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Zeolite nanoparticles with immobilized metal ions: isolation and MALDI-TOF-MS/MS identification of phosphopeptides†

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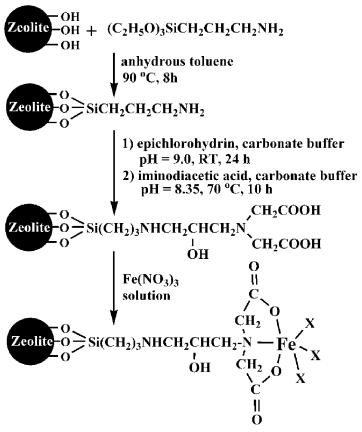
Metal-ion-immobilized zeolite nanoparticles have been applied for the first time to isolate phosphopeptides from tryptic β-casein digest; the phosphopeptides enriched on the modified zeolite nanoparticles could be effectively identified by MALDI-TOF-MS/MS.

Phosphorylation, the most important and ubiquitous post-translational modification of proteins, is able to regulate almost all aspects of cell life in both prokaryotes and eukaryotes.[1] Widespread interest in protein phosphorylation has led to the development of methods to map phosphorylation sites of proteins. The existing preferred approach for phosphorylation site mapping mainly relies on the use of tandem mass spectrometry to sequence individual peptides after proteolysis. However, this method still remains challenging because the signals for phosphorylated peptides are strongly suppressed by other abundant peptides contained in protein digests during positive mass spectrometric analysis.[2] Therefore, the separation of phosphorylated peptides from unphosphorylated ones is highly desirable. Immobilized metal ion affinity chromatography (IMAC) has been often used for the selective enrichment of phosphopeptides from proteolytic digest mixtures.[3,4] In this method, the acidic phosphopeptides were retained by binding to the metal ion (usually Fe^{3+} or Ga^{3+}) immobilized on a porous resin, while other peptides were eluted.[3] One of the advantages of the method is that the phosphopeptides on IMAC beads could be directly detected by MALDI (matrix-assisted laser desorption ionization)-TOF (time of flight)-MS (mass spectrometry), avoiding possible sample loss during the elution process.[4] However, because of the unavoidable "shadow effect"[5] of the porous resin beads at the micron scale, most of the phosphopeptides bound in the pores would be probably inaccessible during the direct laser desorption. One strategy to solve this problem is to make all the chelation occur on the surface of a carrier with a large external surface area. Nanoscale zeolite particles should be a promising candidate because they not only provide a large external surface area but can also be easily modified for the purpose of effective binding with target species. Moreover, due to their high dispersibility and small size, the zeolite nanoparticles could disperse easily in various peptide/protein solutions of any volume to facilitate the chelation process and subsequent identification of the species bound to their surface. Herein, utilizing their easy modifiability of surface hydroxyl groups, zeolite-beta nanoparticles with immobilized Fe^{3+} ions were employed to enrich and isolate phosphopeptides from tryptic β-casein digest, and the resulting phosphopeptides chelated on zeolite nanoparticles could be directly identified by MALDI-MS/MS. Because the phosphopeptides are only chelated on the external surface of the zeolite nanoparticles this is expected to facilitate desorption/ionization of phosphopeptides relative to commercial porous IMAC beads.

Zeolite-beta nanoparticles were hydrothermally synthesized according to the literature,[6] and the size of obtained zeolite particles is below 100 nm. The immobilization of Fe^{3+} ions on the surface of zeolite nanoparticles was achieved by a multi-step reaction (Scheme 1). The surface of zeolite nanoparticles was activated by 3-aminopropyltriethoxysilane in toluene solution, and then the metal chelating ligand (iminodiacetic acid) was coupled on the surface-activated zeolite *via* epichlorohydrin. Finally, Fe^{3+} ions were immobilized by chelating with iminodiacetic acid. The SEM images (ESI†) of zeolite nanoparticles before and after immobilizing metal ions show that this covalent modification has no influence on the morphology and dispersibility of nanoparticles. In the FT-IR spectrum (ESI†) of the Fe^{3+} ion-immobilized zeolite nanoparticles (Fe^{3+}-nanozeolites), the absorptions at 1604 and 1458–1394 cm^{-1}, which can, respectively, be assigned to antisymmetric and symmetric stretching vibration of carboxylates, imply that metal ions have been successfully immobilized on the zeolite nanoparticles. EDS analysis (ESI†) verifies the existence of Fe on the zeolite nanoparticles.

An Fe^{3+}-nanozeolite suspension (10 μL; 3 mg mL^{-1}) was added to 0.2 mL of 0.2 nmol mL^{-1} tryptic β-casein digest, and vibrated for 90 min at 37 °C. After centrifugation and redispersion, the slurry of phosphopeptide-chelating Fe^{3+}-nanozeolites was mixed with the MALDI matrix (α-cyano-4-hydroxycinnamic acid) solution, and finally the mixture was applied onto the MALDI plate for the purpose of MS analysis. For comparison, the tryptic β-casein digest without any prefractionation was also analyzed by MALDI-TOF-MS. Only a weak signal of phosphopeptide (*m*/*z* 2061.8) was observed without any pre-separation process (Fig. 1(a)). After isolation by Fe^{3+}-nanozeolites (Fig. 1(b)), the most prominent signal in the MS is that of phosphopeptide (*m*/*z* 2061.9), while two other signals at *m*/*z* 1967.3 and 1981.9 could be assigned to dephosphorylated fragments of phosphopeptide by loss of H_3PO_4 and HPO_3, respectively. The difference of 94.6 Da instead of the normal 98 Da between *m*/*z* 2061.9 and 1967.3 could



Scheme 1 Immobilization of Fe^{3+} ions on the surface of zeolite-beta nanoparticles for the purpose of isolation of phosphopeptides; X represents the chelated species.

† Electronic supplementary information (ESI) available: Experimental details, SEM images, FT-IR spectrum and EDS analysis of zeolite-beta nanoparticles with immobilized metal ions. See http://www.rsc.org/suppdata/cc/b4/b411336e/

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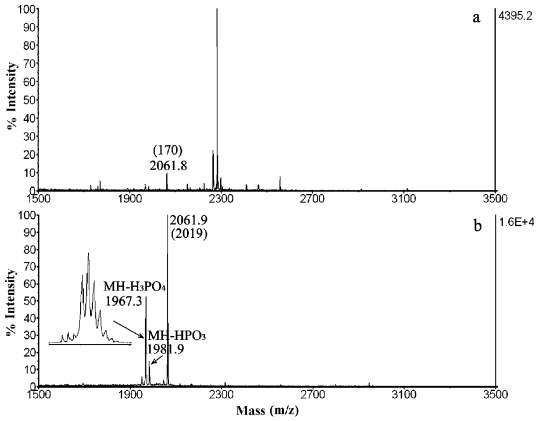

Fig. 1 MALDI-TOF mass spectra of a tryptic-casein digest (0.2 nmol mL^{-1}) without any pretreatment (a) and isolated by Fe^{3+}-nanozeolites (b). The data in parentheses are S/N of the corresponding peptides.

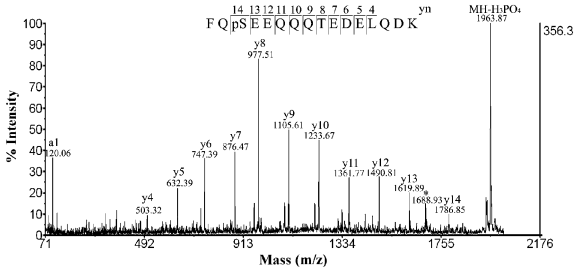

Fig. 2 MALDI-TOF-MS/MS spectra of the parent ion *m/z* 2061.9 signal isolated by Fe^{3+}-nanozeolites obtained on a TOF-TOF instrument. The amino acid sequence coverage is shown by y*n* ions. A prominent loss of 98 Da (*m/z* 1963.87) clearly shows its monophosphorylation. The mass difference 167 Da between fragment ions y13 (*m/z* 1619.9) and y14 (*m/z* 1786.9) corresponds to a phosphoserine residue, indicating the phosphorylation site at serine. The appearance of the peptide fragment (asterisk) by the loss of phosphoric acid (H_3PO_4, 98 Da) from the y14 ion further confirms this assignment.

be attributed to metastable loss of H_3PO_4 from the parent ions, which could be verified by the broadening and low-resolution of the signal of *m/z* 1967.3 (Fig. 1(b)).[7] The MALDI-MS/MS result of the parent ions (*m/z* 2061.9) affirmed its phosphorylation site at serine (Fig. 2). Compared to those obtained from the unisolated peptide mixture (Fig. 1(a)), the intensity and signal-to-noise ratio (S/N) of the MS signal of the phosphorylated peptides isolated by Fe^{3+}-nanozeolites were remarkably improved. Almost no other unphosporylated peptides or unidentified peaks were seen in Fig. 1(b), indicating that the Fe^{3+}-nanozeolite particles could specifically isolate and enrich the phosphorylated peptide from a peptide mixture in this case. Furthermore, the existence of zeolite nanoparticles seems to introduce no serious impurities during the MS analysis. Tryptic digest of bovine β-casein has been reported to contain two phosphorylated peptides: monophosphopeptide FQpSEEQQQTEDELQDK (*M* 2062.0 Da) and tetraphosphopeptide RELEELNVPGEIVEpSLpSpSpSEESITR (*M* 3122.9 Da).[8] However, the MS signal from the tetraphosphopeptide, *i.e.* 3122.9 Da. is not observed in Fig. 1, which could be the result of strong signal suppression and difficult ionization of tetraphosphopeptide under the positive MS mode.[2,9]

Interestingly, when the Fe^{3+}-nanozeolite suspension was employed to isolate and enrich the phosphopeptide from the β-casein digest at a more dilute level (0.02 nmol mL^{-1}), the intensity and S/N of the phosphorylated peptides after isolation (Fig. 3(b)) were still improved by more than 100 times compared

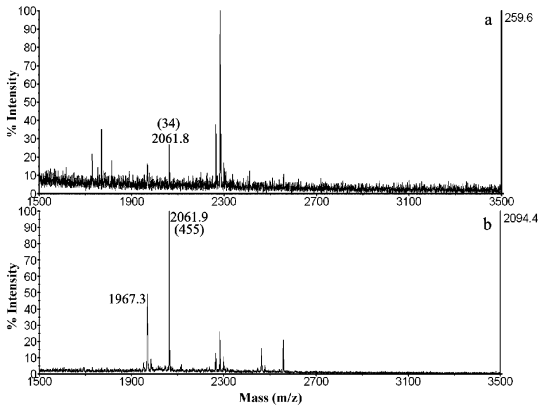

Fig. 3 MALDI-TOF mass spectra of a tryptic-casein digest (0.02 nmol mL^{-1}) without any pretreatment (a) and isolated by Fe^{3+}-nanozeolites (b). The data in the parentheses are S/N of the corresponding peptides.

with those obtained from corresponding unisolated peptide mixture (Fig. 3(a)), although some unphosporylated peptides are also bound in this case. This fact indicates that the Fe^{3+}-nanozeolites could enrich and identify phosphopeptides even when present at a very low level. In this case, the maximum amount of digest applied to the MALDI target is only 0.2 pmol after complete enrichment, which is lower than that after isolation by traditional IMAC beads.[4]

In summary, zeolite nanoparticles with immobilized Fe^{3+} ions have been used, for the first time, to isolate and identify phosphopeptides from tryptic β-casein digest *via* a direct MALDI-TOF-MS/MS analysis. These Fe^{3+}-nanozeolites with large external surface area and high dispersibility could facilitate the MALDI identification of phosphopeptides due to the affinity of their external surface for the phosphopeptides and good compatibility with the MALDI matrix. These results are expected to open up a new possibility for the isolation of phosphopeptides as well as a new application of zeolite nanoparticles.

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