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Hydrophilic Carboxyl Cotton Chelator for Titanium(IV) Immobilization and Its Application as Novel Fibrous Sorbent for Rapid Enrichment of Phosphopeptides

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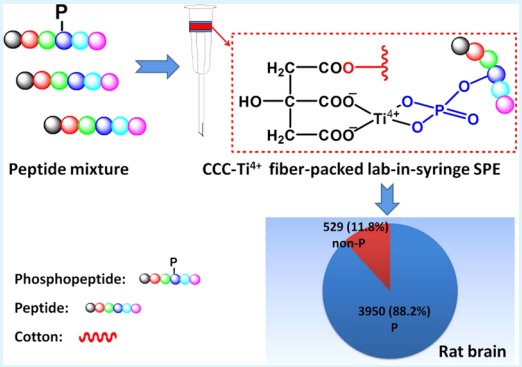
Supporting Information

ABSTRACT: Sample preparation methods with high selectivity, efficiency, and matrix resistance are essential for phosphoproteomic analysis. In this study, carboxyl cotton chelator-titanium(IV) (CCC-Ti^{4+}) fibers, a novel CCC-based fibrous sorbent with excellent biocompatibility, were successfully synthesized on the basis of the coordination effect between double carboxyl groups on CCC and Ti^{4+}. The synthesis of CCC-Ti^{4+} fibers was easy, and the incorporated titanium content was high. On the basis of immobilized metal ion affinity chromatography (IMAC), CCC-Ti^{4+} fibers were used for specific capture of phosphopeptides using a lab-in-syringe solid-phase extraction (SPE) from multiple biological samples, including standard protein digests, nonfat milk digests, human serum, and animal tissue. The proposed sorbent exhibited high selectivity (β-casein/BSA = 1:1000) and good sensitivity (10 fmol) in phosphopeptides analysis. Meanwhile, the lab-in-syringe SPE greatly simplified the entire process of enrichment. Thanks to the good biocompatibility of CCC-based material, CCC-Ti^{4+} fibers showed excellent performance in phosphopeptide enrichment from protein-rich human serum. Finally, CCC-Ti^{4+} fibers were applied for selective capture of phosphopeptides from tryptic digests of rat brain lysate followed by LC-MS/MS analysis. Using the proposed method, we identified 3950 unique phosphosites from 1 mg of rat brain in a single experiment, which is much better than previously reported IMAC-based strategies. Taken together, this efficient method will find broad application in large-scale phosphoproteomics analysis because of the rapid (3 min) convenient procedure and excellent performance.



KEYWORDS: *carboxyl cotton chelator-Ti^{4+}, immobilized metal ion affinity chromatography, phosphopeptide enrichment, human serum, rat brain tissue*

1. INTRODUCTION

Protein phosphorylation, a kind of reversible post-translational modifications, plays a key role in diverse physiological functions, including signaling-regulated differentiation, apoptosis, intercellular communication, and immunological stress.[1,2] Mass spectrometry (MS)-based techniques have been widely used in phosphoproteome study because of their high sensitivity, high-throughput, and capability in identification of thousands of phosphorylation sites and cross-comparison of the changes in different phosphorylation states.[2−4] However, the low abundance of phosphoproteins in biological samples and lower ionization efficiency of phosphopeptides than non-phosphopeptides in MS detection make it impossible to achieve direct analysis. In this respect, a method to enrich phosphopeptides prior to MS detection is essential for MS-based phosphoproteome analysis.[5,6]

So far, numerous strategies have been proposed for selective enrichment of phosphopeptides before MS detection. Therein, immobilized metal affinity chromatography (IMAC) is one of the most effective and commonly used methods for phosphopeptide enrichment, wherein the metal ions are immobilized on different substrates via iminodiacetic acid (IDA),[7,8] nitrilotriacetic acid (NTA),[9,10] phosphate groups,[11,12] or polydopamine.[13,14] In some previous works,[12,15] IMAC techniques have been carried out in a dispersive solid-phase extraction format during sample preparation, which is time-consuming, and high-speed centrifugation may also result in cosedimentation of impurities and loss of target molecules, thus restricting their applications in high-throughput phosphoproteome analysis.[16,17] Therefore, rapid and convenient pretreatment methods are required to improve the selectivity and sensitivity during phosphoproteome analysis.

Recently, magnetic sorbents have been developed for selective enrichment of phosphopeptides, which make the

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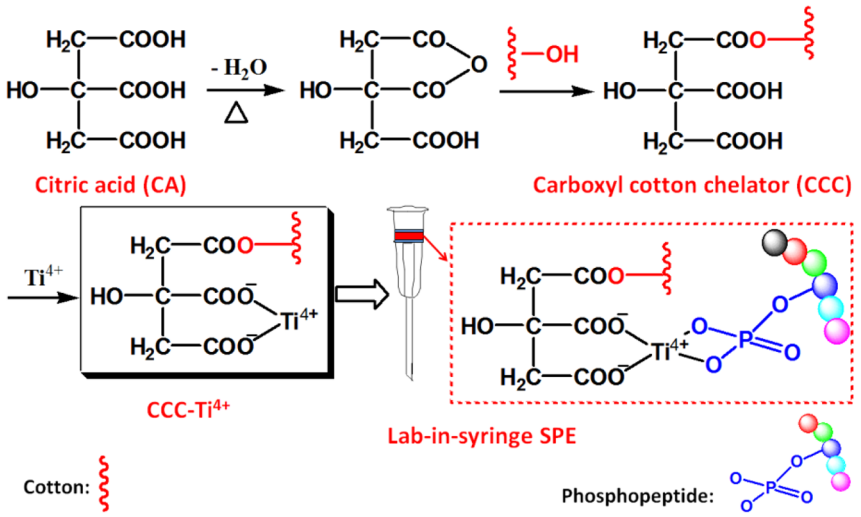

Figure 1. Schematic diagram of the preparation of CCC-Ti^{4+} fibers and the lab-in-syringe SPE.

extraction procedure simple and fast.[17] However, a stable and hydrophilic shell is usually required to endow the magnetic sorbents with high environmental stability and good dispersibility in water. In this respect, a variety of biologically compatible magnetic carbonaceous-based[18,19] or magnetic polydopamine-based[14,16] IMAC sorbents were prepared, and they have been demonstrated to possess better performance in biological analysis. Nonetheless, the synthesis methods for most magnetic materials are tedious.[20−23]

Carboxyl cotton chelator (CCC), prepared by thermochemical esterification of cotton fiber with citric acid (CA), has two carboxyl groups on each structure unit that serve as chelating ligands for bonding metal ions in previous work.[24] Taking into account the advantages inherited from cotton, such as good biocompatibility and high mechanical strength and stability, hydrophilic CCC obtained by a simple synthesis method can be used as a fibrous substrate for metal ion immobilization, which can further serve as a novel IMAC platform. In addition, fibrous sorbents could be easily packed into syringe or pipet tip for miniaturized SPE in previous works, which was simple, rapid, and cost-effective.[25−30]

In this study, we reported a method to prepare CCC-based material by employing the coordination effect between carboxyl group and metal ion. Here, a novel CCC-based fibrous sorbent, CCC-Ti^{4+} fiber, was successfully fabricated and applied to selectively enrich phosphopeptides from standard protein digests, nonfat milk digests, human serum, and tryptic digests of rat brain lysate in a lab-in-syringe mode as described in a previous publication.[25] The results showed that CCC-Ti^{4+} fibers was easily prepared and cost-effective; the CCC substrate has merits of hydrophilicity and biocompatibility. Moreover, the titanium content of CCC-Ti^{4+} fibers was high, which would ensure a large binding capacity for phosphopeptides.

2. EXPERIMENTAL SECTION

2.1. Reagents and Materials. Citric acid, aqueous ammonia solution and titanium(IV) sulfate ($Ti(SO_4)_2$) were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). TiO_2 (T104936) and ZrO_2 (Z104401) were purchased from Aladdin Chemical Reagent Co. (Shanghai, China). Phosphoric acid (H_3PO_4), trifluoroacetic acid (TFA), 2,5-dihydroxybenzoic acid (2,5-DHB), bovine β-casein, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) was purchased from Fisher Scientific (Pittsburgh, PA, USA). TPCK-treated trypsin was purchased from Worthington Biochemical Co. (New Jersey, USA). Milli-Q water was purified by a Milli-Q apparatus (Millipore, Bedford, MA, USA). Nonfat milk was purchased from a local supermarket. Human serum was obtained from The Hospital of Wuhan University according to standard clinical procedures.

2.2. Preparation of the CCC. CCC was prepared according to previous work with a slight modification (Figure 1).[24] Briefly, 5 g of degreasing cotton was totally immersed in 60 mL of citric acid (CA) aqueous solution (0.5 M), and the mixture was put into a watch glass and oven-dried at 50 °C for 24 h, then kept in the oven while raising the temperature to 120 °C within 90 min. After cooling, the resulting CCC was washed with distilled water and collected for the following experiments.

2.3. Preparation of the CCC-Ti^{4+} Adsorbent. As shown in Figure 1, CCC has two free carboxyl groups on each structural unit, which can chelate with metal ions to generate CCC-based IMAC material. Here, Ti^{4+} was immobilized by chelation with carboxyl groups of CCC to obtain CCC-Ti^{4+} fibers (Figure 1). Briefly, 400 mg of CCC was mixed with 40 mL of titanium ion solution (100 mM $Ti(SO_4)_2$). After incubation at 40 °C for 4 h, the immobilized-Ti^{4+} CCC (CCC-Ti^{4+}) was washed thoroughly with distilled water to remove residual Ti^{4+} and dried at 50 °C for 6 h.

2.4. Characterization of Fibers. The microscopic morphology and chemical composition of fibers were examined by scanning electron microscopy (SEM) and energy-dispersive X-ray analysis (EDX), respectively. Detailed information on the instruments can be found in previous work.[26] Fourier transform infrared spectroscopy (FTIR) was carried out with a Thermo Nicolet 670 FT-IR instrument (Boston, MA, USA).

2.5. Sample Preparation. Sample preparation of bovine β-casein digests, BSA digests, nonfat milk digests, and human serum were carried out according to previous work.[26] All of the samples were stored at −20 °C until use.

A Sprague Dawley (SD) male rat (about 220 g in weight), purchased from Hubei Provincial Center for Disease Control and Prevention (Wuhan, Hubei, China) was sacrificed, and the brain was quickly excised. After removing blood with ice-cold physiological saline, the brain tissue was minced and homogenized in a homogenizer (Pottter-Elvejhem) with a Teflon piston on ice 20 times, using 8 mL of RIPA lysis buffer (P0013D, Beyotime Biotechnology) per 1 g of tissue. The suspension was then vortexed on ice for 30 min, followed by centrifugation at 15 000 g and 4 °C for 30 min. The protein concentration of the supernatant was measured using Coomassie light blue staining. Proteins were precipitated with some precipitant (50% acetone, 49.9% ethanol, 0.1% acetic acid, v/v/v) at 4 °C for 3 h and then centrifuged at 4000 g and 4 °C for 30 min. The precipitate was

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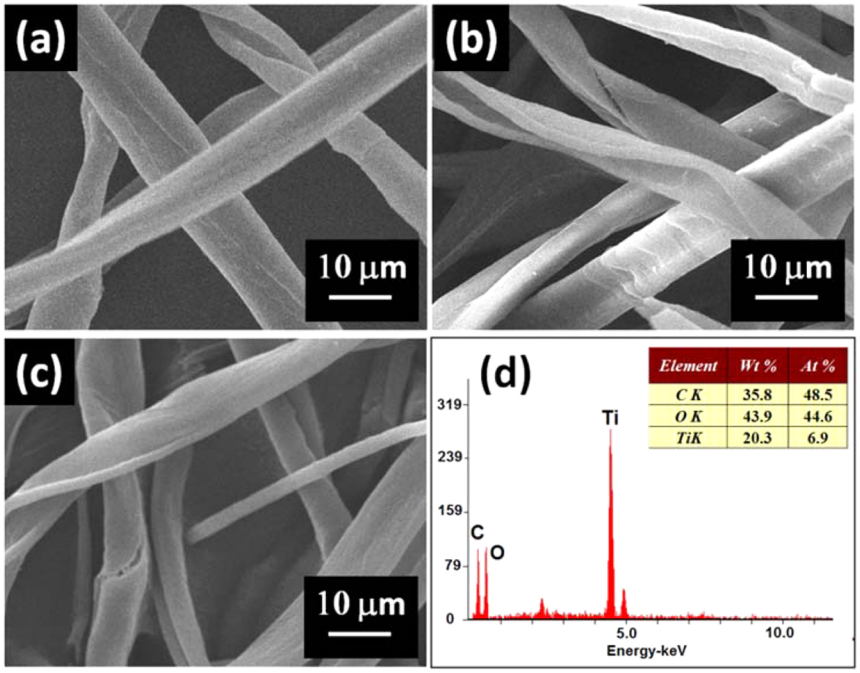

Figure 2. SEM images of (a) cotton, (b) CCC, and (c) CCC-Ti^{4+} fibers. (d) EDX spectrum and chemical composition (inset table) of CCC-Ti^{4+} fibers.

resuspended in Tris-HCl buffer (8 M urea, 0.2 M Tris, 4 mM $CaCl_2$, pH 8.0) to bring the final concentration of protein to 5 mg/mL.[31] The digestion procedure was the same as that of BSA,[26] and the digested products were desalted by C18 cartridge and stored at −20 °C until use.

2.6. Preparation of the Lab-In-Syringe SPE. Lab-in-syringe SPE was prepared according to previous work with a slight modification.[25] Briefly, CCC-Ti^{4+} fibers (5 mg) were packed in a hub of a 1 mL plastic syringe for extraction (Figure 1).

2.7. Selective Enrichment of Phosphopeptides from Protein Digests, Human Serum, and Tryptic Digests of Rat Brain Lysate. CCC-Ti^{4+}-fiber-packed syringe SPE was used for phosphopeptide enrichment. For the standard proteins, the tryptic digests of proteins were first diluted to a certain concentration by loading buffer (50% ACN, 45% H_2O, 5% TFA, v/v/v). The enrichment was carried out by pipetting up and down to ensure full adsorption of peptides. After washing twice with loading buffer, the trapped peptides were eluted with 50 μL of 5% aqueous ammonia solution. The extraction could be completed within 3 min. For the phosphopeptide enrichment from nonfat milk digests, 2 μL of milk digests was diluted 1000-fold with loading buffer and 100 μL of the diluent (containing 0.1 μL of original milk digests) was used as sampling solution. For endogenous phosphopeptide enrichment from human serum, 2 μL of original serum was diluted to 100 μL with loading buffer, and the sample processing was the same as that of peptide mixture enrichment. Then, the eluted solution was lyophilized to dryness, and the residue was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

For phosphopeptide enrichment from tryptic digests of rat brain lysate, 1 mg of tryptic digests was diluted to 500 μL with loading buffer, and the sample processing was the same as that of peptide mixture enrichment. The eluted solution was then lyophilized to dryness, desalted with Ziptip C18, and used for RPLC-ESI-MS/MS analysis.

For comparison, commercial TiO_2 powder and ZrO_2 powder were applied for phosphopeptide enrichment from the mixture of tryptic digests of β-casein and BSA with a molar ratio of 1:500, and the extraction process was the same as that used in our previous work.[29]

2.8. Mass Spectrometry Analysis. All MALDI-TOF-MS spectra were acquired according procedures in our previous work.[25]

Reversed phase liquid chromatography-electrospray ionization tandem mass spectrometry (RPLC-ESI-MS/MS) was used to analyze the sample from rat brain, and the analysis was carried out according to previous work.[31]

Data were searched against the Uniprot rat reference proteome database (version 201412), and the parameters followed can be found in our previous work.[31]

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of Fibers. The infrared spectra of cotton and CCC are shown in Figure S1. Compared with the infrared spectrum of cotton (Figure S1a), a stretching vibration absorption band of a carbonyl group at 1718 cm^{-1} could be observed in infrared spectrum of CCC (Figure S1b), indicating the successful synthesis of CCC.

The preparation of CCC-Ti^{4+} fibers was carried out on the basis of the coordination effect between double carboxyl groups and Ti^{4+}, and the schematic diagram is shown in Figure 1. The SEM images showed that the diameters of cotton (Figure 2a), CCC (Figure 2b), and CCC-Ti^{4+} (Figure 2c) were almost the same (ranging from 10 to 20 μm), indicating that the modification did not impact the fibrous morphology of cotton and that the fiber strength was also well-maintained.

The composition of CCC-Ti^{4+} fibers was examined by EDX spectroscopy. As shown in Figure 2d, CCC-Ti^{4+} fibers were composed of carbon, oxygen, and titanium, demonstrating successful immobilization of Ti^{4+} on CCC. The specific composition of the elements is listed in the inset table of Figure 2d. The weight and atom percentage of titanium were estimated to be 20.3 and 6.9%, respectively, indicating that the prepared CCC-Ti^{4+} fibers possessed high titanium content and therefore would result in a large binding capacity for phosphopeptides.

3.2. Evaluation of the Property of CCC-Ti^{4+} Fiber-Packed Lab-In-Syringe SPE toward Phosphopeptides. Considering the specific interaction between Ti^{4+} and phosphopeptide, the CCC-Ti^{4+} fibers were used to extract

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phosphopeptides from the tryptic digests of β-casein (containing a small amount of α-casein). After optimization, an acidic solution (50% ACN, 45% H_2O, 5% TFA, v/v/v) was used as sampling solution, and an alkaline solution (5% aqueous ammonia solution) was used for the desorption of captured phosphopeptides. The results showed that three phosphopeptides were observed by direct analysis; however, many abundant nonphosphopeptides were observed that dominated the spectrum (Figure 3a). However, after enrichment with CCC-Ti^{4+} fibers, seven phosphopeptides (detailed information in Table S1) with enhanced signal-to-noise ratio (S/N) could be observed (Figure 3b). In addition, the signals of nonphosphopeptides dramatically decreased. To investigate the capture capability of CCC-Ti^{4+}, the sampling eluate was also collected for analysis. The result showed that only nonphosphopeptides were detected (Figure 3c), indicating the good affinity of the prepared material toward phosphopeptides. Therefore, the prepared CCC-Ti^{4+} fibers could be employed for specific capture of phosphopeptides.

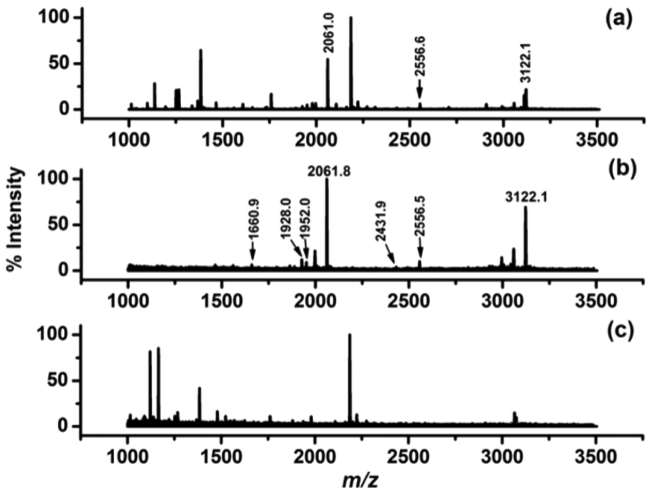

Figure 3. MALDI mass spectra of tryptic digest of β-casein. (a) Direct analysis, (b) analysis after enrichment with CCC-Ti^{4+} fibers, and (c) analysis of sampling eluate. Phosphopeptides are labeled with their observed m/z.

As shown in Figure 4a, when the molar ratio of β-casein and BSA was 1:100, nonphosphopeptides dominated the spectrum, whereas the signals of phosphopeptides were totally unobservable. Nonetheless, after enrichment by CCC-Ti^{4+} fibers, the signals of phosphopeptides remarkably improve, resulting in the identification of seven phosphopeptides with good resolution (Figure 4b). The enrichment specificity of CCC-Ti^{4+} fibers toward phosphopeptides was further evaluated by increasing the amount of nonphosphopeptides. As shown in Figure 4c–f, after enrichment by CCC-Ti^{4+} fibers, six phosphopeptides could be observed in each spectrum with the molar ratios of β-casein and BSA being 1:500 (Figure 4d) and 1:1000 (Figure 4f), which showed significant improvement of the selectivity for phosphopeptides than some reported IMAC materials.[7,15,32] The results indicated that CCC-Ti^{4+} fibers could be used in the enrichment of phosphopeptides even in the presence of plenty of nonphosphopeptides. Comparison of CCC-Ti^{4+} fibers with the previous three IMAC materials is listed in Table S2. Compared with the previous methods, the proposed method was rapid with high selectivity, which should be attributed to the good permeability and hydrophility of CCC-Ti^{4+} fibers,

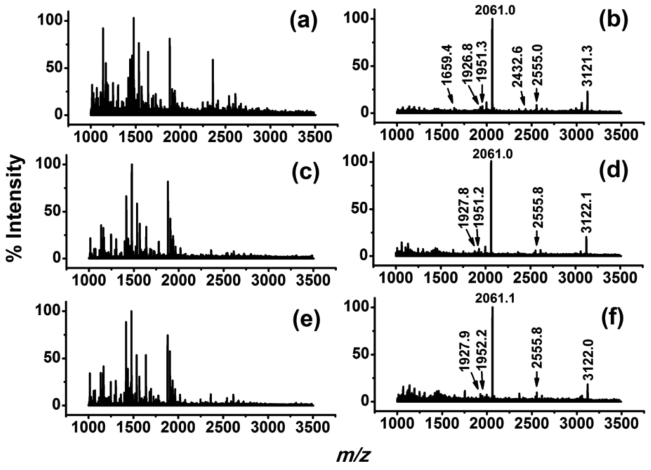

Figure 4. MALDI mass spectra of the tryptic digest mixtures of β-casein and BSA (a, c, and e) without or (b, d, and f) with the CCC-Ti^{4+} fiber enrichment. Molar ratios of β-casein to BSA are 1:100 (a and b), 1:500 (c and d), and 1:1000 (e and f). The concentration of β-casein was 1.0×10^{-8} M (2 pmol). Phosphopeptides are labeled with their observed m/z.

effectively decreasing extraction time as well as improving the identification capacity for phosphopeptides by resisting interference from hydrophobic nonphosphopeptides. Meanwhile, the superior performance of CCC-Ti^{4+} fibers might also benefit from the high amount of immobilized Ti^{4+} and strong chelation action of CCC with Ti^{4+}. In addition, tryptic digests of β-casein and BSA with molar ratios of 1:1 and 1:10 were also used to evaluated the selectivity of CCC-Ti^{4+} fibers, and seven phosphopeptides could be seen in each spectrum after treating by CCC-Ti^{4+} fibers (Figure S2a,b).

To investigate the sensitivity of this method, CCC-Ti^{4+} fibers were applied to enrich phosphopeptides from different amounts of β-casein digests (1000, 100, and 10 fmol; Figure 5). The results suggested that the signal of one phosphopeptide (m/z = 3122.0) could still be well-detected using 10 fmol of β-casein digests (concentration = 1×10^{-10} M) with S/N over 3 (Figure

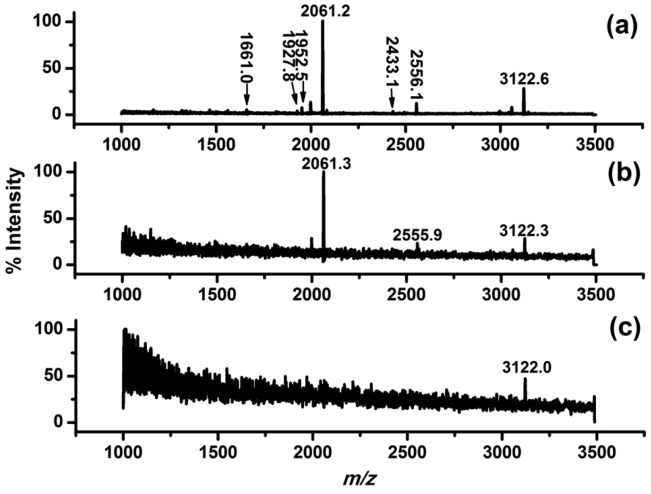

Figure 5. MALDI mass spectra of tryptic digest of β-casein after enrichment with CCC-Ti^{4+} fibers. The amounts of tryptic digested β-casein used in the experiments were (a) 1000 fmol, (b) 100 fmol, and (c) 10 fmol. Phosphopeptides are labeled with their observed m/z.

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5c), demonstrating the high detection sensitivity of this strategy.

In some previous works, commercially available TiO_2 and ZrO_2 particles were frequently used to purify phosphopeptides. For example, TiO_2 beads have been packed in microcolumns for phosphopeptide enrichment of a peptide mixture[33] or plasma membrance protein digests;[34] Hsieh's group has developed a TiO_2-nanoparticle-embeded pipet tip that can selectively enrich phosphopeptides from a standard peptide mixture with the molar ratio of β-casein, α-casein, and BSA being 1:1:10.[35] Here, commercial TiO_2 and ZrO_2 particles were used to enrich phosphopeptides from a peptide mixture (β-casein/BSA= 1:500) in a dispersive SPE format for comparison. After optimization (data not shown), 5% TFA-50% ACN (v/v) was used as sampling solution. The results showed that, after enrichment by TiO_2 or ZrO_2, only four or three phosphopeptides could be identified with poor resolution, respectively, whereas a number of nonphosphopeptides with good resolution could be observed in the mass spectra (Figure S3 and Table S1). Compared to TiO_2 and ZrO_2, CCC-Ti^{4+}-fiber-packed SPE showed better selectivity in complex samples (compare Figure 6b with Figure S3), which might be attributed

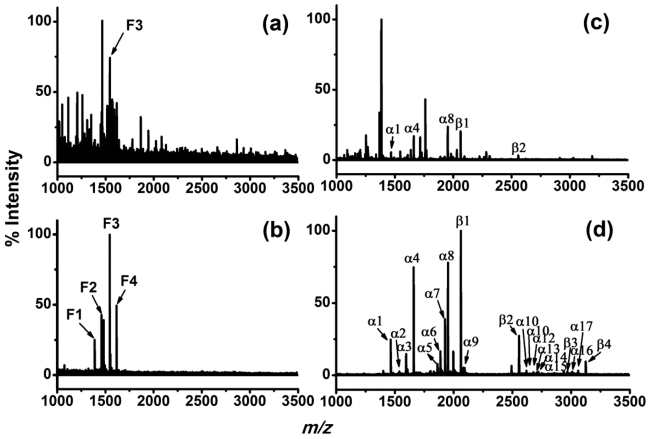

Figure 6. MALDI mass spectra of human serum obtained by (a) direct analysis or (b) after enrichment with the CCC-Ti^{4+} fibers. MALDI mass spectra of tryptic digest of nonfat milk obtained by (c) direct analysis or (d) after enrichment with the CCC-Ti^{4+} fibers.

to the good biocompatibility of CCC-based materials.[29,36] In addition, the dispersive SPE format used here required repeated centrifugation steps in the extraction experiment using commercial TiO_2 and ZrO_2 powder as adsorbents, which might lead to the coprecipitation of nonphosphopeptides and the loss of phosphopeptides.[17] To further verify the feasibility of the proposed method, CCC-Ti^{4+} fibers can be applied to specifically capture phosphopeptides from more complex samples.

3.3. Enrichment of Phosphopeptides from Human Serum and Nonfat Milk by CCC-Ti^{4+} Fiber-Packed Lab-In-Syringe SPE. CCC-Ti^{4+} fibers were further used to enrich phosphopeptides from complex biological samples, including human serum and nonfat milk. Human serum contains endogenous phosphopeptides that correlate with some certain diseases.[12,37] However, because of the high complexity of matrix, including massive nonphosphopeptides and coexisting proteins, the determination of phosphopeptides from human serum is difficult. Here, we used CCC-Ti^{4+} fibers to selectively capture endogenous phosphopeptides from 2 μL of original human serum. MALDI mass spectrum showed that only one phosphopeptide along with many nonphosphopeptides were identified by direct analysis (Figure 6a). After enrichment with CCC-Ti^{4+} fibers, four endogenous phosphopeptides derived from fibrinopeptides could be distinctly identified. (Figure 6b; detailed information on the detected phosphopeptides is listed in Table S3.) This result indicated the excellent performance of CCC-Ti^{4+} fibers for the selective enrichment of phosphopeptides from complex matrix, suggesting good biocompatibility of CCC-based fibers for enrichment of target analytes from protein-rich biological samples. Meanwhile, all the above results demonstrated that CCC-based fibers had a promising application in endogenous phosphopeptide profiling for peptidome research.

We then further used CCC-Ti^{4+} fibers to enrich phosphopeptides from nonfat milk digests. Nonfat milk contains abundant nonphosphoproteins and some phosphoproteins, including α-casein and β-casein. Nonfat milk digests were gradually diluted 1000-fold with loading buffer (5% TFA, 50% ACN, v/v) and subjected to analysis. Only five phosphopeptides along with plenty of nonphosphopeptides with strong signal were observed through direct analysis (Figure 6c). However, after enrichment with CCC-Ti^{4+} fibers, 21 phosphopeptides that were found previously[26,38,39] could be detected with good resolution. (Figure 6d; detailed information on the observed phosphopeptides is listed in Table S4.) The result indicated that the proposed CCC-Ti^{4+} fibers have excellent selectivity toward phosphopeptides in the real in-solution digested samples.

3.4. Phosphopeptide Enrichment from Tryptic Digests of Rat Brain Lysate. Encouraged by the high specificity of CCC-Ti^{4+} fibers, we further investigated its performance using a tryptic digest of rat brain lysate as a complex proteomic mixture. A total of 1 mg of tryptic digest of proteins of rat brain lysate were treated with CCC-Ti^{4+} fibers, and the eluate was analyzed by RPLC-ESI-MS/MS. As shown in Figure 7, 4479

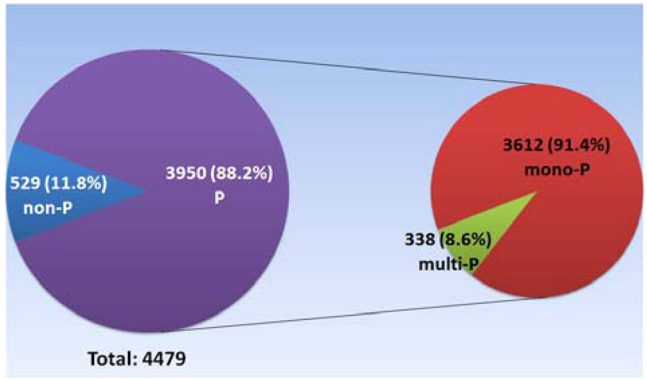

Figure 7. Number of identified peptides from tryptic digests of rat brain lysate after enrichment with CCC-Ti^{4+} fibers. "P" represents phosphopeptide.

unique peptides including 3950 phosphopeptides were identified after enrichment with CCC-Ti^{4+} fibers (Table S6), and the phosphopeptide ratio[31] was 88.2%, which showed better performance in phosphopeptide enrichment from tryptic digests of rat brain lysate than that in previous works.[18,40] A comparison of CCC-Ti^{4+} fibers with the previous strategies is listed in Table S5. The results showed that CCC-Ti^{4+} fibers had a great advantage in highly selective enrichment of

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phosphopeptides from complex biosamples, which might be because of the inherent biocompatibility of CCC-based material. In addition, the number of singly and multiply phosphorylated peptides identified by CCC-Ti^{4+} fibers was 3612 (91.4%) and 338 (8.6%), respectively. These results suggested CCC-Ti^{4+} fibers have high selectivity and great capacity for phosphopeptide enrichment from a complex mixture, and CCC-Ti^{4+} fibers have been proven a promising material for phosphoproteomics analysis.

4. CONCLUSIONS

In the current study, a novel CCC-Ti^{4+} fibrous sorbent was successfully synthesized and applied for phosphoproteomics research. The synthesis of CCC-Ti^{4+} fibers was easy, and the titanium content was high. CCC-Ti^{4+} fibers exhibited specific biological compatibility and good performance in the selective and effective enrichment of phosphopeptides from multiple samples, including standard protein digests, nonfat milk digests, human serum, and tryptic digests of rat brain lysate. Thanks to the good biocompatibility of the CCC-based material, CCC-Ti^{4+} fibers showed excellent performance in phosphopeptide enrichment from protein-rich human serum, indicating its impressive application prospect in endogenous phosphopeptide profiling for peptidome research. In addition, with 1 mg of rat brain lysate, we identified 3950 phosphopeptides and 4304 phosphorylation sites, demonstrating the good performance of this approach. Furthermore, the lab-in-syringe SPE approach greatly simplified the entire process of enrichment. We believe that this novel method will be applicable for phosphopeptide enrichment in large-scale phosphoproteomics studies and that hydrophilic CCC-based fibrous materials are promising candidates for complex biological analysis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b04572.

IR spectra of fibers; MALDI mass spectra of the tryptic digest mixtures of β-casein and BSA before and after enrichment; detailed information on phosphopeptides obtained from β-casein digest, human serum, nonfat milk digests, and rat brain lysate; comparison of CCC-Ti4+ fibers with the previous materials. (PDF)

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Notes

The authors declare no competing financial interest.

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