Magnetic Affinity Microspheres with Meso-/Macroporous Shells for Selective Enrichment and Fast Separation of Phosphorylated Biomolecules

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Supporting Information

ABSTRACT: The flowerlike multifunctional affinity microspheres prepared by a facile solvothermal synthesis and subsequent calcination process consist of magnetic cores and hierarchical meso-/macroporous TiO2 shells. The hierarchical porous structure of the flowerlike affinity microspheres is constructed by the macroporous shell from the stacked mesoporous nanopetals which are assembled by small crystallites. The affinity microspheres have a relatively large specific surface area of 50.45 m² g⁻¹ and superparamagnetism with a saturation magnetization (Ms) value of 30.1 emu g⁻¹. We further demonstrate that they can be applied for rapid and effective purification of phosphoproteins, in virtue of their selective affinity, porous structure, and strong magnetism. In addition, the affinity microspheres can also be used for enrichment of phosphopeptides, and the selectivity is greatly improved due to the increase of mass transport and prevention of the possible “shadow effect” resulting from the smaller and deeper pores by taking advantage of the unique porous structure. Overall, this work will be highly beneficial for future applications in the isolation and identification of phosphorylated biomolecules.

KEYWORDS: affinity probe, porous structure, protein separation, peptide enrichment, biotechnology

INTRODUCTION

Multifunctional hierarchical architectures with designed structures, controlled morphologies, large surface area, and desirable components have fueled research on the construction of affinity probes and the interaction of biomolecules.¹⁻³ Hierarchical architectures with tailored textures would facilitate interaction between the affinity materials and target biomolecules because the unique chemical, physical, and surface properties of nanostructured materials may effectively contribute to shorten the biomolecule transfer-distance and provide an affinity-mediating function.⁴⁻⁻⁷ Especially, various metal oxide nanostructures have been demonstrated to be effective for selective separation and adsorption of biomolecules owing to their selective affinity, large specific surface area, and modifiable surface properties.⁸⁻⁻¹⁰ However, significant challenges remain in the development of affinity nanostructures that show large surface area, easy biomolecule transfer, superior specificity, and high sensitivity.

Protein phosphorylation is one of the most important post-translated modifications involved in regulating various cellular processes such as metabolism, signaling, cell growth, and division. It is reported that many diseases (e.g., cancer and Alzheimer) are relevant to the abnormal phosphorylation,¹¹,¹² and some phosphoproteins and phosphopeptides play important roles in medical inspection.¹³⁻⁻¹⁵ Thus, the identification of phosphorylated biomolecules is a fundamental step toward understanding and detecting the signaling pathways in normal and disease states. Nevertheless, the detection of phosphorylated biomolecules remains a major challenge due to the small quantity of phosphoproteins, the low ratio of phosphorylation, and the signal suppression effect by nonphosphopeptides or proteins.¹⁶,¹⁷ In recent years, significant efforts have been expended to the development of affinity materials that can rapidly and effectively enrich or isolate phosphopeptides,¹⁸,¹⁹ and metal-ion immobilized and metal oxide materials are the most commonly used affinity materials.²⁰⁻⁻²⁴ Especially, mesoporous metal oxide nanostructures have been developed as affinity probes and represent current state of the art for selective capture of low-abundance phosphopeptides, due to their high surface area, superior

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chemicophysical behavior and excellent surface properties.\textsuperscript{25,26} However, the “shadow effect” from the smaller and deeper pores would be unavoidable and hamper the release of target biomolecules.\textsuperscript{27–30} Therefore, only a few of them can be applied for extraction of target peptides from the complex real biosamples. Furthermore, few of them could be used for purification of phosphoproteins, because the single-sized mesoporous materials often suffer from the size exclusion effect due to the relative large size of target proteins, resulting in the low adsorption efficiency and significant sample losses during the washing steps.\textsuperscript{31–33} In addition, the centrifugal separation used for isolating the nanostructures carrying target biomolecules from complex biological samples is a time-consuming and inconvenient procedure. Considering the facile separation of magnetic particles under an external magnetic field, magnetic particles have been applied in bioseparation.\textsuperscript{34–37}

Although some flowerlike TiO\textsubscript{2} microspheres with hierarchical structures were prepared via solvothermal/hydrothermal method using TiCl\textsubscript{4} or titane as precursor and pure ethanol or isopropanol with organic additive (e.g., glycerol, glycerine) as solvent,\textsuperscript{38–41} little work has been carried out for the core–shell Fe\textsubscript{3}O\textsubscript{4}@TiO\textsubscript{2} microspheres with similar hierarchical flowerlike TiO\textsubscript{2} shell based on the above method.\textsuperscript{42,43} For example, Li et al.\textsuperscript{42} reported synthesis of the flowerlike Fe\textsubscript{3}O\textsubscript{4}@TiO\textsubscript{2} yolk–shell microspheres by means of Fe\textsubscript{3}O\textsubscript{4}@SiO\textsubscript{2}@TiO\textsubscript{2} semi-finished product. Chen et al.\textsuperscript{43} reported preparation of the flowerlike magnetic nanostructures by reducing the as-synthesized α-Fe\textsubscript{3}O\textsubscript{4}@TiO\textsubscript{2}–NS which was obtained via the solvothermal method using α-Fe\textsubscript{3}O\textsubscript{4}@SiO\textsubscript{2}@SnO\textsubscript{2} and titanium isopropanoxide as precursors. By this token, it is difficult to directly coat the affinity shells with hierarchical porous structure on the surface of Fe\textsubscript{3}O\textsubscript{4} particles. Herein, to address above issues, we report a facile fabrication of the flowerlike core/shell affinity microspheres with magnetic Fe\textsubscript{3}O\textsubscript{4} cores and hierarchical meso-/macroporous TiO\textsubscript{2} shells (denoted as Fe\textsubscript{3}O\textsubscript{4}@fTiO\textsubscript{2}) for selective enrichment and fast separation of phosphorylated biomolecules. Both the phosphoproteins and phosphopeptides can be effectively enriched, and the enrichment efficiency and selectivity were highly improved by taking advantage of the meso-/macroporous affinity structure for increasing mass transport of target biomolecules. Meanwhile, such porous structure holding the merit of large specific surface area can further increase adsorption efficiency and MS detection sensitivity of the phosphorylated proteins/peptides due to the more adsorption sites for target biomolecules. In addition, compared with the bioseparation techniques used frequently (e.g., filtration, ion exchange, centrifugation, and gravitational separation), the magnetic bioseparation technique with high efficiency and low cost can realize the fast, simple, and effective isolation of biosamples. It is expected that this work could provide new insights for the design of the affinity probes for extracting and separating low-abundance biomarkers from complex biosamples in biomedical applications.

## EXPERIMENTAL SECTION

**Chemicals.** Ferric chloride (FeCl\textsubscript{3}·6H\textsubscript{2}O), ethylene glycol (EG), isopropanol (IPA), dimethylformamide (DMF), and ammonium hydroxide (NH\textsubscript{4}·H\textsubscript{2}O) were obtained from Beijing Chemical Regent Co., Ltd. (Beijing, China). Tetraethyl titanate (TBOT), trifluoroacetic acid (TFA), acetonitrile (ACN), ammonium bicarbonate (NH\textsubscript{4}HCO\textsubscript{3}), sinapic acid (SA), 2,5-dihydroxybenzoic acid (2,5-DHB), and dithiothreitol (DTT) were purchased from Aladdin (Shanghai, China). Bovine β-casein, bovine serum albumin (BSA), myoglobin (from equine heart), horseradish peroxidase, and trypsin (from bovine pancreas, TPCK treated) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iodoacetamide (IAA) was provided by Alfa Aesar (USA).

**Preparation of Core/Shell Structured Fe\textsubscript{3}O\textsubscript{4}@fTiO\textsubscript{2} Microspheres.** The magnetic Fe\textsubscript{3}O\textsubscript{4} particles were prepared according to our previous reported synthetic process.\textsuperscript{44} Typically, FeCl\textsubscript{3}·6H\textsubscript{2}O (2.72 g) and NaAc (7.20 g) were completely dissolved in EG (80 mL) under vigorously magnetic stirring. The obtained homogeneous solution was transferred to a Teflon-lined stainless-steel autoclave (120 mL) and sealed to heat at 180 °C for 6 h. Then, it was cooled to room temperature naturally. The obtained product was washed with ethanol and deionized water in sequence with the help of a magnet and, then, dried in vacuum at 60 °C for 12 h.

The flowerlike Fe\textsubscript{3}O\textsubscript{4}@fTiO\textsubscript{2} hierarchical microspheres were synthesized by a facile solvothermal method. Typically, the as-synthesized Fe\textsubscript{3}O\textsubscript{4} particles (40 mg; see Figure S1) were dispersed in DMF (10 mL) and IPA (30 mL) under ultrasonication. The mixture was transferred into a Teflon-lined stainless-steel autoclave (50 mL) and heated to 200 °C for 5 min. The mixture was transferred into a Teflon-lined stainless-steel autoclave (50 mL) and heated to 200 °C for 5 min. The mixture was transferred into a Teflon-lined stainless-steel autoclave (50 mL) and heated to 200 °C for 5 min. Finally, the products were washed with ethanol, dried overnight at 60 °C, and calcined at 400 °C for 2 h.

**Characterization of Fe\textsubscript{3}O\textsubscript{4}@fTiO\textsubscript{2} Microspheres.** A field emission scanning electron microscope (FESEM, 8400, Hitachi) equipped with an energy-dispersive X-ray spectrum (EDX, JEOLJXA-840) was applied to determine morphologies and compositions of the as-prepared samples. Transmission electron microscopy (TEM) and high-resolution TEM (HRTEM) images were taken with a FEI Tecnai G2 S-Twin transmission electron microscope operated at 200 kV. Powder X-ray diffraction (XRD) patterns were collected on a Bruker D8 Focus X-ray diffractometer using Cu Kα radiation (λ = 1.5405 Å). Fourier-transform infrared spectroscopy (FTIR) analysis was carried out on a Perkin-Elmer 580B infrared spectrophotometer using the KBr pellet technique. Nitrogen adsorption isotherms were measured at a liquid nitrogen temperature (77 K) with a Micromeritics ASAP 2010 M apparatus. The specific surface area was determined by the Brunauer–Emmett–Teller (BET) method. The total pore volume was evaluated by the t-pot method, and pore size distribution was analyzed with the supplied BJH software package from the adsorption branch of the isotherms. Magnetization measurement was carried out with a superconducting quantum interface device (SQUID) magnetometer (Quantum Design MPMS XL) at 300 K.

**Purification of Phosphoproteins.** To evaluate selectivity and efficiency of the Fe\textsubscript{3}O\textsubscript{4}@fTiO\textsubscript{2} microspheres for enrichment of phosphoproteins, the Fe\textsubscript{3}O\textsubscript{4}@fTiO\textsubscript{2} microspheres (3 mg) were incubated in a phosphoprotein (β-casein) solution with different concentration (3 mL buffer solution containing 50% acetonitrile and 0.5% TFA) with shaking. After adsorption, the Fe\textsubscript{3}O\textsubscript{4}@fTiO\textsubscript{2} microspheres captured phosphoproteins were isolated from the suspension with a magnet. The concentrations of residual proteins in the supernatant were detected, and the adsorption capacities were calculated indirectly. The collected supernatant was lyophilized to dryness and subsequently dissolved in PBS solution. The resulting protein solution was measured using a UV–visible spectrophotometer under monitored at 280 nm. The UV–vis absorption spectroscopy analysis was carried out with a Shimadzu UV-1750 UV–visible spectrophotometer.

To further evaluate selectivity and efficiency of the Fe\textsubscript{3}O\textsubscript{4}@fTiO\textsubscript{2} microspheres for enrichment of phosphoproteins, a complex protein solution (prepared in the 50% acetonitrile and 0.5% TFA buffer solution) containing β-casein (1×10\textsuperscript{−2} M), myoglobin (2×10\textsuperscript{−2} M), horseradish peroxidase (1×10\textsuperscript{−5} M), and BSA (2.5×10\textsuperscript{−5} M) was used. Fe\textsubscript{3}O\textsubscript{4}@fTiO\textsubscript{2} microspheres (0.2 mg) were incubated in 60 μL of the protein mixture solution with shaking for 30 min. The Fe\textsubscript{3}O\textsubscript{4}@fTiO\textsubscript{2} microspheres were isolated from the suspension with a magnet and, then, washed with the buffer solution two times. To elute the captured proteins, the Fe\textsubscript{3}O\textsubscript{4}@fTiO\textsubscript{2} microspheres were redispersed into 40 μL of 10% ammonium hydroxide with shaking for 5 min. After
magnetic separation, the supernatant was collected and lyophilized to dryness. According to the same procedure, the controlling test was performed using commercial TiO₂ particles as the substitute of the Fe₃O₄@pTiO₂ microspheres.

**Selective Enrichment of Phosphopeptides.** To evaluate selectivity and practice service performance of the Fe₃O₄@pTiO₂ microspheres for enrichment of phosphopeptides, the digests of β-casein and BSA were used as the complex peptide testing solutions. A 1.0 mg portion of β-casein or BSA was dissolved in 1 mL of 50 mM NH₄HCO₃ solution. Then, trypsin was added into the solution with a ratio of 50:1 (protein/trypsin, w/w). After that, the solution was incubated at 37 °C for 16 h. Finally, the obtained tryptic digests were diluted to the target concentration with 0.5% TFA solution in acetonitrile/deionized water (1/1, v/v).

Enrichment experiments of the phosphopeptides are described as follows. A 100 μL portion of protein digest solution was mixed with 5 μL of 20 mg/mL Fe₃O₄@pTiO₂ microspheres suspending solution and then shaken for 2 min. Subsequently, the microspheres captured phosphopeptides were collected and isolated from the mixture with the help of a magnet. The obtained microspheres were washed with 50 μL 0.5% TFA solution in acetonitrile/deionized water (1/1, v/v) three times. For the peptide mixture of β-casein and BSA, three aqueous solutions of 0.5%, 1.0%, or 1.5% TFA in acetonitrile/deionized water (1/1, v/v) were used to optimize the affinity selectivity. After that, the trapped phosphopeptides were eluted with 20 μL of 10% ammonium hydroxide. After magnetic separation, the supernatant was collected and lyophilized to dryness.

For enriching and isolating the phosphopeptides from human serum, the pristine human serum (0.5 μL) used as the real sample was dissolved in 50 μL 0.5% TFA solution in acetonitrile/deionized water (1/1, v/v) and, then, treated according to the same procedure as the standard protein digests.

**Mass Spectrometry Analysis.** Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The above products obtained from the elution step were dissolved in 2 μL of matrix solution containing 20 mg/mL SA (for proteins) or DHB (for peptides) in 50% acetonitrile and 1% H₃PO₄ aqueous solution (v/v) by pipetting, and 0.5 μL of the mixture was deposited onto the MALDI target. MALDI-TOF MS experiment were performed on Bruker Autoflex III MALDI-TOF MS (Bruker Daltonics Bremen, Germany) equipped with a 355-nm Nd:YAG laser. The acceleration voltage and repetition rate were set at 19 kV and 200 Hz, respectively, while the laser power was optimized. MS spectra were acquired as an average of 100 laser shots for three times, which was obtained in positive ion reflection mode and analyzed by Bruker Daltonics flex Analysis software.

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**RESULTS AND DISCUSSION**

The morphologies, elemental analysis, crystal and surface structures, and magnetism of the flowerlike Fe₃O₄@fTiO₂ microspheres were characterized using modern testing instruments. A possible formation mechanism is proposed based on time-dependent evolution experiments. The selectivity and efficiency of the Fe₃O₄@fTiO₂ microspheres for phosphorylated protein/peptide separation were evaluated using a commercial phosphoprotein (β-casein) and a normal protein (BSA) as model proteins. Finally, the affinity microspheres were applied to enrich phosphopeptides from the real biological sample (the pristine human serum).

**Morphologies and Structures of the Flowerlike Fe₃O₄@fTiO₂ Microspheres.** As illustrated in Scheme 1a, the hierarchical flowerlike Fe₃O₄@fTiO₂ microspheres were synthesized by a facile solvothermal synthesis and subsequent calcination process. The flowerlike precursor composites with macroporous shell (denoted as Fe₃O₄@pTiO₂) were first prepared through a solvothermal reaction using the as-synthesized Fe₃O₄ particles and TBOT as the precursors and the mixed solution of DMF and IPA as the organic solvent. Then, the hierarchical flowerlike Fe₃O₄(and/or γ-Fe₂O₃)@fTiO₂ microspheres with meso-/macroporous shell were obtained after the flowerlike Fe₃O₄@pTiO₂ microspheres were calcined at 400 °C for 2 h in air. To conveniently depict the microspheres, they were still denoted as the Fe₃O₄@fTiO₂.

Morphologies and structures of the Fe₃O₄@fTiO₂ microspheres were characterized using scanning electron microscopy (SEM). The precursor Fe₃O₄@pTiO₂ (Figure 1a and b) microspheres with an average diameter of about 1 μm have the flowerlike heterogeneous TiO₂ shell. The flower-like TiO₂ shell with a thickness of about 270 nm, as revealed in the SEM image of a broken Fe₃O₄@pTiO₂ microsphere, is composed of many TiO₂ nanopetals wrapped around the Fe₃O₄ core. Importantly, the petallike TiO₂ nanosheets assembled together constructed many macropores with pore size 50–200 nm, which help to avoid the “shadow effect” and the size exclusion effect of the smaller and deeper pores by means of effective mass transport.
High magnification SEM images (Figure 1d and Supporting Information Figure S2a) further demonstrated that the macroporous structure is composed of the cross-linked nanopetals with thickness of about 10 nm, which erectly grew on the surface of the Fe3O4 microspheres.

Figure 2 shows the SEM and TEM images of the Fe3O4@fTiO2 microspheres from the calcined Fe3O4@pTiO2 microspheres. Compared with the precursor Fe3O4@pTiO2 microspheres, the flowerlike structures and sizes of the Fe3O4@fTiO2 microspheres (Figure 2a, b) are almost unchanged. After careful observation and comparison, we found that the nanopetals of the Fe3O4@fTiO2 microspheres have become very coarse, which may be ascribed to the recrystallization of titania. TEM images of the Fe3O4@fTiO2 microspheres (Figure 2c) confirm that the Fe3O4@fTiO2 microspheres are still core−shell structures and the macroporous shells are constructed from curved nanopetals. High-resolution TEM images (Figure 2d) further reveal that the nanopetal composed of numerous nanocrystallites assembled together has many shallow mesopores on it, which remarkably increase the specific surface area and active sites. In addition, the lattice fringes of these nanocrystallites with a spacing of 0.351 nm (Figure 2d, inset) can be apparently observed, indicating the existence of many hydroxyls on the surface, and large adsorptive capacity, which can facilitate the transport of target biomolecules, effectively avoid the “shadow effect” and improve the affinity selectivity and efficiency.

The elemental composition and the crystal structure of the Fe3O4@fTiO2 microspheres can be verified by energy-dispersive X-ray spectrum (EDX), powder X-ray diffraction (XRD), and Fourier-transform infrared (FTIR) spectroscopic analysis. Comparison of EDX spectra between the Fe3O4@fTiO2 microspheres and the Fe3O4 particles (as shown in Figure 3a) reveals the existence of the TiO2 shell due to the presence of Ti element in the EDX spectrum of the Fe3O4@fTiO2 microspheres besides the Fe and O elements. It should be pointed out that the C element in the EDX spectra comes from the conductive tape used to fix the sample.

Compared with diffraction peaks of the Fe3O4 particles, diffraction peaks in the XRD pattern of Fe3O4@fTiO2 microspheres (Figure 3b) can be respectively assigned to the characteristic diffraction peaks from iron oxide (Fe3O4 and/or γ-Fe2O3: JCPDS card no. 75−0449 or 25−1402) and those from the crystalline anatase TiO2 phase (JCPDS card no. 21−1272) (marked asterisk). The XRD result indicates that the Fe3O4 phase could be entirely or partially transformed into γ-Fe2O3 phase after calcination at 400 °C for 2 h in air under protection of the TiO2 coating. It is well-known that it is difficult to distinguish between the two oxides by means of XRD due to their very similar structures. However, in this work, we mainly focused on magnetism of iron oxide for magnetic separation of the target biomolecules.

FTIR spectra of the Fe3O4 particles and the Fe3O4@fTiO2 microspheres are shown in Figure 2c. The characteristic absorption peak at around 560 cm−1 can be assigned to stretching vibration of Fe−O bonds of the Fe3O4, while the characteristic absorption peak at around 460 cm−1 in FTIR spectra of Fe3O4@fTiO2 microspheres can be attributed to stretching vibration of Ti−O bonds of the TiO2 shell. In addition, a broad band at around 3400 cm−1 can be obviously observed, indicating the existence of many hydroxyls on the surface of Fe3O4@fTiO2 microspheres. Above results further confirm the formation of the Fe3O4@fTiO2 microspheres.

Possible Formation Mechanism of the Flowerlike Fe3O4@fTiO2 Microspheres. To understand the formation process of the flowerlike core−shell Fe3O4@fTiO2 microspheres, time-dependent evolution experiments were carried out and their SEM images were shown in Supporting Information Figure S3. On the basis of the results of time-dependent evolution experiments, a possible formation mechanism is proposed.38 First, after hydrothermal reaction for 1 h, we found that surface of the Fe3O4 microspheres has become slightly smooth (Figure S3a), suggesting deposition of titania oligomers. Thus, we speculated the titanium oxyhydrate
species (i.e., titania oligomers), which would play an important role in nucleation of titania, may be produced through alcoholysis reactions or hydrolysis—condensation reactions of TBOT during the initial stage of the solvothermal process. Next, when the reaction time reached 2.5 h, some sparsely thin curved nanosheets grown on the surface of the Fe₃O₄ microspheres can be observed (Figure S3b), indicating the emergence of the oriented crystallization growth. This indicated that the titania oligomers continuously grew along the scraggly surface of Fe₃O₄ microspheres, finally leading to the formation of many curved and cross-linked together heterogeneous crystalline nuclei. As the reaction time is prolonged from 2.5 to 10 h, the petallike nanosheets cross-linked together grown on the surface of Fe₃O₄ microspheres become thicker and denser gradually (Figure S3c), finally resulting in the feature of the flowerlike TiO₂ architecture (Figure S3d). Thus, the continuous oriented crystallization growth and self-assembly process of these heterogeneous nuclei of titania could be responsible for the formation of the hierarchical flowerlike TiO₂ architectures. Finally, the hierarchical meso-/macroporous Fe₃O₄@fTiO₂ microspheres were obtained by calcination of the flowerlike Fe₃O₄@pTiO₂ microspheres after removing the adsorbed water and organic additives (Figure S3e), resulting in high specific surface area and more active sites.

Porous Structure and Magnetism of the Flowerlike Fe₃O₄@fTiO₂ Microspheres. Porous affinity materials are beneficial to increase the affinity selectivity and efficiency of target capturing due to their high surface areas, appropriate pore sizes, and numbers of affinity sites. Nitrogen adsorption—desorption isotherm study was carried out to evaluate the porous structures of the Fe₃O₄@fTiO₂ microspheres. As shown in Figure 4a, the N₂ adsorption—desorption isotherm of the Fe₃O₄@fTiO₂ exhibits a little hysteresis, thus it could be approximately categorized as type IV with a type-H3 hysteresis loop according to the IUPAC classification, indicating the porous characteristic of the Fe₃O₄@fTiO₂ microspheres. The conclusion is fully consistent with that of refs 38, 39, and 41. The average Brunauer—Emmett—Teller (BET) specific surface areas and the total pore volume of the Fe₃O₄@fTiO₂ microspheres are calculated to be 50.45 m²/g and 0.25 cm³/g, respectively. The pore-size distribution derived from the adsorption branch using the Barrett—Joyner—Halenda (BJH) method (Figure 4a, inset) shows two sets of different pores with sizes concentrating at 2.4 and 15.0 nm for the flowerlike Fe₃O₄@fTiO₂ microspheres, indicating their hierarchical porous structure. From these results together with the unique morphology observed in the TEM images, it can be concluded that the hierarchical porous structure of the Fe₃O₄@fTiO₂ microspheres is constructed by the macroporous shell from the stacked nanopetals and the mesoporous nanopetal from the assembled small crystallites. It should be noted that the hierarchical meso-/macroporous shell would contribute to the capture and release of biomolecules of different sizes because the suitable porous structure can improve the affinity efficiency due to existence of more active sites. More importantly, some larger biomolecules like proteins can also be effectively enriched by means of the macroporous structure, which is often restricted by single mesoporous structure.

The magnetic properties of the Fe₃O₄ particles as magnetic cores and the Fe₃O₄@fTiO₂ affinity microspheres were examined at 300 K via a superconducting quantum interface device (SQUID) magnetometer. As revealed in the hysteresis loops (Figure 4b), the microspheres show a strong superparamagnetism at room temperature. The saturation magnetization (Mₛ) values of Fe₃O₄ and Fe₃O₄@fTiO₂ microspheres are 75.5 and 30.1 emu/g, respectively. The superparamagnetism of the Fe₃O₄@fTiO₂ affinity microspheres is beneficial to the rapid and convenient separation. They can be separated from the mixture within 1 min by applying an external magnetic field and rapidly and easily dispersed by shaking after removing the magnetic field (as shown in Figure 4b inset). The result reveals that the affinity microspheres possess the good magnetic response and can meet the requirement of the bioseparation application.

Selective Binding and Magnetic Separation of Phosphoproteins. To evaluate adsorption capacity of the Fe₃O₄@fTiO₂ microspheres for target proteins, β-casein, and BSA were used as model proteins. As illustrated in Scheme 1b, the affinity microspheres were first incubated with the protein solution for affinity and then separated from the supernatant by a magnet (Scheme 1b). The adsorption capacity of the Fe₃O₄@fTiO₂ microspheres for model proteins with various concentrations (0.2–1.0 mg/mL) is shown in Supporting Information Figure S4. The adsorption capacities of the Fe₃O₄@fTiO₂ microspheres for the β-casein and BSA (1 mg/mL) are 141.4 and 31.1 mg/g, respectively. This indicates that the Fe₃O₄@fTiO₂ microspheres have larger adsorption efficiency to the phosphoprotein (β-casein) than that to the nonphosphoprotein (BSA). Compared with commercial TiO₂ microspheres, the adsorption capacity of the Fe₃O₄@fTiO₂ microspheres for phosphoprotein is superior to that of the commercial TiO₂ microspheres in our experimental condition obviously.
To further evaluate selectivity of the Fe₃O₄@fTiO₂ microspheres for phosphoproteins, a complex protein mixture containing one kind of phosphoprotein (β-casein 23.8 kDa) and three kinds of nonphosphoproteins (myoglobin 16.7 kDa; horseradish peroxidase 42.9 kDa; and BSA 66.1 kDa) was used. The experiments were performed by incubating the Fe₃O₄@fTiO₂ microspheres with the protein mixture, separating by a magnet, washing off the nonspecifically bound proteins, and eluting the phosphoproteins. The original and residual protein mixture solution and the purified phosphoproteins were then analyzed by MALDI-TOF MS. Figure 5 shows the successful separation of phosphoproteins from the protein mixture. Apparently, the original protein mixture which contained four proteins of various molecular weights shows multiple peaks (Figure 5a), and the signal of β-casein was very poor due to the low concentration and the suppression of abundant nonphosphoproteins. However, after treatment with the Fe₃O₄@fTiO₂ microspheres, (c) the phosphoproteins enriched from the protein mixture by the commercial TiO₂ microspheres, (c) the phosphoproteins enriched from the protein mixture by the Fe₃O₄@fTiO₂ microspheres, and (d) the proteins enriched from the protein mixture by the commercial TiO₂ microspheres. “M”, “β”, “H”, and “B” indicate myoglobin, β-casein, horseradish peroxidase, and BSA, respectively. “β(2+)”, “H(2+)”, and “B(2+)” represent the doubly charged ions of the β-casein, horseradish peroxidase, and BSA, respectively. The peaks of β-casein with a strong intensity and a high S/N ratio can be easily detected and no nonphosphoprotein peak can be observed for the captured proteins which were purified and released before MS detection (Figure 5c), indicating that the Fe₃O₄@fTiO₂ microspheres are effective for the purification and enrichment of phosphoproteins. For comparison, an enrichment test was also performed using commercial TiO₂ particles as a substitute for the Fe₃O₄@fTiO₂ microspheres under the same experimental conditions except centrifugal separation. As shown in Figure 5d, although the phosphoprotein can be enriched effectively, a weak peak of myoglobin can also be observed (Figure 5d). The above results demonstrate the advantages of the Fe₃O₄@fTiO₂ microspheres in the selective purification and enrichment of the phosphoproteins from the complex protein mixture.

Selective Enrichment of Low-Abundance Phosphopeptides. β-Casein digest, a digest mixture of BSA and β-casein, and the pristine human serum were used to investigate the effectiveness of the Fe₃O₄@fTiO₂ microspheres for enrichment of phosphopeptides. As shown in Scheme 1c, the phosphopeptidites not only can be enriched by the affinity sites of outer surface of the TiO₂ shell, but also can be enriched by the affinity sites inside the porous TiO₂ nanopetals due to easy permeation of peptides through the macropores. Meanwhile, the “shadow effect” could be prevented when the enriched phosphopeptidites release from the pores. Therefore, high selectivity and affinity efficiency for phosphopeptide enrichment can be obtained by taking advantage of the high specific surface area of the porous structure.

β-Casein is a common model phosphoprotein, and its tryptic digest contains three phosphopeptides with molecular masses of 2061.8, 2556.0, and 3122.2 Da, respectively. Supporting Information Table S1 lists the molecular masses and corresponding sequences of the enriched phosphopeptides. Figure 6 shows the MALDI mass spectra of the diluted β-casein digest before and after enrichment by the Fe₃O₄@fTiO₂ affinity microspheres. Apparently, a few nonphosphopeptides can be detected before enrichment using the affinity microspheres, while phosphopeptides almost cannot be observed due to the low concentration and ionization efficiency (Figure 6a). However, three expected phosphopeptides (marked with \(\beta\)-casein digest (10⁻⁷ M) and (b) β-casein digest (10⁻⁹ M) after enrichment with the Fe₃O₄@fTiO₂ affinity microspheres. “☆” and “#” indicate phosphorylated peptides and their dephosphorylated counterparts, respectively.
“#” with strong intensity and free from disturbances of nonphosphopeptides after enrichment were evidently detected (Figure 6b). In addition, the dephosphorylated phosphopeptide MS peaks (marked with “#”) were also observed. Above results indicate that Fe₃O₄@fTiO₂ affinity microspheres can selectively capture the phosphopeptides. To evaluate the sensitivity of the used Fe₃O₄@fTiO₂ affinity microspheres for enrichment of phosphopeptides, we applied the affinity microspheres to enrich the phosphopeptides from β-casein digest with different concentrations (5 × 10⁻⁸, 5 × 10⁻⁹, and 5 × 10⁻¹⁰ M). The experiment results show that the ion signals from the phosphopeptides can still be detected in concentration of low to 5 × 10⁻¹⁰ M, which indicates the high detection sensitivity of Fe₃O₄@fTiO₂ affinity microspheres (Supporting Information Figure S5).

To further evaluate the selectivity, the Fe₃O₄@fTiO₂ microspheres were used to capture phosphopeptides from a more complex peptide mixture consisting of β-casein and BSA with different molar ratio (β-casein:BSA = 1:100, 1:500, 1:1000). For comparison, the precursor Fe₃O₄@pTiO₂ microspheres without calcination and commercial TiO₂ microspheres were also used to enrich the protein mixture. Figure 7a shows the MALDI mass spectrum of the tryptic digest mixture of β-casein and BSA (molar ratio 1:100). MALDI-TOF mass spectra of the tryptic digest mixture of β-casein and BSA (molar ratio 1:100) after enrichment with (b) the Fe₃O₄@fTiO₂ affinity microspheres and (c) the commercial TiO₂ microspheres, respectively. “#” and “*” indicate phosphorylated peptides and their dephosphorylated counterparts, respectively.

Figure 7. (a) MALDI-TOF MS of the tryptic digest mixture of β-casein and BSA (molar ratio 1:100). MALDI-TOF mass spectra of the tryptic digest mixture of β-casein and BSA (molar ratio 1:1000) after enrichment with diﬀerent molar ratio (β-casein:BSA = 1:100, 1:500, 1:1000). For comparison, the precursor Fe₃O₄@pTiO₂ microspheres, only two phosphopeptides as well as many nonphosphopeptides were also observed. Above results indicate that Fe₃O₄@fTiO₂ affinity microspheres can selectively capture the phosphopeptides from β-casein digest with different concentrations (5 × 10⁻⁸, 5 × 10⁻⁹, and 5 × 10⁻¹⁰ M). The experiment results show that the ion signals from the phosphopeptides can still be detected in concentration of low to 5 × 10⁻¹⁰ M, which indicates the high detection sensitivity of Fe₃O₄@fTiO₂ affinity microspheres (Supporting Information Figure S5).

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The Fe₃O₄@fTiO₂ multifunctional affinity microspheres prepared by a facile solvothermal synthesis consist of magnetic cores and flowerlike hierarchical meso-/macroporous TiO₂ shells. The unique TiO₂ shell structure with large specific surface area not only can markedly improve the selectivity and sensitivity for enrichment of the phosphorylated proteins/peptides from complex biosamples, but also can effectively increase mass transport and prevent the “shadow effect”. The fast magnetic response of the superparamagnetism of the...
Fe₃O₄@fTiO₂ affinity microspheres also can realize the rapid and convenient separation. Meanwhile, it can be expected that the Fe₃O₄@fTiO₂ multifunctional affinity materials can be extended to selective enrichment of other phosphate-containing biomolecules. Therefore, this work will be highly beneficial for future applications in the isolation and identification of biomolecules, in particular, low-abundance phosphopeptide biomarkers.

ASSOCIATED CONTENT

Supporting Information
More sample characterization (SEM) as well as details of the MS analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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