization of the acidic residues, and thus reduce non-specific adsorption. In addition to low pH, additives such as lactic acid, glycolic acid or 2,5-dihydroxybenzoic acid were also added to the loading buffer to reduce non-specific binding of acidic peptides.

Recently, mesoporous metal oxide nanoparticles were utilized in phosphopeptide enrichment. The dimensions of the nanomaterials were in the range of 10–1000 nm. With a channeled structure and high surface area, the nanoparticles showed high efficiencies in phosphopeptide enrichment.
Compared with solid core metal oxide microspheres, nanoparticles possess many advantages such as lower cost, higher loading capacity and comparable enrichment efficiency. In this experiment, we evaluated three types of mesoporous metal oxide nanospheres, ZnSn(OH)₆, P₀.₅–Ti-40 and P₀.₅–Ti-50, for their efficacy in phosphopeptide enrichment. ZnSn(OH)₆ was synthesized using ZnCl₂, 5H₂O, SnCl₄·5H₂O and anhydrous ethanol, and SEM images revealed that ZnSn(OH)₆ has a cubic structure with a diameter of ~1–2 μm (Fig. 1a). P₀.₅–Ti-40 and P₀.₅–Ti-50 were synthesized by a sol–gel method using tetrabutyl titanate and ethanol at different aging temperatures. SEM images showed that both P₀.₅–Ti-40 and P₀.₅–Ti-50 had a granulated cluster structure, and the diameter of the particles was less than 1 μm (Fig. 1b and c).

When evaluating the performance of phosphopeptide enrichment materials, most reports used a β-casein digest as a model system and complex samples, like a cell lysate or total protein digest, were rarely used. Such a simple single protein digest lacks the complexity for comprehensive evaluation of enrichment materials. In this study, we used Arabidopsis thaliana leaf protein digest as a model in addition to β-casein digest and evaluated the enrichment efficiency of the three types of mesoporous nanoparticles. The complexity of Arabidopsis thaliana leaf protein provides an excellent model for the evaluation of such materials in a close to reality environment. Experimental conditions such as the composition and concentration of the loading buffer and peptide to bead ratios were optimized to achieve the highest level of enrichment efficiency and specificity. The results were compared with those of commercially available materials such as TiO₂, ZnSn(OH)₆, SnCl₄·5H₂O and the PolyMAC-Ti reagent. After optimization, the enrichment capabilities of these three types of nanoparticles were comparable with those of commercially available materials. Thus, we evaluated a series of mesoporous metal oxide nanomaterials for phosphopeptide enrichment from complex protein samples. The results showed that the nanoparticles can be used as a low cost alternative to commercial beads with comparable enrichment efficiency.

2. Materials and methods

2.1 Chemicals and reagents

β-Casein from bovine milk (98%), 2,5-dihydroxybenzoic acid (DHB, 98%), β-dithiothreitol (DTT, 99%), iodoacetamide (IAM, 99%), lactic acid, glycolic acid solution, and ammonium bicarbonate (ABC, 99%) were purchased from Sigma. KCl (99.5%), NaOH (96%), sucrose, and ammonium acetate were obtained from Sinopharm Chemical Reagent Co. Ltd. Protease inhibitors were from Roach. Sequence grade trypsin was supplied from Promega. The PolyMAC-Ti phosphopeptide enrichment Kit, Titansphere phos-TiO Kit and Tiansphere phos-TiO bulk material were purchased from Tymora-Analytical and GL Sciences. Chromatographic grade water and acetonitrile (ACN) were purchased from Fisher Scientific. HPLC grade trifluoroacetic acid (TFA) and formic acid were obtained from Dikma Technology.

2.2 Plant materials

Arabidopsis thaliana wild type Columbia-0 seeds were disinfected with 2% NaClO and 70% ethanol and vernalized at 4 °C for 3 days. The seeds were transferred to 1/2 Murashige and Skoog medium, and cultured at 23 °C under 24 h illumination, 70% humidity and a light intensity of 50 μM m⁻² s⁻¹ for two weeks. The leaves were collected and stored at −80 °C until protein extraction.

2.3 Protein extraction and digestion

Protein extraction was adopted from a published method with slight modification, i.e. 2 g Arabidopsis thaliana leaves were ground to fine powder with a mortar in liquid nitrogen. 6 mL extraction buffer (500 mM Tris–HCl, 500 mM EDTA, 700 mM sucrose, 100 mM KCl, pH 8.0, 1% protease inhibitor cocktail, 1% phospho-STOP) was added and ground for 10 min. 6 mL Tris–phenol was added and ground for another 10 min. The phenol layer was collected after centrifugation and protein was precipitated with 0.1 M ammonia acetate in methanol. The protein precipitate was further washed with cold acetone and dried under a vented hood, and the dried protein was stored at −20 °C.

The protein was dissolved in 7 M urea and 2 M thiourea. The concentration of the protein solution was measured by the Bradford assay. The protein was digested using a modified FASP method. In short, the protein solution was loaded onto an ultrafiltration device (30 000 MWCO, 500 μL, Sartorius, Germany), washed with 50 mM NH₄HCO₃ (ABC), reduced with 200 mM DTT at 56 °C and alkylated with 200 mM iodoacetamide in the dark. The protein was digested with trypsin at 37 °C overnight (enzyme : protein ratio = 1 : 50). The digested peptides were lyophilized and stored at −20 °C. 50 μg β-casein
was dissolved in 50 mM ABC and digested with 1 μg trypsin at 37 °C overnight.

2.4 Phosphopeptide enrichment

The nanomaterials were washed with washing buffer (80% ACN, 2% TFA) and loading buffer (washing buffer with additives) twice prior to use. Peptide samples were dissolved in loading buffer and mixed with the nanospheres. After incubation with mild agitation for 30 min, the nanoparticles were collected by centrifugation. Non-phosphopeptides were removed by washing the nanoparticles with loading buffer and washing buffer twice. 5% ammonium hydroxide was added and incubated for 10 min to elute the bonded phosphopeptides. The collected peptides were dried in a SpeedVac and dissolved in 0.1% FA for nano LC-MS analysis.

Enrichment using TiO₂-tips/microbeads (GL Sciences) or PolyMAC-Ti agarose beads (Tymora Analytical) was performed according to the manufacturers’ protocols. The phosphopeptides were dried and reconstituted in 0.1% FA for nano LC-MS analysis.

2.5 Mass spectrometry analysis

A Bruker Daltonics Autoflex II MALDI-TOF mass spectrometer (Framingham, MA, USA) was used for MALDI-TOF analysis. 1 μL peptides and 1 μL matrix (α-cyano-4-hydroxycinnamic acid, Sigma, 1 mg mL⁻¹ in 50% acetonitrile and 0.1% TFA) were mixed on a stainless steel MALDI plate. Positive ions in the mass range of m/z 500 to 5000 were collected. 500 laser shots were accumulated from each sample spot. Flexanalysis 2.2 was used for data analysis.

Nano LC-MS analysis was performed on a Waters nanoAcquity nano HPLC (Milford, MA, USA) coupled with a Thermo Q-Exactive high resolution mass spectrometer (Thermo Scientific, Waltham, MA, USA). 7 μL sample was loaded onto a 100 μm I.D. fused silica capillary trap column filled with 2 cm of C18 stationary phase (Aqua C18, 5 μm, 125 Å Phenomenex, Torrance, CA, USA). The analytical column was a 50 μm I.D. fused silica capillary filled with 10 cm of C18 stationary phase (Aqua C18, 3 μm, 125 Å, Phenomenex). A 95 min gradient was used to elute the peptides. Mobile phase A was 0.1% FA in water, B was 0.1% FA in acetonitrile, and the flow rate was 200 nL min⁻¹. The nano ESI spray voltage was 2.0 kV and a full mass scan in the range of m/z 300 to 1800 was obtained with a resolution of 70 000 at m/z 200. The 10 most intensive peptide signals from the full scan were selected for the MS/MS scan at a resolution of 17 500 at m/z 200, and the dynamic exclusion time was 10 s.

2.6 Data analysis

Raw data were preprocessed with Mascot Distiller 2.4 for peak picking and searched with the Mascot 2.5 search engine (Matrix Science, London, UK) using the Arabidopsis database TAIR 10. Trypsin was selected as the specific enzyme with two missed cleavages. Fixed modification was carbamidomethyl (C). Oxidation (M) and phosphorylations (S, T, and Y) were set as variable modifications. MS mass tolerance was 10 ppm and MS/MS mass tolerance was 0.02 Da. A decoy database was used and peptides were filtered at a 1% false discovery rate (FDR). Peptides with Mascot score higher than 30 were selected for further analysis. Scaffold PTM (Proteome Software, USA) was used to analyze phosphorylation sites using the Ascore algorithm with a score threshold of 13.²⁵

3. Results and discussion

3.1 Preliminary evaluation of phosphopeptide enrichment

β-Casein is commonly used in evaluating phosphopeptide enrichment materials. Here, we also used the β-casein digest to obtain a preliminary view of the enrichment capability of the three types of nanomaterials. 80% ACN/2% TFA was used as the loading buffer with a peptide to particle ratio of 1 : 5. MALDI-TOF results showed that the raw digest of β-casein had very weak signals corresponding to phosphopeptides and strong signals from non-phosphorylated peptides (Fig. 2). After enrichment with ZnSn(OH)₆, the mono-phosphopeptide (FQpSEEQQTQDELDQK) dominated the mass spectrum at m/z 2061, but multi-phosphopeptides were not observed (Fig. 2). In the case of P₀.₅–Ti-40 and P₀.₅–Ti-50, both the mono-phosphopeptide and multi-phosphopeptides (REELNVPGEIVEpS₉₅pSpS₉EESTR) were captured (Fig. 2). The three types of nanoparticles showed good sensitivity towards β-casein; with ZnSn(OH)₆, phosphopeptides from 4 pmol of β-casein digest can be detected by MALDI-TOF MS (Fig. S1†). While for P₀.₅–Ti-40 and P₀.₅–Ti-50, 40 fmol of β-casein digest can still be detected (Fig. S2†). These preliminary results demonstrated that the three types of nanoparticles had selectivity towards phosphopeptides, but non-phosphorylated peptides can still be enriched with them, and thus further optimization of the enrichment conditions is needed to assess the full potential of these nanoparticles.

Tryptic digest of Arabidopsis thaliana leaf protein was used to further assess the enrichment capability of the nanoparticles. Arabidopsis leaf protein provides a better model than β-casein due to its complexity and closeness to day-to-day samples. To simplify the experimental process, we used a loading buffer containing only 80% ACN and 2% TFA, and the ratio between peptides and nanoparticles was set at 1 : 1. Under such conditions, the performances of the three types of nanoparticles were not satisfactory. With ZnSn(OH)₆, only 721 phosphoproteins were identified, while for P₀.₅–Ti-40 and P₀.₅–Ti-50, the numbers were even lower at 503 and 331 phosphoproteins respectively. Thus a simple loading buffer containing only ACN with TFA and a 1 : 1 sample to nanoparticle ratio cannot unleash the full potential of the mesoporous nanoparticles. Optimization of the enrichment conditions is needed to improve the enrichment efficiency of the nanoparticles.

The three types of nanoparticles had different core metal ions, were synthesized under different reaction conditions, so the chemical/physical properties were distinct.²⁶,²⁷ Given the chemical and physical properties of the nanoparticles, the composition of the loading buffer and the ratio between samples and the nanoparticles are two major factors that may affect enrichment efficiency. The addition of acidic modifiers can reduce non-specific adsorption of acidic peptides,¹³,¹⁴ while
the sample to particle ratio can impact binding selectivity. Thus the two conditions were further optimized.

### 3.2 Effect of loading buffer composition on enrichment efficiency

As shown in Section 3.1, when 80% ACN/2% TFA was used as the loading buffer, the three types of nanomaterials can capture phosphopeptides from simple sample matrices such as β-casein digests. However, when enriching phosphopeptides from complex sample matrices, such as *Arabidopsis* leaf protein digests, such a loading buffer was not capable of dealing with interference from non-specific adsorption of acidic peptides and resulted in poor enrichment efficiency. DHB, lactic acid, and glycolic acid with various concentrations were added to the loading buffer to enhance the selectivity of the nanoparticles. It can be seen that different additives had different effects on the three types of nanoparticles (Fig. 3A). For ZnSn(OH)_6, DHB had higher enrichment efficiency; with DHB in the loading buffer, 37.1% of the detected peptides were phosphopeptides; while for lactic acid and glycolic acid, this value was only 2.2% and 2.9% respectively. For P_{0.5-Ti-40} and P_{0.5-Ti-50}, DHB was not as good as lactic acid or glycolic acid, as only 18.5% and 25.4% of the peptides detected were phosphopeptides with DHB. Lactic acid and glycolic acid showed similar enrichment efficiency for P_{0.5-Ti-40} and P_{0.5-Ti-50}. For P_{0.5-Ti-40}, 52.2% of the detected peptides were phosphopeptides with lactic acid and 54.2% with glycolic acid; while for P_{0.5-Ti-50}, the values were 44.5% and 46.0% respectively.

On the basis of optimized loading buffer, we tried various concentrations of the additives to further improve binding of phosphopeptides. The concentrations of DHB were 50 mg mL\(^{-1}\), 80 mg mL\(^{-1}\) and 100 mg mL\(^{-1}\). The concentrations of lactic acid were 25%, 35% and 40%. The concentrations of glycolic acid were 21%, 26% and 35%. For ZnSn(OH)_6, increased DHB concentration resulted in increased enrichment efficiency. However, 100 mg mL\(^{-1}\) DHB not only decreased binding of non-phosphopeptides, but also decreased the absorption of phosphopeptides. So we chose 80 mg mL\(^{-1}\) DHB as the final additive in the loading buffer for ZnSn(OH)_6 (Fig. 3B).

For P_{0.5-Ti-40} and P_{0.5-Ti-50}, lactic acid and glycolic acid showed similar results in eliminating the adsorption of non-phosphopeptides: increased concentration of additives resulted in an increased level of phosphopeptide enrichment. When 40% lactic acid was added to the loading buffer, the percentages of phosphopeptides were 60.1% and 63%, and the percentages of phosphoprotein were 65.7% and 71% for P_{0.5-Ti-40} and P_{0.5-Ti-50}, respectively. When 35% glycolic acid was used, the percentages of phosphopeptides were 68.2% and 56% and the percentages of phosphoproteins were 74.3% and 63.6%, respectively (Fig. 3B). So 40% lactic acid and 35% glycolic acid in 80% ACN/2% TFA were chosen as the final loading buffers for P_{0.5-Ti-40} and P_{0.5-Ti-50}.

### 3.3 Peptide-to-nanomaterial ratio also affects enrichment efficiency

When enriching phosphopeptides with TiO_2 microbeads, the ratio between peptides and enrichment materials is generally in the range of 1 : 2 and 1 : 8. That is, the amount of TiO_2 material is in excess of peptides. In contrast to conventional TiO_2 beads, nanoparticles have mesoporous structure, higher surface area, and more binding sites, and thus more phosphopeptides were needed to saturate the binding sites. Thus, the ratios between peptides and nanoparticles were set at 1 : 1, 2 : 1, and 3 : 1, and 250 μg of nanoparticles was used for each enrichment experiment with varying amounts of *Arabidopsis* leaf protein digest. The results showed that different materials had different loading capacities and increasing the amount of protein won’t always increase the recovery of phosphopeptides. For ZnSn(OH)_6, the optimal peptide/nanomaterial ratio was 2 : 1. Under this

![Fig. 2](image-url)  
MALDI-TOF MS results of enrichment of β-casein with the three types of nanoparticles. 1P represents the mono-phosphopeptide FQPSEEQQQTEDELQDK. Multi-phosphopeptides were REELNVPGEIVEpSpSpSpSEESITR.
condition, 1384 phosphoproteins and 2925 phosphopeptides can be detected. For $P_{0.5}$–Ti-40 and $P_{0.5}$–Ti-50, the optimal ratio was 1 : 1. 1088 phosphoproteins and 2979 phosphopeptides were captured with $P_{0.5}$–Ti-40, and 1086 phosphoproteins and 2474 phosphopeptides were detected with $P_{0.5}$–Ti-50 (Fig. 4).

3.4 Robustness of the nanoparticles in phosphopeptide enrichment

After optimization, the optimal enrichment conditions for ZnSn(OH)$_6$ were as follows: ratio between peptides and materials: 2 : 1 and loading buffer: 80 mg mL$^{-1}$ DHB in 80% ACN with 2% TFA. For $P_{0.5}$–Ti-40 and $P_{0.5}$–Ti-50: peptide-to-nanoparticle ratio: 1 : 1 and loading buffer: 40% lactic acid or 35% glycolic acid in 80% ACN with 2% TFA. Under such conditions, three technical replicates were carried out to evaluate the reproducibility of the methods. A 250 μg Arabidopsis leaf protein digest was used for each replicate. Unique phosphopeptides and phosphosites were analyzed and validated by using Scaffold PTM software. For ZnSn(OH)$_6$, a total of 1611 phosphoproteins were identified, corresponding to 3557 phosphopeptides and 3826 phosphosites, and among them, 820

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Fig. 3  (A) The number of phosphopeptides with the three types of nanoparticles using different loading buffers. (B) Distribution of peptides with three types of nanoparticles using different additives.
phosphoproteins were identified in all three replicates. When 40% lactic acid was used as the loading buffer, for P₀.₅-Ti-40, 1474 phosphoproteins were identified with 3850 phosphopeptides and 4589 phosphosites and 785 phosphoproteins were identified in all three replicates. For P₀.₅-Ti-50, the total number of phosphoproteins was 1449 with 3454 phosphopeptides and 3872 phosphosites. 803 phosphoproteins were identified in all three runs. Glycolic acid resulted in similar results with slightly lower reproducibility (Table S1†). The detected phosphopeptides

![Fig. 4](image1.png)

**Fig. 4** Number of phosphopeptides detected with the three types of nanoparticles at different peptide-to-bead ratios.

![Fig. 5](image2.png)

**Fig. 5** (A) Number of phosphorylation sites per phosphopeptide with the three types of nanoparticles. (B) Two motifs were enriched with the nanoparticles.
and phosphosites with the three types of nanoparticles are presented in the ESI.†

We analyzed the number of phosphorylation sites per phosphopeptide with the three types of nanoparticles using scaffold PTM software (Fig. 5A). It can be seen that phosphopeptides with a single phosphorylation site dominated the enrichment, especially for ZnSn(OH)₆, while P₀.₅–Ti-40 and P₀.₅–Ti-50 captured more multiple phosphorylated peptides than ZnSn(OH)₆. We also mapped the motif distribution of the phosphorylation sites (Table 1), and SP and RxxS were two major types of motifs with all nanoparticles suggesting that the three types of nanoparticles had no preference towards a certain type of motif of phosphopeptides (Fig. 5B).

### 3.5 Comparison with commercially available materials

Three commercially available phosphopeptide enrichment materials were also tested with the Arabidopsis leaf protein digest and the results were compared with those of the nanoparticles. The three commercial materials included pre-packed TiO₂-tips (GL science, 3 mg 200 μL), TiO₂ powder (3 mg for each replicate, GL science) and PolyMAC-Ti. These materials all used Ti as the core metal ion to capture phosphopeptides. PolyMAC-Ti used a unique strategy to capture phosphopeptides from solution using a dendrimer based polymer material to further enhance specificity and efficiency.²⁹ All experiments used a 250 μg protein digest, with TiO₂-tips, 1847 phosphoproteins and 4807 phosphopeptides were detected. For the TiO₂ powder, we identified 4171 phosphopeptides from 1699 phosphoproteins. 932 phosphoproteins and 3801 phosphopeptides were captured with PolyMAC-Ti. The number of phosphoproteins identified with the three types of nanoparticles was slightly lower than that with TiO₂-tips or TiO₂ powder, but higher than that with PolyMAC-Ti, and the number of phosphopeptides with the three types of nanoparticles was slightly lower than the three benchmarks, but the overall percentages of phosphoproteins in all detected proteins were comparable when using the three types of nanoparticles (Fig. 6). So, after optimization, the three nanomaterials can be utilized in complex protein digests for selective enrichment of phosphopeptides. The three types of nanoparticles were easy and economical to synthesize and much less materials were needed to process the same amount of protein digest, and thus, the mesoporous nanoparticles provide an economical and efficient alternative to commercially available phosphoprotein enrichment materials.

### 4. Conclusion

In this study, three types of mesoporous nanoparticles were applied to enrich phosphopeptides from an Arabidopsis leaf protein digest. The nanospheres showed high specificity in phosphopeptide enrichment. To improve the coverage of phosphoproteins, a series of optimization processes were carried out, which included optimization of the peptide-to-bead ratio and different additives in loading buffer. After optimization, the results were compared with those of three commercially available phosphopeptide enrichment materials, and all the nanoparticles showed comparable enrichment efficiency with much less enrichment materials used per experiment. The nanomaterials can be synthesized at a much lower cost, which greatly reduces the cost of phosphopeptide enrichment. The results showed that mesoporous metal oxide nanoparticles are promising materials for high-throughput phosphoproteomic research with high specificity, high efficiency and low analysis cost.

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### Notes and references
