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Facile Preparation of Biocompatible Sulfhydryl Cotton Fiber-Based Sorbents by "Thiol−ene" Click Chemistry for Biological Analysis

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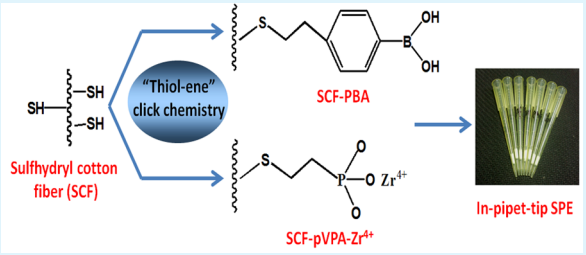
Supporting Information

ABSTRACT: Sulfhydryl cotton fiber (SCF) has been widely used as adsorbent for a variety of metal ions since 1971. Thanks to the abundant thiols on SCF, in this study, we reported a universal method for the facile preparation of SCF-based materials using "thiol−ene" click chemistry for the first time. With the proposed method, two types of SCF-based materials, phenylboronic acid grafted sulfhydryl cotton fiber (SCF-PBA) and zirconium phosphonate-modified sulfhydryl cotton fiber (SCF-pVPA-Zr^{4+}), were successfully prepared. The grafted functional groups onto the thiol group of SCF were demonstrated by X-ray photoelectron spectroscopy (XPS) and energy dispersive X-ray spectroscopy (EDX). The prepared fibrous materials exhibited excellent fiber strength, good stability in aqueous or nonaqueous solutions, and great biocompatibility. Moreover, we developed filter-free in-pipet-tip SPE using these SCF-based materials as adsorbent for the enrichment of ribonucleosides, glycopeptides and phosphopeptides. Our results showed that SCF-PBA adsorbent can selectively capture ribonucleosides and glycopeptides from complex biological samples. And SCF-pVPA-Zr^{4+} adsorbent exhibited high selectivity and capacity in the enrichment of phosphopeptides from the digestion mixture of β-casein and bovine serum albumin (BSA), as well as human serum and nonfat milk digest. Generally, the preparation strategy can be a universal method for the synthesis of other functionalized cotton-based adsorbents with special requirement in microscale biological analysis.



KEYWORDS: *sulfhydryl cotton fiber (SCF), "thiol−ene" click chemistry, boronate affinity, immobilized metal ion affinity chromatography (IMAC), biological analysis*

1. INTRODUCTION

Cotton fiber, a kind of natural material, has been applied as solid phase extraction (SPE) absorbent for sample preparation over the past few decades due to its high adsorption capability toward hydrophobic compounds.[1−5] Very recently, Chen and co-workers used cotton fiber as a SPE sorbent to extract seven kinds of polycyclic aromatic hydrocarbons (PAHs) in environmental water samples.[6] On the other hand, the abundant hydroxyl groups on the surface of cotton provide good hydrophilic property. In 2011, Selmen and co-workers developed a hydrophilic interaction liquid chromatography (HILIC) microtip using cotton wool as adsorbent for microscale purification and enrichment of glycans and glycopeptides before mass spectrometric detection.[7] These cotton-based adsorbents showed good biocompatibility, high mechanical strength and stability in aqueous or nonaqueous solutions. However, the functional groups on the cotton are monotonous, which restricts its further application.

Sulfhydryl cotton fiber (SCF), prepared by esterification of cotton fiber, is a commonly used fibrous adsorbent for trace heavy metal ions from water sample.[8,9] Nowadays, SCF is commercially available and the preparation method for SCF is also simple and low-cost. In addition to the advantages inherited from cotton, such as good biocompatibility, high mechanical strength and stability, SCF also has abundant thiol group on the surface, which offers the potential for modification. However, to the best of our knowledge, until now almost all investigations about SCF has focused on its adsorption capacity toward heavy metal ions, while little effort was taken to develop SCF-based adsorbents through further modification of its thiol group. As we know, thiol group has high reactivity and easy to be modified. In this regard, due to the abundant thiol groups on the surface, SCF might be considered as a basic material for further modification with different functional groups by "thiol−ene" click chemistry.

"Thiol−ene" reaction has been well-known since 1900s.[10] It possesses many merits, such as simplicity, good selectivity, being insensitive to oxygen or water and high conversion under mild conditions.[11−13] This reaction also has been widely utilized in the preparation of separation materials.[13,14] In our previous works, organic-silica hybrid monoliths[15] and magnetic

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polymer material[16] were successfully prepared using the "thiol−ene" click chemistry.

In the current study, we reported a universal method for the facile preparation of SCF-based materials using "thiol−ene" click chemistry for the first time. Two types of novel SCF-based adsorbents, sulfhydryl cotton fiber-phenylboronic acid (SCF-PBA) and zirconium phosphonate-modified sulfhydryl cotton fibers (SCF-pVPA-Zr^{4+}), were successfully prepared using 4-vinylphenylboronic acid and vinyl phosphonic acid as organic monomers, respectively. Based on boronate affinity interaction,[17−19] SCF-PBA adsorbent was applied to specific capture of *cis*-diol-containing compounds, including ribonucleosides and glycopeptides. On the other hand, SCF-pVPA-Zr^{4+} adsorbent was used as an immobilized metal ion affinity chromatography (IMAC)[16,20−23] sorbent for selective enrichment of phosphopeptides from complex biological samples, human serum and nonfat milk digest.

To shorten the extraction time, simplify the operation and minimize the sample volume, in-pipet-tip SPE format was employed in this study. In previous works, monolithic materials,[24] fibrous materials,[7,25] and even powder materials[26,27] could be packed into pipet tip for SPE. This miniaturized SPE format is simple, rapid, cost-effective, does not require any intricate equipment and need only a small amount of sample, which make it quite suitable for bioanalysis.

2. EXPERIMENTAL SECTION

2.1. Reagents and Materials. Aqueous ammonia solution ($NH_3·H_2O$, 25 wt %), methanol, acetic acid, sulfuric acid (98%), mercaptoacetic acid, acetic anhydride, *N*,*N*-dimethylformamide (DMF), 2,2′-azobid(2-methylpropionitrile) (AIBN), zirconyl chloride octahydrate ($ZrOCl_2·8H_2O$) were all of analytical grade and purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). AIBN was purified by recrystallization from ethanol at 40 °C. 4-Vinylphenylboronic acid (4-VPBA) was purchased from Soochiral Chemical & Technology Co. (Jiangsu, China).Vinyl phosphonic acid (VPA), TiO_2 (T104936) and ZrO_2 (Z104401) were purchased from Aladdin Chemical Reagent Co. (Shanghai, China), respectively. Phosphoric acid (H_3PO_4), trifluoroacetic acid (TFA), 2,5-dihydroxybenzoic acid (2,5-DHB), horse radish peroxidase (HRP), bovine β-casein, bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Sequencing grade trypsin was purchased from Promega (Madison, WI, USA). Purified water was obtained with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Nonfat milk was purchased from a local supermarket. 2′-deoxyadenosine (dA), thymidine (T), cytidine (C), guanosine (G), adenosine (A), and uridine (U) were purchased from Sigma-Aldrich (Beijing, China).

2.2. Preparation of the Sulfhydryl Cotton Fiber (SCF). Sulfhydryl cotton fiber (SCF) was prepared according to previous work with a slight modification.[28] Briefly, 1.5 g of degreasing cotton was shredded and weighed. Then 10 mL of mercaptoacetic acid, 6 mL of acetic anhydride, 4 mL of 36% acetic acid, and 0.03 mL of concentrated sulfuric acid were sequentially added into a brown jar. The mixture was swirled and then cooled to room temperature. Subsequently, cotton was added into the solution and the jar was well-sealed and placed in an oven for 3 days under 45 °C. The resulting fibers were then washed with 500 mL of deionized water and 20 mL of absolute ethanol followed by placing in a shallow bowl covered with aluminum foil for 24 h at 45 °C under vacuum condition. The dried fibers were stored in a centrifuge tube that was kept from light at 4 °C before use.

2.3. Preparation of the Sulfhydryl Cotton Fiber-Phenylboronic Acid (SCF-PBA) Adsorbent. The SCF-PBA adsorbent was synthesized by "thiol−ene" click chemistry. Briefly, 250 mg of SCF was shredded and placed in a 50 mL centrifuge tube. Then 100 mg of 4-VPBA, 20 mL of DMF and 1 wt % AIBN were added sequentially. After completely mixing, the mixture was degassed by a 10 min ultrasonication and then incubated at 60 °C for 20 h under nitrogen atmosphere. The as-prepared SCF-PBA adsorbent was subsequently washed with DMF, distilled water and methanol to remove residual monomers followed by drying under vacuum at 60 °C for 8 h.

2.4. Preparation of Zirconium Phosphonate-Modified Sulfhydryl Cotton Fiber (SCF-pVPA-Zr^{4+}) adsorbent. The SCF-pVPA-Zr^{4+} adsorbent was synthesized by "thiol−ene" click chemistry. Briefly, 0.805 g of $ZrOCl_2·8H_2O$, 20 mL of H_2O, 5.0 mL of CH_3OH, 0.4 g of vinylphosphonic acid, 0.2 g of SCF and 1 wt % AIBN were added in a 50 mL centrifuge tube. After completely mixing, the mixture was degassed by a 10 min ultrasonication and incubated at 60 °C for 20 h under nitrogen atmosphere. The as-prepared SCF-pVPA-Zr^{4+} fibers were subsequently rinsed with distilled water and methanol to remove residual monomers and nonspecifically adsorbed Zr^{4+} followed by drying under vacuum at 60 °C for 8 h.

2.5. Characterizations of SCF-PBA and SCF-pVPA-Zr^{4+}. The chemical composition of SCF-PBA and SCF-pVPA-Zr^{4+} were examined by X-ray photoelectron spectroscopy (XPS) using a Thermo VG Multilab 2000 electron spectrometer and Shimadzu EDX-720 energy-dispersive X-ray analysis (EDX, Kyoto, Japan) using Mg Kα radiation as the excitation source, respectively. The microscopic morphology of fibers was determined by a Quanta 200 scanning electron microscopy (SEM) (FEI, Holand).

2.6. Sample Preparation. HRP (1 mg) was dissolved in NH_4HCO_3 solution (50 mM, 1 mL) and denatured at 95 °C for 5 min. To the above solution, trypsin (1 mg/mL, 40 μL) was added and the mixture was incubated at 37 °C for 16 h. The obtained HRP digestion mixture was diluted 250 times with sampling solution before use.

Bovine β-casein was originally made up into stock solutions of 1 mg/mL. Proteins were digested with trypsin using an enzyme to substrate ratio of 1:50 (w/w) in 100 mM Tris-HCl pH 8.5, and the digestion was performed at 37 °C for overnight. BSA (1 mg) was dissolved in 100 μL of denaturing buffer solution (8 M urea in 100 mM Tris-HCl pH 8.5). The protein solution was mixed with 5 μL of 100 mM tri(2-chloroethyl)phosphate (TCEP) and incubated for 20 min at room temperature to reduce protein disulfide bonding. Iodoacetamide (IAA) (3 μL of 500 mM stock) was added to the solution and incubated for an additional 30 min at 25 °C in dark. The reduced and alkylated protein mixture was diluted with 300 μL 100 mM Tris-HCl (pH 8.5). Nine μL of 100 mM $CaCl_2$ was then added to the above solution and the mixture (50 μL in volume) was digested with trypsin at an enzyme to substrate ratio of 1:50 (w/w) by incubating at 37 °C for overnight. All the tryptic digests were lyophilized to dryness and stored at −80 °C for future use.

For in-solution digestion, nonfat milk (50 μL) was first denatured by the ammonium bicarbonate solution (50 mM, 250 μL) containing urea (8 M) and incubated at 37 °C for 30 min. Then, dithiothreitol (DTT) solution (200 mM, 25 μL) was added and incubated at 55 °C for 1 h. After cooling to room temperature, the IAA solution (200 mM, 50 μL) was added and the mixture was kept in the dark for 3 h. Finally, the resulting mixture was incubated with trypsin (2 mg/mL, 5 μL) at 37 °C for 24 h. All the tryptic digestions were stored at −80 °C for future use.

Human urine samples and serum samples were collected from healthy people from The Hospital of Wuhan University according to the standard clinical procedures. The utilization of human urine and serum complied with guidelines of Ethics Committee of the Institute, and all participants gave their informed consent. The samples were stored at −80 °C until use. For the urine sample analysis, 0.3 mL urine was diluted with 0.7 mL of ACN. After centrifugation, the supernatant was collected for the extraction process.

2.7. Preparation of In-Pipet-Tip SPE. Two mg of the as-prepared fibrous materials were packed into a 200-μL commercial pipet tip using a blunt needle and pressed to a fixed length (~4.0 mm) (Figure S1).

Before extraction with in-pipet-tip SPE, 50 μL of sampling solution was used to condition the sorbent by aspirating and dispensing the

solution with a 200-μL manual pipettor. Owing to the good permeability of SCF-based fibrous materials, the in-pipet-tip SPE system was found to be fast and smooth.

2.8. Extraction of Ribonucleosides and Glycopeptides by SCF-PBA Fiber-Packed SPE. SCF-PBA fiber-packed SPE was used to enrich *cis*-diol-containing ribonucleosides and glycopeptides. The sample mixture (200 ng mL^{-1}, 50 μL) was pipetted up and down 40 times. After washing once with 50 μL of sampling solution (1% $NH_3 \cdot H_2O$, 99% ACN (v/v)), the adsorbed nucleosides were eluted by 50 μL of 1% TFA-99% H_2O (v/v). The whole procedure can be accomplished within 3 min. The LC-UV experiments were carried out on a HPLC system of LC-20A (Shimadzu, Japan). HPLC separation was performed on a Hisep C18 column (150 mm × 4.6 mm i.d., 5 μm, Weltech Co., Ltd., Wuhan, China) with a flow rate of 1 mL/min at 35 °C. The injection volume was 20 μL. Water (solvent A) and methanol (solvent B) were employed as mobile phases. A gradient of 10 min 5−30% B, 0.01 min 30−80% B, 5 min 80% B, 0.01 min 80−5% B and 10 min 5% B was used. The detection wavelength was 254 nm.

For extraction of glycopeptides, the sampling and eluted solution were 50 μL of 1% $NH_3 \cdot H_2O$-90% ACN (v/v) and 1% TFA (v/v) in H_2O, respectively. The concentration of glycopeptides in sampling solution was 1.0×10^{-7} M. Before analysis, the eluted solution (50 μL) of glycopeptides was lyophilized to dryness and redissolved in 50% ACN (5 μL). Then, glycopeptides mixture (1 μL) and matrix solution (1 μL) were applied for MALDI-TOF MS analysis, and the matrix solution was 20 mg/mL DHB in 50% ACN containing 0.1% TFA. All MALDI-TOF-MS spectra were recorded with an Axima TOF^2 mass spectrometry equipped with a 337 nm nitrogen laser with a 3 ns pulse width (Shimadzu, Kyoto, Japan). The detection was performed in positive ion reflector mode with an accelerating voltage of 20 kV. Typically, 200 laser shots were averaged to generate each spectrum.

For comparison, cotton fiber-packed SPE and SCF-packed SPE were also used for nucleosides and glycopeptides enrichment. The extraction procedure was performed in the same way as the SCF-PBA fiber-packed SPE.

2.9. Extraction of Phosphopeptides by SCF-pVPA-Zr^{4+} Fiber-Packed SPE. SCF-pVPA-Zr^{4+} fiber-packed SPE was used for phosphopeptides enrichment. The sampling solution was 50 μL of 5% TFA-50% H_2O (v/v). 50 μL of peptides mixture in buffer I (5% TFA, 50% ACN (v/v)) was pipetted up and down to ensure full adsorption of peptides. After washing twice with 50 μL of buffer I, the trapped peptides were eluted with 50 μL of 5% aqueous ammonia solution. The whole procedure can be accomplished within 3 min. Then the eluted solution was lyophilized to dryness. Finally, 1.2 μL of matrix solution (mixture of 20 mg/mL 2,5-DHB in 50% (v/v) ACN, 1% (v/v) phosphoric acid) was introduced into the residue and the mixture was directly applied to MALDI-TOF MS analysis. For comparison, cotton fiber-packed SPE and SCF-packed SPE were also used for phosphopeptides enrichment. The extraction procedure was performed in the same way as the SCF-pVPA-Zr^{4+} fiber-packed SPE.

The phosphopeptide enrichment with commercial TiO_2 powder (Aladdin, T104936) and ZrO_2 powder (Aladdin, Z104401) were also performed using dispersive SPE. Briefly, 300 μg of TiO_2 (or ZrO_2) powder were dispersed in 100 μL of peptides mixture in buffer I (5% TFA, 50% ACN (v/v)) and incubated at 37 °C for 15 min. The supernatant was removed after centrifugation at 12,000 r/min for 3 min. After washing twice with 100 μL of buffer I, the trapped peptides were eluted with 50 μL of 5% aqueous ammonia solution. The whole procedure should cost approximate 30 min. Then the eluted solution was lyophilized to dryness and applied to MALDI-TOF MS analysis.

For endogenous phosphopeptide enrichment from human serum, 2 μL of original serum was diluted to 50 μL with buffer I, and the following steps were the same as that of peptides mixture enrichment.

For the phosphopeptide enrichment from tryptic digest nonfat milk, 2 μL of milk digests was diluted 500 folds with buffer I and 50 μL of the diluent (containing 0.1 μL of original milk digests) was used as sampling solution. The following sample processing was the same as that of peptides mixture enrichment.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of SCF-PBA and SCF-pVPA-Zr^{4+} Fibers. Both SCF-PBA and SCF-pVPA-Zr^{4+} fibers were synthesized by "thiol−ene" click chemistry based on the abundant thiol groups on SCF. The schematic diagram is shown in Figure 1a. The SEM images showed that the

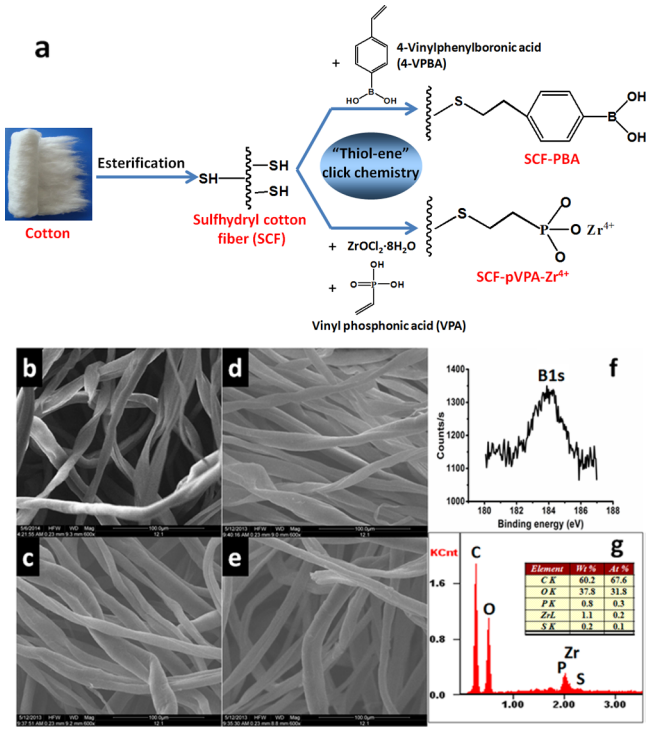

Figure 1. Schematic diagram for the preparation of SCF-PBA and SCF-pVPA-Zr^{4+} fibers (a); SEM images of cotton (b), SCF (c), SCF-PBA (d) and SCF-pVPA-Zr^{4+} (e); XPS spectrum of B 1s of SCF-PBA (f); EDX spectrum and chemical composition (the inset table) of SCF-pVPA-Zr^{4+} (g).

diameters of cotton, SCF, SCF-PBA and SCF-pVPA-Zr^{4+} were almost the same (ranging from 10 to 20 μm) (Figure 1), indicating that the modification did not impact the fibrous morphology of cotton, and the fiber strength was also well maintained.

XPS analysis of SCF-PBA was performed for B 1s (Figure 1f). The single peak at 184.0 eV suggested the existence of boron (B) element. The elemental analysis was also performed by XPS (Table S1, ESI), which showed that 4-VPBA was successfully imported and the B atomic percentage of SCF-PBA was 1.5%.

The composition of SCF-pVPA-Zr^{4+} fibers was examined by EDX. As shown in Figure 1g, the fibers were clearly constituted by C, O, P, S and Zr^{4+} elements, and the atom percentage of zirconium was 0.2% for SCF-pVPA-Zr^{4+}.

3.2. Evaluation of the Property of SCF-PBA Fiber-Packed SPE Toward Ribonucleosides. Boronate groups can form reversible five or six-membered cyclic esters with *cis*-diol-containing molecules under either basic aqueous or non-aqueous environments, and the complexes will be dissociated in acidic condition.[29] Therefore, the recognition of *cis*-diol containing adenosine (A) against 2-deoxyadenosine (dA) was examined to evaluate the specificity of SCF-PBA fiber. To achieve the best performance of SCF-PBA, the content of

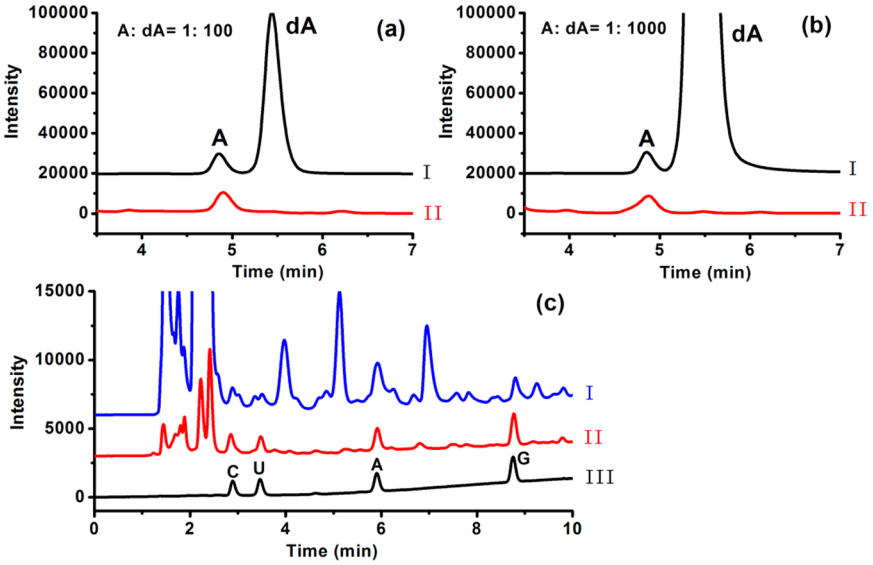

Figure 2. LC-UV chromatograms of adenosine (A) and 2′-deoxyadenosine (dA) without (a I, b I) or with (a II, b II) SCF-PBA fibers enrichment. Mass ratios of A to dA are 1:100 (a) and 1:1000 (b), and the amount of adenosine was 10 ng. (c) LC-UV chromatograms of four ribonucleosides in urine sample. (I) direct analysis of a spiked urine sample; (II) spiked urine sample after enrichment with SCF-PBA; (III) direct analysis of ribonucleoside standards (200 ng mL^{-1}).

sampling solution and the amount of sorbent were optimized. Accordingly, 1% $NH_3 \cdot H_2O$-99% ACN (v/v) was used as sampling solution (Figures S2a and S2b) and the optimized amount of sorbent was 3 mg (Figures S2c).

According to the results, the prepared SCF-PBA fibers exhibited specific selectivity to adenosine (A) over 2-deoxyadenosine (dA). In this study, the selectivity of SCF-PBA toward adenosine was evaluated using adenosine and 2-deoxyadenosine with different ratios (A: dA= 1:100 and 1:1000). As shown in Figure 2, even when the amount of 2-deoxyadenosine was 1000-fold higher than adenosine (Figure 2b I), only adenosine could be captured by SCF-PBA fibers (Figure 2b II). And the signal intensities of adenosine after enrichment were similar to those of the control groups. In addition, the breakthrough curve (Figure S 2d) suggested that the adsorption capacity of SCF-PBA was estimated to be 65 $\mu g\ g^{-1}$ for adenosine. The results demonstrated that SCF-PBA fibers have high specificity and good adsorptive capacity toward *cis*-diol containing adenosine.

We further performed the enrichment of ribonucleosides from human urine sample. Obviously, there were many high-intensity interference peaks through direct analysis of spiked-urine sample (Figure 2c I), which made the identification of ribonucleosides difficult. However, all the four target ribonucleosides could be distinctly detected after enrichment with SCF-PBA fibers from the spiked-urine sample (Figure 2c III), demonstrating the excellent purification capability of SCF-PBA fibers in complex physiological condition.

3.3. Evaluation of the Property of SCF-PBA Fiber-Packed SPE Toward Glycopeptides. To evaluate the enrichment efficiency of SCF-PBA fibers toward glycopeptides, a tryptic digest of HRP was used. 1% $NH_3 \cdot H_2O$-90% ACN (v/v) was used as sampling and washing solution to achieve the best performance. The results showed that 7 glycopeptides with poor intensity were observed by direct analysis (Figure 3a). After enrichment by SCF-PBA, 15 glycopeptides were found with high resolution (Figure 3b). However, only 6 and 4 glycopeptides could be captured by cotton (Figure 3c) and

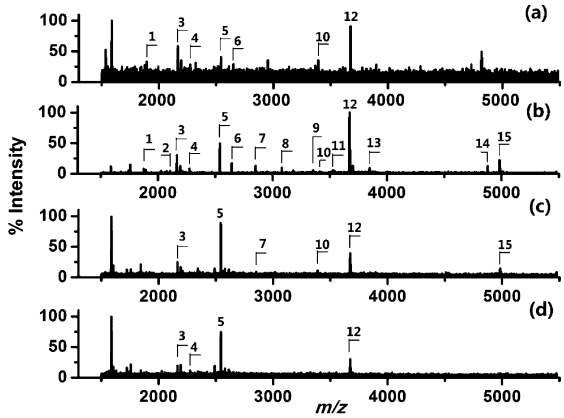

Figure 3. MALDI mass spectra of tryptic digest of HRP. Direct analysis (a), after enrichment with SCF-PBA fibers (b), cotton (c) and SCF (d).

SCF (Figure 3d), respectively. The results indicated the high selectivity and affinity of SCF-PBA toward glycopeptides mainly come from boronate affinity mechanism. The oligosaccharide composition and amino acid sequence of these detected glycopeptides are listed in Table S2 (ESI).

3.4. Evaluation of the Property of SCF-pVPA-Zr^{4+} Fiber-Packed SPE Toward Phosphopeptides. The extraction performance of SCF-pVPA-Zr^{4+} fibers for phosphopeptide was first investigated by the tryptic digest of bovine β-casein. After optimization, buffer I (5% TFA, 50% ACN (v/v)) was used as sampling and washing solution to achieve the best performance, and 5% aqueous ammonia solution was used to elute the captured phosphopeptides. The results showed that 4 phosphopeptides with poor resolution were observed by direct analysis, while nonphosphopeptides dominated the spectrum (Figure 4a). Whereas, after enrichment with SCF-pVPA-Zr^{4+} fibers, 7 phosphopeptides (the sequences were listed in Table S3) with good resolution can be observed (Figure 4b). To

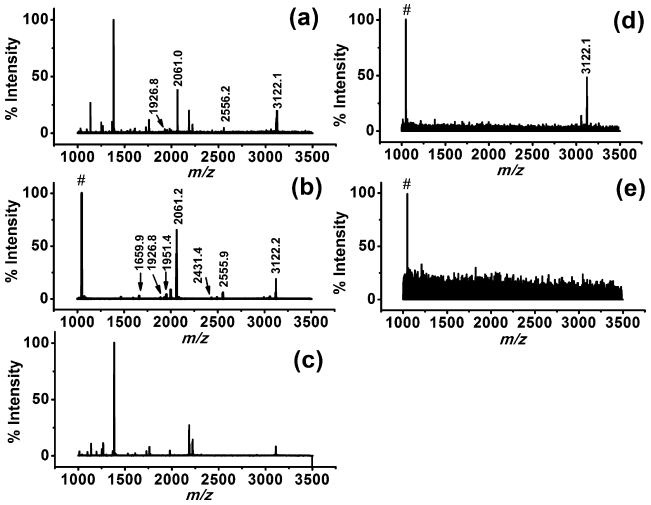

Figure 4. MALDI mass spectra of tryptic digest of β-casein. Direct analysis (a), analysis after extraction with SCF-pVPA-Zr^{4+} fibers (b), and analysis of the flow-through after sampling (c); after enrichment with cotton (d), SCF (e). Phosphopeptides are labeled with their observed m/z and the internal standard is marked with '#'.

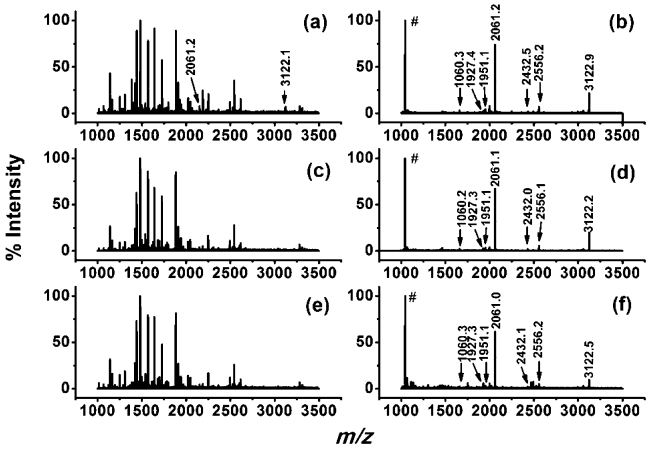

Figure 5. MALDI mass spectra of the tryptic digest mixtures of β-casein and BSA without (a, c, e) or with (b, d, f) the SCF-pVPA-Zr^{4+} fibers enrichment. Molar ratio of β-casein to BSA are 1:1 (a, b), 1:10 (c, d), and 1:100 (e, f). The concentration of β-casein was 4.0×10^{-8} M (2 pmol). Phosphopeptides are labeled with observed m/z and the internal standard is marked with '#'.

investigate the capture capability of SCF-pVPA-Zr^{4+} fibers, the sampling eluate after enrichment was also analyzed. The result showed that only nonphosphopeptides were detected (Figure 4c), indicating the high affinity of the prepared material toward phosphopeptides. For comparison, cotton and SCF were also applied to the enrichment experiment using the same condition as that of SCF-pVPA-Zr^{4+} fibers. Obviously, only 1 phosphopeptide with poor intensity could be captured by cotton (Figure 4d) and no phosphopeptide was observed when SCF (Figure 4e) was used. Therefore, the high selectivity and affinity of SCF-pVPA-Zr^{4+} fibers toward phosphopeptides come from the particular affinity of zirconium.

The enrichment specificity of SCF-pVPA-Zr^{4+} fibers toward phosphopeptides was further evaluated using tryptic digests of β-casein and BSA with different molar ratios (β-casein: BSA= 1:1, 1:10 and 1:100). With the increase of digested BSA, the signals of nonphosphopeptides dramatically enhanced and the identification of phosphopeptides became impossible by direct analysis (Figures 5a, 5c and 5e). However, even with the existence of high amount of BSA digests, 7 phosphopeptides could be distinctly detected after SCF-pVPA-Zr^{4+} fibers enrichment (Figures 5b, 5d, 5f). The results indicated the excellent selectivity of SCF-pVPA-Zr^{4+} fibers toward phosphopeptides even in the presence of a large amount of interfering nonphosphopeptides.

For comparison, TiO_2 and ZrO_2, which were frequently used to purify phosphopeptides,[30] were also used to enrich phosphopeptides from the mixture of tryptic digest of β-casein and BSA with molar ratios of 1:1, 1:10 and 1:100. The results showed that, TiO_2 performed well when the molar ratios of β-casein and BSA were 1:1 (Figure S3a) and 1:10 (Figure S3b). However, when the molar ratios of β-casein and BSA was 1:100, numbers of nonphosphopeptides with good resolution could be observed in the mass spectrum, while only 3 phosphopeptides with poor resolution could be identified (Figure S3c and Table S3). On the other hand, ZrO_2 could capture 6 phosphopeptides when the molar ratios of β-casein and BSA was 1:1 (Figure S 3d), but it could not work well when the molar ratios of β-casein and BSA were 1:10 and 1:100 (Figures S3e and S 3f). Compared to TiO_2 and ZrO_2, SCF-pVPA-Zr^{4+} fiber-packed SPE showed better selectivity from complex samples (compare Figure 5 with Figure S3, and see Table S3), which might be attributed to the suitable Lewis acidity of zirconium in SCF-pVPA-Zr^{4+} fiber and good biocompatibility of SCF substrate.[6,16] In addition, the repeated centrifugation steps in the extraction experiment using commercial TiO_2 and ZrO_2 powder as adsorbents might lead to the loss of phosphopeptides.[31]

The sensitivity of this approach toward phosphopeptides was also investigated. The MALDI mass spectra of phosphopeptides enriched from β-casein digests with different amounts (1000, 100, 10, and 2.5 fmol) were presented in Figure 6. The results showed that the signal of 1 phosphopeptide (m/z = 3122.3) with signal-to-noise ratio over 3 could still be well detected even in 2.5 fmol of β-casein digests (the concentration was 2 ×

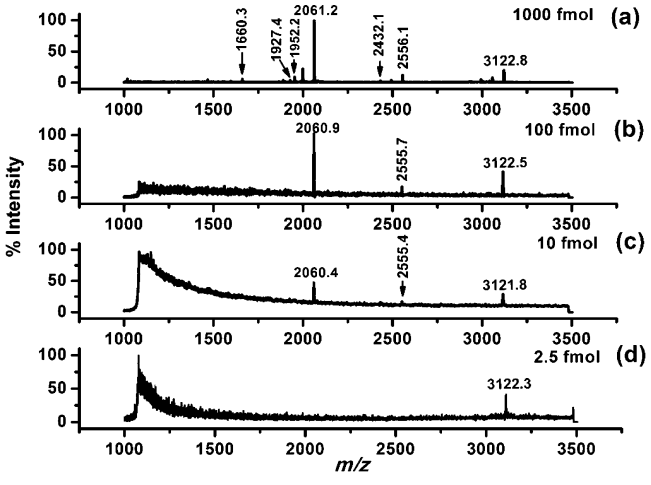

Figure 6. MALDI mass spectra of tryptic digest of β-casein after enrichment with the SCF-pVPA-Zr^{4+} fibers. The amounts of tryptic digested β-casein used in the experiments were (a) 1000 fmol, (b) 100 fmol, (c) 10 fmol and (d) 2.5 fmol. Phosphopeptides are labeled with their observed m/z.

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10^{-9} M) (Figure 6d), demonstrating the high detection sensitivity of this strategy.

Comparison of our material with the previous four materials[32−35] was listed in Table S4. Compared with the previous methods, the performance of SCF-pVPA-Zr^{4+} possessed good selectivity and high sensitivity, which might due to the hydrophilic nature of SCF that could effectively resist the interference from hydrophobic nonphosphopeptides. In addition, the proposed approach could be completed within 3 min, which might reduce the degradation of phosphopeptides.[36]

3.5. Enrichment of Phosphopeptides from Human Serum and Nonfat Milk by SCF-pVPA-Zr^{4+} Fiber-Packed SPE. SCF-pVPA-Zr^{4+} fibers were further used to enrich phosphopeptides from complex biological samples, human serum and nonfat milk. Human serum contains endogenous phosphopeptides that are associated with certain diseases.[37,38] However, due to the high complexity of matrix, such as thousands of nonphosphopeptides and coexisting proteins, the determination of phosphopeptides from human serum is difficult. Here, we used SCF-pVPA-Zr^{4+} fibers to selectively enrich endogenous phosphopeptides from a small volume of original human serum (2 μL). MALDI mass spectrum showed that no phosphopeptide but plenty of nonphosphopeptides were identified with direct analysis (Figure 7a). After

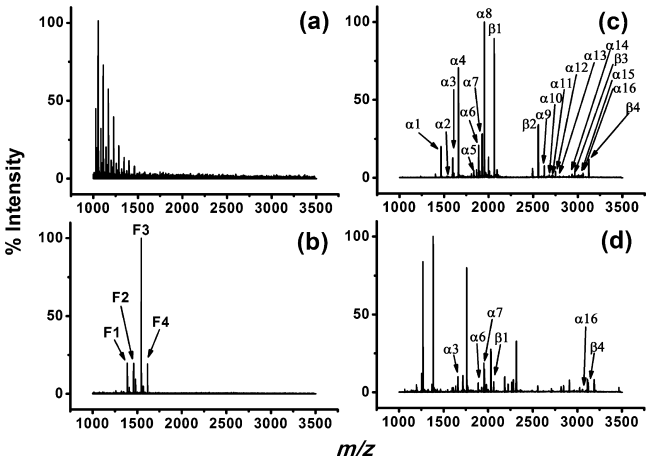

Figure 7. MALDI mass spectra of human serum obtained by direct analysis (a) or after enrichment with the SCF-pVPA-Zr^{4+} fibers (b); MALDI mass spectra of tryptic digest of nonfat milk obtained by direct analysis (c) or after enrichment with the SCF-pVPA-Zr^{4+} fibers (d).

enrichment with SCF-pVPA-Zr^{4+} fibers, 4 phosphopeptides derived from fibrinopeptides could be distinctly identified (Figure 7b). The sequence information on the detected endogenous phosphopeptides was listed in Table S5. This result, which was consistent with previous studies,[39−42] demonstrated the excellent performance of SCF-pVPA-Zr^{4+} fibers for the selective enrichment of phosphopeptides from small volume serum with complex biomatrix.

We then further used SCF-pVPA-Zr^{4+} fibers to enrich phosphopeptides from nonfat milk. Nonfat milk contains abundant proteins including phosphoproteins of α-casein and β-casein. Tryptic digest of nonfat milk was gradually diluted 500 folds with buffer I (5% TFA, 50% ACN (v/v)) and subjected to analysis. Only 6 phosphopeptides along with lots of nonphosphopeptides with strong signal were observed through direct analysis (Figure 7c). However, after enrichment with SCF-pVPA-Zr^{4+} fibers, 20 phosphopeptides (16 from α-casein and 4 from β-casein)[33,35,36] could be detected with good resolution (Figure 7d). The sequence information on the observed phosphopeptides was listed in Table S6. The result indicated the proposed SCF-pVPA-Zr^{4+} fibers have excellent selectivity toward phosphopeptides in the real in-solution digested sample.

4. CONCLUSION

In the current study, a general approach for facile preparation of functionalized SCF-based materials has been developed using "thiol−ene" click chemistry for the first time. Two types of novel fibrous sorbents, SCF-PBA and SCF-pVPA-Zr^{4+}, were successfully prepared. Subsequently, SCF-PBA was applied for specific capture of *cis*-diol-containing biomolecules (ribonucleosides and glycopeptides) and SCF-pVPA-Zr^{4+} was used to enrich phosphopetides from human serum and nonfat milk, respectively. Both of the two sorbents exhibited high selectivity and good sensitivity in extraction experiments. In addition, benefiting from the in-pipet-tip SPE system, simple, rapid and microminiaturized methodology was achieved for the enrichment experiments. Taken together, the proposed method represents a new option to prepare a variety of SCF-based materials with desirable functional groups for specific applications in biological analysis.

■ ASSOCIATED CONTENT

Supporting Information

XPS of fibers; oligosaccharide composition and the sequences of the glycopeptides; detailed information on phosphopeptides obtained from β-casein digest, human serum and nonfat milk digests; photographs of the fibrous materials and preparation of in-pipet-tip SPE; optimization of conditions for SCF-PBA; enrichment capacity of SCF-PBA. 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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This paper was published on the Web on October 10, 2014, with errors in the TOC/Abstract graphic, along with Figure 1. The corrected version was reposted on October 22, 2014.