Ti$^{4+}$-immobilized multilayer polysaccharide coated magnetic nanoparticles for highly selective enrichment of phosphopeptides

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Highly selective and efficient enrichment of trace phosphorylated proteins or peptides from complex biological samples is of profound significance for the discovery of disease biomarkers in biological systems. In this study, a novel immobilized metal affinity chromatography (IMAC) material has been synthesized to improve the enrichment specificity and sensitivity for phosphopeptides by introducing a titanium phosphate moiety on a multilayer polysaccharide (hyaluronate (HA) and chitosan (CS)) coated Fe$_3$O$_4$@SiO$_2$ nanoparticle (denoted as Fe$_3$O$_4$@SiO$_2$@(HA/CS)$_{10}$–Ti$^{4+}$ IMAC). The thicker multilayer polysaccharide endows excellent hydrophilic properties and a higher binding capacity of the titanium ion to the IMAC material. Due to the combination of uniform magnetic properties, highly hydrophilic properties and enhanced binding capacity of the titanium ion, the Fe$_3$O$_4$@SiO$_2$@(HA/CS)$_{10}$–Ti$^{4+}$ nanoparticle possesses many merits, such as high selectivity for phosphopeptides (phosphopeptides/non-phosphopeptides at a molar ratio of 1:2000), extreme detection sensitivity (0.5 fmol), large binding capacity (100 mg g$^{-1}$), high enrichment recovery (85.45%) and rapid magnetic separation (within 10 s). Moreover, the as-prepared IMAC nanoparticle provides effective enrichment of phosphopeptides from real samples (human serum and nonfat milk), showing great potential as a tool for the detection and identification of low-abundance phosphopeptides in biological samples.

Introduction

In recent years, extensive research has been carried out on the biomedical application of multifunctional nanomaterials.$^{1-5}$ Among various nanomaterials, magnetic nanoparticles have been intensively investigated for their application in proteomic research due to their unique biocompatibility, easy preparation, controlled size, versatile modification and quick magnetic response. As one of the most important and ubiquitous post-translational modifications, protein phosphorylation plays vital roles in regulating many complex biological processes, such as cell division and growth, signaling transduction and metabolic pathways.$^{6-10}$ Currently, mass spectrometry (MS) based techniques have been the premier technology for the characterization of phosphorylation. However, low abundance, low ionization efficiency, high complexity and severe ion suppression caused by the co-existence of abundant non-phosphopeptides make the direct MS analysis of phosphopeptides still a challenge. Thus, the selective enrichment of phosphoproteins/phosphopeptides from a highly complicated mixture prior to MS analysis is indispensable.

To date, various materials and techniques including metal oxide affinity chromatography (MOAC),$^{11-14}$ immobilized metal ion affinity chromatography (IMAC),$^{15-19}$ strong cation exchange$^{20}$ and strong anion exchange$^{21}$ have been developed for the selective enrichment of phosphoproteins/phosphopeptides. Among them, IMAC is one of the most commonly used techniques,$^{22}$ in which the metal ions are immobilized on a polymer bead, porous bead and nanoparticle using a linker molecule, and much efforts have been devoted to developing the IMAC material. Traditionally, linkers such as iminodiacetic acid and nitrilotriacetic acid were used to chelate the metal ion.$^{23-25}$ However, the bound metal ions were easily lost during the sample loading and washing procedure due to the relatively weaker interaction, which greatly reduced the enrichment efficiency. Recently, a new ligand of the phosphate group was introduced to immobilize the Ti$^{4+}$ or the Zr$^{4+}$ ion to overcome the above drawback, and has been applied for...
phosphoproteome research.\textsuperscript{26,27} Nevertheless, most of the conventional IMAC materials still require a laborious separation procedure (e.g. centrifugation), which is not only inconvenient, but also may lead to undesirable non-specific peptides and loss of low-abundance phosphopeptides. Therefore, the design and synthesis of a novel IMAC material to improve the phosphopeptide enrichment efficiency is still attracting attention.

A combination of a magnetic nanomaterial and a covalently bonding functional group could simultaneously achieve the simple and efficient separation of the target biomolecule from the complex mixture by magnetic separation.\textsuperscript{8} The functionalized magnetic nanoparticles have been widely used in proteomic research including protein digestion,\textsuperscript{28,29} removal of abundant proteins,\textsuperscript{18} extraction of low-abundance peptides/proteins,\textsuperscript{31,32} specific enrichment of glycopeptides and phosphopeptides,\textsuperscript{33,34} and capture of histidine-tagged peptides/proteins.\textsuperscript{35} In phosphopeptide enrichment, several kinds of IMAC magnetic nanoparticles (e.g. Fe$_3$O$_4$@SiO$_2$–Zr\textsuperscript{4+},\textsuperscript{36} Fe$_3$O$_4$@C–Zr\textsuperscript{4+},\textsuperscript{37} Fe$_3$O$_4$@mSiO$_2$–Zr\textsuperscript{4+},\textsuperscript{38} and Fe$_3$O$_4$@PD–Ti\textsuperscript{4+} (ref. 39)) were developed which showed selectivity to capture phosphopeptides. However, the grafted monolayer and the relatively low density of the ligand on the surface for the immobilization of metal ions have limited the specificity, sensitivity and binding capacity for phosphopeptides. Therefore, it is highly desirable for an IMAC material with abundant ligands to achieve the immobilization of metal ions and to improve the specificity and sensitivity for phosphopeptides.

Recently, core–shell magnetic polymer nanoparticles with thick polymer shells, abundant functional groups and good magnetic responsiveness which make them promising candidates for sample preparation in proteomic research have been synthesized.\textsuperscript{40–43} Ma et al. fabricated double polymer shell coated magnetic nanoparticles and anchored numerous Ti\textsuperscript{4+} ions to the phosphate group and the material was utilized to selectively enrich phosphopeptides from complex biological samples.\textsuperscript{42} Zhao et al. prepared PEG brush grafted magnetic nanoparticles and immobilized abundant Ti\textsuperscript{4+} ions with the reactive hydroxyl group.\textsuperscript{43} The PEG polymer enhanced the binding amount of Ti\textsuperscript{4+} ions and binding capacity for phosphopeptides. The design and synthesis of novel polymer coated magnetic nanoparticles for improving the enrichment specificity and sensitivity for phosphopeptides still remains a challenge.

In this work, a novel IMAC material, the Fe$_3$O$_4$@SiO$_2$@([HA/CS])$_{10}$–Ti\textsuperscript{4+} nanoparticle, has been designed and fabricated via a simple and reliable synthetic route (as shown in Scheme 1). The thicker polysaccharide polymer shell endows the magnetic nanoparticle not only with excellent hydrophilic properties, but also with numerous titanium phosphate moieties for high binding capacity and detection sensitivity for phosphopeptides. Moreover, the uniform magnetic properties will facilitate the rapid and complete separation of the IMAC nanoparticle. The performance of the IMAC nanoparticle in phosphopeptide enrichment has been evaluated by using different biological samples. The high selectivity, excellent sensitivity, high enrichment recovery and large binding capacity of the Fe$_3$O$_4$@SiO$_2$@([HA/CS])$_{10}$–Ti\textsuperscript{4+} magnetic nanoparticles clearly indicated its great potential as a high-performance IMAC material in phosphoproteome research.

\section*{Experimental section}

\subsection*{Materials}

Iron(III) chloride hexahydrate (FeCl$_3$–6H$_2$O), ethylene glycol (EG), sodium acetate (NaAc), isopropanol and dimethyl sulfoxide (DMSO) were obtained from Tianjin Chemical Plant (Tianjin, China). Tetraethyl orthosilicate (TEOS), 3-aminopropyltrimethoxysilane (APTS), ammonia solution (NH$_3$–H$_2$O, 28–30 wt%), N-hydroxysuccinimide (NHS), 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (CDI), chitosan (CS, low molecular weight), 1,1’-carbonyldimidazole (CDI), 2,4,6-collidine, β-casein (from bovine milk), bovine serum albumin (BSA), trypsin (TPCK treated), dithiothreitol (DTT), iodoacetamide (IAA), urea, 2,5-dihydroxy benzoic acid (DHB) and sodium bicarbonate (NaHCO$_3$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), trifluoroacetic acid (TFA) and formic acid (FA) were provided by Merck (Darmstadt, Germany). Sodium hyaluronate (HA) (M$_w$ = 100 kDa) was obtained from Zhenjiang Dong Yuan Biotech Co., Ltd., (Zhenjiang, China). Ti(SO$_4$)$_3$ was purchased from Sinopharm Chemical Reagents Co. Ltd (Shanghai, China). Human serum from a healthy volunteer was provided by the Dalian Medical University and stored at –80 °C before analysis. Nonfat milk was obtained from a local supermarket. The standard phosphopeptide (LRRAPSLGGK) was from Shanghai Apeptide Co., Ltd., (Shanghai, China). Water (18.4 MΩ cm) used in all experiments was purified by a Milli-Q system (Millipore, Milford, MA, USA). All other chemicals were of analytical grade and used without purification.

\subsection*{Preparation of Fe$_3$O$_4$@SiO$_2$@([HA/CS])$_{10}$–Ti\textsuperscript{4+} magnetic nanoparticles}

Fe$_3$O$_4$ particles were prepared by a solvothermal reaction according to previous work.\textsuperscript{24} 100 mg of Fe$_3$O$_4$ particles were dispersed in a mixture solution containing ethanol (200 mL), water (50 mL) and NH$_3$–H$_2$O (1.5 mL) with 30 min sonication, and then the mixture was stirred for 30 min at room
temperature. TEOS (0.4 mL) was added into it and stirred for another 12 h. The resulting product was collected and successively washed with ethanol, water and isopropanol, then redispersed in isopropanol (30 mL). APTS (0.5 mL) was added dropwise and stirred for 24 h at room temperature. The obtained product (denoted as Fe3O4@SiO2-NH2) was washed using a magnet, washed three times with ethanol and dried at 50 °C.

Fe3O4@SiO2@[(HA/CS)10] nanoparticles were synthesized according to our previous work.23 Typically, 50 mg of Fe3O4@SiO2-NH2 nanoparticles were activated with ethanol and dispersed in sodium hyaluronate solution (1 mg mL−1, 0.135 mol L−1 NaCl, pH = 5), stirred for 20 min, and the product was collected using a magnet and washed three times with water to remove excess sodium hyaluronate. Then the nanoparticles were redispersed in chitosan solution (1 mg mL−1, 0.135 mol L−1 NaCl, pH = 5) and stirred for 20 min, followed by magnetic separation and washing with water. After ten cycles, the product was collected, and immersed in PBS solution (10 mmol L−1, pH = 5.5) containing EDC (2 mg mL−1) and NHS (2 mg mL−1). The mixture was stirred at room temperature overnight.

50 mg of Fe3O4@SiO2@[(HA/CS)10] nanoparticles were dispersed in dried DMSO (10 mL), and CDI (500 mg) was added and the mixture was stirred for 24 h at room temperature. The resulting nanoparticles were collected and washed with DMSO, then redispersed in ethylenediamine (25 mL), stirred for 8 h at 60 °C. The obtained product (denoted as Fe3O4@SiO2@[(HA/CS)10-NH2]) was collected and washed three times with ACN. Fe3O4@SiO2@[(HA/CS)10-NH2] nanoparticles were dispersed in ACN solution (60 mL) containing POCl3 (60 mmol L−1) and 2,4,6-collidine (60 mmol L−1), and the mixture was stirred at room temperature for 12 h under a nitrogen atmosphere. After rinsing with ACN and water, the resulting nanoparticles (denoted as Fe3O4@SiO2@[(HA/CS)10-PO43−]) were incubated in Ti(SO4)2 (60 mL, 50 mmol L−1) aqueous solution at room temperature overnight under gentle stirring. The obtained nanoparticles (denoted as Fe3O4@SiO2@[(HA/CS)10-Ti4+] were collected and washed with CH3COOH–H2O (10 : 90, v/v, 200 mmol L−1 NaCl) and pure water six times to remove residual titanium ions. The obtained nanoparticles were dispersed in TFA–H2O (0.1:99.9, v/v) before use.

Material characterization

Field emission scanning electron microscopy (FE-SEM) images were recorded on a JSM-7001F scanning electron microscope and transmission electron microscopy (TEM) images were obtained using a JEOL JEM-2000 EX transmission electron microscope (JEOL, Tokyo, Japan). Fourier-transform infrared spectroscopy (FT-IR) characterization was performed on a Thermo Nicolet 380 spectrometer using KBr pellets (Nicolet, Wisconsin, USA). Thermogravimetric (TG) analysis was carried out under a nitrogen atmosphere at a heating rate of 10 °C min−1 from 30 °C to 1000 °C (NETZSCH, Selb, Germany). The saturation magnetization curve was recorded on a Physical Property Measurement System 9T (Quantum Design, San Diego, USA) at room temperature. Inductively coupled plasma-atomic emission spectrometry (ICP-AES) was used to determine the amount of titanium ions immobilized on the nanoparticle (Shimadzu Scientific Instruments, Kyoto, Japan). Zeta (ζ) potential measurements were performed on a Nano-ZS90 instrument in water at 25 °C (Malvern, Worcestershire, United Kingdom).

Tryptic digestion of standard protein

1 mg of β-casein was dissolved in NH4HCO3 (1 mL, 50 mmol L−1, pH = 8.3) and digested with trypsin (an enzyme/protein ratio of 1 : 40, w/w) at 37 °C for 16 h. BSA (2 mg) was denatured in urea (1 mL, 8 mol L−1) and the NH4HCO3 solution (50 mmol L−1), after the addition of DTT (20 μL, 1 mol L−1), the mixture was incubated at 56 °C for 1 h. Subsequently, IAA (7.4 mg) was added and incubated at room temperature in the dark for 45 min. The mixture was further diluted ten-fold with NH4HCO3 (50 mmol L−1), and incubated with trypsin (an enzyme/protein ratio of 1 : 40, w/w) at 37 °C for 16 h.

Tryptic digestion of proteins extracted from nonfat milk

30 μL of nonfat milk was added into NH4HCO3 (1 mL, 25 mmol L−1), and this solution was centrifuged at 16 000 rpm for 15 min. The supernatant was collected and then denatured at 100 °C for 10 min. The supernatant was digested with trypsin (40 μg) at 37 °C for 16 h.

Selective enrichment of phosphopeptides

50 μg of Fe3O4@SiO2@[(HA/CS)10–Ti4+] nanoparticles were added into the loading buffer (ACN–H2O–TFA, 60 : 34 : 6, v/v/v, 400 μL) containing β-casein trypsic digest, BSA tryptic digest or proteins extracted from nonfat milk, and the mixture was gently incubated at room temperature for 20 min. After removing the supernatant, the nanoparticles were washed three times with washing buffer 1 (ACN–H2O–TFA, 60 : 34 : 6, v/v/v, 200 mmol L−1 NaCl) and washing buffer 2 (ACN–H2O–TFA, 30 : 69.9 : 0.1, v/v/v). The captured phosphopeptides were eluted with NH3·H2O (2 × 10 μL, 10 wt%) by powerful shaking for 5 min. The eluate was directly analyzed by MALDI-TOF MS.

A certain amount of the standard phosphopeptide (LRRAPSLGGK) was divided equally into two parts and labelled with light and heavy isotopes by using a stable isotope dimethyl labeling approach according to a previously reported procedure.44 Then the heavy labelled phosphopeptide (1 pmol) was
enriched with Fe₃O₄@SiO₂@(HA/CS)₁₀–Ti⁴⁺ nanoparticles (50 µg) according to the procedure mentioned above. The eluted section was mixed with the same amount of the light labelled phosphopeptide (1 pmol), and the mixed peptides were analyzed by MALDI-TOF MS. The recovery of the standard phosphopeptide was calculated by the MS intensity ratio of the heavy labelled phosphopeptide to the light labelled phosphopeptide.

Mass spectrometry analysis

All MALDI-TOF MS experiments were performed in the reflector positive mode on an AB Sciex 5800 MALDI-TOF/TOF mass spectrometer (AB Sciex, CA) with a pulsed Nd/YAG laser at 355 nm. The DHB matrix was dissolved in ACN–H₂O–H₃PO₄ (70 : 29 : 1, v/v/v), 25 mg mL⁻¹. A 0.5 µL aliquot of the eluate and 0.5 µL of the DHB matrix were sequentially dropped onto the MALDI plate for MS analysis.

Results and discussion

The preparation procedure of the Fe₃O₄@SiO₂@(HA/CS)₁₀–Ti⁴⁺ nanoparticle with Fe₃O₄ as the magnetic core, silica as the intermediate layer, the cross-linked polysaccharide as the outer shell, and Ti⁴⁺ as the immobilized affinity ion is illustrated in Scheme 1. Firstly, the Fe₃O₄ particle was synthesized by a solvothermal reaction, and coated with a silica layer via the sol–gel process, then reacted with APTES to obtain the Fe₃O₄@SiO₂–NH₂ nanoparticle; secondly, a thick and cross-linked polysaccharide layer was coated onto the nanoparticle via the layer-by-layer approach to form the Fe₃O₄@SiO₂@(HA/CS)₁₀ nanoparticle; thirdly, the terminal amine and hydroxyl groups on the polymer were converted into the phosphate group by successive reactions with CDI, 1,2-ethanediamine and POCl₃; finally, the titanium cations (Ti⁴⁺) were immobilized onto the nanoparticle by the coordination reaction between Ti⁴⁺ and the phosphate group to obtain the Fe₃O₄@SiO₂@(HA/CS)₁₀–Ti⁴⁺ IMAC nanoparticle. For comparison, the Fe₃O₄@SiO₂ nanoparticle without the polysaccharide shell was also modified with titanium phosphate (designated as Fe₃O₄@SiO₂–Ti⁴⁺) according to the above procedure.

Characterization of Fe₃O₄@SiO₂@(HA/CS)₁₀–Ti⁴⁺ magnetic nanoparticles

Representative TEM and FE-SEM images are shown in Fig. 1. The Fe₃O₄@SiO₂ nanoparticles (Fig. 1a) were composed of a magnetic core (ca. 220 nm) and a thin layer of SiO₂ (ca. 3 nm). The TEM image of Fe₃O₄@SiO₂@(HA/CS)₁₀ nanoparticles (Fig. 1b) clearly indicates that the cross-linked polysaccharide shell of hyaluronic acid and chitosan has been successfully coated on the surface of Fe₃O₄@SiO₂ nanoparticles, and the thickness of the polymer shell in the dry state is around 10 nm. After modifying the composite with the phosphate group and the Ti⁴⁺ ion, its structure and morphology has no significant change (Fig. 1c), indicating the robust polymer shell structure. The FE-SEM image in Fig. 1d shows that the Fe₃O₄@SiO₂@(HA/CS)₁₀–Ti⁴⁺ nanoparticles have a uniform shape and narrow size distribution.

FT-IR spectroscopy and thermogravimetric (TG) analyses were used to inspect the cross-linked polysaccharide shell on the Fe₃O₄@SiO₂ nanoparticle. Compared to the FT-IR spectrum of Fe₃O₄@SiO₂ (581 cm⁻¹, νFe–O–Fe, 1091 cm⁻¹, νSi–O–Si), some new characteristic adsorption peaks (1420 cm⁻¹, νC=O of hyaluronic acid; 2923 cm⁻¹, νC–H of –CH₃) in the spectrum of Fe₃O₄@SiO₂@(HA/CS)₁₀–Ti⁴⁺ nanoparticles.
Fe₂O₃@SiO₂@(HA/CS)₁₀ (Fig. 2a) could demonstrate the formation of the cross-linked polysaccharide shell. TGA curves indicated that the weight loss of 4.06% of Fe₂O₃@SiO₂ is attributed to the adsorbed water. It could be calculated that the weight loss of Fe₂O₃@SiO₂@(HA/CS)₁₀ was 22.2% (Fig. 2b), which further demonstrated that the cross-linked polysaccharide polymer was successfully coated onto the nanoparticle.

Energy dispersive X-ray (EDX) spectra and zeta potential measurements were conducted to confirm the introduction of amine, the phosphate group and the titanium ion. As shown in Fig. 2c, the C, O, Fe, Si, P and Ti element peaks were found, indicating the successful modification with the phosphate group and the titanium ion. In addition, the zeta potential values of four kinds of functionalized nanoparticles have also given evidence for the successful immobilization of the Ti⁺⁺ ion on the Fe₂O₃@SiO₂@(HA/CS)₁₀ nanoparticles (Table S1, ESI†). The amounts of immobilized Ti⁺⁺ on Fe₂O₃@SiO₂@(HA/CS)₁₀–Ti⁺⁺ with a thick shell of cross-linked polysaccharide and Fe₂O₃@SiO₂–Ti⁺⁺ without the polymer shell measured by ICP-AES are 44.38 μg mg⁻¹ and 10.87 μg mg⁻¹, respectively, indicating the contribution of the high density of hydroxyl and amine groups on the cross-linked polysaccharide shell for the larger amount of immobilized titanium ions.

The magnetic properties of the two kinds of nanoparticles were studied by using a vibrating sample magnetometer at room temperature (Fig. 2d). The magnetic hysteresis loop curves show that the two kinds of materials have no obvious remanence or coercivity at room temperature, suggesting that they all could be supermagnetic. As a comparison, the saturation magnetization (Mₛ) value of the Fe₂O₃@SiO₂ nanoparticle was 68.08 emu g⁻¹. After coating the polymer layer and modification with the phosphate group and the titanium ion, the Mₛ value strikingly decreased to about 52.05 emu g⁻¹. A testing experiment showed that the Fe₂O₃@SiO₂@(HA/CS)₁₀–Ti⁺⁺ nanoparticle can be easily dispersed in water in the absence of a magnetic field. Thanks to the high magnetic response of Fe₂O₃, the final product of the Fe₂O₃@SiO₂@(HA/CS)₁₀–Ti⁺⁺ nanoparticle can also be easily and rapidly separated from the solution in only 10 s when a magnet was applied (inset in Fig. 2d).

**Application in selective enrichment of phosphopeptides from the tryptic digest of standard protein**

To demonstrate the practicability of the Fe₂O₃@SiO₂@(HA/CS)₁₀–Ti⁺⁺ nanoparticle as the IMAC stationary phase for the enrichment of phosphopeptides, a standard phosphoprotein (bovine β-casein) tryptic digest was used to evaluate its performance. β-casein tryptic digest was incubated with Fe₂O₃@SiO₂@(HA/CS)₁₀–Ti⁺⁺ in loading buffer, after isolating the nanoparticle from the solution and washing with washing buffer, the captured phosphopeptides were eluted and deposited on the MALDI target for MALDI-TOF MS analysis. As shown in Fig. 3a, for the direct analysis of β-casein tryptic digest, only one phosphopeptide with weak MS signal intensity and low signal-to-noise (S/N) ratio was detected due to the low-concentration of phosphopeptides and severe signal suppression by the abundant non-phosphopeptides. However, after enrichment by Fe₂O₃@SiO₂@(HA/CS)₁₀–Ti⁺⁺, three expected phosphopeptides (b₁, b₂, and b₃) could be clearly detected with strong MS signal intensities and S/N ratios, along with their dephosphorylated peptides (Fig. 3b), which were likely formed during the MALDI ionization process. The detailed information of the captured phosphopeptides from β-casein is displayed in Table S2 (ESI†). For comparison, the tryptic digest of β-casein was also treated with Fe₂O₃@SiO₂@(HA/CS)₁₀ and no peak representing the phosphopeptide was observed (Fig. 3c). These results demonstrated the enrichment selectivity of the Fe₂O₃@SiO₂@(HA/CS)₁₀–Ti⁺⁺ nanoparticle for phosphopeptides.

![Fig. 3 MALDI-TOF mass spectra of the tryptic digest of β-casein (0.5 pmol). (a) Direct analysis and after enrichment by (b) Fe₂O₃@SiO₂@(HA/CS)₁₀–Ti⁺⁺ and (c) Fe₂O₃@SiO₂@(HA/CS)₁₀ nanoparticles. * indicates phosphopeptides and # indicates dephosphorylated peptides.](image)

![Fig. 4 MALDI-TOF mass spectra of the tryptic digest mixture of β-casein (0.5 pmol) and BSA. (a) Direct analysis of the peptide mixture at a molar ratio of 1 : 100, after enrichment by Fe₂O₃@SiO₂@(HA/CS)₁₀–Ti⁺⁺ nanoparticles at molar ratios of (b) 1 : 100, (c) 1 : 500, and (d) 1 : 2000. * indicates phosphopeptides and # indicates dephosphorylated peptides.](image)
To evaluate the high selectivity of the IMAC nanoparticle for the enrichment of phosphopeptides, a mixture of β-casein and BSA tryptic digest was used as the testing sample. As shown in Fig. 4a, when the molar ratio of β-casein and BSA was 1 : 100, no phosphopeptide was detected, while non-phosphopeptide peaks with high MS intensities were observed. However, after treatment with Fe3O4@SiO2@(HA/CS)10–Ti4+, all the three phosphopeptides could be easily detected (Fig. 4b). Even when the molar ratio of β-casein and BSA was decreased to 1 : 500 and 1 : 2000, the three target phosphopeptides still can be distinctly identified with a clean background (Fig. 4c and d). These results indicated that the Fe3O4@SiO2@(HA/CS)10–Ti4+ nanoparticle has high selectivity for the capture of phosphopeptides from a complex peptide mixture.

As the level of phosphopeptides in a complex biological sample could be much lower, the ability to enrich and detect phosphopeptides from a highly diluted solution is a key parameter to evaluate the enrichment performance of the IMAC material. Therefore, β-casein tryptic digests with three low concentrations were treated with Fe3O4@SiO2@(HA/CS)10–Ti4+, and the eluates were analyzed by MALDI-TOF MS. As shown in Fig. 5a, two phosphopeptides were clearly detected in 10 fmol of β-casein tryptic digest after enrichment. Even though the total amount of β-casein tryptic digest was decreased to as low as 0.5 fmol (Fig. 5c), one phosphopeptide could still be identified at a S/N ratio of 19 with a m/z of 2061.70. The resulting detection sensitivity was higher than those of many previously reported IMAC and MOAC nanomaterials such as Fe3O4@mTiO2 (10 fmol), Fe3O4@PD-Ti4+ (2 fmol), Fe3O4@PMMA@PEGMP-Ti4+ (50 fmol), mesoporous γ-Fe2O3 (50 fmol) and Al2O3 hollow spheres (5 fmol). The lower detection limit may be attributed to the excellent hydrophilicity, large amounts of immobilized Ti4+ ions and absolute magnetic separation of the Fe3O4@SiO2@(HA/CS)10–Ti4+ nanoparticle. This result shows that the prepared Fe3O4@SiO2@(HA/CS)10–Ti4+ IMAC nanoparticle has high detection sensitivity for phosphopeptides.

To test the contribution of the cross-linked polysaccharide shell to the enrichment of phosphopeptides, the binding capacity of Fe3O4@SiO2@(HA/CS)10–Ti4+ and Fe3O4@SiO2–Ti4+ was investigated. Different amounts of nanoparticles were added to a fixed amount of β-casein tryptic digest (1 μg). After enrichment, the eluates (0.5 μL) were analyzed by MALDI-TOF MS. When the signal of one selected phosphopeptide (β1) reached the maximum value, the total phosphopeptides were bonded onto the nanoparticle. As illustrated in Fig. 6a, the binding capacity of the Fe3O4@SiO2–Ti4+ nanoparticle was calculated to be about 40 mg g−1, and the Fe3O4@SiO2@(HA/CS)10–Ti4+ nanoparticle showed a binding capacity as high as 100 mg g−1. The results indicated that the cross-linked polysaccharide shell coated on the Fe3O4@SiO2 has a significant effect on the immobilization capacity of the titanium ion, resulting in a larger binding capacity for phosphopeptides.

The enrichment recovery of the Fe3O4@SiO2@(HA/CS)10–Ti4+ nanoparticle for phosphopeptides was measured by using the quantitative approach of stable isotope dimethyl labelling. Two samples containing the same amount of standard phosphopeptide were labelled with light and heavy isotopes. The first part was treated with formaldehyde, and a 28 Da mass increase was produced by introducing two 12CH3 at the N-terminal of the lysine. The second part was reacted with deuterium formaldehyde, then two hydrogen atoms were replaced by two 14CH3 and a 32 Da mass increase was produced. Then, the second part was treated with Fe3O4@SiO2@(HA/CS)10–Ti4+, and the eluate was mixed with the first part and analyzed by MALDI-TOF MS. The MS intensity ratio of heavy to light labelled peptides reflects the recovery yield. As shown in Fig. 6b, the enrichment recovery of Fe3O4@SiO2@(HA/CS)10–Ti4+ for phosphopeptides was as high as 85.45%. The results reveal that the Fe3O4@SiO2@(HA/CS)10–Ti4+ nanoparticle could act as an ideal IMAC material for the enrichment of phosphopeptides.

Application in highly specific enrichment of phosphopeptides from human serum and nonfat milk

To further demonstrate the applicability of the Fe3O4@SiO2@(HA/CS)10–Ti4+ nanoparticle in selective enrichment of low-abundance phosphopeptides from practical biological samples, a serum sample containing low abundant phosphopeptides was used. After magnetic separation, the eluate was analyzed by MALDI-TOF MS. As shown in Fig. 7, three phosphopeptide peaks at 82, 95 and 108 were detected after magnetic separation, while the background peak was decreased to a clean background. This result shows that the Fe3O4@SiO2@(HA/CS)10–Ti4+ IMAC material has high selectivity and enrichment capacity for phosphopeptides.
samples, human serum and nonfat milk were applied as real samples. For diluted human serum, as shown in Fig. 7a, only one phosphopeptide with weak MS signal intensity appeared owing to the low-abundance phosphopeptides and high salt content. After treatment with Fe$_3$O$_4$@SiO$_2$@[HA/CS]$_{10}$–Ti$^{4+}$, four peaks of phosphopeptides with higher MS intensities and a clean background can be clearly detected (Fig. 7b). The detailed information of the four phosphopeptides from human serum are given in Table S3 (ESI†). Similarly, the direct analysis results of the digested nonfat milk by MALDI-TOF MS are shown in Fig. 7c, where only three weak MS signal intensities of phosphopeptides can be detected due to the interference of the abundant non-phosphopeptides. However, eleven peaks of phosphopeptides were distinctly observed with a clean background (Fig. 7d). The detailed information of the eleven phosphopeptides from the tryptic digest of proteins extracted from nonfat milk is given in Table S4 (ESI†). The results suggested that the Fe$_3$O$_4$@SiO$_2$@[HA/CS]$_{10}$–Ti$^{4+}$ nanoparticle shows great practicability in identifying low-abundance phosphopeptides from complex biological samples. We believe that this work would help to design and prepare more efficient and sensitive tools for phosphoproteome research.

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**Notes and references**