We herein demonstrate superparamagnetic Fe$_3$O$_4$ nanoparticles coated with nitrolotriacetic acid derivative (NTA) that can bind with different immobilized metal ions are capable of probing diverse target species. Immobilized Ni(II) on the surfaces of the NTA–magnetic nanoparticles have the capability of selectively trapping histidine (His)-tagged proteins such as a mutated streptokinase tagged with 6× His, i.e., C192S (MW ~42 kDa), from cell lysates. Enrichment was achieved by vigorously mixing the sample solution and the nanoparticles by pipetting in and out of a sample vial for only 30 s. After enrichment, the probe—target species could be readily isolated by magnetic separation. We also characterized the proteins enriched on the affinity probes using on-probe tryptic digestion under microwave irradiation for only 2 min, followed by matrix-assisted laser desorption/ionization mass spectrometry analysis. Using this enrichment and tryptic digestion, the target species can be rapidly enriched and characterized, reducing the time required for carrying out the complete analysis to less than 10 min. Furthermore, when either Zr(IV) or Ga (III) ions are immobilized on the surfaces of the NTA–magnetic nanoparticles, the nanoparticles have the capability of selectively enriching phosphorylated peptides from tryptic digests of α-, β-caseins, and diluted milk. The detection limit for the tryptic digests of α- and β-caseins is ~50 fmol.

Columns packed with particles immobilized with metal ions, which is so-called immobilized metal affinity chromatography (IMAC), have been used to selectively enrich diverse target species from complex samples. One of the advantages of IMAC for purification is that the target species being concentrated on the column depend on the metal ions immobilized based on Pearson's hard soft acid/base principle. The metal ions trapped on the IMAC adsorbents can be easily altered because the ligand, such as nitrolotriacetic acid (NTA), immobilized on the column can chelate with a variety of metal ions. For example, IMAC columns with immobilized ions such as Fe(III) and Ga(III) ions have been used to selectively purify phosphate peptides, while immobilized Ni(II) ions have been used to enrich histidine (His)-tagged proteins. On the same principle, magnetic nanoparticles immobilized with metal ions have recently been used as an alternative method for selective enrichment of various target species. Using magnetic nanoparticles as IMAC adsorbents has several advantages, including a high trapping capacity due to

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their high surface/volume ratio and easy isolation on the basis of their magnetic property. Recombinant proteins genetically expressed from bacterial cells are generally tagged with a polyhistidine tag, which is designed for the convenience of isolation after protein expression from complex cell lysates. Using magnetic particles as affinity probes to selectively trap His-tagged proteins from cell lysates has been demonstrated to be a promising method for protein isolation.\(^{20-24}\) Xu and co-workers\(^{20,21}\) have successfully prepared magnetic nanoparticles with immobilized NTA, which were capable of chelating Ni(II) ions. These nanoparticles have the capacity to selectively concentrate His-tagged proteins from complex samples. They demonstrated two approaches to bind NTA covalently on the surface. Pt–S and Fe–S bonds were used to link NTA derivatives on the FePt magnetic nanoparticles.\(^{20}\) Another approach was using dopamine as a linker to bind covalently with the NTA derivative molecules onto the iron oxide shell of the magnetic particles.\(^{17}\) Shieh et al.\(^{22}\) employed chemical methods to bind NTA–Ni onto Fe\(_3\)O\(_4\)–NH\(_4\)^+ magnetic nanoparticles for protein purification and cell targeting. They demonstrated that the average binding capacity for a 42-kDa protein, streptopain, is 230 mg/g (~5.5 μmol/g) (protein/Fe\(_3\)O\(_4\)–NTA–Ni(II)). We generated silanized Fe\(_3\)O\(_4\) nanoparticles modified with NTA on their surface bridged by a long spacer molecule to reduce nonspecific binding. These were then chelated with Ni(II) ions (Fe\(_3\)O\(_4\)/NTA–Ni(II)), which could be used to selectively bind with His-tagged proteins from cell lysates. We used pipetting as the mixing method for rapid trapping and enrichment of target species from low volumes of sample solutions.\(^{25}\) The proteins trapped on the surface of the affinity probes were digested using microwave-assisted tryptic digestion\(^{26,27}\) recently developed in our group. The species concentrated on the nanoparticles were eluted and characterized by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). The analysis time could be dramatically reduced by combining these methods.

It has been demonstrated that either zirconia-coated magnetic nanoparticles\(^{28}\) or zirconium dioxide microparticles\(^{28}\) can effectively enrich phosphorylated peptides from complex samples. We hypothesized that Zr(IV) ions immobilized on the surface of the Fe\(_3\)O\(_4\) magnetic nanoparticles (Fe\(_3\)O\(_4\)/NTA–Zr(IV)) should have a similar capability of selectively binding with phosphopeptides. Thus, the feasibility of using Fe\(_3\)O\(_4\)/NTA–Zr(IV) magnetic nanoparticles to selectively enrich phosphopeptides from the tryptic digestion products of α- and β-caseins and milk was examined. Additionally, Ga(III) immobilized affinity chromatography\(^{3}\) had previously demonstrated effectiveness in the purification of phosphopeptides. However, the use of Ga(III) immobilized magnetic nanoparticles in the enrichment of phosphopeptides has not been reported. Thus, we herein examine the trapping performance of Zr(IV) and Ga(III) immobilized magnetic nanoparticles for phosphopeptides.


**EXPERIMENTAL SECTION**

**Reagents.** Iron(III) chloride hexahydrate, zirconium(IV) oxide chloride, and ammonium bicarbonate were obtained from Riedel-de Haën (Seelze, Germany), while iron(II) chloride tetrahydrate, 3-(2-aminoethylamino)propyltrimethoxysilane (EDAS), 2-cyanooxyhydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), iodoacetic acid (IAA), gallium(III) chloride, and succinic anhydride were purchased from Aldrich (Milwaukee, WI). Dimethylformamide (DMF) was obtained from J. T. Baker (Phillipsburg, NJ), and methanol was obtained from Tedia (Fairfield, OH). Tetraethoxysilane (TEOS), N\(_6\)N\(_6\)N\(_6\)bis(carboxymethyl)-L-lysine hydrate (NTA), and N-hydroxy succinimide (NHS) were obtained from Fluka (Buchs, Switzerland). Hydrochloric acid, trifluoroacetic acid (TFA), nickel(II) chloride, and acetonitrile were purchased from Merck (Darmstadt, Germany). N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), bradykinin, trypsin, 3,5-dimethoxy-4-hydroxycinnamic acid, dithiothreitol (DTT), and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma (Steinheim, Germany).

**Preparation of Fe\(_3\)O\(_4\) Magnetic Nanoparticles.** FeCl\(_3\) (1 M) and FeCl\(_2\) (0.5 M) were dissolved in aqueous hydrochloric acid (0.4 M, 10 mL) at room temperature under sonication. After the salts were completely dissolved in solution, the mixture was degassed using a pump. Aqueous sodium hydroxide (0.5 M, 100 mL) was slowly added under nitrogen with stirring at room temperature. The mixture was left to react, heated in an oil bath at 80 °C for 30 min. After cooling to room temperature, the magnetic nanoparticles were rinsed with aqueous hydrochloric acid (0.1 M) and ethanol to remove any unreacted impurities followed by resuspension in deionized water (40 mL) under sonication for 30 min. The supernatant was collected after centrifugation at 13 500 rpm for 30 min at 4 °C.

**Preparation of Functional Magnetic Nanoparticles.** Scheme 1 shows the steps for fabrication of functional iron oxide magnetic nanoparticles. The details of the experiment were described as follows. The supernatant, containing iron oxide magnetic nanoparticles (0.3 mg/mL, 100 mL), was mixed with ethanol (400 mL) and put in a water bath at 40 °C. TEOS (0.375 mL) was added and mixed well, followed by slowly adding aqueous ammonia solution (10%, 20 mL) with stirring. After stirring for 12 h, the mixture was centrifuged at 13 500 rpm for 30 min and the supernatant was discarded. The nanoparticles remaining in the vial were rinsed with ethanol and methanol (280 mL) and resuspended in methanol (200 mL) under sonication for 30 min. The suspension was degassed using a pump followed by covering with nitrogen gas. It was heated in an oil bath at 110 °C with reflux before the addition of EDAS solution (80%, 2 mL) with stirring. After 12 h, the mixture was centrifuged at 13 500 rpm for 25 min. The supernatant was removed, and the isolated particles were rinsed with methanol. The particles were resuspended in DMF (50 mL) under sonication for 30 min.

The suspension was stirred with succinic anhydride (0.2 g/mL, 10 mL) dissolved in DMF under nitrogen for 10 h. The mixture was centrifuged at 13 500 rpm for 25 min, and the supernatant was removed. The nanoparticles remaining in the vial were resuspended in methanol under sonication for 30 min followed by centrifugation at 13 500 rpm for 25 min. The supernatant was removed, and the rinsing was repeated three times. The rinsed
nanoparticles were resuspended in deionized water under sonication for 30 min followed by centrifugation at 13,500 rpm for 25 min. This rinsing was repeated twice. The nanoparticles were resuspended in deionized water (40 mL) under sonication for 30 min. The suspension (20 mL) was mixed with EDC (100 mg) and NHS (100 mg) with stirring for 30 min, followed by the addition of NTA derivative (10 mg). The mixture was left to react at room temperature for 24 h and centrifuged at 13,500 rpm for 30 min. The supernatant was removed, while the isolated particles (Fe₃O₄) were rinsed with deionized water three times and resuspended in deionized water (20 mL).

**Magnetic Separation and Rinse of Nanoparticles.** Holding a magnet (~5000 G) to the outside of the sample vial caused the nanoparticles to aggregate on the side of the vial. The solution was discarded by pipet, leaving the particles in the vial. The particles were rinsed by adding the rinse solution and pipetting it vigorously in and out of the sample vial for 30 s. They were resuspended and mixed in the same way. These methods were used in the manipulations described below.

**Immobilizing Ni(II) Ions onto the Surface of Fe₃O₄/NTA Magnetic Nanoparticles.** The nanoparticles (120 µg) were vortex-mixed in aqueous nickel chloride solution (0.1 M, 200 µL) for 1 h. The solution was removed by magnetic separation, and the nanoparticle–Ni(II) conjugates (Fe₃O₄/NTA–Ni(II)) were rinsed with deionized water (200 µL × 3) and resuspended in deionized water (200 µL) before use. The concentration of the nanoparticle in the suspension was ~0.6 µg/µL. When estimating the binding capacity of Ni(II) ions on the nanoparticles, the Fe₃O₄/NTA–Ni(II) nanoparticles (120 µg) in the suspension was collected by magnetic separation and the nanoparticles were resuspended in an EDTA solution (0.125 mg/mL, 100 µL) in methanol/0.05% ammonia (1/1, v/v) solution, with vortex mixing for 30 min. The solution, containing the EDTA–metal ion complex from the particle suspension, was collected for analysis by electrospray/sheetless ESI MS.

Using Fe₃O₄/NTA–Ni(II) Magnetic Nanoparticles to Selectively Enrich His-Tagged Species. Analytes including bradykinin, HHHHHH (6×His), KAEEKAA (Ka-6), BSA, C192S prepared in deionized water, and cell lysate containing C192S were used as the samples. C192S was a 6×His tagged recombinant protein, a mutant form of streptopain, expressed in Escherichia coli. The details for the expression of C192S have been described elsewhere. The solution was removed from the Fe₃O₄/NTA–Ni(II) suspension (0.6 µg/µL, 50 µL) by magnetic separation. The sample solution (50 µL) was mixed with the particles remaining in the vial, and then the solution was removed, leaving the particle–target species conjugates. The isolated particles were rinsed vigorously with deionized water (50 µL × 3) and mixed with a saturated CHCA matrix solution (0.5 µL) prepared in acetonitrile/water (2/1, v/v), which was then pipetted onto a MALDI sample target. After evaporation of the solvent, the sample was ready for MALDI MS analysis.

**On-Probe Microwave-Assisted Tryptic Digestion.** After enrichment by the Fe₃O₄/NTA–Ni(II) magnetic nanoparticles, the proteins on the nanoparticles could be directly subjected to tryptic digestion under microwave irradiation. Briefly, the nanoparticle–target species conjugates were rinsed with ammonium bicarbonate (50 mM, pH 8) followed by the addition of trypsin (10 µg/mL, 2 µL) prepared in ammonium bicarbonate solution (50 mM, pH 8). The suspension was put in a domestic oven (power, 900 W) for 2 min. About 0.5 µL of digestion product solution was pipetted into saturated CHCA solution (0.5 µL) for MALDI MS analysis.

**Immobilizing Zr(IV) (or Ga(III)) Ions onto the Surface of Fe₃O₄/NTA Magnetic Nanoparticles.** Fe₃O₄/NTA magnetic nanoparticles (3 mg) were vortex-mixed in zirconium(IV) oxide chloride aqueous solution (0.1 M, 5 mL) in hydrochloric acid (1 M) for 2 h, and the solution was discarded. The nanoparticle–Zr(IV) conjugates (Fe₃O₄/NTA–Zr(IV)) were rinsed with hydrochloric acid (1 M) three times and resuspended in hydrochloric acid (1 M, 5 mL) before use. Fe₃O₄/NTA–Ga(III) nanoparticles were generated by using the same preparation steps as shown.

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**Scheme 1. Steps for Fabrication of Functional Fe₃O₄ Magnetic Nanoparticles**

**Scheme 1. Steps for Fabrication of Functional Fe₃O₄ Magnetic Nanoparticles.**

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on above. However, zirconium(IV) oxide chloride was replaced by gallium(III) chloride (0.1 M, 5 mL). Gallium(III) chloride is very reactive. It should be handled in a hood with care.

**Using Fe$_3$O$_4$/NTA–Zr(IV) (or –Ga(III)) Magnetic Nanoparticles to Enrich Phosphorylated Peptides.** The mixture of α- and β-caseins (10$^{-4}$ M, 100 μL) was digested enzymatically by mixing with trypsin (1 μg/μL, 4.8 μL) with a weight ratio of 50 : 1 in aqueous ammonium bicarbonate (50 mM, pH 8) and incubated at 37 °C for 24 h. The mixture was diluted in 0.15% TFA/water solution. The proteins in the nonfat milk were denatured prior to tryptic digestion. That is, the milk sample (0.25 mL) was mixed with aqueous ammonium bicarbonate (50 mM, 0.25 mL) containing urea (8 M), followed by incubation at 38 °C for 30 min. DTT (50 μL, 90 mM) prepared in ammonium bicarbonate solution (50 mM) was added to this mixture in a heating block maintained at 55 °C for 1 h. After being cooled to room temperature, the sample solution was mixed with IAA solution (50 μL, 200 mM) in aqueous ammonium bicarbonate (50 mM) in an ice bath. The sample vial was wrapped with aluminum foil and vortex-mixed for 2.5 h at room temperature, when it was ready for tryptic digestion. The denatured milk (0.6 mL) and trypsin (1 μg/μL, 10 μL) prepared in aqueous ammonium bicarbonate (50 mM) solution were incubated at 38 °C for 16 h. The tryptic digestion products were diluted in 0.15% TFA/water solution.

The acidified digestion solution (50 μL) was mixed vigorously with the magnetic nanoparticles, previously isolated from the particle suspension (0.6 μg/μL, 50 μL). The mixture was vigorously mixed by pipetting in and out of a sample vial for 30 s. The particles conjugated with their target species were isolated by magnetic separation. The isolated particles were rinsed with 0.15% TFA solution (50 μL) in an ice bath. The sample vial was wrapped with aluminum foil and vortex-mixed for 30 s. The particle suspension (0.6 mL) containing urea (8 M), followed by incubation at 38 °C for 30 min. DTT (50 μL, 90 mM) prepared in ammonium bicarbonate solution (50 mM) was added to this mixture in a heating block maintained at 55 °C for 1 h. After being cooled to room temperature, the sample solution was mixed with IAA solution (50 μL, 200 mM) in aqueous ammonium bicarbonate (50 mM) in an ice bath. The sample vial was wrapped with aluminum foil and vortex-mixed for 2.5 h at room temperature, when it was ready for tryptic digestion. The denatured milk (0.6 mL) and trypsin (1 μg/μL, 10 μL) prepared in aqueous ammonium bicarbonate (50 mM) solution were incubated at 38 °C for 16 h. The tryptic digestion products were diluted in 0.15% TFA/water solution.

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**Instrumentation.** ESI mass spectra were obtained using an Esquire 2000 ion trap mass spectrometer (Bruker Daltonics, Leipzig, Germany). MALDI mass spectra were obtained using a Biflex III (Bruker Daltonics) time-of-flight mass spectrometer equipped with a 337-nm nitrogen laser. The acceleration voltage was set at 19 kV, while the laser power was carefully adjusted during MS analysis to obtain the optimized mass resolution. Absorption spectra were obtained using a Varian Cary 50 spectrophotometer (Melbourne, Australia). Transmission electron microscope (TEM) images were obtained using a JEOL 2000FX TEM. The magnetic measurements were obtained from a superconducting quantum interference device (MPMS XL-7) in the cofacility center at National Chiao Tung University.

**RESULTS AND DISCUSSION.**

The Fe$_3$O$_4$/NTA magnetic nanoparticles we generated have superparamagnetic properties with a particle size of ~50 nm. In order to clarify the binding capacity of Fe$_3$O$_4$/NTA magnetic nanoparticles to Ni(II) ions, we saturated the surfaces of Fe$_3$O$_4$/NTA magnetic nanoparticles with sufficient Ni(II) ions. EDTA was used to elute Ni(II) ions from the surfaces of the particles because the complex of Ni(II) with EDTA has a higher formation constant (log $K_{Ni(II)-EDTA} = 18.62$) than that with NTA (log $K_{Ni(II)-NTA} = 11.50$). On the basis of this experiment, we estimated that 1 g of the magnetic particles can trap 6.8 × 10$^{-5}$ mol of Ni(II) ion. We first demonstrated the capability of the Fe$_3$O$_4$/NTA–Ni(II) magnetic nanoparticles for selectively trapping 6×His from a peptide mixture. Figure 1a displays the direct MALDI mass spectrum of the peptide mixture containing 6×His (3.3 × 10$^{-7}$ M, MH$^+$ = 841.2), bradykinin (3.3 × 10$^{-7}$ M, MH$^+$ = 1060.6), and Ka-6 (3.3 × 10$^{-6}$ M, MH$^+$ = 1617.6). (b) MALDI mass spectrum obtained using Fe$_3$O$_4$/NTA–Ni(II) magnetic nanoparticles (30 μg) as the affinity probes to selectively enrich their target species from the same peptide mixture (50 μL).

To investigate the binding affinity of the Fe$_3$O$_4$/NTA–Ni(II) nanoparticles for His-tagged species, we used a His-tagged protein, C192S, as the sample. Figure 2a displays the direct MALDI mass spectrum of the protein mixture containing C192S (10$^{-6}$ M) and 6×His at m/z 841.2. The peak at m/z 841.2 corresponds to the protonated pseudomolecular ion of 6×His at m/z 841.2 does not appear in the same mass spectrum. Many ions generated from the matrix appear in the mass spectrum. Figure 1b presents the MALDI mass spectrum obtained using the Fe$_3$O$_4$/NTA–Ni(II) nanoparticles as the affinity probes to selectively enrich their target species from the same peptide mixture. Only the peak at m/z 841.2 corresponding to the protonated pseudomolecular ion of 6×His appears in the mass spectrum. Furthermore, the background ions generated from the MALDI matrix disappear. It is owing to ion suppression effects that are generally observed in mass spectra. That is, because 6×His molecules have been concentrated on the affinity probes after enrichment, a high concentration of 6×His presents in the MALDI sample. Therefore, abundant protonated pseudomolecular ions of 6×His dominate the MALDI mass spectrum, and the ions with relatively low intensities derived from the MALDI matrix are suppressed. The results indicate that our affinity probes have the capability of selectively binding with 6×His from a peptide mixture.

BSA (10^{-5} M), which appear at \( m/z \) 42 k and 66 k, respectively. Figure 2b displays the MALDI mass spectrum obtained using the \( \text{Fe}_3\text{O}_4/\text{NTA–Ni(II)} \) magnetic nanoparticles to selectively enrich their target species from the same protein mixture. Only the peak at \( m/z \) 42 k representing C192S appears in the mass spectrum. The results indicate that \( \text{Fe}_3\text{O}_4/\text{NTA–Ni(II)} \) magnetic nanoparticles have the capacity to selectively enrich His-tagged proteins from a cell lysate containing C192S (0.115 \( \mu g/mL \), 50 \( \mu L \)).

![Figure 2](image2.png)

Figure 2. (a) Direct MALDI mass spectrum of the protein mixture containing C192S (10^{-5} M, MW = ~42 000) and BSA (10^{-5} M, MW = ~66 000). (b) MALDI mass spectrum obtained using \( \text{Fe}_3\text{O}_4/\text{NTA–Ni(II)} \) magnetic nanoparticles (30 \( \mu g \)) to selectively enrich their target species from the same protein mixture (50 \( \mu L \)). (c) MALDI mass spectrum obtained using \( \text{Fe}_3\text{O}_4/\text{NTA–Ni(II)} \) magnetic nanoparticles (30 \( \mu g \)) to selectively enrich their target species from a cell lysate containing C192S (0.115 \( \mu g/mL \), 50 \( \mu L \)).

For confirmation, we employed on-probe tryptic digestion to characterize the protein concentrated on the probes. The MALDI mass spectrum (Figure 3) shows peaks at \( m/z \) 1123.4, 1370.4, 1716.3, 1725.7, 1907.9, 2017.9, 2046.6, 2169.7, 2191.9, 2289.9, 3970.2, and 4133.0 marked with asterisks, which are derived from C192S. The details of the sequence for each peak are listed in Table 1. The sequence coverage is 68.2%. The peak at \( m/z \) 2272.5 marked with a circle is generated from autolysis of trypsin. Those at \( m/z \) 1772.1, 2304.4, 2322.3, 2713.7, and 3152.9 are unidentified. The results demonstrated that, in the combination of rapid enrichment and on-probe microwave-assisted tryptic digestion followed by MALDI MS analysis, the target species can be easily identified.

We also examined the trapping capacity of the magnetic nanoparticles for C192S. Thus, we employed absorption spectroscopy to investigate the absorption change of a solution containing purified C192S, before and after incubation, with the \( \text{Fe}_3\text{O}_4/\text{NTA–Ni(II)} \) magnetic nanoparticles. We used the difference of the optical density (OD) of the C192S solution (2.45 \( \times \) 10^{-6} M, 1 mL) obtained before (OD 0.029 35) and after (OD 0.013 22) incubation with beads at wavelength 280 nm to determine the C192S trapped on the magnetic particles (300 \( \mu g \)).

Table 1. Peptide Ion Peaks Observed in the MALDI Mass Spectrum of Tryptic Digest of C192S

<table>
<thead>
<tr>
<th>calculated ( m/z )</th>
<th>observed ( m/z )</th>
<th>start–end sequence</th>
<th>peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1123.5</td>
<td>1123.4</td>
<td>1–11</td>
<td>ASMTGGQQMGR</td>
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<td>1370.7</td>
<td>1370.4</td>
<td>329–342</td>
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<td>1961.8</td>
<td>125–142</td>
<td>KLDITYGATAEIKQPVK</td>
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<td>50–88</td>
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</tr>
</tbody>
</table>

of a calibration curve ($y = 13.085x - 0.00271, R^2 = 0.9999$) obtained by plotting the absorption intensity of C192 at 280 nm as a function of the concentration of C192S, we determined the trapping capacity is 200 mg/g (50 μM, 50 μL). The nanoparticle–target species conjugates after enrichment were mixed with 0.5 μL of DHB (50 mg/mL in acetonitrile/deionized water (2/1, v/v) containing 1% phosphoric acid prior to MALDI MS analysis. “i.s.” stands for internal standard, which was used for internal mass calibration. The peaks marked with asterisks are from α-casein, while the peaks marked with circles are obtained from β-casein.

We immobilized Zr(IV) ions on the surface of Fe3O4/NTA nanoparticles to make affinity probes for the enrichment of phosphopeptides. Figure 4a displays the direct MALDI mass spectrum of the tryptic digest of the mixture of α- and β-caseins (10⁻⁷ M). The peaks marked with asterisks at m/z 1928.0 and 1952.2 are generated from α-casein, while the peaks marked with a circle at m/z 2062.1 is obtained from β-casein. Figure 4b presents the MALDI mass spectrum obtained using the Fe3O4/NTA–Zr(IV) magnetic nanoparticles as the affinity probes to selectively enrich their target species from the tryptic digest of the mixture of α-casein (10⁻⁸ M, 50 μL) and (b) β-casein (10⁻⁹ M, 50 μL). The nanoparticle–target species conjugates were mixed with 0.5 μL of DHB (30 mg/mL) containing 1% phosphoric acid prior to MALDI MS analysis. The peaks marked with asterisks are phosphopeptides, while the corresponding sequences are marked aside the peaks. The peak at m/z 1943.7 was obtained from the sequence of DIgEsTEDQAMEDIK (51/43–58), while methionine (M) has been oxidized. “s” stands for phosphorylated serine.

Fe3O4/NTA–Zr(IV) magnetic nanoparticles can selectively bind with phosphorylated peptides.

Additionally, we also examined the trapping performance of Fe3O4/NTA–Ga(III) for phosphopeptides. Figure 5a presents the MALDI mass spectrum obtained using Fe3O4/NTA–Ga(III) magnetic nanoparticles as the affinity probes to selectively enrich their target species from the tryptic digest of α-casein (10⁻⁸ M, 50 μL). Three peaks at m/z 1237.4, 1660.8, and 1943.7 marked with asterisks are phosphopeptides derived from α-casein. However, when we lowered the concentration of the casein sample to 10⁻⁹ M and performed the same enrichment steps, there were no analyte signals appearing in the MALDI mass spectrum (results not shown). Figure 5b shows the MALDI mass spectrum obtained using Fe3O4/NTA–Ga(III) magnetic nanoparticles as the affinity probes to selectively enrich their target species from the tryptic digest of β-casein (10⁻⁹ M, 50 μL). Only a peak at m/z 2061.8 marked with an asterisk appears in the MALDI mass spectrum. Similarly, when the concentration of the sample was lowered to 10⁻¹⁰ M, no analyte peaks were observed in the MALDI mass spectra after employing the same enrichment steps. We also examined the trapping capacity of these two types of affinity probes for phosphopeptides. We first generated a calibration curve ($y = 0.00592x + 0.01591$) by plotting the absorbance at 200 nm as a function of the concentration of a phosphopeptide solution purified from the tryptic digest of β-casein. The phosphopeptides derived from β-casein correspond to the sequences of 33–48, 33–52, and 1–25 (see Table S1 in Supporting Information). The absorbance at 200 nm of a phosphopeptide solution (0.1678 mg/mL, 0.3 mL) was 0.5564. After the sample solution enriched by the nanoparticles (120 μg), the absorbance of the remaining solution after separation from the Fe3O4/NTA–Ga(III) and Fe3O4/NTA–Zr(IV) magnetic nanoparticles decreased to 0.33741 and 0.06747, respectively. On the bases of these results, we estimated that 1 μg of Fe3O4/NTA–Ga(III) and Fe3O4/NTA–Zr(IV) could trap 0.1091 and 0.2435 μg of phosphopeptides, respectively. The

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**Figure 4.** (a) Direct MALDI mass spectrum of the tryptic digest of the mixture of α- and β-caseins (10⁻⁷ M). MALDI mass spectra obtained using Fe3O4/NTA–Zr(IV) magnetic nanoparticles (30 μg) as the affinity probes to selectively enrich their target species from the tryptic digest of the mixture (50 μL) of α- and β-caseins at concentrations of (b) 10⁻⁷ and (c) 10⁻⁹ M. The nanoparticle–target species conjugates after enrichment were mixed with 0.5 μL of DHB (30 mg/mL) containing 1% phosphoric acid prior to MALDI MS analysis. “i.s.” stands for internal standard, which was used for internal mass calibration. The peaks marked with asterisks are from α-casein, while the peaks marked with circles are obtained from β-casein.

**Figure 5.** MALDI mass spectra obtained using Fe3O4/NTA–Ga(III) magnetic nanoparticles (30 μg) as the affinity probes to selectively enrich their target species from the tryptic digest of (a) α-casein (10⁻⁸ M, 50 μL) and (b) β-casein (10⁻⁹ M, 50 μL). The nanoparticle–target species conjugates were mixed with 0.5 μL of DHB (30 mg/mL) containing 1% phosphoric acid prior to MALDI MS analysis. The peaks marked with asterisks are phosphopeptides, while the corresponding sequences are marked aside the peaks. The peak at m/z 1943.7 was obtained from the sequence of DIgEsTEDQAMEDIK (51/43–58), while methionine (M) has been oxidized. “s” stands for phosphorylated serine.
Results indicated that the trapping performance of Fe\textsubscript{3}O\textsubscript{4}/NTA–Ga(III) nanoparticles is worse than that of Fe\textsubscript{3}O\textsubscript{4}/NTA–Zr(IV) nanoparticles. The coordination number of Zr(IV) ions is 8,\textsuperscript{34} while it is 6 for Ga(III).\textsuperscript{35} Presumably, the coordination number of the metal ions may play a role in affecting the trapping performance. Zr(IV) ions may be capable of providing more binding sites for phosphopeptides.

To demonstrate that the affinity probe is capable of selectively trapping phosphopeptides from a real complex sample, we used the tryptic digest of milk, which contains abundant proteins including phosphoproteins such as \( \alpha \)- and \( \beta \)-caseins, as the sample. Figure 6a presents the direct MALDI mass spectrum of the tryptic digestion product of milk. Among the peaks, only the peak at \( m/z \) 1952.2 marked with an asterisk is generated from \( \alpha \)-casein. Figure 6b displays the MALDI mass spectrum obtained using Fe\textsubscript{3}O\textsubscript{4}/NTA–Zr(IV) magnetic nanoparticles to selectively trap their target species from the tryptic digest of milk. The peaks marked with asterisks are derived from \( \alpha \)-casein, while the peaks marked with circles are derived from \( \beta \)-casein.

**CONCLUSIONS**

We have established an approach for generating Fe\textsubscript{3}O\textsubscript{4}/NTA magnetic nanoparticles, which are capable of probing multitargets simply by altering the metal ions immobilized on the surface. We demonstrated three types of affinity probes, i.e., Fe\textsubscript{3}O\textsubscript{4}/NTA–Ni(II), Fe\textsubscript{3}O\textsubscript{4}/NTA–Zr(IV), and Fe\textsubscript{3}O\textsubscript{4}/NTA–Ga(III), which can be used to trap selectively His-tagged proteins and phosphopeptides, respectively, from real complex samples. To the best of our knowledge, this is the first time that magnetic nanoparticles immobilized with Zr(IV) and Ga(III) ions are used for the enrichment of phosphopeptides. On the basis of our results, the Fe\textsubscript{3}O\textsubscript{4}/NTA–Zr(IV) nanoparticles have better trapping performance than that of Fe\textsubscript{3}O\textsubscript{4}/NTA–Ga(III) nanoparticles. Furthermore, we also combined the use of microwave-assisted enzymatic digestion using the magnetic nanoparticles as both of the trapping agents and microwave absorbers. Because the approaches of quick enrichment and rapid tryptic digestion were employed, the analysis time can be dramatically reduced. On the basis of this straightforward approach, this method can be readily used to enrich diverse target species from complex samples by immobilizing different metal ions on the surfaces of Fe\textsubscript{3}O\textsubscript{4}/NTA magnetic nanoparticles.

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**SUPPORTING INFORMATION AVAILABLE**

Additional information related to this work. This material is available free of charge via the Internet at http://pubs.acs.org.

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