Low-cost iron oxide magnetic nanoclusters affinity probe for the enrichment of endogenous phosphopeptides in human saliva†

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The reversible phosphorylation of proteins plays a key role in numerous biological processes, while abnormal phosphorylation is a cause or consequence of many human diseases. Selective enrichment and MALDI-TOF MS or MS/MS characterization and identification of endogenous phosphopeptides fished-out from human saliva sample were achieved using simple and low-cost iron oxide magnetic nanoclusters (Fe 3O 4 MNCs) affinity probe in this work. The physicochemical properties of the prepared MNCs material was characterized by SEM, TEM, XRD, EDS, SQUID, etc. Based on their merits of high affinity, rapid separation, good biocompatibility, excellent dispersibility and superior specificity for capturing phosphopeptides, the performances of Fe 3O 4 MNCs were assessed by selectively and efficiently isolating phosphopeptides from standard phosphoprotein and actual non-fat milk tryptic digests, and intricate human saliva, respectively. Moreover, we demonstrated that the probe can be applied for rapid and effective purification and enrichment of phosphoproteins. The Fe 3O 4 MNCs affinity probe, which possesses the merits of integration of solid Lewis acids function and magnetic property, together with the “double economical” (both time and money) synthesis strategy, should be a promising material for the large scale investigation of endogenous phosphoproteomics.

1. Introduction

As one of the most important post-translational modifications (PTM), reversible phosphorylation of proteins regulates almost all aspects of cell life. 1 The cycle processes of phosphorylation and dephosphorylation can result in conformational changes in structures of many enzymes and receptors. 2 Meanwhile, as a function of electrostatic switches, it also can modulate the on and off states of many signalling pathways. 3 Moreover, the abnormal phosphorylation of proteins is now known to be a cause or consequence of major diseases, such as cancer, diabetes and rheumatoid arthritis. 4,5 Likewise, unusual endogenous phosphopeptides may also be related with certain human diseases. For example, many studies have demonstrated that increased endogenous fibrinopeptide A levels are found in ovarian, hepatocellular and urothelial cancers. 6–10 Therefore, analysis of the change of endogenous phosphopeptides is beneficial for understanding the development and diagnosis of some diseases.

Recently, post-translational salivary protein modifications (such as glycosylation, phosphorylation, and proteolysis) have been extensively studied. 11 Especially, a number of reports showed that one can readily detect antibodies in saliva to mumps, hepatitis A virus (HAV), hepatitis B virus (HBV), human immunodeficiency virus (HIV) infections, measles and rubella virus. 12 Results indicated that saliva plays an important role in oral and systemic diseases. Furthermore, salivary testing is becoming more common as clinicians have begun to appreciate its advantages and as investigators have defined its clinical conditions in which saliva can contribute to the diagnosis worth. 13 Its advantages over blood are clear: the simplicity of obtaining samples, hazard-free nature, and economy of use, acceptance by the patient, ability and convenience to be used in field studies. Consequently, investigation of human saliva endogenous phosphopeptides may be of great significance to the diagnosis of diseases.

Currently, mass spectrometry (MS) is the most suitable and powerful tool for identifying and recognizing phosphorylated proteins/peptides. Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is frequently...
used for methodology demonstration. However, due to the sub-
stoichiometry nature, high dynamic range and low ionization
efficiency of endogenous phosphopeptides as well as the
interference and suppression of signal by high-abundance
proteins and endogenous non-phosphopeptides in the
complex sample, the MS detection of endogenous phospho-
peptides still remains challenging. Therefore, it is a strong
requirement to explore new channels to capture and purify
endogenous phosphopeptides from non-phosphopeptides
before MS analysis.

Various enrichment techniques, as representative method
embracing both immobilized metal-ion affinity chromatog-
raphy (IMAC)\textsuperscript{14-16} and metal oxide affinity chromatog-
raphy (MOAC)\textsuperscript{17,18} have been widely employed to purify and enrich phosphoproteins/phosphopeptides. Remarkably, affinity mate-
rials for enrichment of phosphorylated proteins/peptides have
attracted tremendous attention in endogenous phosphopro-
teomics research.\textsuperscript{19-21} For IMAC, however, it has the weakness of
possible metal-ion leakage or loss and nonspecific binding for
non-phosphopeptides and acidic peptides in complex sample.\textsuperscript{22}
Due to the occupation and cover of affinity sites on the surface
of affinity materials by some non-target biomolecules carrying
acidic moieties, the enrichment selectivity and efficacy towards
endogenous phosphopeptides would often be highly affected.
Recently, thanks to the rapid development in material science,
especially the occurrence of nanotechnologies, the emergence
of a batch of novel affinity materials with composite structure
have been masterly designed to provide more affinity sites to
enhance the efficiency of enrichment. For these materials, such
as graphene (G),\textsuperscript{23-25} polydopamine (PDA),\textsuperscript{26} polymer,\textsuperscript{27,28} metal–
organic frameworks (MOFs),\textsuperscript{29,30} as a substrate further to modify
or a ligand to chelate metal-ions, have been developed for the
enrichment of different biomolecules. Compared with other
enrichment methods, MOAC has been reported to demonstrate
higher binding sensitivity and selectivity to phosphate group
based on the robust Lewis acid–base interaction between metal
oxides (MOs) and phosphate moieties. In its nascent stage,
some pure or binary metal-oxide nanoparticles (NPs) or aerosol,
such as NiO,\textsuperscript{31} TiO\textsubscript{2},\textsuperscript{31,32} ZrO\textsubscript{2},\textsuperscript{33} Al\textsubscript{2}O\textsubscript{3},\textsuperscript{32} SnO\textsubscript{2},\textsuperscript{34,35} Fe\textsubscript{3}O\textsubscript{4},\textsuperscript{36} TiO\textsubscript{2}–
ZnO,\textsuperscript{37} TiO\textsubscript{2}–ZrO\textsubscript{2},\textsuperscript{38,39} SnO\textsubscript{2}–ZnSn(OH)\textsubscript{6},\textsuperscript{40} and so on, which were
utilized as the affinity probes towards phosphopeptides, often
suffer from the cumbersome, cockamamie and time-consuming
centrifugation procedure that readily leads to sample loss.
Therefore, the practical application of these materials beneficial
for efficient and selective enrichment of endogenous phos-
phoproteins is limited. To address the issue, some novel
magnetic core–shell structured nanocomposites (Fe\textsubscript{3}O\textsubscript{4}–
TiO\textsubscript{2},\textsuperscript{41} Fe\textsubscript{3}O\textsubscript{4}@ZrO\textsubscript{2},\textsuperscript{42} Fe\textsubscript{3}O\textsubscript{4}@ZnO\textsubscript{4},\textsuperscript{43} Fe\textsubscript{3}O\textsubscript{4}@Al\textsubscript{2}O\textsubscript{3},\textsuperscript{44} Fe\textsubscript{3}O\textsubscript{4}@Ga\textsubscript{2}O\textsubscript{3},\textsuperscript{45} Fe\textsubscript{3}O\textsubscript{4}@Ta\textsubscript{2}O\textsubscript{5},\textsuperscript{46} and Fe\textsubscript{3}O\textsubscript{4}@TiO\textsubscript{2}–ZrO\textsubscript{2} (ref. 49)) have
been elaborately designed and fabricated as an effective way
to improve the sensitivity and precision for selective enrichment
of endogenous phosphopeptides since the magnetic property of
Fe\textsubscript{3}O\textsubscript{4} endows them to simplify the process of sample separa-

However, it is necessary to introduce harsh reaction
conditions or deposit most of MOs on the surface of Fe\textsubscript{3}O\textsubscript{4} core
NPs via additional calcination process. Recently, bare or doped
magnetic ferrites (Fe\textsubscript{3}O\textsubscript{4}, \(\gamma\)-Fe\textsubscript{2}O\textsubscript{3}, NiFe\textsubscript{2}O\textsubscript{4}, ZnFe\textsubscript{2}O\textsubscript{4} and
NiZnFe\textsubscript{2}O\textsubscript{4}, etc.),\textsuperscript{20,51} which possess the merits of integration of
solid Lewis acids function and magnetic property, have been
applied for phosphopeptides enrichment. Results showed that
these ferrites better fulfilled the requirement than other MOs
for selective capture of phosphopeptides, especially NiZnFe\textsubscript{2}O\textsubscript{4}
towards multi-phosphopeptides. These works also provided
a new thought to enhance the enrichment efficiency of crystal-
line MOs by tailoring their exposed crystal facets, increasing
area or decreasing magnetic field of exposed octahedral
sublattices.\textsuperscript{52} Almost all of above mentioned affinity materials have
been applied in phosphopeptides enrichment. Unexpect-
edly, however, few have reported the direct application to
phosphoproteins yet.\textsuperscript{53}

In this present study, iron oxide magnetic nanoclusters
(Fe\textsubscript{3}O\textsubscript{4} MNCs) affinity probe, composed of several mono-
crystallines with the size of 30 nm, were prepared and proposed
to apply as the absorbent for endogenous phosphopeptides
enrichment and phosphoproteins purification. For Fe\textsubscript{3}O\textsubscript{4}
MNCs, on one hand, the magnetic response can facilitate the
process of sample separation and provides us with simple, rapid
and effective capture of phosphopeptides/phosphoproteins
from complex mixture. On the other hand, the affinity probe
possesses good biocompatibility, excellent dispersibility, high
affinity and selective enrichment towards phosphopeptides/
phosphoproteins. Attributed to all above unique properties,
the superior performance of the material was demonstrated by
its specific adsorption of phosphopeptides from tryptic digests of
\(\alpha\)-casein, \(\beta\)-casein and BSA at different molar ratios, and non-
fat milk. Moreover, the Fe\textsubscript{3}O\textsubscript{4} MNCs affinity probe also could be
applied for capturing endogenous phosphopeptides from
human saliva. Meanwhile, the characteristic of the endogenous
phosphopeptides was further recognized and identified \textit{via}
MALDI tandem mass spectrometry (MS/MS). In addition, we
explored the possibility to employ the Fe\textsubscript{3}O\textsubscript{4} MNCs affinity
probe directly in the purification and enrichment of phospho-
proteins in this work. It was interestingly found that the affinity
probe also can be applied to rapidly, effectively and selectively
enrich phosphoproteins from protein mixtures.

2. Materials and methods

2.1. Chemicals and materials

Bovine \(\alpha\)- and \(\beta\)-casein (MW = 23.9 kDa), bovine serum albumin
(BSA, MW = 68.4 kDa), myoglobin (MW = 16.9 kDa), 2,5-di-
hydroxybenzoic acid (DHB) (puriss, p.a.), sinapic acid (SA), adeno-
sine 5’-triphosphate (ATP) (99%), acetonitrile (ACN) and
trifluoroacetic acid (TFA) of HPLC grade, and phosphoric acid
(85%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).
Sequencing grade modified trypsin was purchased from Prom-
ega (Madison, WI, USA). Ferric chloride (FeCl\textsubscript{3}·6H\textsubscript{2}O), sodium
acetate (NaAc), ethylene glycol (EG), anhydrous ethanol and
ammonium hydroxide (NH\textsubscript{3}·H\textsubscript{2}O) were obtained from Nanj-
ing Chemical Reagent (Nanjing, China). Two commercial Fe\textsubscript{3}O\textsubscript{4} NPs
(labelled 20 nm and 100–300 nm, respectively), and TiO\textsubscript{2} NPs
(labelled 100 nm) were bought from Aladdin (Aladdin Industrial
Corporation, Shanghai, China). All these reagents were used as
received without further purification. Non-fat milk was bought
from Jiangsu Province Educational Supermarket in the campus of Nanjing University. Human saliva sample from healthy volunteer was provided in Nanjing University and obtained by standard “drooling method”. This study was approved by the Ethics Committee of Nanjing Drum Tower Hospital affiliated Nanjing University Medical College (Nanjing, China) and conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participating individuals. The samples were stored at -80 °C before analysis. Deionized water (DIW) was prepared with a Milli-Q water purification system (Millipore, Billerica, MA, USA). All of other chemicals were of analytical grade unless otherwise noted.

2.2. Synthesis of Fe3O4 MNCs affinity probe
Fe3O4 MNCs were prepared according to our previously reported procedure32 with certain small modifications (Scheme 1A). Briefly, in view of the different solubility, NaAc (3.6 g) was first completely dissolved in EG (40 mL) via ultrasonication for 30 min to form clear solution, followed by the addition of FeCl3·6H2O (1.35 g). The obtained yellowish mixture was transferred into a Teflon-lined stainless-steel autoclave and sealed to heat at 200 °C for 8 h. After cooling to room temperature naturally, the black magnetite Fe3O4 MNCs particles were collected with the help of a magnet. The products were rinsed with DIW and ethanol for three times, respectively, and finally dried under vacuum at 60 °C for 8 h.

2.3. Characterizations of Fe3O4 MNCs affinity probe
Scanning electron microscopic (SEM) images were recorded with a Hitachi S-3000 N SEM (Hitachi, Japan) at an acceleration voltage of 10 kV. The transmission electron microscopic (TEM) images were obtained on a JEM-2100 TEM (JEOL, Japan). The composition of the prepared Fe3O4 MNCs was analyzed on an energy dispersive X-ray spectroscopy (EDX) (Hitachi, Japan). Fourier-transform infrared (FT-IR) spectra of the materials were collected on a NEXUS 870 FTIR spectrometer ( Nicolet, USA) using KBr pellets. Crystallographic phases of Fe3O4 MNCs were investigated by an XTRA X-ray power diffraction method (XRD) (ARL, Switzerland) at a scanning rate of 10°·min⁻¹ and a detection range from 10° to 80°. The magnetic properties were measured by using a vibration sample magnetic properties measurement system (VS-MPMS) utilizing a superconducting quantum interference device (SQUID) equipped with SQUID VSM (Quantum Design, USA). Dynamic light scattering (DLS) of Fe3O4 MNCs in different synthesis time (4, 8, and 12 h) were performed in Milli-Q water using a BIC 90Plus (New York, NY, USA).

2.4. Sample preparation
α/-β-Casein or BSA (1.0 mg) were respectively dissolved in 1.0 mL of NH4HCO3 aqueous solution (50 mM, pH 8.2) to form a substrate solution, and digested at 37 °C for 18 h with trypsin at the ratio of enzyme-to-substrate of 1:50 (wt/wt).

30 μL of NH4HCO3 aqueous solution (50 mM, pH 8.2) and centrifugated at a speed of 16 000 rpm for 15 min. Then, the albuminous supernatant was degenerated at 100 °C for 15 min, and 20 μL mL⁻¹ of trypsin was added for proteolysis at 37 °C for 16 h.

All of above aliquots of proteolytic digests were frozen at −80 °C for standby application. The frozen digests were thawed and diluted to the target concentration with an aqueous solution of 50% ACN and 0.1% TFA (v/v, denoted as A-T solution) prior to use.

2.5. Selective enrichment of phosphopeptides from tryptic digestion of standard proteins and non-fat milk
A schematic representation of the workflow to enrich phosphopeptides is shown in Scheme 1B. For enrichment of the phosphopeptides from standard protein digests, 100 μg Fe3O4 MNCs were added into 100 μL of a peptide mixture, which was originated from the mixture of standard peptides (both α-casein and β-casein tryptic digests) or practical sample (non-fat milk tryptic digest) (2.5 μL) diluted to 1.0 mL with A-T solution. The mixture was then vortexed at room temperature for 30 min, and the phosphopeptide-loaded Fe3O4 MNCs were collected with the help of a magnet. Next, the particles were washed three times with 100 μL of A-T solution to remove any unbound impurities. Afterwards, the trapped phosphopeptides were eluted with 5.0 μL of 10 mM NH4H2O by sonications for 10 min. Finally, the supernatants were taken after magnetic separation for further MALDI-TOF MS analysis.

For convincing comparison, some peptide mixtures were used to test with two commercial Fe3O4 NPs (labelled 20 nm and 100–300 nm, respectively) and TiO2 NPs (labelled 100 nm) for enrichment of the phosphopeptides under the same experimental conditions as described above, while the TiO2 NPs were isolated by centrifugation at a speed of 10 000 rpm for 5 min. It was worth mentioning that the commercial Fe3O4 NPs were pretreated with dilute hydrochloric acid before use.

2.6. Selective enrichment of endogenous phosphopeptides from human saliva
For enrichment of endogenous phosphopeptides from human saliva, 100 μg Fe3O4 MNCs were added into 100 μL of a 50% acetonitrile and 0.1% TFA solution which contain 5 μL human saliva. The specific enrichment procedures (vortexing, isolation, collection, washing, elution and analysis) were just the same as the above described.

Similarly, for convincing comparison, human saliva sample was used to test the commercial TiO2 NPs for enrichment of the phosphopeptides under the same experimental conditions as described above, while the TiO2 NPs were isolated by centrifugation at a speed of 10 000 rpm for 5 min.

2.7. Selective enrichment of phosphoproteins from protein mixture
To extend practical application in phosphoproteomics study, the Fe3O4 MNCs affinity probe was employed to directly enrich and purify phosphoprotein from protein mixture. To evaluate the selectivity and efficiency of the affinity probe for purification of phosphoprotein, 200 μg Fe3O4 MNCs were added into 100 μL of
a protein mixture (prepared in the A-T solution), which contained β-casein (1.2 mM), myoglobin (0.8 mM), and BSA (8.0 mM) for vortex to incubate for 30 min. Then, the Fe₃O₄ MNCs bound with phosphoprotein were isolated from the suspension with a magnet. After washing off the non-specifically bound proteins three times with the A-T buffer solution, the trapped phosphoproteins were eluted with 10.0 mL of 10 mM NH₄·H₂O by sonication for 10 min. Finally, the supernatants were taken after magnetic separation for further MALDI-TOF MS analysis. As a comparison, the commercial TiO₂ NPs were employed for enrichment of phosphoproteins by the same way but centrifugation.

2.8. MALDI-TOF MS or MS/MS analysis

The 0.5 μL of eluate was deposited on the MALDI plate and then another 0.5 μL of DHB (for peptides) or SA (for proteins) aqueous solution (20 mg mL⁻¹, 50% acetonitrile and 1% H₃PO₄) was introduced as a matrix to perform the MALDI-TOF MS or MS/MS analysis in a positive ion mode on a 4800 Proteomics Analyzer (Applied Biosystems, USA) with the Nd-YAG laser at 355 nm, a repetition rate of 200 Hz and an acceleration voltage of 20 kV. During tandem MS (MS/MS) analysis, air was used as the collision gas for collision-induced dissociation.

Scheme 1  Schematic illustration of the synthesis strategy of Fe₃O₄ MNCs affinity probe (A); workflow of selective enrichment and magnetic separation of endogenous phosphopeptides (B).
(CID) with a collision voltage of 1 kV. MS spectra were obtained by accumulation of 2000–3000 consecutive laser shots.

2.9. Investigation of post-enrichment recovery by ATP

Some small phosphate molecules, such as p-nitrophenylphosphate (pNPP)33 and adenosine mono-phosphate (AMP),34 were employed as models to evaluate phosphopeptides enrichment performance. ATP was chosen here as the test compound for post-enrichment recovery investigation. Because ATP molecule contains both UV chromophoric group and phosphate, the employment of ATP as a substitute will greatly simplify the experimental procedures and readily calculate post-enrichment recovery in quantification. A stock solution of ATP (1 mg mL\(^{-1}\)) was prepared in A-T solution in advance. Then, 0.15 mL of ATP solution (20 mg L\(^{-1}\)) was mixed with 200 µg Fe\(_3\)O\(_4\) MNCs and incubated for 30 min at room temperature. The resulting solution was rapidly treated by Fe\(_3\)O\(_4\) MNCs and incubated for 30 min at room temperature.

3. Results and discussion

3.1. Synthesis and characterization of Fe\(_3\)O\(_4\) MNCs affinity probe

Magnetic Fe\(_3\)O\(_4\) nanoclusters were synthesized through a solvothermal reaction as shown in Scheme 1. The simple method, which has low energy consumption, is easy to produce preferably dispersible and less agglomerative particles with good shape. Experiments showed that the role of the solvent EG is to control the growth of crystals in the reaction process. In addition, the different morphologies of products can be controllable.

The morphology and size of as-synthesized magnetite Fe\(_3\)O\(_4\) nanoclusters (MNCs) were examined by SEM and TEM. The SEM image of the MNCs exhibited a very approximate spherical morphology (Fig. 1A). The average diameter of the MNCs was calculated to be around 200 ± 10 nm based on statistical analysis for size measurement of 100 nanoclusters as shown in the inset of Fig. 1A. The SEM image also showed that the materials are endowed with narrow size distribution and possess good dispersity. TEM image (Fig. 1B) confirmed that the magnetic Fe\(_3\)O\(_4\) materials are uniform and the surface is rough and sag. The particle size measured from TEM images is 192 ± 5 nm, which is in very close agreement with the SEM result. The feature surface structure of the Fe\(_3\)O\(_4\) MNCs affinity probe makes it a good candidate for the enrichment of phosphopeptides, because the Fe\(_3\)O\(_4\) nanoclusters could offer abundant affinity sites and the well-dispersed property could facilitate to enlarge adsorption capacity. In addition, the inset of Fig. 1B showed that the fringe spacing of 0.150 nm and 0.202 nm are respectively in good conformity with the \(d\) (440) and \(d\) (400) plane of cubic phase iron oxide (JCPDS card no. 19-0629). The composition and content, and bonding of MNCs were further tested and verified by EDX (see the ESI Fig. S1A†) and FT-IR spectra (see the ESI Fig. S1B†). The EDX energy peak of elemental Fe/O illustrated the exact distribution of fundamental compositions and contents. In Fig. S1B†, the characteristic FT-IR absorption peak for Fe\(_3\)O\(_4\) at around 575.8 cm\(^{-1}\) was assigned to Fe–O vibration. According to the XRD analysis (see the ESI Fig. S1C†), all diffraction peaks of magnetic nanoclusters can be perfectly indexed to the magnetite cubic phase of Fe\(_3\)O\(_4\) (JCPDS 19-0629).

Magnetic characterization was carried out by magnetometry at 300 K using a superconducting quantum interference device (SQUID). The magnetization value was measured to 62.4 emu g\(^{-1}\) for Fe\(_3\)O\(_4\) MNCs affinity probe. Fig. S1D† shows its hysteresis loop, and it was apparent that this affinity probe has superparamagnetic property with large coercivity and remanence at 300 K. Every Fe\(_3\)O\(_4\) nanocluster here is a secondary particle aggregated together by several primary Fe\(_3\)O\(_4\) single crystals (see the ESI Fig. S2†), i.e., the Fe\(_3\)O\(_4\) MNCs materials are polycrystalline particles rather than monocrystalline ones. The mean crystal size of the primary Fe\(_3\)O\(_4\) nanoparticle is calculated to be around 50 nm using the broadening XRD peaks (220), (311), (400), (511), and (440) of the spinel iron oxide based on the Scherrer equation (see the ESI Table S1†). The inset in Fig. S1D† indicated that the Fe\(_3\)O\(_4\) MNCs are fast and conveniently separated by using an external magnetic field, and easily redispersed by shaking the sample solution after removing the external magnetic field.

3.2. Optimization of loading buffer solution (A-T solution)

To evaluate the efficiency of the Fe\(_3\)O\(_4\) MNCs affinity probe for enrichment of phosphopeptides, we employed respectively standard phosphoprotein (bovine \(a\)-casein) and mixture of phosphoprotein tryptic digest (\(a\)-casein and \(b\)-casein), which are frequently proofed the selective effectiveness of affinity materials toward phosphopeptides because of their having been identified phosphorylation sites (http://www.phosphosite.org/).
molar ratio at 1 : 1) as the models to optimize experiment conditions with MALDI-TOF MS detection. The amount of α-casein digest was 10 pmol and remained constant in all test samples. To obtain the best performance of phosphopeptides enrichment, A-T solution as the loading buffer with different ACN and TFA concentration were investigated. The procedure of separation and enrichment for phosphopeptides is illustrated in Scheme 1B. The efficiency and specificity of phosphopeptide enrichment were evaluated respectively from the peak intensity and signal to noise ratio (S/N) of phosphopeptides isolated from peptide mixture and from interference of non-phosphopeptides as a result of nonspecific competitive binding. During the process of selective enrichment of phosphopeptides, pH of the loading buffer is one of the critical factors. From a theoretical point of view, the main factor is Lewis acidity of Fe₃O₄ MNCs. With increasing acidity, they display a stronger binding property towards Lewis bases. We adopted the different volume of concentrate TFA to adjust the acidity. As we all know, a low pH can result in the protonation of acidic residues and thereby reduce the nonspecific binding of acidic peptides. However, a high TFA concentration can damage the surface nature of Fe₃O₄ MNCs affinity probe. The effect of TFA on the capture of phosphopeptides from the model of α-casein tryptic digest was studied at the concentrations of 0.05%, 0.1%, 1.0%, and 5.0%, respectively. At different concentrations of TFA, we observed that all these peaks detected are phosphopeptides as shown in the mass spectra Fig. 2. For the same peak at m/z 1951.7 Da as example, the S/N values were 1514.03, 2404.02, 1909.52, and 514.47 respectively, corresponding to TFA concentrations (0.05%, 0.1%, 1.0%, and 5.0%). Considering the higher S/N value, finally, the concentration of TFA was thus 0.1% chosen as the optimum concentration for the acidity of loading buffer.

An appropriate proportion of ACN in A-T solution is essential since it reduces hydrophobic interaction between Fe₃O₄ MNCs affinity probe and non-phosphopeptides, thereby improving the selectivity of the affinity material. Several ACN concentrations, 30%, 50%, 70%, and 90%, respectively, were studied for phosphopeptides enrichment performance (Fig. 3). The results of our experiment showed that increasing the ACN concentration produced increasing peaks intensity and S/N value. However, the peaks of non-phosphopeptides, at m/z 1759.8 Da with the S/N of ca. 2657.79, dominated the mass spectra at 90% ACN (Fig. 3D). Similarly, for instance, the peak at m/z 1951.8 Da, a lower or higher concentration of ACN (30% and 90%) could result in detecting at low S/N values (1262.39 and 1307.12, respectively). While compared between Fig. 3B and C, even though the S/N of 2683.36 at m/z 1951.8 Da at 70% ACN is slightly higher than that at 50% ACN (S/N = 2381.40), however, for the peaks at m/z 1660.7 Da and 1466.5 Da, their S/N values are 1368.36 and 755.62, respectively, for 70% ACN, and 1459.46 and 924.45 for 50% ACN. Considering a compromise between the whole peak intensities and S/N values, the concentration of 50% ACN was selected as optimum for the loading buffer. Thus, we conducted experiments to select the appropriate acidity by adjusting the concentrations of TFA and found that the most efficient isolation of phosphopeptides was made with 0.1% TFA in 50% ACN loading buffer.

For better optimizing enrichment performance of the Fe₃O₄ MNCs affinity probe in selective capture towards phosphopeptides, the factors of particle size resulting from synthetic time or commercial Fe₃O₄ NPs influencing the overall recognition process are discussed here. Firstly, we investigated the influence of synthetic time of Fe₃O₄ MNCs (4, 8, and 12 h) on the enrichment performance for mixture of bovine α-casein digest

![Fig. 2](image_url) MALDI-TOF MS spectra of α-casein tryptic digest at different TFA concentrations in loading solution (A-T solution) treated with Fe₃O₄ MNCs affinity probe. (A) 0.05%; (B) 0.1%; (C) 1.0%; (D) 5.0%.
(10 pmol) and β-casein digest (10 pmol). Synthetic time primarily affects the size of Fe₃O₄ MNCs materials. According to the practical experiment, the longer heat time in synthetic process, the greater particle size. For small particle size of Fe₃O₄ MNCs, they can provide more binding sites towards target analytes due to the larger specific surface area. However, they are also prone to aggregation. As shown in the mass spectrum (see the ESI Fig. S3†), phosphopeptides marked with “*” and part of relevant dephosphorylation ions marked with “#” were detected evidently and the S/N values of representative phosphopeptides adsorbed by Fe₃O₄ MNCs are listed in Table S2.† The satisfactory results (S/N > 1000) were obtained if the objective samples were treated by the Fe₃O₄ MNCs, the preparation time of which is 8 h. Generally, the smaller particles size, the better enrichment effect. It stands to reason that the particle size for Fe₃O₄ MNCs of synthetic time 4 h was smaller than that of 8 h, and it would bring about a good result due to the larger specific surface area. However, actually, the enrichment effect of 4 h was in keeping with that of 12 h but was inferior to that of 8 h. It could come to a conclusion that Fe₃O₄ MNCs probe apparently underwent aggregation (see the ESI Fig. S4†).

For comparison, then, commercial Fe₃O₄ affinity NPs materials labelled sizes of 20 and 100–300 nm, respectively, were utilized for treatment of bovine α-casein tryptic digest. The results are shown in the MS spectra (see the ESI Fig. S5†) and the S/N values of representative phosphopeptides are enumerated in Table S3.† It was found that the enrichment effect of labelled 20 nm was superior to that of labelled 100–300 nm due to the larger specific surface area. However, self-prepared Fe₃O₄ MNCs exhibited an outstanding performance in phosphopeptides enrichment procedure. On one hand, by the way of self-preparation, the uniformity and controllability of nanoclusters size as well as their good dispersibility could be gained with a powerful guarantee; on the other hand, surface modification is often carried out on commercial Fe₃O₄ NPs in order to prevent their oxidization and aggregation. In addition, the acidification process of the commercial Fe₃O₄ NPs will increase the operational complexity. Above results indicated that as-prepared Fe₃O₄ MNCs for synthetic time of 8 h were devoted to selectively capture target phosphopeptides from samples and provide higher enrichment performance.

### 3.3. Enrichment of phosphopeptides from standard phosphoprotein tryptic digests

To examine the enrichment efficiency of the Fe₃O₄ MNCs, we took advantage of the standard phosphoprotein hydrolysate mixture as objective sample solution. The peptides mixture, which consisted of 10 pmol bovine α-casein tryptic digests with 10 pmol bovine β-casein tryptic digests (molar ratio, α : β = 1 : 1), was incubated with 100 μg of Fe₃O₄ MNCs affinity probe and the phosphopeptides were selectively isolated. Only 7 phosphopeptides with low S/N values were observed without enrichment in the mass spectrum. In addition, the signal intensity of these phosphopeptides was seriously suppressed by non-phosphopeptides (Fig. 4A). However, after being enriched with Fe₃O₄ MNCs affinity materials, 30 phosphopeptides and part of correlative doubly charged ion peaks were detected. Almost all the signals can be assigned to phosphopeptides in the spectra (Fig. 4B). Among them, the peaks at mass-to-charge ratios (m/z) 2062.5, 2432.9, 2556.9, and 3123.2 were derived from bovine β-casein tryptic digests. Besides, the peaks at m/z marked with 1031.8, 1279.5, and 1562.2 represented the doubly charged ions derived from m/z 2062.5, 2556.9, and 3123.2,
respectively. Other ion peaks were derived from bovine α-casein tryptic digests, as well as the peaks at m/z 1683.4, 1855.9, 2084.5, and 2578.9 represented the Na⁺ adducted ions (Δ22 Da) for corresponding phosphopeptides (1661.4, 1833.5, 2062.5, and 2556.9, respectively). In addition, the peaks at m/z 1254.1, 1483.1, 1611.3, and 1944.3 and 2721.8 indicated oxidation on methionine ([Mo] Δ16 Da) and deletion or truncation of N-terminal on glutamine (*Q, Δ17 Da) for corresponding phosphopeptides (1238.1, 1467.1, 1595.3, and 1928.3, and 2704.8, respectively). The corresponding peptide sequences of these ions are listed in Table 1. Moreover, six peaks marked with “#” at m/z 1370.1, 1564.5 and 1768.4, 1873.9 and 1982.6 can be assigned to metastable ion peaks with a mass loss of approximately 98 Da (1467.1, 1661.4 and 2556.9), and dephosphorylate fragments of phosphopeptide with a mass loss of 80 Da (1848.4, 1952.6 and 2062.5), respectively. It is worthwhile to note that the mean enrichment factor (EF) was over 7 by comparing the ratios of S/N for α/β-phosphopeptides enrichment as listed in Table S4.† Remarkably, the Fe₃O₄ MNCs affinity probe had a predilection for mono-phosphopeptides enrichment with high peak intensity since these ions peaks dominated the mass spectrum. The above results implied that the Fe₃O₄ MNCs affinity material is very effective in selectively capturing phosphopeptides, especially for mono-phosphopeptides compared to weakly bound multi-phosphopeptides, which is similar to the results by using Fe₃O₄, NiFe₂O₄ and ZnFe₂O₄ NPs as the absorbents.  

The enrichment specificity of the Fe₃O₄ MNCs affinity probe toward phosphopeptides was further evaluated with tryptic digests of bovine α-casein, β-casein and BSA at molar ratios of 1 : 1 : 1, 1 : 1 : 10, 1 : 1 : 50, respectively. With the increase of digested BSA in the mixed samples, the signals of high-abundant non-phosphopeptides dramatically increased as shown in Fig. S6A1, B1 and C1.† Through directly spotting sample analysis, only 6 phosphopeptides with low peak intensity were detected from the mixture sample solution (α : β : BSA = 1 : 1 : 1), while 18 phosphopeptides with high-S/N values were detected after being enriched with Fe₃O₄ MNCs (see the ESI Fig. S6A2†). In the same way, for the other two distinct proportions mixture sample solutions (α : β : BSA = 1:1:10 and α : β : BSA = 1 : 1 : 50), no phosphopeptide was observed before Fe₃O₄ MNCs treatment. After extracting the phosphopeptides from these two mixture sample solutions, 18 phosphopeptides were remained to detect in the mass spectra (Fig. S6B2†). And even though the molar concentration of interfering substance (BSA) increased to 50-fold, 12 phosphopeptides were still detected and their peaks dominated the mass spectra (see the ESI Fig. S6C2†). For comparison, commercial TiO₂ affinity NPs materials were employed for treatment of the three diverse mixed samples respectively and the results are shown in the spectra (see the ESI Fig. S6A3, B3 and C3†). The enrichment performance as a whole was parallel to Fe₃O₄ MNCs. With the increase of digested BSA, the signals of phosphopeptides were dramatically suppressed by non-phosphopeptides, i.e., the more interference, the greater suppression. We propose that Fe₃O₄ has the Lewis acidity much higher than TiO₂ and the pH tolerance is inferior to TiO₂, making it selective to mono-phosphopeptides.
above results, it is further revealed that highly efficient trapping and enrichment of phosphopeptides from complex mixture samples were realized with Fe$_3$O$_4$ MNCs materials treatment.

To verify the sensitivity of the Fe$_3$O$_4$ MNCs affinity probe for phosphopeptide enrichment, we applied the materials to enrich the phosphopeptides from standard mixture $\alpha$-casein and $\beta$-casein tryptic digest (molar ratio at 1:1) at 500, 50, 20 and 10 fmol, respectively. As shown in Fig. 5, the intensity and S/N ratio of target phosphopeptides decreased with the decrease of protein concentration. The peaks of 10 phosphopeptides, originating from $\alpha$-casein tryptic digest (six peaks) and $\beta$-casein tryptic digest (four peaks), were found when the molar concentrations of $\alpha$-/$\beta$-casein were respective 500 fmol. Six phosphopeptides, including four peaks of $\alpha$-casein and two peaks of $\beta$-casein, were observed from the concentration of standard mixture decreased to 50 fmol. For 20 fmol digest, two phosphopeptides ($m/z$ 2061.8 and 2556.1) were at S/N ratios of 6.41 and 4.57 obviously, respectively. More importantly, the results showed that the ion signals from the phosphopeptides ($m/z$ 2061.8) can still be detected in protein concentration as low as 10 fmol with the S/N ratio of ca. 3.60, which demonstrated the high detection sensitivity of the Fe$_3$O$_4$ MNCs affinity probe for phosphopeptides.

Table 1  Identified phosphopeptides from proteolytic mixture digests of $\alpha$-casein and $\beta$-casein

<table>
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<tr>
<th>Position</th>
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</tbody>
</table>

$^a$ “[ps]” shows phosphorylation on serine; “[Mo]” indicates oxidation on methionine; “$^c$” denotes doubly charged peak; “Q$^*$” represents deletion or truncation of N-terminal on glutamine.

Fig. 5  MALDI-TOF MS spectra of mixture of bovine $\alpha$-casein digest and $\beta$-casein digest with molar ratio at 1:1, treated with Fe$_3$O$_4$ MNCs affinity probe. (A) 500 fmol; (B) 50 fmol; (C) 20 fmol; (D) 10 fmol. “$^*$” indicates phosphopeptides; the data in parentheses represent S/N ratios.
The recyclability and reproducibility of the Fe₃O₄ MNCs affinity probe for phosphopeptides was also investigated by taking advantage of the α-casein and β-casein digests mixture (molar ratio at 1 : 1) as objective analytes. The same batch of Fe₃O₄ MNCs were used three times and they were rinsed fully by A-T solution for re-use before each sample loading step. As shown in Fig. S7† there was no attenuation in analytical performance during the sequential three extractions times. The results indicated the specificity and affinity as well as good reusability of the Fe₃O₄ MNCs affinity probe towards phosphopeptides.

In addition, the post-enrichment recovery of Fe₃O₄ MNCs was investigated by using ATP as a substitute for the phosphopeptides. Satisfied result was obtained to be 68.8% of the standard ATP with 20 mg L⁻¹ (0.15 mL). Such a high adsorption rate (92.4%) was benefited from the high affinity between the surface of the Fe₃O₄ MNCs and phosphate group of ATP, while the relatively low elution rate (74.5%) perhaps was resulted from the sample losing in washing/elution processes.

3.4. Enrichment of phosphopeptides from non-fat milk

To investigate practical application potential of Fe₃O₄ MNCs affinity probe, non-fat milk was chosen as a real sample for isolating phosphopeptides. Only two weak phosphopeptides peaks marked with ** at m/z 1952.5 (S/N = 29.58) and 2062.4 (S/N = 61.64) were detected in the direct analysis because of the non-phosphopeptides suppression effect as shown in the mass spectrum (see the ESI Fig. S8A†). In contrast, after treated with Fe₃O₄ MNCs affinity probe, ten of phosphopeptides with high MS peak intensity and S/N ratio can be easily observed without any interference from the high-abundant non-phosphopeptides (see the ESI Fig. S8B and Table S5†). The S/N of peaks at m/z of 1660.9, 1952.1, 2061.9 and 3122.3 were 191.25, 498.76, 843.86 and 124.78, respectively. It should be pointed out that the EFs of m/z 1952.1 and 2061.9 were close to 17 and 14, respectively. For comparison, commercial TiO₂ NPs were used to capture phosphopeptides from the real non-fat milk sample under the same experimental conditions. Apparently, only five of phosphopeptides with low intensity were isolated in the mass spectrum (see the ESI Fig. S8C†). However, the EF of 2062.3 (S/N = 307.99) was only 5. Above results demonstrated the selectivity and the efficiency of the Fe₃O₄ MNCs are superior to those of commercial TiO₂ NPs for enrichment of phosphopeptides.

3.5. Enrichment of endogenous phosphopeptides from human saliva

Human saliva containing various informative endogenous peptides including the phosphopeptides released by diseased tissue and these peptides have gained considerable interest for the disease biomarker discovery. In order to further prove the practical effect of the Fe₃O₄ MNCs affinity probe, human saliva was employed as a real biosample for the selective enrichment of endogenous phosphopeptides. No phosphopeptides was detected from the saliva samples resulting owing to the suppression effect of non-phosphopeptides (Fig. 6A). Nevertheless, after treated with the Fe₃O₄ MNCs affinity materials, 11 phosphopeptides (marked with **), which can be identified by the MALDI tandem mass spectrometry (MS/MS) (see the ESI Fig. S9†), were observed in the mass spectrum and free of the interference of non-phosphopeptides (Fig. 6B). A prominent loss of 80 Da (HPO₄²⁻)/98 Da (H₃PO₄) or 196 Da (2H₃PO₄) is characteristic of a facile phosphoric acid loss during CID of the phosphopeptide and is generally the most dominant fragment ion in the MS/MS spectrum. The presence of a single or double phosphoric acid losses indicates that it is a mono- or di-phosphorylated peptide. As shown in Fig. S9III† for instance, the peptide at m/z 1270.4 is a phosphopeptide due to the presence of the fragment ions at m/z 1172.5 and 1074.5 adjacent to the parent ion with a mass difference of 98 Da and 196 Da in MS/MS spectra. It is well worth noting that the presence of the fragment ion at m/z 1092.5 (80 Da) further confirmed that the peptide at m/z 1270.4 is recognized a di-phosphopeptide. Similarly, all these peptides at m/z 1426.5, 1539.6, 1752.6, and 1898.7 were identified as di-phosphopeptides. We also found that some other losses of 43 Da, 17 Da, and 16 Da (m/z) are respectively characteristic of existence of acetylation, deletion or truncation of N-terminal on glutamine, and oxidation on methionine. This special information on these phosphopeptides is listed in Table S6†. Similarly, for comparison, we also utilized commercial TiO₂ affinity NPs for enriching phosphopeptides from human saliva. Only five of phosphopeptides were captured under the same experimental conditions in the mass spectrum (Fig. 6C). Above results further confirmed the effectiveness of the Fe₃O₄ MNCs affinity materials for capturing phosphopeptides from saliva.

3.6. Enrichment of phosphoprotein from protein mixture

To extend practical application in phosphoproteomics study, the prepared Fe₃O₄ MNCs affinity probe was employed directly
to the phosphoproteins enrichment and purification from protein mixture. To further evaluate selectivity and suitability of the affinity probe for phosphopeptides, a complex protein mixture containing a sort of phosphoprotein (β-casein, 23.9 kDa) and two sorts of non-phosphoproteins (myoglobin, 16.9 kDa and BSA, 68.4 kDa) was employed. The specific enrichment procedures (vortexing, isolation, collection, washing, elution and analysis) were just the same as phosphopeptides enrichment. While the original and residual protein mixture solutions and the purified phosphoproteins were then analyzed by MALDI-TOF MS. Fig. 7 shows the successful phosphoproteins separation from the protein mixture. Due to the suppression of abundant non-phosphoproteins (myoglobin and BSA), the signal and intensity of β-casein was low, with the S/N ca. 302.25 (Fig. 7A). However, after treatment with the Fe₃O₄ MNCs affinity probe, only the peaks of myoglobin and BSA in the residual protein mixture solution were observed in the mass spectrum (Fig. 7B). It reminded that β-casein has been selectively adsorbed by the Fe₃O₄ MNCs. In contrast, only the peaks of β-casein along with its doubly charged ions with a strong intensity can be easily detected with the S/N of ca. 733.67. Simultaneously, non-phosphoprotein peaks cannot be detected because they were removed before MS detection (Fig. 7C), suggesting that the Fe₃O₄ MNCs were effective for the purification and enrichment of phosphoproteins.

For comparison, an enrichment test was also performed using commercial TiO₂ NPs under the same experimental conditions except using centrifugal separation. The peak of β-casein with a high S/N ratio (ca. 1751.57) and a strong intensity can be detected (Fig. 7E). However, using TiO₂ NPs as probe suffers from laborious centrifugal operation. All results above demonstrated that the Fe₃O₄ MNCs are also potential in applying for the selective purification and enrichment of the phosphoproteins from complicate protein mixture, and therefore can provide a rapid magnetic separation, convenient and effectual method for enriching phosphoprotein.

3.7. Comparison with other previously reported affinity probes

The enrichment capability of some partial previous materials (different types of metal oxides (MOs) nanoparticles or functionalized magnetic-MOs affinity probes) is listed in Table S7.† From Table S7, we can find that our material has comparable enrichment effect with materials reported in these previous works, especially, the high magnetization value (\( Mₐ = 62.4 \text{ emu g}^{-1} \)), separation time (just 15 s to complete), and enrichment sensitivity (10 fmol) of the affinity probe are significantly superior to other materials. In conclusion, it provides a rapid, convenient, and high sensitive alternative for phosphopeptides enrichment.

4. Conclusions

In this work, we described a simple, rapid enrichment process for selective isolation of endogenous phosphopeptides from real-world complex human body fluids, by taking advantage of unfancy Fe₃O₄ MNCs affinity probe, which was prepared by a facile solvothermal method without any complementary and superfluous procedures. Fe₃O₄ MNCs were characterized with various tools, and the result obtained revealed that one Fe₃O₄ MNC is composed of several monocrystallines. In this material, the Fe₃O₄ MNCs affinity probe can provide abundant affinity sites for adsorbing phosphopeptides. In addition, taken together with good biocompatibility, superior dispersibility, readily releasable, high affinity and selectivity towards phosphopeptides, the excellent performance of Fe₃O₄ MNCs were evaluated by enriching model standard phosphopeptides from the mixtures of tryptic digests of α-casein and β-casein (molar ratio, 1 : 1), the complex samples mixtures of (α-casein, β-casein and BSA) tryptic digests with distinct molar ratio (1 : 1 : 1, 1 : 1 : 10, and 1 : 1 : 50), and actual non-fat milk. Moreover, Fe₃O₄ MNCs affinity probe were successfully applied for the effective separation and enrichment of endogenous phosphopeptides from intricate human saliva. These endogenous phosphopeptides can be further identified by MALDI tandem

![Fig. 7 MALDI-TOF mass spectra of the original protein mixture before enrichment (A), the residual protein mixture after enrichment (B) and the phosphoprotein enriched from the protein mixture (C) by the Fe₃O₄ MNCs affinity probe, and the residual protein mixture after enrichment (D) and the phosphoprotein enriched from the protein mixture (E) by the commercial TiO₂ NPs. \( *^{\text{a}} \text{Mb}^{*+} \), \( ^{\text{a}}\beta^{+} \), and \( ^{\text{a}}B^{+} \) indicate myoglobin, β-casein and BSA, respectively. \( ^{\text{a}}\beta^{2+} \) represent the doubly charged ions of the β-casein. \( ^{\text{a}}\text{S/N} \) indicates phosphoprotein. The data in parentheses represent S/N ratios.](image-url)
mass spectrometry (MS/MS) by the presence of prominent losses of 80 Da (HPO$_3$)$^-$/98 Da (H$_2$PO$_4$)$^-$ or 196 Da (2H$_3$PO$_4$)$^-$ characteristic fragment ions in the MS/MS spectra. Our research using a simple and low cost Fe$_3$O$_4$ MNCs affinity material provided a powerful approach for the enrichment of endogenous phosphopeptides in human saliva and identification with MS/MS. At last, we further demonstrated that the affinity probe can be applied to rapidly, effectively and selectively purify phosphoproteins from protein mixtures. We foresee more potential direct applications of the Fe$_3$O$_4$ MNCs affinity probe in phosphoproteome analysis.

Acknowledgements

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References