Facile Synthesis of Guanidyl-Functionalized Magnetic Polymer Microspheres for Tunable and Specific Capture of Global Phosphopeptides or Only Multiphosphopeptides

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Supporting Information

ABSTRACT: The highly selective and efficient capture of heterogeneous types of phosphopeptides is critical for comprehensive and in-depth phosphoproteome analysis, but it still remains a challenge since the lack of affinity material with large binding capacity and controllable specificity. Here, a new affinity material was prepared to improve the enrichment capacity and endure the tunable specificity by introducing guanidyl onto poly(glycidyl methacrylate) (PGMA) modified Fe3O4 microsphere (denoted as Fe3O4@PGMA-Guanidyl). The thick polymer shell endows the composite microsphere with large amount of guanidyl and is beneficial to enhancing the affinity interaction between phosphopeptides and the material. Interestingly, the Fe3O4@PGMA-Guanidyl possesses tunable enriching ability for global phosphopeptides or only multiphosphopeptides through simple regulation of buffer composition. The composite has large enrichment capacity (200 mg g⁻¹), extremely high detection sensitivity (0.5 fmol), high enrichment recovery (91.30%), great specificity, and rapid magnetic separation. Moreover, the result of the application to capture of phosphopeptides from tryptic digest of nonfat milk has demonstrated the great potential of Fe3O4@PGMA-Guanidyl in detection and identification of low-abundance phosphopeptides of interest in biological sample.

KEYWORDS: magnetic microspheres, guanidyl, tunable enrichment ability, phosphorylated peptides, mass spectrum

1. INTRODUCTION

In recent years, nanostructured materials with controlled morphologies, tailored structures, and desired functionalities have drawn considerable attention for both fundamental researches and practical applications. As an important component type, magnetic nanoparticles have gained immense interest due to their unique properties and potential applications in biomedical field including magnetic resonance imaging, drug delivery system, and separation technique. The integrated biomolecules could be tagged and detected magnetically in vivo and in vitro. Along this line, the application of functionalized magnetic nanoparticles to proteomics research has also acquired much attention.

Reversible protein phosphorylation is one of the most common and crucial post-translational modifications and the analysis of the phosphorylated proteins and peptides are of great significance for understanding the disease formation and act as the biomarkers in cell regulatory procedures including cell division, growth, migration, and signal transduction. Currently, mass spectrometry (MS) based techniques have become the most important and powerful tools for the characterization of phosphorylation. Unfortunately, the inherent low abundance and serious signal suppression by the nonphosphorylated peptides make direct MS analysis of phosphopeptides very difficult. Hence, an efficient isolation and enrichment step prior to MS analysis is urgently needed. Global phosphopeptides enrichment gives information on both monophosphopeptides and the IMAC inclines to enrich global phosphopeptides. Among them, the MOAC material appears to have a stronger selectivity for multiphosphopeptides and the IMAC inclines to enrich multiphosphopeptides. The two methods share the same features that they identify different but partially overlapping segments of the phosphoproteome, and no single method is capable of providing a whole phosphoproteome.
The activity of regulatory proteins was found associated with multiphosphorylation, and one of the challenges in comprehensive phosphoproteome is the analysis of multiphosphoproteins. However, the poor ionization efficiency of multiphosphopeptides and severe suppression by the coexistence of nonphosphorylated peptides and monophosphopeptides, extensive efforts have been made to facilitate the detection of multiphosphopeptides. Particular noteworthy is that the polyarginine coated diamond nanoparticles and guanidyl functionalized graphene show high affinity for multiphosphopeptides due to strong arginine (guanidyl)—phosphate interaction. Based on this, design and synthesis of novel functionalized materials with high enrichment efficiency for the specific capture of global phosphopeptides or only multiphosphopeptides are still attracting research attention.

Polymeric coating is an attractive means to tailor surface property. Due to the tunable composition, dispersibility, grafting density, and functionality, polymeric coating is also a promising candidate for biological applications. The abundance of reactive site on the termination enhances binding amount of pendant and provides multipoint attachment between the target and the matrix. The functionalized polymer coated magnetic microspheres have been widely used in anchoring proteins and enriching peptides with high capacity. Qi et al. synthesized poly(glycidyl methacrylate)(methacrylic acid) and poly(ethylene glycol methacrylate) and immoblized the trypsin, and the prepared enzyme reactor was utilized to fast digest of protein. Zou et al. fabricated multilayer polysaccharide (hyaluronate and chitosan) shells coated magnetic nanoparticles for glycogepptide enrichment. Wang et al. developed a novel route to obtained double polymer shells (poly(ethylene glycol methacrylate) and poly(ethylene glycol methacrylate phosphate)) coated magnetic nanoparticles and anchored Ti4+ for phosphopeptides enrichment. The high binding amounts of functionalized groups have improved the detection sensitivity of biomolecules. In addition, the preparation of polymeric coating with required functionalities by a simple and convenient polymerization method is also propitious to further application.

Herein, a new kind of affinity material, the Fe3O4@PGMA-Guanidyl microsphere, was prepared by reflux-precipitation polymerization and a postgrafting modification (as shown in Scheme 1). The thick polymer shell endows the composite with high density of guanidyl for a large enrichment capacity and high detection sensitivity for phosphopeptides. Moreover, the unique magnetic property will facilitate the rapid and complete separation of the affinity material. Besides, the composite can be used for not only capture global phosphopeptides but also only enrich multiphosphopeptides. The specificity, enrichment capacity, detection sensitivity, and enrichment recovery of Fe3O4@PGMA-Guanidyl microsphere in phosphopeptides enrichment have been evaluated by using different biological samples (standard phosphopeptide, phosphoproteins of α- and β-casein, and BSA). Furthermore, the practical applicability of Fe3O4@PGMA-Guanidyl was demonstrated by capture global phosphopeptides or only multiphosphopeptides in tryptic digests of proteins extracted from nonfat milk.

2. EXPERIMENTAL SECTION

2.1. Materials. Iron(III) chloride hexahydrate (FeCl3·6H2O), ethylene glycol (EG), sodium acetate (NaAc), and aqueous ammonia solution (25 wt %) were obtained from Shanghai Chemical Reagents Company (Shanghai, China). γ-Methacryloxypropyltrimethoxysilane (γ-MPS), glycylid methacrylate (GMA), N,N′-methylenebis(acrylamide) (MBA), α- and β-caseins (from bovine milk), bovine serum albumin (BSA), trypsin (TPCK treated), dithiothreitol (DTT), iodoacetamide (IAA), urea, 2,5-dihydroxyl benzoic acid (DHB), and sodium bicarbonate (NaHCO3) were purchased from Sigma-Aldrich (St. Louis, MO). 2,2-Azobis(isobutyronitrile) (AIBN) was supplied by Sinopharm Chemical Reagents Company (Shanghai, China). 2-Ethyl-2-thiopseudourea hydrobromide was received from TCI (St, Louis, MO). 2,2-Azobis(isobutyronitrile) (AIBN) was supplied by Sinopharm Chemical Reagents Company (Shanghai, China). 2-Ethyl-2-thiopseudourea hydrobromide was received from TCI Moving Your Chemistry Forward (Shanghai, China). Acetonitrile (ACN) and trifluoroacetic acid (TFA) were provided by Merck (Darmstadt, Germany). The nonfat milk was obtained from a local supermarket. Standard phosphopeptide (LRRApSLGGK) was from Shanghai Aptide Co., Ltd., (Shanghai, China). Pure water (18.4 MΩ cm) was used in all experiments was purified by a Milli-Q system (Millipore, Milford, MA). All other chemicals including anhydrous ethanol, ethylenediamine, and acetic acid were of analytical grade and used without purification.

2.2. Synthesis of Fe3O4@PGMA microspheres. The Fe3O4 magnetic superparticles were prepared by a solvothermal reaction similar to our previous work. Typically, 2.7 g of FeCl3·6H2O and 7.2 g of NaAc were dissolved in 100 mL of EG. The mixture was stirred vigorously for 1 h at room temperature to form a homogeneous yellow solution and then transferred into a 150 mL Teflon-lined stainless-steel autoclave. The autoclave was heated to 200 °C and maintained for 6 h, before it was cooled to room temperature. The resulted magnetic superparticles were washed several times with ethanol and dried overnight at 80 °C.

Modification of magnetic superparticles with γ-MPS was achieved as follows: 40 mL of ethanol, 10 mL of pure water, 1.5 mL of NH4H2O, and 800 μL of γ-MPS were mixed with 300 mg of Fe3O4. Then, the mixture was stirred vigorously for 24 h at 60 °C. The obtained product was separated by a magnet and washed with anhydrous ethanol to remove excess γ-MPS and dried overnight at 50 °C.

The core−shell Fe3O4@PGMA microspheres were synthesized by an one-step reflux-precipitation polymerization (RPP) of GMA, with MBA as the cross-linker and AIBN as the initiator, in ACN. Specifically, 60 mg of Fe3O4-MPS microspheres were dispersed in 40 mL of ACN in a dried 100 mL single-necked flask. Then, 140 μL of GMA, 150 mg of MBA, and 5 mg of AIBN were added to the flask under ultrasonic condition for 10 min. The flask was submerged in a heating oil bath and heated from room temperature to the boiling state within 30 min, and the reaction was completed after 1.5 h. The obtained Fe3O4@PGMA microspheres were collected by magnetic separation and washed repeatedly with ethanol and water. Finally, the product was dried at 50 °C for 24 h.

2.3. Synthesis of Fe3O4@PGMA-Guanidyl Microspheres. Fe3O4@PGMA microspheres (200 mg) were dispersed in 80 mL of anhydrous ethylenediamine and heated at 80 °C for 3 h. The obtained
amine-terminated microspheres (denoted as Fe3O4@PGMA-NH2) were washed with ethanol and water. Guanidinylation of the pendant amine groups on the microspheres was then carried out as follows: 200 mg of Fe3O4@PGMA-NH2 was dispersed in degassed 10 mmol L−1 of PBS (30 mL) containing 100 mg of 2-ethyl-2-thiopseudourea hydrobromide and reacted at 70 °C under a positive pressure of inert nitrogen gas. After 3 h, the reaction mixture was cooled to room temperature. The final product (denoted as Fe3O4@PGMA-Guanidyl) was separated and washed vigorously with ethanol and water for further use.

2.4. Material Characterization. Transmission electron microscopy (TEM) images were obtained by JEOL JEM-2000 EX transmission electron microscope (JEOL, Tokyo, Japan), and field emission scanning electron microscopy (FE-SEM) images were recorded on JSM-7001F scanning electron microscope. Fourier-transformed infrared spectroscopy (FT-IR) characterization has been performed on Thermo Nicolet 380 spectrometer using KBr pellet (Nicolet, WI). Zeta (ζ) potential measurement was operated on Nano-ZS90 instrument in water at 25 °C (Malvern, Worcestershire, U.K.). Thermogravimetric analysis (TGA) was carried out under nitrogen atmosphere at a heating rate of 10 °C min−1 from 30 to 800 °C (NETZSCH, Selb, Germany). The saturation magnetization curve was conducted on the Physical Property Measurement System 9T (Quantum Design, San Diego, CA) at room temperature.

2.5. Preparation of Tryptic Digests of Standard Proteins. One milligram of α- or β-casein was dissolved in 1 mL of NH4HCO3 solution (50 mmol L−1, pH = 8.3) and digested at 37 °C for 16 h with trypsin at the mass ratio of enzyme to protein of 1:40 (w/w). BSA (2 mg) was dissolved in 1 mL of buffer containing urea (8 mol L−1) and NH4HCO3 solution (50 mmol L−1). After the addition of 20 μL of DTT (1 mol L−1), the mixture was incubated at 60 °C for 1 h to reduce the disulfide bonds of proteins. Subsequently, 7.4 mg of IAA was added and the mixture was incubated at room temperature in the dark for 45 min. Finally, the mixture was diluted 10-fold with NH4HCO3 (50 mmol L−1) and incubated at 37 °C for 16 h with trypsin at the mass ratio of enzyme to protein of 1:40 (w/w).

2.6. Preparation of Tryptic Digests of Proteins Extracted from Nonfat Milk. Nonfat milk (30 μL) was dissolved in 1 mL of NH4HCO3 (25 mmol L−1), and this solution was centrifuged at 16 000 rpm for 10 min. The supernate was saved; then denaturation proceeded at 100 °C for 10 min. The supernate was digested with trypsin (40 μg) at 37 °C for 16 h. This tryptic digest of nonfat milk was diluted by loading buffer for further use.

2.7. Selective Capture Global Phosphopeptides or Only Multiphosphopeptides with Fe3O4@PGMA-Guanidyl. Fe3O4@PGMA-Guanidyl microspheres were washed with loading buffer 1 (50% ACN-H2O, 0.1 mol L−1 HAc, for global phosphopeptides) or loading buffer 2 (66% ACN-H2O, 0.02% TFA, for multiphosphopeptides) and then suspended in corresponding loading buffer. Tryptic digests of α-casein, β-casein, BSA, or proteins extracted from nonfat milk were dissolved in loading buffer; then, Fe3O4@PGMA-Guanidyl microspheres were added, and the mixture was incubated at room temperature for 20 min. After removing the supernatant, the microspheres were washed three times with loading buffer (400 μL) to remove the nonspecifically adsorbed peptides. The captured phosphopeptides were eluted by 50% ACN-H2O containing 2% TFA (2 × 10 μL) under powerful shaking for 10 min. The eluate was analyzed by MALDI-TOF MS.

2.8. Evaluation of the Enrichment Capacity of Fe3O4@PGMA-Guanidyl for Phosphopeptides. Different amounts of Fe3O4@PGMA-Guanidyl microspheres (2–200 μg) were mixed with a fixed amount of tryptic digest of β-casein (1 μg) in loading buffer 1 (50% ACN-H2O, 0.1 mol L−1 HAc) and the mixture was incubated for 20 min. After the washing and elution procedure, the eluted fraction (0.5 μL from 20 μL total) was analyzed with MALDI-TOF MS. When the mass signal intensity of the target phosphopeptide reached the maximum value, the material was enough to capture of almost the total phosphopeptides. The enrichment capacity was calculated by the amount of β-casein (1 μg) divided by the amount of the microspheres.

2.9. Recovery Test of Phosphopeptide Enrichment. A certain amount of standard phosphopeptide (LRRApSLGGK) was divided equally into two parts and labeled with light and heavy isotopes by using a stable isotope dimethyl labeling approach according to our previous reported procedure.33 Then, the heavy labeled phosphopeptide (0.5 pmol) was enriched with Fe3O4@PGMA-Guanidyl (50 μg) according to the procedure mentioned above. The eluted section was mixed with the same amount of light labeled phosphopeptide (0.5 pmol), and the mixed peptides were analyzed by MALDI-TOF MS. The recovery of standard phosphopeptide was calculated by the MS intensity ratio of the heavy labeled phosphopeptide divided by the light labeled phosphopeptide.

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

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of Fe3O4@PGMA-Guanidyl Microspheres. The procedure for the fabrication of composite microsphere containing a supermagnetic core and a functional polymer shell with abundant guanidyl was schematically illustrated in Scheme 1. First, Fe3O4 particle was synthesized by a solvothermal reaction. Second, the particle was modified with γ-MPS to form abundant available double bonds in order to promote the polymer coating in the next step. Third, a layer of GMA polymer was coated onto the Fe3O4-MPS surface by one-step reflux-precipitation polymerization and the epoxide groups were introduced to the material. Fourthly, the epoxide groups on the polymer reacted with anhydrous ethylenediamine to obtain a hydrophilic intermediate with amino groups through ring open reaction. Finally, the abundant amino groups reacted with 2-ethyl-2-thiopseudourea hydrobromide and the guanidyl was immobilized onto the microsphere to obtain the Fe3O4@PGMA-Guanidyl microsphere. For comparison, the Fe3O4 microsphere without the polymer shell was also modified with guanidyl according to the above procedure.

The morphology and characteristic of the as-prepared magnetic microspheres were measured by various characterization methods. Representative TEM images of Fe3O4 and Fe3O4@PGMA are shown in Figure 1a, b. The Fe3O4 particles are spherical in shape and have an average diameter of about 220 nm. After encapsulated with GMA polymer by RPP method, the particles showed obvious core–shell structure and the size of the composite microspheres increased to around 340 nm with the shell thickness of about 60 nm in dry state. After the material was modified with anhydrous ethylenediamine and 2-ethyl-2-thiopseudourea hydrobromide, its structure and morphology have no significant change (Figure S1a, b in the Supporting Information), indicating the robust polymer shell structure. In addition, the surface of Fe3O4@PGMA microspheres was smoother than that of the Fe3O4 cores (Figure 1c, d).

The functional groups of the polymer shell were analyzed by FT-IR spectroscopy. As shown in Figure 2a, the peaks at 1720 and 908 cm−1 are attributed to the stretching vibration of C=O of the ester group and the epoxy group of GMA, respectively. The peak at 1530 cm−1 corresponds to the bending vibration of N–H in the MBA, proving successful coating of GMA polymer shell on the surface of Fe3O4. Compared with the FT-IR spectrum of Fe3O4@PGMA, the peak at 908 cm−1 disappears in
the spectrum of Fe₃O₄@PGMA-Guanidyl, implying that the epoxy groups reacted completely with ethylenediamine in the postgrafting process. However, the characteristic adsorption peaks of guanidyl group are not obvious. Therefore, ζ-potential measurement was conducted to confirm the postgrafting procedure. The ζ-potential value of Fe₃O₄@PGMA-NH₂ in pure water (pH = 7.0) is +8.96 mV. After modification with guanidyl groups, the value changes to +17.4 mV (Figure 2b), indicating the successful functionalization with guanidyl group.

Thermogravimetric analysis (TGA) was executed to quantitatively determine the composition of the composite microsphere. The TGA curves in each step are shown in Figure 2c. The 9.7 wt % loss of Fe₃O₄-MPS is attributed to the MPS, indicating Fe₃O₄ content of 90.3 wt %. After coating by GMA layer, the Fe₃O₄ content in composite microsphere dramatically declined to 31.8 wt %. Compared with the previous microsphere, the excess weight losses of Fe₃O₄@PGMA-NH₂ and Fe₃O₄@PGMA-Guanidyl are about 4.8 and 8.7 wt %, respectively, indicating that high amounts of amino and guanidyl groups were grafted on the polymer shell in the postgrafting procedure.

The magnetic properties of the three kinds of microspheres were studied using vibrating sample magnetometer at room temperature (Figure 2d). The magnetic hysteresis curves show the three kinds of microspheres have no obvious remanence or coercivity at room temperature, suggesting that they all could be supermagnetic. As a comparison, the saturation magnet-
fragment of phosphopeptides through loss of $\text{H}_3\text{PO}_4$ was marked with a circumflex. It was found that the $\textrm{Fe}_3\textrm{O}_4@\textrm{PGMA-Guanidyl}$ not only captures monophosphopeptides and multiphosphopeptides simultaneously without the interference of nonphosphorylated peptides with high abundance but also can separate the multiphosphopeptides from the monophosphopeptides and nonphosphorylated phosphopeptides. As shown in Figure 3b, two monophosphopeptides ($\beta1$, $\beta2$) and one multiphosphopeptide ($\beta4$) (Table S1 in the SI) could be clearly identified after enrichment in 50% ACN, 0.1 M HAc buffer. The S/N ratio of the peak of $\beta1$ was increased nearly 6 folds. When the loading buffer was changed to 66% ACN, 0.02% TFA, only one multiphosphopeptide ($\beta4$) was detected (Figure 3c). Compared with the S/N ratio of the peak of $\beta4$ in Figure 3b, the value was further enhanced nearly 3 times. The adjacent arginine residues (guanidine moieties) and the phosphate groups are able to generate stable complexes having “covalent-like” stability.\(^{36}\) Hence, the phosphopeptides were captured from the mixture by the guanidyl-functionalized particles. In loading buffer with lower acidity (50% ACN, 0.1 M HAc), mono- and multiphosphopeptides were simultaneous captured by the composite through the strong affinity interaction between phosphate group and guanidyl group. However, in buffer with higher acidity (66% ACN, 0.02% TFA), the affinity interaction between monophosphopeptides and the guanidyl groups was weakened, and the multiphosphopeptides were still adsorbed by the material due to the strong multivalent interaction. The results demonstrate the $\textrm{Fe}_3\textrm{O}_4@\textrm{PGMA-Guanidyl}$ microspheres possess tunable and specific enriching ability for global phosphopeptides or only multiphosphopeptides through simple regulation of loading buffer composition.

To further confirm the tunable enriching ability for phosphopeptides, tryptic digest of $\alpha$-casein with more phosphorylation sites was employed. A direct MALDI-TOF mass spectrum of $\alpha$-casein is illustrated in Figure 4a. The MS signals of phosphopeptides were severely suppressed by the nonphosphorylated peptides. Nevertheless, the situation dramatically changed when $\textrm{Fe}_3\textrm{O}_4@\textrm{PGMA-Guanidyl}$ was used for extraction and enrichment (Figure 4b and c). The peaks of 8 monophosphopeptides and 6 multiphosphopeptides (Table S1 in the SI) were observed after enrichment in 50% ACN, 0.1 M HAc buffer while 7 multiphosphopeptides were found in 66% ACN, 0.02% TFA buffer. The result was in agreement with the enrichment selectivity toward mono- and multiphosphopeptides in two buffers using $\beta$-casein tryptic digest.

The $\textrm{Fe}_3\textrm{O}_4@\textrm{PGMA-Guanidyl}$ was further utilized in the selective enrichment of phosphopeptides with the interference of abundant nonphosphorylated peptides. A mixture of $\beta$-casein and BSA tryptic digests was used as the testing sample. As shown in Figure 5a, when the molar ratio of $\beta$-casein and BSA was 1:400, no phosphopeptide peak was observed. However, after enrichment in 50% ACN, 0.1 M HAc buffer, three phosphopeptides could be clearly detected (Figure 5b). It is similar to the result of the enrichment in 66% ACN, 0.02% TFA buffer (Figure 5c). Obviously, the result demonstrates that $\textrm{Fe}_3\textrm{O}_4@\textrm{PGMA-Guanidyl}$ could specifically capture phosphopeptides.

3.3. Evaluation of the Enrichment Capacity, Enrichment Recovery, and Detection Sensitivity of $\textrm{Fe}_3\textrm{O}_4@\textrm{PGMA-Guanidyl}$ in Phosphopeptides Enrichment. In the synthesis process of guanidyl functionalized magnetic affinity
Phosphopeptides from Nonfat Milk. Encouraged by its unique properties, we used nonfat bovine milk digest to further examine the selectivity and effectiveness of the prepared Fe₃O₄@PGMA-Guanidyl microspheres in the specific capture of low-abundant phosphopeptides from a real complex sample. Figure 6a displays the direct analysis of the trypic digest of milk. Nonphosphorylated peptides dominated the spectrum and only one weak MS signal intensity of phosphopeptide was detected. From one aspect, after treatment with Fe₃O₄@PGMA-Guanidyl in 50% ACN, 0.1 M HAc buffer, 6 monophosphopeptides and 5 multiphosphopeptides with higher MS intensities were distinctly observed with a clean background (Figure 6b). From other aspect, 8 multiphosphopeptides were identified in 66% ACN, 0.02% TFA buffer (Figure 6c). The detailed information on the 14 phosphopeptides from trypic digest of proteins extracted from nonfat bovine milk is given in Table S2 in the Supporting Information. The results suggest that Fe₃O₄@PGMA-Guanidyl is capable of simultaneous trapping monophosphopeptides and multiphosphopeptides or highly selective capture of multiphosphopeptides from tryptic digest of α-casein and β-casein is in accordance with our results, but the unique magnetic property, high amount of guanidyl groups of the Fe₃O₄@PGMA-Guanidyl affinity microspheres, which resulting in convenient separation procedure, high enrichment capacity, high enrichment recovery and extremely high detection sensitivity, make it a more capable candidate for phosphopeptides enrichment. Besides, the SAX particles (quaternary amonium groups modified particles) and TiO₂ particles were also adopted for the enrichment and separation. For one thing, the numbers and S/N ratios of the identified phosphopeptides from α-casein and β-casein were far less than that of guanidyl-functionalized particles in loading buffer 1 and loading buffer 2. The result further demonstrated that the enrichment recovery and separation. For one thing, the numbers and S/N ratios of the identified phosphopeptides from α-casein and β-casein were far less than that of guanidyl-functionalized particles in loading buffer 1 and loading buffer 2. The result further demonstrated that the affinity interactions between guanidyl groups and phosphopeptides are not electrostatic interactions but are more stable interactions. Additionally, fewer monophosphopeptides and multiphosphopeptides could be detected with an inferior enrichment capacity and S/N ratios in the case of using TiO₂ as the adsorbent (Figure S4 in the SI), indicating its superiority of guanidyl-particle not only in global phosphopeptides but also in phosphopeptides.

3.4. Application in Highly Specific Enrichment of Phosphopeptides from Nonfat Milk. Encouraged by its unique properties, we used nonfat bovine milk digest to further examine the selectivity and effectiveness of the prepared Fe₃O₄@PGMA-Guanidyl microspheres in the specific capture of low-abundant phosphopeptides from a real complex sample. Figure 6a displays the direct analysis of the trypic digest of milk. Nonphosphorylated peptides dominated the spectrum and only one weak MS signal intensity of phosphopeptide was detected. From one aspect, after treatment with Fe₃O₄@PGMA-Guanidyl in 50% ACN, 0.1 M HAc buffer, 6 monophosphopeptides and 5 multiphosphopeptides with higher MS intensities were distinctly observed with a clean background (Figure 6b). From other aspect, 8 multiphosphopeptides were identified in 66% ACN, 0.02% TFA buffer (Figure 6c). The detailed information on the 14 phosphopeptides from trypic digest of proteins extracted from nonfat bovine milk is given in Table S2 in the Supporting Information. The results suggest that Fe₃O₄@PGMA-Guanidyl is capable of simultaneous trapping monophosphopeptides and multiphosphopeptides or highly selective capture of multiphosphopeptides from a naturally obtained complex biological sample.

4. CONCLUSIONS

To summarize, the Fe₃O₄@PGMA-Guanidyl, a tunable and specific affinity material, was successfully synthesized by introducing guanidyl onto a GMA polymer modified Fe₃O₄.
microsphere for the enrichment of phosphopeptides with large enrichment capacity, extremely high detection sensitivity and high enrichment recovery. The thick GMA polymer increases the amount of guanidyl and endows the material with enhanced enrichment capacity and detection sensitivity. Importantly, the results have confirmed that the Fe3O4@PGMA-Guanidyl microsphere has tunable enriching ability for global phosphopeptides or only multiphosphopeptides through simple regulation of loading buffer. Furthermore, in the selective enrichment of phosphopeptides from nonfat milk, the Fe3O4@PGMA-Guanidyl microspheres show great practicability in identifying low-abundant global phosphopeptides or only multiphosphopeptides from complex biological sample. We believe that this work could offer a novel material for comprehensive phosphoproteome research.

■ ASSOCIATED CONTENT
1 Supporting Information
TEM images of the Fe3O4@PGMA-NH2 and Fe3O4@PGMA-Guanidyl microspheres, detailed information on the observed phosphopeptides from α-casein, β-casein, and nonfat milk, binding capacity, enrichment recovery, and detection sensitivity of phosphopeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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