The synthesis of Ti-hexagonal mesoporous silica for selective capture of phosphopeptides†

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Ti-hexagonal mesoporous silica (Ti-HMS) with high titanium content has been synthesized. The Ti-HMS has been applied as a potential adsorbent for the selective capture of phosphopeptides, due to the strong affinity interaction between the incorporated titanium in the framework and the phosphoryl groups of phosphopeptides.

Reversible protein phosphorylation is one of the most important protein post-translational modifications, which affects an estimated one-third of whole proteins and plays a crucial role in the regulation of cellular processes of signal transduction, cell proliferation, differentiation and transcription. The examination of phosphorylated proteins has attracted great interests in various fields not only because of their importance but also the difficulty in the analysis of phosphorylated proteins resulting from their low abundance and substoichiometry. Specific capture of the phosphorylated proteins and peptides is an effective approach for the analysis of phosphorylated proteins. Immobilized metal affinity chromatography (IMAC) adsorbents with immobilization of Fe³⁺, Ga³⁺, Ni²⁺ and Ti⁴⁺ or Zr⁴⁺ have been widely utilized for the enrichment of phosphopeptides in phosphoproteome analysis. The weakness of IMAC enrichment is the relatively complex process along with the preloading of metal ions on the IMAC materials and the possible consequent loss of preloaded metal ions from IMAC materials. Metal oxides, such as TiO₂ and ZrO₂, have been also used for the enrichment of phosphopeptides. Due to the strong surface Lewis acidity of these metal oxides, the possible consequent loss of preloaded metal ions from their low abundance and substoichiometry. Ti⁴⁺ or Zr⁴⁺ have been widely utilized for the enrichment of phosphopeptides.

Specific capture of the phosphorylated proteins and peptides is an effective approach for the analysis of phosphorylated proteins. Immobilized metal affinity chromatography (IMAC) adsorbents with immobilization of Fe³⁺, Ga³⁺, Ni²⁺ and Ti⁴⁺ or Zr⁴⁺ have been widely utilized for the enrichment of phosphopeptides in phosphoproteome analysis. The weakness of IMAC enrichment is the relatively complex process along with the preloading of metal ions on the IMAC materials and the possible consequent loss of preloaded metal ions from IMAC materials. Metal oxides, such as TiO₂ and ZrO₂, have also been used for the enrichment of phosphopeptides. Due to the strong surface Lewis acidity of these metal oxides, the selectivity for phosphopeptides analysis was found to be highly dependent on the solvents used in loading and washing steps. Using additive acids including 2,5-dihydroxybenzoic acid (DHB), β-hydroxypropanoic acid, phthalic acid and glutamic acid in loading and washing buffer were able to improve the chemoselectivity of phosphopeptide enrichment, though these additives also complicated the LC-MS analysis.

Since the early 1990s, mesoporous materials have attracted intense research attentions and exhibited great potential as catalysts, adsorbents, sensors and nanodevices due to the large surface-to-volume ratio, well-defined pore structure, narrow pore size distribution and chemical versatility. Hexagonal mesoporous silica (HMS), synthesized via the hydrogen-bonding interaction (SHT⁴⁻) assembly pathway, shows small domain size, short channels, large textural mesoporosity and high thermal stability. These distinguishing features of HMS could provide better transport channels for molecules to access the active sites inside the materials. The incorporation of metal ions (i.e. titanium) in the framework of HMS would not only offer the metal affinity to phosphopeptides, but also provide lower Lewis acidity as compared to TiO₂, which would in turn give an appropriate chemoselectivity for Ti-HMS to circumvent the nonspecific adsorption of acidic peptides on TiO₂ in phosphoproteome analysis.

Ti-HMS samples with lower and relatively higher Ti-content (2 and 8 mol%) were synthesized in this work for the capture of phosphorylated peptides. Small-angle XRD patterns of the synthesized Ti-HMS materials exhibit single diffraction peaks corresponding to the (100) plane at 2Θ of 1.8° (ESI, † Fig. S1), which represent the typical characteristic of HMS materials with short-range hexagonal symmetry. It can also be observed that the diffraction intensity of Ti-HMS-008 is slightly weaker than that of Ti-HMS-002, which indicates that the increase of Ti content has partially decreased the mesoporosity of Ti-HMS-008. Additionally, the wormhole mesoporous structures of these HMS materials were observed by TEM (ESI, † Fig. S2).

Fig. 1 shows the N₂ adsorption–desorption isotherm and pore size distribution curve of the synthesized Ti-HMS. Fig. 1(a) exhibits the type IV isotherms with hysteresis loops for Ti-HMS-008 and Ti-HMS-002 as defined by IUPAC for mesoporous materials. An abrupt increase of P/P⁰ from 0.40 to 0.60 (Fig. 1(b)) was clearly observed, suggesting uniform pore size distributions of both materials. The BET surface area, pore volume and pore radius for Ti-HMS-002 are 925 m² g⁻¹, 2.02 cm³ g⁻¹ and 3.4 nm, respectively, while the corresponding values for Ti-HMS-008 are 662 m² g⁻¹, 0.89 cm³ g⁻¹ and 3.8 nm, respectively. The apparent decrease of surface area and pore volume manifest that the free channel space was reduced for Ti-HMS-008 due to the increase of Ti content.

To investigate the coordination geometry of titanium ions incorporated in Ti-HMS, UV-Vis diffuse reflectance spectra (UV-Vis DRS) characterization was carried out. The appearance of a band near 210 nm were observed in the spectra (ESI, † Fig. S3), which is usually assigned to the electron transfer from oxygen ligands to tetra-coordinated Ti (iv) ions. This result indicates that the majority of titanium ions are coordinated within the framework of HMS. In addition, absorption bands in the region of 250 and 300 nm emerge for both materials,
intensities of bands at 459 and 1052 cm$^{-1}$ in the obtained UV-Raman spectra (ESI, Fig. S4), the intensities of bands at 459 and 1052 cm$^{-1}$ of Ti-HMS-008 are much stronger than those of Ti-HMS-002, which are attributed to the contribution of Si–O–Ti asymmetric stretching vibration of isolated titanium species in silica framework.

To examine the performance of the Ti-HMS material in the selective capture of phosphopeptides with the interference of abundant nonphosphorylated peptides, the tryptic digest of bovine $\beta$-casein (1 pmol, 1 $\mu$l) by (a) direct analysis and enrichment by (b) HMS, (c) Ti-HMS-002 and (d) Ti-HMS-008. Peaks $\beta_1$, $\beta_2$ and $\beta_3$ represent the phosphopeptides with m/z of 2062.42, 2556.28 and 3121.24, respectively. The peak intensities of $\beta_1$, $\beta_2$ and $\beta_3$ in (d) are increased ca. 5.8, 3.8 and 5.2 fold, respectively, as compared with (a).

Fig. 2 MALDI-TOF mass spectra of tryptic digest of $\beta$-casein (1 pmol, 1 $\mu$l) by (a) direct analysis and enrichment by (b) HMS, (c) Ti-HMS-002 and (d) Ti-HMS-008. Peaks $\beta_1$, $\beta_2$ and $\beta_3$ represent the phosphopeptides with m/z of 2062.42, 2556.28 and 3121.24, respectively. The peak intensities of $\beta_1$, $\beta_2$ and $\beta_3$ in (d) are increased ca. 5.8, 3.8 and 5.2 fold, respectively, as compared with (a).

which are attributed to the presence of octahedral coordination of titanium ions. Moreover, no significant absorption bands around 300–350 nm are observed for either material, indicating the absence of segregated TiO$_2$ anatase phase. Furthermore, UV-Raman spectroscopy has also been carried out to identify the titanium atoms in the framework of Ti-HMS. As shown in the obtained UV-Raman spectra (ESI, Fig. S4), the intensities of bands at 459 and 1052 cm$^{-1}$ of Ti-HMS-008 are much stronger than those of Ti-HMS-002, which are attributed to the contribution of Si–O–Ti asymmetric stretching vibration of isolated titanium species in silica framework and manifest that the amount of isolated titanium species in the silica framework increases with the increase of Ti content.

Owing to the well known good metal affinity of titanium towards phosphoryl groups we expect that the Ti-HMS would provide the desirable specificity toward phosphopeptides due to the presence of the titanium in the framework. To examine the specificity of Ti-HMS in trapping phosphopeptides, the tryptic digest of bovine $\beta$-casein (with five known phosphorylation sites) was selected as the standard phosphoprotein. Fig. 2 shows the MALDI-TOF mass spectra of the tryptic digest of $\beta$-casein (1 pmol) obtained by direct analysis and after enrichment by HMS (without Ti-incorporation) and Ti-HMS materials (Ti-HMS-002 and Ti-HMS-008). As shown in Fig. 2(a), peaks of phosphorylated peptides are merged with the peaks of nonphosphorylated peptides arising from the tryptic digestion of $\beta$-casein by direct analysis (i.e. directly spotting the sample on a plate without the enrichment treatment by Ti-HMS). For isolating the phosphopeptides from the nonphosphopeptides, we first attempted to utilize the HMS material as the adsorbent to capture the phosphopeptides. As presented in Fig. 2(b), there are no peaks detected that can be assigned to the corresponding peptides after washing procedures for HMS treatment, which indicates that HMS itself does not possess the ability to capture either phosphorylated or nonphosphorylated peptides. Interestingly, when using the Ti-HMS-002 as the adsorbent, there is a peak ($\beta_3$) detected by the MALDI-TOF-MS (Fig. 2(c)), which corresponds to the tetraphosphorylated peptide of $\beta$-casein with m/z at 3122.56. This result indicates that the incorporated titanium in the framework of Ti-HMS demonstrates affinity toward the phosphorylated peptide, due to the specific coordination occurring between the incorporated titanium and the phosphoryl group of phosphopeptides. However, the peak ($\beta_3$) detected in this MALDI-TOF mass spectrum is fairly weak in intensity, which can be attributed to the low content of titanium incorporated in the silica framework of Ti-HMS-002.

Based on this investigation, Ti-HMS-008 with higher titanium content was thus applied in the capture of phosphorylated peptides. The obtained MALDI-TOF mass spectrum is illustrated in Fig. 2(d). Compared to Fig. 2(c), three significant peaks of $\beta_1$, $\beta_2$ and $\beta_3$ were detected with higher intensities. These three peaks of $\beta_1$, $\beta_2$ and $\beta_3$ can be identified as the phosphopeptides of bovine $\beta$-casein with amino acid sequence pattern of $\text{FQ}[\text{PS}]\text{EEQQTEDELQK}$ ($m/z$, 2061.94), $\text{FQ}[\text{PS}]\text{EEQQTEDELQDKHPF}$ ($m/z$, 2556.93) and $\text{RELEELNVPGEIV}[\text{PS}]\text{L}[\text{PS}]\text{[PS]}\text{EESITR}$ ($m/z$, 3122.56), respectively. The enhanced peak intensities of the corresponding phosphopeptides is due to the contribution of the increased titanium content in the framework of Ti-HMS-008 as compared to Ti-HMS-002, which further confirmed the specificity of Ti-HMS materials to phosphopeptides.

To examine the performance of the Ti-HMS material in the selective capture of phosphopeptides with the interference of abundant nonphosphorylated peptides, the tryptic digest of a mixture of $\beta$-casein and BSA with a molar ratio of 1 : 50 via direct analysis by a MALDI-TOF mass spectrometer, where the abundant nonphosphorylated peptides dominate the spectrum and make the identification of the phosphorylated peptides of $\beta$-casein
impossible. After the enrichment by the Ti-HMS material, the characteristic phosphorylated peptides (β₁, β₂ and β₃) of the β-casein could be clearly detected (Fig. 3(b)), while the nonphosphorylated peptides of the mixture disappeared. Even when the molar ratio of β-casein to BSA was increased up to 1 : 100 (Fig. 3c), these phosphopeptides of β-casein could be detected, though with a lowering of the peak intensity. These results further indicated the reliable performance of the Ti-HMS material in the selective capture of phosphopeptides from a complex peptide mixture.

Inspired by the selective capture of phosphopeptides from the tryptic digest of β-casein with five known phosphorylation sites, we further applied the Ti-HMS-008 in the selective capture of phosphopeptides from the tryptic digest of α-casein, a standard phosphoprotein with more phosphorylation sites. The MALDI-TOF mass spectra (ESI† Fig. S5) of the tryptic digest of α-casein were obtained, where panels (a) and (b) (ESI† Fig. S5) show MALDI mass spectra of the tryptic digest of α-casein (1 pmol, 2 μl) with and without the treatment of Ti-HMS-008. By comparing panel (a) and (b) (ESI† Fig. S5), it is clearly seen that the phosphopeptides can be distinctly isolated from the strong background of nonphosphopeptides after the treatment of Ti-HMS-008.

It can be concluded that the Ti-HMS materials showed specific affinity toward phosphorylated peptides, which provides great potential in the specific capture of phosphopeptides. Compared to the IMAC materials, this composite Ti-HMS material does not need the preloading of metal ion while the possible loss of the preloaded metal ion from IMAC materials could be avoided. In addition, the non-specific adsorption caused by Lewis acid interaction can be reduced as compared with the metal dioxide affinity adsorbents such as (TiO₂) (ESI† Fig. S6; Table S1).

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Notes and references

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