Published in final edited form as:

Anal Chem. 2020 November 03; 92(21): 14790–14797. doi:10.1021/acs.analchem.0c03557.

Glass Fiber-Supported Hybrid Monolithic Spin Tip for Enrichment of Phosphopeptides from Urinary Extracellular Vesicles

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Abstract

Extracellular vesicles (EVs) are attracting increasing interest with their intriguing role in intercellular communications. Protein phosphorylation in EVs is of great importance for understanding intercellular signaling processes. However, the study of EV phosphoproteomics is impeded by their relatively low amount in limited clinical sample volumes, and it is necessary to

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.0c03557>.

Additional experimental methods, characterization results of hybrid monoliths, Western blot analysis of CD9 in urinary EVs, numbers of identified EVs phosphopeptides enriched by TiO₂, overlap of identified unique phosphopeptides enriched from different volumes of urinary EV samples, and protein network analysis of up-regulated phosphoproteins in the prostate cancer group obtained from STRING (PDF)

LC-MS phosphoproteomics data for different sample volumes and quantitative phosphoproteomics results of LC-MS analysis for healthy control and prostate cancer samples (XLSX)

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acs.analchem.0c03557>

The authors declare the following competing financial interest(s): EVTRAP is manufactured and sold by Tymora Analytical Operations which was co-founded by A.B.I. and W.A.T.

The proteomics raw data generated in this work have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifiers PXD020861 and 10.6019/PXD020861.

have a sensitive and efficient enrichment method for EV phosphopeptides. Herein, a novel Ti(IV)-functionalized and glass fiber-supported hybrid monolithic spin tip, termed PhosTip, was prepared for enriching phosphopeptides from urinary EVs. Glass fiber as the stationary phase positions the hybrid monolith in a standard pipet tip and prevents the monolith from distortion during experiments. The preparation procedure for the new PhosTip is simple and time-saving. The hybrid monolithic PhosTip provides excellent enrichment efficiency of low-abundance phosphopeptides from cell digests and urinary EVs with minimum contamination and sample loss. Using the PhosTip, we demonstrate that 5373 and 336 unique phosphopeptides were identified from 100 and 1 μ g of cell lysates, while 3919 and 217 unique phosphopeptides were successfully identified from 10 and 1 mL of urinary samples, respectively. The PhosTip was finally applied to enrich phosphopeptides in urine EVs from prostate cancer patients and healthy controls and quantify 118 up-regulated proteins with phosphosites in prostate cancer samples. These results demonstrated that the PhosTip could be a simple and convenient tool for enriching phosphopeptides from clinical samples and for broader applications in biomarker discovery.

Graphical Abstract

Extracellular vesicles (EVs), including exosomes and other membranous vesicles, are secreted into the extracellular space by most cell types and can be found in bodily fluids, such as urine, saliva, blood, and cerebrospinal fluid.^{1,2} Over the past years, EVs have emerged as an important mediator of cell–cell communication because they transport molecular information such as proteins, regulatory RNAs, and other biomolecules from donor to recipient cells.^{3–6} Among them, cargo proteins possess unique features over conventional serological makers that may be detected in higher sensitivity compared to the proteins directly detected in bodily fluids. Meanwhile, EV proteins are highly stable because they are protected from chemical and enzymatic degradation and can evade recognition and subsequent removal by the immune system.⁷ Therefore, EV proteins have been considered as potential circulating biomarkers to diagnose or monitor disease progress.^{8–12}

Protein post-translational modifications (PTMs) in nature represent a high level of molecular regulation of signaling pathways and *in vivo* functions. Reversible protein phosphorylation is one of the most widespread and important PTMs, which plays a key role in the regulation of intracellular biological processes, such as the cell cycle, apoptosis, metabolism, cellular signaling, and proliferation.^{13,14} Only recently, extensive phosphorylation has been observed in EVs from biofluids such as plasma^{15,16} and from urine.^{17–19} Gonzales et al. first reported

phosphoproteomic study of EVs in 2009, and exosome was obtained from human urine by differential centrifugation.¹⁷ In the study, 1132 proteins were identified from urinary EVs. However, after TiO₂ enrichment only 14 phosphoproteins corresponding to 19 phosphorylation sites were identified by liquid chromatography–mass spectrometry (LC–MS). In 2018, Wu et al. developed a rapid extracellular vesicles total recovery and purification (EVTRAP) method to efficiently capture EVs in human urine samples.¹⁸ These results showed that the crucial factors for phosphoproteomic study of EVs were the efficiency of EV isolation and phosphopeptide enrichment.

Over the past decades, multiple methods have been developed to enrich phosphopeptides from complex samples, such as TiO₂, immobilized metal ion affinity chromatography (IMAC), PolyMAC, etc.^{20–25} These approaches exhibit overall efficient enrichment for phosphopeptides with their high selectivity and sensitivity.^{26–29} However, one of the main challenges in enriching phosphopeptides from limited amounts of biomedical samples is relatively lower yield due to loss of sample from multiple transfer steps and the residual solution during the enrichment process. The spin tip, as a robust and scalable method, has been widely applied for microscale complex sample analysis because it largely eliminates contamination or sample loss.³⁰ Liu et al. developed an organic monolithic polypropylene pipet tip grafted with porous Ti(IV)-immobilized monolithic materials.³¹ It enabled fast and efficient capture of phosphopeptides from microscale cell digests via centrifugation mode. However, it requires modifying the surface of pipet tips with functional groups before preparation. This limited the enrichment capacity to 25 μ g of HeLa cell digests, which restricted the range of applications. Yao et al. developed a novel solid-phase extraction (SPE) tip assisted by centrifugation using Ti(IV)-IMAC material made in their lab.³² This method was fitted to analyze minute amounts of sample. However, the preparation process of this kind of Ti(IV)-IMAC material was not trivial and was time-consuming.

Melamine–formaldehyde (MF) sponge has been used as a skeleton to stabilize the silica-monolith sorbents for SPE³³ and oil–water separation.^{34,35} The flexible framework was able to keep its shape during use. However, the MF sponge was difficult to cut into small enough pieces to be adapted to the size of tips. In this work, we employed glass fiber, which has been frequently used as a catalyst stationary phase and chromatography support, as the monolithic framework to prevent the monoliths from shrinking. In the preparation process, methacryl-substituted polyhedral oligomeric silsesquioxane (POSS-MA), valproic acid (VPA), and 1,4-dithiothreitol (DTT) were selected as monomers to prepare Ti(IV)-IMAC hybrid monolithic spin tips via “one-step” thermo-initiated thiol–ene free radical polymerization. This preparation process was very simple and accessible, and the resulting spin tips, that we named PhosTip, demonstrated high selectivity and sensitivity for phosphopeptides from limited amounts of biological samples such as cell digests and urinary EVs.

EXPERIMENTAL SECTION

Experimental details of cell culture and sample preparation, urine samples collection, optimization of the PhosTip prepared condition, enrichment of phosphopeptides by commercial TiO₂, cell samples LC–MS/MS analysis, database searching, phosphopeptides

identification, and label-free quantitation analysis are included in the Supporting Information.

Materials.

POSS-MA (cage mixture, $n = 8, 10, 12$) was obtained from Hybrid Plastics, Inc. (Hattiesburg, MS, U.S.A.). VPA (97%), DTT, sodium chloride (NaCl), trifluoroacetic acid (TFA), methacryloxypropyltrimethoxysilane (γ -MAPS), sodium deoxycholate (SDC, BioXtra, 98%), *N*-lauroysarcosine sodium salt (SLS), trizma-HCl, tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), 2-chloroacetamide (CAA), tetraethylammonium bromide (TEAB), protease inhibitor cocktail II (for use with mammalian cell and tissue extracts), phosphatase inhibitor (1 mM NaF and 1 mM Na₃VO₄), TPCK-treated trypsin, urea, iodoacetamide (IAA), triethylamine (TEA), and ammonia-water (NH₃·H₂O) were obtained from Sigma (St. Louis, MO, U.S.A.). Lys-C was from Wako (Japan). 2,2-Azobis(isobutyronitrile) (AIBN), Triton 100, NP 40, 1-butanol, and 1,4-butylene glycol were obtained from Aladdin (Shanghai, China). Glass fiber (XH-SYM, 1–3 μ m) was obtained from Xihu (Shanghai, China). Titanium sulfate was from Shanghai Chemical Plant (Shanghai, China). RPMI-1640 cell culturing medium and PBS were purchased from Gibco, Invitrogen Corporation (Carlsbad, CA). Formic acid (FA) was obtained from Fluka (Buchs, Germany). Acetonitrile (ACN, mass grade) was purchased from Thermo Fisher. The water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore Inc., Milford, MA, U.S.A.).

Samples from a total of 10 patients with prostate cancers and 10 healthy donors were collected from the First Affiliated Hospital of Nanjing Medical University with the approval from its ethics committee. Three replicates (10 mL of samples in total normalized by creatinine assay) were processed and analyzed with LC–MS/MS.

Preparation of the Glass Fiber-Supported Hybrid Monolithic PhosTip.

Prior to preparation of the PhosTip, the glass fiber was first modified with γ -MAPS to immobilize a layer of methacrylate groups on its surface. In brief, glass fiber was immersed sequentially into 0.1 mol/L NaOH, H₂O, 0.1 mol/L HCl, H₂O, and methanol. After that, the washed glass fiber was immersed into γ -MAPS/methanol (50%, v/v) solution, and then put in a water bath at 50 °C for 16 h. Finally, the modified glass fiber was washed with methanol and dried out.

To prepare the PhosTip, a certain amount of γ -MAPS-modified glass fiber was loaded into the pipet tips and settled in a fixed position of the pipet tips. POSS-MA, VPA, and DTT were then dissolved in the porogenic agents (1-butanol and 1,4-butylene glycol, 11/3, v/v) by sonication for 5 min. After that, 1% AIBN (wt %) was added into the solution, and it was sonicated for 3 min to remove the gas in solution. Then, several microliters of the solution were loaded onto the glass fiber in pipet tips with an injection needle. The tips were sealed in a centrifuge tube, and the polymerization reaction was carried out at 60 °C for 12 h. Finally, the obtained phosphate-functionalized hybrid monoliths in pipet tips were washed three times with methanol (200 μ L) by centrifugation at 1000g to remove the porogenic agents and monomer residuals.

In order to chelate Ti(IV) on the surface of phosphonate-functionalized hybrid monoliths in the PhosTip, 200 μL of titanium sulfate aqueous solution (50 mg/mL) was transferred five times into the tips by centrifugation at 1000g for at least 15 min to ensure complete binding. Then, the tips were washed sequentially three times by 200 mM NaCl aqueous solution and three times by 0.1% TFA (v/v) by centrifugation at 1000g to remove the excess Ti(IV).

Characterization of the Hybrid Monolithic PhosTip.

The specific surface area of the hybrid monolith was calculated from nitrogen adsorption/desorption measurement using an ASAP 2460 physisorption analyzer (Micromeritics, U.S.A.). Hybrid monolith was first treated at 120 °C for 6 h in a vacuum before analysis. The specific surface area was calculated via the Brunauer–Emmett–Teller (BET) method, and the pore size distribution was determined from the adsorption isotherm by the Barrett–Joyner–Halenda (BJH) method. The total pore volume was determined at $P/P_0 = 0.99$. Fourier transform infrared spectroscopy (FT-IR) characterization was carried out on Thermo Nicolet 380 spectrometer (Nicolet, Wisconsin, U.S.A.) using KBr pellets containing approximately 1 mg of sample and 100 mg of KBr. The microscopic morphology of hybrid monolithic materials was obtained by scanning electron microscopy (SEM, Hitachi, S-3000N). The macropores size distribution of the hybrid monolith was measured by the mercury intrusion porosimetry (MIP) method on PoreMasre GT-60 (Quantachrome Boynton Beach, U.S.A.). The analysis of S, P, and Ti contents in prepared Ti(IV)-IMAC hybrid monolith was carried out by inductively coupled plasma atomic emission spectrometry (ICP-AES) on a Vista-MPX (Varian, U.S.A.). Thermogravimetric (TG) data were collected in air atmosphere with heating at 10 °C/min on a Discover TGA (TA, U.S.A.).

Preparation of Urinary EVs Samples.

Urinary samples were collected from healthy individuals without digital rectal examination (DRE) after informed consent and centrifuged at 2500g for 1 h to remove cell debris and large apoptotic bodies. The supernatant was collected for subsequent processing. Urinary EVs were isolated using EVTRAP beads (Tymora Analytical Operations, West Lafayette, Indiana, U.S.A.).¹⁸ The enrichment method for EVs was based on the manual instruction: EVTRAP beads were added into the urinary sample at a beads-to-urine ratio of 1:50 (v/v). The sample was incubated for 1 h with end-over-end rotation at room temperature, and then the solution was removed using the magnetic separator. After washing steps, 50–200 μL of fresh 100 mM TEA solution was used twice to elute the EVs from beads by shaking for 10 min, and the eluate was dried out in a vacuum centrifuge (Labconco SpeedVac).

The digestion was performed with a phase transfer surfactant-aided procedure (PTS).^{15,16} The dried EVs were solubilized in 100 μL of lysis buffer (50 mM Tris–HCl, 12 mM SDC, 12 mM SLC, 10 mM TCEP, 40 mM CAA, and 1% phosphatase inhibitor mixture). The solution was boiled at 95 °C for 10 min, and then diluted 5-fold with 50 mM TEAB. The proteins were digested with Lys-C at a 1:100 (w/w) enzyme-to-proteins ratio for 3 h at 37 °C. Subsequently, trypsin was added to the digest at a 1:100 (w/w) enzyme-to-proteins ratio overnight. Then, 10% TFA was added to acidify the solution which contained digested peptides, and 500 μL of ethyl acetate was added. After vortexing for 2 min, the mixture solution was centrifuged at 10 000g for 3 min to obtain aqueous and organic phases. The

organic phase was removed. The ethyl acetate extraction was repeated one more time. The obtained aqueous phase was dried completely in a vacuum centrifuge. The digested peptides were desalted using a homemade C18 stage-tip (3 M Empore).

Phosphopeptide Enrichment Using the Ti(IV)-IMAC Hybrid Monolithic PhosTip.

Before enrichment of phosphopeptides, the Ti(IV)-IMAC hybrid monolithic PhosTip was equilibrated by the loading solution (80% ACN, 6% TFA). Peptide mixtures from Bel cells or EVs were redissolved in 200 μL of loading solution. Then, the peptide solution was transferred into the PhosTip. To achieve complete binding, peptides were loaded into the hybrid monolithic PhosTip by centrifugation at 800g for at least 30 min. The PhosTip was then washed twice with 200 μL of washing solution I (50% ACN, 6% TFA, 200 mM NaCl) and another washing solution II (30% ACN, 0.1% TFA) separately to remove nonspecifically bound peptides by centrifugation. Finally, the captured phosphopeptides were eluted twice by 100 μL of 10% $\text{NH}_3\text{-H}_2\text{O}$. The eluents containing phosphopeptides were collected in the centrifuge tube and dried in a vacuum centrifuge.

LC-MS/MS Analysis of EV Samples.

Phosphopeptides of EVs after enrichment with the PhosTip were analyzed with a timsTOF Pro mass spectrometer (Bruker) coupled with a Nanoelute LC system. Phosphopeptides were separated on a 25 cm in-house packed column (360 μm o.d. $\times$ 75 μm i.d.) containing C18 resin (2.2 μm , 100 Å, Michrom Bioresources), and the flow rate was adjusted to 300 nL/min with a linear 120 min gradient. The mass spectrometer was operated in the datadependent acquisition (DDA) mode with a full-scan MS (m/z was 100–1700 with the resolution at the “target value” of 40 000) and one full frame accompanied by 10 PASEF MS/MS frames, each containing on average 12 MS/MS spectra [MS precursors for PASEF MS/MS spectra until the intensity value and an ion mobility range ($1/K_0$) of 0.6–1.6 Vs/cm² were all reached]. For all experiments the quadrupole isolation width was set to 2 Th for m/z < 700 and 3 Th for m/z > 700. Collision energy was changed in five steps within a TIMS elution ramp: 52 eV for 0–19%, 47 eV for 19–38%, 42 eV for 38–57%, 37 eV for 57–76%, and 32 eV for 76–95% of the ramp time.

The obtained MS data were searched directly against the Uniprot database version downloaded November 2019 with no redundant entries, using PEAKS Studio X+ software (Bioinformatics Solutions Inc.). Initial precursor mass tolerance was set at 15 ppm, the final tolerance was set at 6 ppm, and MS/MS tolerance was set at 0.6 Da. Search criteria included a static carbamidomethylation of cysteine (+57.0214 Da), variable modifications of methionine oxidation (+15.9949 Da), N-terminal acetylation (+42.011 Da), and serine/threonine/tyrosine phosphorylation (+79.996 Da). The search was performed with full trypsin/P digestion allowing a maximum of two missed cleavages on the peptides analyzed from the sequence database. The false-discovery rates (FDR) of proteins, peptides, and phosphosites were all set to 0.1%. The phosphosite localization was also completed with PEAKS Studio X+ based on the Ascore, a probability-based score that measures the probability of correct phosphorylation site localization based on the presence and intensity of site-determining ions in MS/MS spectra.³⁶ All protein and peptide identifications were

grouped without any redundant entries. Only unique peptides/phosphopeptides and unique master proteins were reported.

RESULTS AND DISCUSSION

Preparation and Characterization of the Hybrid Monolithic PhosTip.

Spin tips are regularly used for microscale proteomics analysis since they can minimize contamination and sample loss during the sample preparation process.³⁷ The preparation process of Ti(IV)-IMAC hybrid monolithic PhosTip is illustrated in Figure 1. POSS-MA, VPA, and DTT as monomers were dissolved in the mixture of porogenic solvents which was composed of 1-butanol and 1,4-butylene glycol, and the molar ratio of these monomers (POSS-MA/VPA/DTT) was 1:2:1. The polymerization reaction was performed via thermo-initiated thiol-ene polymerization. In this preparation, POSS-MA served as a cross-linker and provided good chemical stability against the acidic environment when chelating with Ti(IV) and sample loading and also against the alkaline media used for the elution of phosphopeptides with 10% $\text{NH}_3\cdot\text{H}_2\text{O}$. VPA as functional monomer provided abundant phosphonate groups to immobilize Ti(IV) for capture of phosphopeptides in complex samples. The employment of VPA could directly merge phosphate groups into the hybrid monolithic materials. The introduction of DTT, which could react with both POSS-MA and VPA by thiol-ene click chemistry, was to eliminate the major obstacle of oxygen inhibition.³⁸ We compared the condition under which no DTT was added, and the polymerization reaction did not occur, likely due to the oxygen inhibition. The porogenic solvents have a great impact on the formation of the monolithic porous structure. After a series of screening experiments, 1-butanol and 1,4-butylene glycol were finally selected as optimal binary porogenic solvents to prepare hybrid monoliths. Before injecting the preparation solution into the pipet tips, a small amount of γ -MAPS-modified glass fiber (skeleton size was 1–3 μm) was sealed into the pipet tips at a set position. The glass fiber could adsorb the solution by a siphon phenomenon and immobilize the prepared hybrid monolith at the set position in the pipet tip. The glass fiber effectively supports the hybrid monolith and prevents it from potential collapse and separation from the inner surface of pipet tips. Meanwhile, it is noteworthy that it is not necessary to modify the surface of pipet tips with functional groups in advance, and the commonly used pipetting spears or most standard tips (with appropriate sizes) are all suitable for the preparation of similar hybrid monolithic PhosTips for different applications.

The glass fiber-supported hybrid monolithic PhosTip was characterized using SEM. It revealed that the hybrid monolith possessed a microlevel of macroporous structure, and the glass fiber was embedded in hybrid monolith as shown in Figure 2, parts a and b. The large size of the macropores could offer a good permeability for peptides. The surface of the prepared Ti(IV)-IMAC hybrid monolith in the PhosTip was also characterized by the energy dispersive X-ray (EDX) spectroscopy mapping images. As shown in Figure 2, parts c and d, and the Supporting Information, the P, Ti, S, and Si were uniformly distributed on the surface of the hybrid monolith. It was deduced that phosphonate groups were evenly distributed on the surface of the hybrid monolith and Ti(IV) was successfully immobilized in the PhosTip. The porosity of the hybrid monolith prepared in pipet tips was further

investigated by nitrogen adsorption–desorption isotherm and MIP measurement. It could be seen that the macropore diameters of the hybrid monolith ranged from 0.1 to 10 μm as shown in Figure 2e. Although the macropore size distribution was slightly broader than we hoped, it provides the hybrid monolith with good permeability and allows solutions to flow through the PhosTip quickly by centrifugation even at low speed, which is desirable for rapid enrichment and elution. Figure 2f and Figure S2 demonstrate the nitrogen sorption isotherm and mesopore size distribution of the hybrid monolith, respectively. The results clearly indicated the existence of mesopores structure, and the hybrid monolith exhibited a pore volume of 0.32 cm^3/g and high surface area of 119 m^2/g with mesopores structure based on the nitrogen adsorption–desorption isotherm. The size of the mesopores ranged from 10 to 60 nm based on BJH analysis of the adsorption isotherm (Figure S2). Because the hybrid monolith possesses high specific surface area and large mesopores size, it facilitates phosphopeptides enrichment with the resulting IMAC PhosTip after chelating with Ti(IV).

The chemical formation of the hybrid monolith by using POSS-MA, DTT, and VPA as the monomers was monitored by FT-IR characterization. Figure S3 clearly shows the strong peak signal at 1637 cm^{-1} (assigned to C=C stretching vibration) for the POSS-MA monomer. After the formation of the hybrid monolith, peak signals of 2954 and 2893 cm^{-1} were remarkably decreased but did not disappear, which indicated that there remained residual unreacted acrylate groups due to the steric hindrance of POSS-MA. Compared to the FT-IR spectrum of VPA (Figure S3b), the strong signal peak at 928 cm^{-1} , which corresponds to the symmetrical stretching vibration of phosphate groups, could be clearly observed in the FT-IR spectrum of the hybrid monolith (Figure S3c). The peak around 1200 cm^{-1} was assigned to the stretching vibration of P=O, while the peak at 984 cm^{-1} was assigned to the stretching vibration of P–O. These results indicated that phosphonate groups were successfully incorporated into the hybrid monolith. Thermogravimetric analysis not only characterized the thermal stability of the hybrid monolith in the PhosTip but could also evaluate the immobilization of Ti(IV). As shown in Figure S4, a significant mass loss only began at 300 $^{\circ}\text{C}$ and continued to 600 $^{\circ}\text{C}$ due to the pyrolysis of organic moieties under air atmosphere. This result indicated that the obtained hybrid monolith exhibited satisfactory thermal stability. Meanwhile, the residual mass of the hybrid monolith before the immobilization of Ti(IV) was 23.7%, and it was increased to 33.7% after the immobilization of Ti(IV), likely due to the fact that the residual mass which contains titanium compounds could not be decomposed at 800 $^{\circ}\text{C}$. This result also indicated that an appropriate amount of Ti(IV) was successfully immobilized on the surface of hybrid monolith. The S, P, and Ti contents in the hybrid monolith were further analyzed by ICP-AES. The S content was measured at 1.14%, while the theoretical value was calculated at 1.76%. Because DTT preferentially reacts with POSS-MA and VPA via thiol–ene click chemistry and one molecule of DTT reacts with two molecules of VPA to form VPA–DTT–VPA molecule, not all DTT monomers were incorporated into the hybrid monolith. Similarly, the actual value of P content (1.60%) was lower than its theoretical value (3.49%), indicating that VPA not only coupled with DTT but also formed oligomer via free radical polymerization. The VPA–DTT–VPA molecules and oligomer of VPA were washed away with ethyl alcohol. The actual Ti content was calculated at 2.02%, which will be sufficient for the PhosTip to have enough chelating sites [Ti(IV)] to capture phosphopeptides in biological samples.

Evaluation of the Ti(IV)-IMAC Hybrid Monolithic PhosTip.

To evaluate the performance of the new hybrid monolithic PhosTip for complex biological samples, Bel cell lysates were first selected. In the experiment, different amounts of starting tryptic digest of Bel cell proteins (1.0, 5.0, 10, 25, 50, and 100 μg) were used, and three technical replicates for each amount were performed. The enriched phosphopeptides were subsequently analyzed by an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). As shown in Figure 3a, more than 2000 unique phosphopeptides were identified from 10 μg of complex protein digest, and the enrichment specificity (the percentage of unique phosphopeptides among all identified peptides) was 87.5%. This result showed that the enrichment performance of the prepared hybrid monolithic PhosTip was comparative to that of the IMAC tips reported.^{31,32} With the protein amount increasing to 25, 50, and 100 μg , the total number of identified phosphopeptides was increased as well (2833, 4073, and 5373 unique phosphopeptides were identified, respectively). Meanwhile, the enrichment specificity for enriching 25, 50, or 100 μg of samples was all close to 90%. A higher amount of protein digest (150 μg) was also performed with the PhosTip, and 5642 unique phosphopeptides were identified, indicating it is likely beyond its capacity. We suggest that the PhosTip is suitable for sample amounts lower than 100 μg .

To sum up, the high number of unique phosphopeptides identified after enriching by the prepared Ti(IV)-IMAC PhosTip attributes to its high specific surface area, which provided adequate chelating sites and improved the adsorption capacity for phosphopeptides. Meanwhile, when further decreasing the amount of cellular digest to 5 and 1 μg , the enrichment ability of the prepared IMAC PhosTip for limited amounts of biological sample was explored. As shown in Figure 3a, 786, 825, and 905 unique phosphopeptides were identified from 5 μg of cell protein digest, while 329, 338, and 377 unique phosphopeptides were successfully identified from 1 μg of cellular digest in triple experiments. This number of phosphopeptides identified from a limited amount of whole cell extract was comparable to that obtained by the spin tips previously reported^{31,32} (where about 336 and 467 unique phosphopeptides were identified from 1 μg of HeLa proteins digest). These data demonstrated that the IMAC PhosTips were capable of enriching phosphopeptides from microscale biological samples.

We also evaluated the percentages of monophosphopeptides and multiphosphopeptides (peptides with multiple phosphorylation sites) enriched by the PhosTip. As shown in Figure 3a, the percentages of multiphosphopeptides were at about 20% when 10 and 25 μg of cellular protein digest (the monolith-to-protein ratio was 1/2 and 1/5, respectively) were used. The percentages were increased to about 30% when the starting amounts of cellular lysate digest were increased to 50 and 100 μg (the monolith-to-protein ratios were 1/10 and 1/20, respectively). The results indicated that the PhosTip had enough active sites to capture both multiphosphopeptides and monophosphopeptides when low ratios of monolith to protein digests (such as 1/0.2, 1/1, 1/2, and 1/5) were used. However, when the amount of loading protein digest was further increased (the ratio of monolith to protein digest was increased to 1/10 and 1/20), there exists competitive adsorption between multiphosphopeptides and monophosphopeptides with the limited surface area of the PhosTip (active sites). It is known that multiphosphopeptides were more tightly bound to

metal ions such as Ti(IV), and therefore the percentage of identified multiphosphopeptides was increased with the limited surface area in the PhosTip.

The overlap of phosphopeptides identified and the relative standard deviations (RSDs) of the unique phosphopeptide identified, the percent of identified monophosphopeptides, and the specificity in repeated experiments were used to investigate the reproducibility of the prepared PhosTip. The RSDs of the phosphopeptides identification numbers, the percent of identified monophosphopeptides, and the specificity from three tips were 4.35%, 0.63%, and 1.40%, respectively. The RSDs of the phosphopeptide abundance for different loading protein amounts (10, 25, 50, and 100 μg) were 5.27%, 11.35%, 8.40%, and 7.33%, respectively. These results indicated overall good reproducibility of the prepared IMAC PhosTip.

Application of the PhosTip for Processing EV Samples from Minute Urinary Volumes.

We then used the Ti(IV)-IMAC hybrid monolith PhosTip to enrich phosphopeptides from urinary EV samples. EVs were first captured from urinary samples by EVTRAP beads. The successful isolation of EVs was initially assessed by Western blot using a known EV marker, CD9 (Figure S5). The EV protein digests from 1, 3, 5, and 10 mL of urine samples from healthy individuals were subjected to phosphopeptide enrichment by the prepared IMAC PhosTip and then analyzed by a timsTOF Pro mass spectrometer (Bruker). As shown in Figure 3b, 217 unique phosphopeptides representing 160 phosphoproteins from just 1 mL (6 μg of proteins) of urine sample (Table S1) were identified. With increased urine volume, the number of identified phosphopeptides was increased as well: 929, 2142, and 3919 unique phosphopeptides, representing 324, 595, and 782 unique phosphoproteins from EV protein digest of 3 mL (18 μg of proteins), 5 mL (30 μg of proteins), and 10 mL (60 μg of proteins) of urine, respectively (Tables S2–S4). As a comparison, we also used commercial TiO_2 beads to enrich phosphopeptides from urinary EVs samples. As shown in Figure S6, the results showed that 137, 800, 1732, and 2666 unique phosphopeptides were identified from 1, 3, 5, and 10 mL of urine, respectively. The overlap of identified unique phosphopeptides enriched from 1, 3, 5, and 10 mL of urinary EVs is shown in Figure S7. More than two-thirds of phosphopeptides identified from 1 mL of urinary EV samples were also identified in the 3 mL samples, while 75.5% of those phosphopeptides could be also seen in 10 mL urine samples. These results indicate that the new PhosTip possesses good reproducibility and provides a convenient and efficient method for microscale phosphopeptide enrichment of EVs from body fluids such as urine.

Finally, we demonstrate the utility of the PhosTip for biomarker discovery. Prostate cancer is one of the most common malignant tumors among men. Recent efforts suggest the utility of urinary EVs for disease diagnosis,^{39–41} indicating the possibility of developing phosphoproteins of EVs as a noninvasive method to evaluate and monitor cancer progression. Urine from healthy donors ($n = 10$) and prostate cancer patients ($n = 10$) was compared in this study. The samples of each group were pooled, and three technical replicates were performed for quantitative EVs phosphoproteomics. The results identified a total of 3819 and 4398 unique phosphopeptides, representing 1323 and 1415 phosphoproteins from the healthy and the prostate cancer groups, respectively (Tables S5

and S6). We performed a heat map and analysis of variance (ANOVA) statistical analysis to screen significantly regulated proteins in each group, as shown in Figure 4, parts a and b. The results highlight 118 proteins with differentially up-regulated phosphorylation in the group of prostate cancer patients compared with healthy group, while there were 77 proteins with down-regulated phosphorylation in the cancer groups. To better understand the biological roles of the up-regulated proteins in this prostate cancer data set, we used STRING (search tool for the retrieval of interacting genes/proteins) to identify enriched gene ontology (GO) categories and protein networks. As shown in Figure 4c, the up-regulated proteins in the prostate cancer group were involved in diverse biological functions but enriched in immune response, regulation, secretion, and signaling related to stimulus. We also found that a total of 58 up-regulated proteins with phosphosites were part of a common network as shown in Figure S8. Among them, multiple phosphoproteins, such as CD14,^{42,43} APOA4,⁴⁴ EGF,^{45,46} and DPP4^{47,48} (shown in Figure 4b), were previously reported to associate with prostate cancer biology. The results indicated that this new developed PhosTip approach is an effective tool for enrichment of phosphopeptides and could be incorporated into the analytical pipeline for EV-based biomarker discovery.

CONCLUSION

In summary, a novel Ti(IV)-functionalized hybrid monolithic PhosTip with embedded glass fiber as the support was conveniently synthesized via thermo-initiated free radical polymerization of POSS-MA and VPA. DTT was introduced to eliminate the major obstacle of oxygen inhibition. The employment of glass fiber allowed us to immobilize the hybrid monoliths at any designated position in the pipet tip, which also prevented the hybrid monoliths from collapsing during sample preparation. This design is relatively straightforward and time-saving with no extra steps needed to functionalize the tip. The resulting Ti(IV)-IMAC PhosTip exhibited excellent enrichment specificity and sensitivity for phosphopeptides from cell digests and, in particular, from urinary EVs. The results showed its great practicability in identifying low-abundant phosphopeptides from minute biological sample amounts. With overall good reproducibility, it is conceivable that PhosTips can be prepared in-house in batches for multiple applications in parallel. It was also quite flexible and convenient to adjust the amount of hybrid monoliths according to the amount of biological sample via different volumes of polymerization solutions introduced into the tips. With the easy preparation procedure and convenient operation protocol, it is conceivable that the glass fiber-supported hybrid monolithic tips functionalized with different groups can provide a universal platform for broad applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Financial support is gratefully acknowledged from the National Key Research and Development Program of China (no. 2017YFA0700404) to H.Z. and C.Y. and the China Postdoctoral Science Foundation (2019T120373 and 2019M651637) to H.Z.

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Figure 1.
Schematic approach for the preparation of the glass fiber-supported hybrid monolithic PhosTip.

Figure 2. Characterization of the hybrid monolithic PhosTip. (a and b) SEM micrographs, (c and d) EDX micrographs, (e) macropore diameters measured by MIP, and (f) nitrogen sorption isotherms (BET) of the prepared hybrid monolithic PhosTip.

Figure 3.
Phosphoproteome analyses of (a) cell lysates and (b) urinary EVs.

Figure 4.

Analysis of phosphoproteomes from urine EV samples. (a) Heat map of the significantly regulated proteins between the healthy and prostate cancer groups. (b) Volcano plot of coefficient of variance between healthy and prostate cancer groups (fold change, $\log_2 > 1$; p -value < 0.05 by ANOVA). Proteins with phosphorylation sites significantly up-regulated in prostate cancer groups are colored in red on the right side, and down-regulated proteins are colored in blue on the left side. (c) Biological functions based on gene ontology annotation of 118 up-regulated proteins. Individual proteins might be annotated to more than one category.