Analytica Chimica Acta xxx (2018) 1–8

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Plasma-assisted alignment in the fabrication of microchannel-array-based in-tube solid-phase microextraction microchips packed with TiO_2 nanoparticles for phosphopeptide analysis

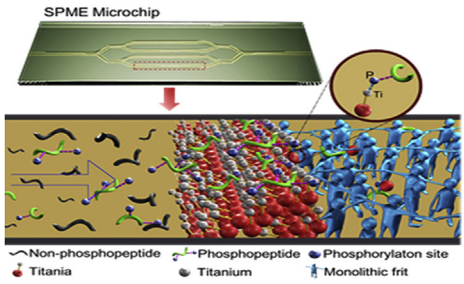
Siyu Wang [a], Xiayan Wang [a], Lin Wang [a], Qiaosheng Pu [b], Wenbin Du [c], Guangsheng Guo [a, *]

[a] Beijing Key Laboratory for Green Catalysis and Separation, Department of Chemistry and Chemistry Engineering, Beijing University of Technology, Beijing 100124, China
[b] Department of Chemistry, Lanzhou University, Lanzhou 730000, Gansu, China
[c] State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China

HIGHLIGHTS

- We first proposed a novel, simple and rapid approach for the fabrication of glass microchip using plasma assistance for precise alignment.
- Channel-array microchip was designed and prepared for in-tube solid phase microextraction.
- Nanomaterials were packed in channel array SPME microchip for selective and sensitive phosphopeptide analysis.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 4 August 2017
Received in revised form
17 January 2018
Accepted 5 February 2018
Available online xxx

Keywords:
Microchannel array microchip
In-tube solid phase microextraction
MALDI-TOF-MS
Phosphopeptides
Nanomaterials

ABSTRACT

Phosphorylated peptides are important for understanding the processes of biological regulation. However, the detection of phosphopeptides remains a challenge because of their low abundance and the suppression by non-phosphopeptides. Here, we report a strategy for the facile and rapid fabrication of TiO_2-nanoparticle-packed microchannel-array glass microchips (TMA-microchips) for in-tube solid-phase microextraction (IT-SPME) using a plasma-assisted method for the precise alignment of the microstructure. This proposed strategy was applied to the selective enrichment of phosphopeptides from a protein digestion mixture, demonstrating the high capacity and selectivity of the SPME microchips. An important feature of this array design is that it fully exploits the advantage of nanoparticles for improving extraction capacity and simultaneously provides an effective way to reduce the pressure for driving solutions; thus, it paves the way for future methods that simultaneously take advantage of nanomaterials and microchips.

* Corresponding author.
E-mail address: gsguo@bjut.edu.cn (G. Guo).

https://doi.org/10.1016/j.aca.2018.02.018
0003-2670/

1. Introduction

Solid-phase microextraction (SPME), invented by Arthur and Pawliszyn [1], is a green analytical chemistry strategy for rapid sample preparation. Compared to traditional sample preparation methods, SPME offers several advantages: it is a non-exhaustive extraction and integrates sampling, extraction, and introduction of the sample in a single step [2]. Thus, it is beneficial to the separation of only a small portion of the target analytes from complex sample matrices [3] such as those of biological, environmental and food samples [4–9], especially in the case of polar and thermally sensitive analytes [10]. With the appropriate design of devices such as fiber-SPME, in-tube SPME (IT-SPME), in-tip SPME, stir-bar SPME and membrane-SPME (M-SPME), this technique is conveniently used in applications such as in vivo detection, automatic sampling systems, and chromatography-coupled techniques [11–17]. Among these SPME methodologies, IT-SPME has unique advantages, including the ability to overcome weak mechanical properties, poor efficiency and selectivity and also providing easy coupling to the final determination systems. The properties of the extraction phase are well known to determine the SPME performance with respect to the selectivity and sensitivity of this method. Nanomaterials are ideal adsorbents because they have a high specific surface area and an adjustable pore size, which result in better extraction efficiency and capacity [18]. However, irrespective of the favorable impression made by this satisfactory performance, the higher back-pressure against the sorbent in packed columns or microchip channels caused by the smaller particles is a problem that cannot be ignored.

Protein phosphorylation plays an important role in modulating the pathways for cellular signal transduction [19–21]. However, the detection of phosphopeptides by mass spectrometry (MS) remains a challenge because of their low abundance and poor ionization efficiency [22] and because the signals are suppressed by non-phosphopeptides. Thus, an enrichment technology for phosphopeptides is particularly important for the improvement of their MS signals. According to the literature, titania (TiO_2) has a sufficiently strong affinity toward phosphopeptides to enable the effective enrichment of phosphopeptides from complex samples [23,24]. However, thus far, the preparation of pure titania monolithic columns in capillaries and microchannels has proven difficult.

Interest in microfluidic devices has grown because of their competitive advantages and wide range of applications, including DNA separation, metal-ion detection and bioanalysis [25–29]. Various methods have been developed to suit different purposes [30–32]. However, the interface between a microchip and the other analytical techniques with tiny dead volume is a challenge. Liu et al. [33] proposed a method which can integrate capillaries to microchannels with almost zero dead volume, which mirror symmetric semicircular structures have to be etched on a pair of wafer and precise alignment should be made before the bonding process to get perfect circular microchannels. To achieve it, the glass should be cleaned and activated with piranha solution which is time-consuming and may lead to serious personal safety risk. The special alignment holder is needed and the process is difficult.

In this article, we report the use of SPME with channel array glass microchips coupled to a matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI-TOF-MS) for detection, where the microchips are packed with TiO_2 nanoparticles for enrichment of phosphopeptides from digestion mixtures of α-casein, β-casein and BSA, also from digestion of egg white. The channel array microchip was fabricated by a simple and rapid approach using plasma-assisted precise alignment of the microstructure. Each SPME microchip is composed of a bottom plate and a cover plate. Both the bottom plate and the cover plate were designed with four parallel microchannels. The channel array structure decreases the requirement for a high driving pressure, which is a consequence of the packing of nanomaterials, and can also increase the extraction efficiency. In this work, four parallel microchannels were sufficient for the analysis. In cases of more complex biological samples, increasing the quantity of array channels under the concept of array-orientation is extremely easy.

2. Material and methods

2.1. Chemicals

Hydrofluoric acid (HF), acetic acid and sodium hydroxide (NaOH) were purchased from the Fuchen Chemical Reagent Factory (Tianjin, China). Methanol, acetone and isopropanol were purchased from Beijing Chemical Works (China). 3-(Trimethoxysilyl) propyl methacrylate (γ-MAPS), butyl methacrylate (BMA), ethylene glycol dimethacrylate (EDMA), 1-propanol, 1,4-butanediol, and 2,2-dimethoxy-2-phenylacetophenone (DMPA) were obtained from J&K Scientific. The mono-phosphopeptide (FQpSEEQQQTEDELQDK) and tetra-phosphopeptide (RELEELNVPGEIVEpSLpSpSpSEESITR) were purchased from AnaSpec (USA). Titanium dioxide (anatase) was obtained from Alfa Aesar. Ammonium fluoride (NH_4F), ammonium hydroxide ($NH_3 \cdot H_2O$), bovine α-casein, bovine β-casein, bovine serum albumin (BSA), 2,5-dihydroxybenzoic acid (2,5-DHB) and phosphoric acid (H_3PO_4) were purchased from Sigma-Aldrich. Sequencing-grade trypsin was purchased from Promega (USA). Trifluoroacetic acid (TFA) and acetonitrile (ACN) were obtained from Fisher Scientific. Purified water (18.2 MΩ cm) was used to prepare all solutions.

2.2. Equipment

The glass substrate layered with chromium and a photoresist was exposed for 5 s with a BG-401A mask aligner (China Electronics Technology Group Corporation No. 45 Research Institute). The dimensions of the channels were measured with a profilometer (Dektak XT, Bruker, USA). The substrate surface was treated with plasma using a PDC-002 plasma cleaner (HARRICK PLASMA). Treatment effect tested with SL200KB optical contact angle & interface tension meter (KINO Industry CO. Ltd., USA). A metallographic microscope used for precise alignment of the microstructure (Nikon LV100ND, Japan). The cross sections of the microchips, the inner surfaces of the microchannels and the microscopic morphology of the monolithic frit were characterized using a Nova NanoSEM 430 (FEI, USA). A dicing machine (ADT Ltd., Israel, MODEL 7122.) was used for cutting the microchip into pieces for SEM. The morphology of the TiO_2 nanoparticles was characterized using a JEM2100 transmission electron microscope (JEOL Ltd., Japan). A MALDI-TOF-MS (Shimadzu Biotech Axima Performance, Kyoto, Japan) equipped with a 337 nm nitrogen laser was used for detection in the positive ion reflector mode.

2.3. Fabrication of TMA-microchip

2.3.1. Fabrication of channel array microchip

Two cycles of photolithographic and chemical wet etching were used, respectively, for the preparation of the SPME array channels and capillary connecting channels on a 2.3 mm thick $40 \times 60\ mm^2$ glass substrate layered with chromium and photoresist. We designed two different photomasks for fabricating round channels of two different diameters. The pattern line width of these two photomasks was both 20 μm. The design for capillary channels on the photomask was transferred onto the photoresist and chrome mask following UV exposure. The microchannels were wet etched

Please cite this article in press as: S. Wang, et al., Plasma-assisted alignment in the fabrication of microchannel-array-based in-tube solid-phase microextraction microchips packed with TiO_2 nanoparticles for phosphopeptide analysis, Analytica Chimica Acta (2018), https://doi.org/10.1016/j.aca.2018.02.018

using a HF/NH_4F solution in a 40 °C well-stirred bath for approximately 3 h. Then, the microchannel array of solid phase microextraction was etched on the same glass substrates with the second photomask. The final dimensions of the array microextraction channels were 50 μm deep and 100 μm wide, with a length of 1 cm. The capillary channels were 200 μm deep and 200 μm wide, with a length of 1.2 cm. Two pieces of etched substrates were rinsed with acetone and isopropanol to remove the photoresist, and the Cr film was eliminated with chrome etching solution. The substrates were then thoroughly brushed with detergent to remove any attached particles and washed with ultrapure water.

The pre-bonding details are as follows: the dry and clean substrate surface was treated with plasma for 10 min under an air atmosphere using a PDC-002 plasma cleaner for surface cleaning and activation. The substrates were put together face-to-face quickly and pre-aligned with naked eyes, and aligned further under a microscope making sure that the edge of every one of the four parallel array channels on each substrate as well as the merging point aligned to form a complete array structure. If mal-alignment happens, we just need to move the cover plate to the exact position under the guiding by the microscope easily and freely, the movement of substrates is flexible under drying condition. When all structures are perfectly aligned, 1 μL ultrapure water was dropped at the edge of substrates to pre-bond the substrates. The substrates were firmly held during the process to keep the alignment. Gentle pressure was applied to any poorly attached area (with interference fringe), the excessive water was removed under vacuum to avoid the problem of microchip burst during subsequent thermal bonding. Lastly, the array microchip was heated in a muffle furnace up to 550 °C for permanent bonding, forming a sealed microchip.

2.3.2. Pretreatment of microchip

Before pretreatment of microchip, capillaries have to be used for external connection by apply epoxy glue to the peripheral surface of the insertion segment [34], then rotatingly inserted. In particular, the outer diameter of the inserted capillary need to match with the outer diameter of the capillary connecting channels of the microchip well, also the cross-section of the capillary insertion end need flattened without significant bevels for less dead volume.

The channels were sequentially washed with 1 mol/L NaOH (2 h), water (30 min), acetone (30 min) and then dried under nitrogen to activate the silanol groups. The channels were subsequently vinylized by being filled with acetone/3-(trimethoxysilyl) propyl methacrylate (v:v = 1:1), which was allowed to react overnight. The channels were then flushed with acetone and dried.

2.3.3. Preparation of monolithic frits

The reaction mixture consisting of 24.0% BMA, 16.0% EDMA, 47.68% 1-propanol, 5.96% 1,4-butanediol, 5.96% H_2O and 0.4% DMPA was sonicated for 10 min to obtain a homogeneous solution and subsequently purged with nitrogen for 5 min. The prepolymerization solution was introduced into the vinylized microchip. The microchip was then sealed at both ends with rubber stoppers. The polymerization location was accurately controlled by wrapping with opaque adhesive tape and cutting windows (<0.5 mm) with a razor blade. The entire microchip system was placed in the UV reaction tank (CBIO-UV8, 254 nm, 18 W) for 25 min. The reaction was terminated by washing with methanol.

2.3.4. TiO_2 slurry packed details

The slurry of TiO_2 nanoparticles was injected into the PEEK pipe. Each side of the PEEK was then connected to the capillary of the microchip, and the capillary used for preventing back suction with a union. Lastly, the end of the passage was connected to a chromatographic pump for filling to a pressure of 3000 psi.

2.4. Enzymolysis of egg white

The eggs were purchased from a local supermarket, then the separated egg white (50 μL) was processed as follow [16]: 50 mmol/L 250 μL NH_4CO_3 solution (with 8 mol/L urea) was introduced and incubated at 37 °C for 30 min; 25 μL dithiothreitol was added and the solution was incubated for 1 h at 55 °C; further, added 50 μL 200 mmol/L iodoacetamide after cool to room temperature, and the solution was maintaining avoiding of light. In the end, the mixture was mixed with 5 μL 2 mg/mL trypsin solution and digested at 37 °C for 24 h. The tryptic digestion was stored at −20 °C for future use, when use the sample was diluted 20 times by sampling solution.

2.5. Enrichment of mono- and tetra-phosphopeptides using TMA-microchip-SPME

Standard samples of mono-and tetra-phosphopeptide were made up into stock solutions of 500 μmol/L using 0.1% formic acid (pH 3); these stock solutions were subsequently diluted to different concentrations (2 μmol/L, 5 μmol/L, 10 μmol/L, 20 μmol/L, 40 μmol/L, and 60 μmol/L). Before extraction, 0.1% formic acid was pumped through the SPME channels in the microchip for conditioning. A solution vial was then filled with a 10 μL sample and driven through the channels at 100 psi. Under the same conditions, 20 μL of formic acid was flowed through the channels to wash them. A 1% NH_4OH (10 μL) solution was then used to desorb the extracted phosphopeptides. The effluent solution was collected from the outlet of the channels after extraction and desorption for further UV detection. The channel eluents were introduced into a capillary UV detector and monitored at a wavelength of 214 nm.

2.6. Enrichment process of tryptic digests of the mixture of α-casein, β-casein and BSA using TMA-microchip-SPME

Digestion samples containing α-casein, β-casein and BSA in molar ratios of 1:1:1, 1:1:10, and 1:1:100 were dissolved in 1% TFA/ACN 50/50 (v/v). The concentration of α-casein was 3.8×10^{-7} mol/L, that of β-casein was 4.0×10^{-7} mol/L, and those of BSA were 5.2×10^{-7} mol/L, 5.2×10^{-6} mol/L and 5.2×10^{-5} mol/L, respectively. The mixed solutions were placed into a nitrogen-pressurized chamber. The loading solution was driven through the microchip under an applied pressure of 100 psi. An acidic loading buffer was flowed through the microextraction channels to eliminate the residual sample solution and to remove the unretained matrix. The 1% NH_4OH (10 μL) solution then desorbed the extracted phosphopeptides. The driving pressure was set to 100 psi at all times. The effluent solution was collected from the outlet. The collection solutions were mixed in a ratio of 1:1 with the MALDI matrix, which was composited of DHB (20 mg/L 2,2-DHB in 50% ACN) with 1% (v/v) phosphoric acid, for MS detection.

2.7. Enrichment of β-casein for evaluating the detection of limit

Ten microliters of tryptic digest of β-casein (4.0×10^{-9} mol/L) was driven through the SPME microchip and the same conditions for evaluating the detection of limit. The 1% NH_4OH (5 μL) solution then desorbed the extracted phosphopeptides. The driving pressure was set to 100 psi at all times.

3. Results and discussion

3.1. Channel arrays microchip design and characterization

Two cycles of photolithographic and chemical wet etching were used for the preparation of the SPME array channels and capillary

Please cite this article in press as: S. Wang, et al., Plasma-assisted alignment in the fabrication of microchannel-array-based in-tube solid-phase microextraction microchips packed with TiO_2 nanoparticles for phosphopeptide analysis, Analytica Chimica Acta (2018), https://doi.org/10.1016/j.aca.2018.02.018

connecting channels, respectively, on a 2.3 mm-thick 40 × 60 mm^2 glass substrate layered with chromium and photoresist. The design on the cover plate is a mirror image of that on the bottom plate, as shown in Fig. S1. One of the most important factors for successfully preparing microchips is cleanliness [35]; thus, all processes were carried out in a clean-room. The photolithographic and chemical wet etching procedures have been described elsewhere [36]. By accurately controlling conditions such as exposure time, maintenance of cleanliness and etching time, we fabricated the channels with the required dimensions and a smooth internal surface that satisfies the requirement for nanoparticle packing (Fig. 1 and Fig. S2). After the wet etching process, the dimensions of the channels were measured using a profilometer. The microextraction channels had a depth of 50–55 μm and a width of 100–120 μm. The capillary connecting channels had a depth of 190–200 μm and a width of 200–220 μm. The lengths of the microextraction channels and capillary connection channels were measured using a digital calliper as ~1.0 cm and ~0.5 cm, respectively. The top-view scanning electron microscopy (SEM) images show that the unbonded microchannels are uniform and smooth (Fig. 1A–D).

The bonding process is one of the critical processes for glass microchip fabrication and still a challenge. Here, we have proposed a new protocol using plasma assistance for precisely aligning the microstructures on the bottom plate with those on the cover plate for pre-bonding. Plasma is often used in the preparation and etching of organic polymer microchips with a good activation effect, such as PMMA and PDMS [37,38], but "piranha solution" (H_2SO_4:H_2O_2 = 3:1) is frequently used for glass substrates. The protocol proposed here avoids the use of the dangerous "piranha solution" and achieves an easy and rapid precise alignment of the microchannels on the different glass plates, which is an attractive feature, especially for the preparation of microchips with a complex microstructure on both the bottom plate and cover plate. In traditional methods, the use of piranha solution for cleaning and activating the glass surface both creates a serious personal safety risk and is time-consuming. However, the proposed plasma-assisted method is an extremely green and safe method that not only rapidly completes the cleaning and alignment processes but also produces excellent surface activation, resulting in an obvious increase in the bonding strength, because the surface treated with plasma exhibit admirable hydrophilic properties as shown in Fig. S3, the contact angle of untreated glass surface is 75.42° and after treated with piranha solution for 30 min is 21.58°, but the contact angle on the glass surface treated with plasma for 10 min is less than 5°.

In addition, the more striking advantage is that the plasma pretreatment method provides an extremely convenient method for precise alignment. In our microchips, capillaries have to be used for external connection, etching on one plate for capillary connection channels may lead to extremely large dead volume at the junction area of the connection channel and the packing channel. It may also cause inappropriate packing of particles at this area. Etching on bottom and cover plate ensure the coaxial connection for extraction and capillary channels. The appearance of them are both circular after permanent bonding which avoid the irregular flow patterns that may occur for semicircular or trapezoidal packing channel of microchip when only etching at one plate. Although plasma may not be efficient for removing particles, the clean process of glass surface with brush can efficiently remove large particles and ensure good surface activation by plasma. The most significant aspect of plasma assisted alignment is its simplicity. It can be conducted with almost 100% success under a microscope without need of any special tools. Avoid of use of dangerous piranha solution is of course another advantages. Fig. 2 shows the optical microscopy images of the microchannels before and after accurate

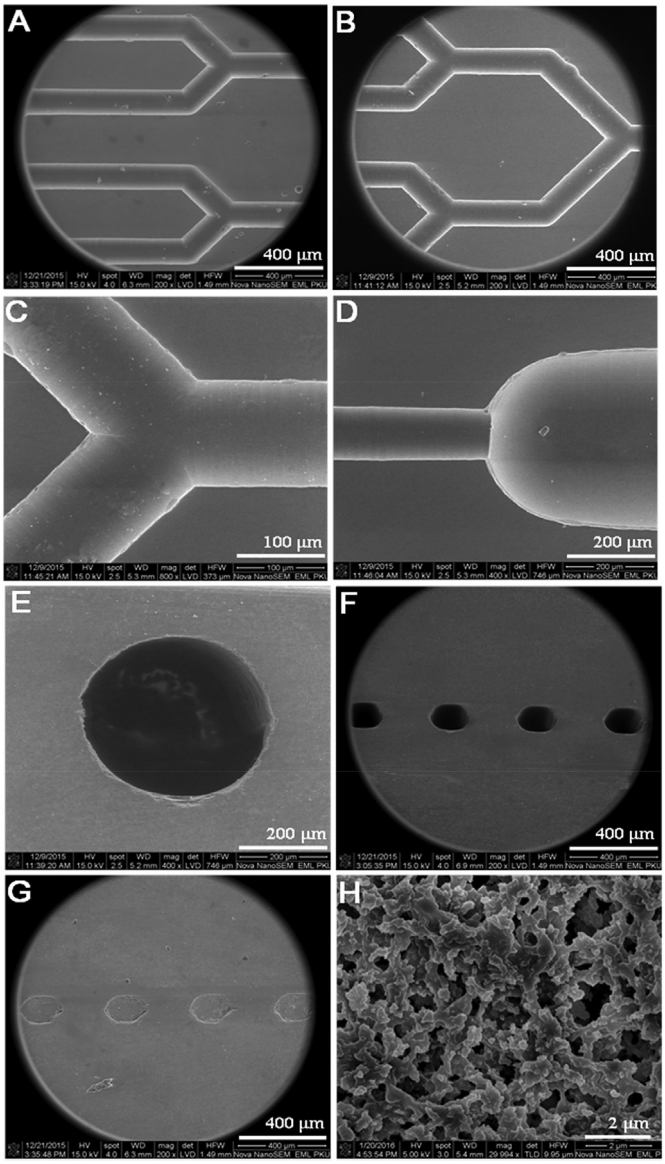


Fig. 1. SEM images of the microchannels. (A)–(C) Top view of the SPME microchannels on the bottom plate before bonding. (D) Top view of the junction region of the SPME microchannel and the capillary connection channel on the bottom plate before bonding. Cross-sectional views of (E) the sealed capillary connection channel and (F) the sealed SPME microchannels. (G) Cross-section view of the sealed SPME microchannels immobilized with monolithic frits. (H) Magnified image of the monolithic frit from (G).

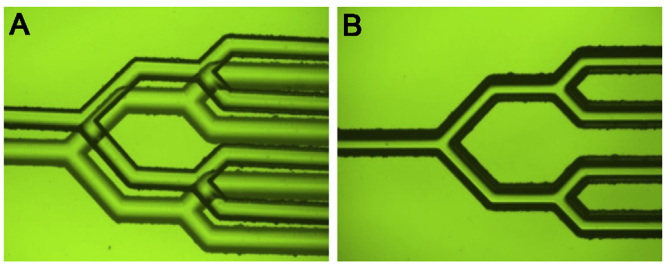


Fig. 2. Optical microscopy images of the microchannels before (A) and after (B) precise alignment.

Please cite this article in press as: S. Wang, et al., Plasma-assisted alignment in the fabrication of microchannel-array-based in-tube solid-phase microextraction microchips packed with TiO_2 nanoparticles for phosphopeptide analysis, Analytica Chimica Acta (2018), https://doi.org/10.1016/j.aca.2018.02.018

alignment. After the plasma treatment, the pre-bonded plates remained flexible and moving throughout the alignment process, which provided a convenient preparation technology for microchips having complex microstructures on both the bottom and cover plates. This protocol overcomes the limitation of the alignment method performed in water [28,39], where accurate alignment of the microstructures on the plates is difficult because the bottom plates are attached too tightly to move under the action of surface tension of water after contacting the cover plate. The pre-bonding details are shown in the Experimental section.

After the pre-bonding process was completed, the microchip was heated in a muffle furnace for permanent bonding to form sealed channels. SEM cross-sectional images of the sealed microchannels are shown in Fig. 1E–G. These images show that the bottom plate and the cover plate are well bonded to each other, merging into an integral structure without any trace of bonding. The microchannels are approximately round, which makes them well matched with the external capillaries and clearly demonstrates that the proposed plasma-assisted precise alignment method can be used to fabricate very high quality microchannels. Impressively, the as-prepared microchip exhibited bonding strength sufficient to withstand extremely high pressures. In later work, we connected the microchip to a chromatography pump for the subsequent packing process. When the pump output pressure reached 3000 psi for 1 h, the microchip remained intact and no leakage or fracture was observed. The microchip withstood a pressure greater than 3000 psi, making it compatible with most experimental chromatographic conditions. Photographs of the channel array microchip are shown in Fig. S4. The microchip has three sets of parallel channels array for analysis. Each set has four parallel microchannels sharing the same inlet and outlet. Fused silica capillaries (100 μm I.D. × 365 μm O.D.), which were used for external connections, were inserted into the ends of the connection channels and sealed in the channels using epoxy adhesive with maximal pressure far more than 3000 psi. The internal diameter of the capillary and that of the extraction channel are similar, which is beneficial for further processes, reducing the risk of nanoparticles accumulating in improper positions as a result of a mismatch between the capillary and the SPME channels.

3.2. Monolithic frit characterization

Next, the microchip was pretreated for the subsequent experiments. The channels were flushed with a sequence of 1 mol/L NaOH, water, and acetone and were dried with nitrogen to activate the silanol groups on their surface. The channels were then vinylized by being filled with a solution of acetone/3-(trimethoxysilyl) propyl methacrylate (v:v = 1:1), which was allowed to react overnight. The channels were then flushed with acetone and dried. Before nanoparticle packing, monolithic frits with a length of approximately 1 mm were synthesized via an in situ UV-initiated polymerization method and were immobilized at the end of the SPME channels (see the Experimental section). A cross-sectional SEM image of the monolithic frits is shown in Fig. 1G. The frits fit tightly within the inner surfaces of the microchannels without any gaps. Fig. 1H displays a magnified image of the monolithic frit, which shows a three-dimensional porous network structure favoring the flow of solutions.

3.3. TiO_2 nanoparticle packing

TiO_2 nanoparticles were packed into the SPME microchannel using a slurry packing method (see the Experimental section). A transmission electron microscopy (TEM) image of the TiO_2 nanoparticles and their Brunauer-Emmett-Teller (BET) measurement results are shown in Fig. S5 and Fig. S6. The TiO_2 nanoparticles exhibit an average diameter of 15 nm. Experiments demonstrated that the monolithic frits with an average pore size of ~1 μm retained the TiO_2 nanoparticles.

3.4. The principle of monolithic frit

A schematic demonstrating the principle by which the monolithic frit holds the nanoparticles is shown in Fig. 3. The monolithic frit has a three-dimensional continuous skeleton with interconnecting micropores, and the cross-linked polymer network acts as the keystone holding the TiO_2 nanoparticles. When the slurry flows through the monolithic frit, some of the nanoparticles are retained in front of the frits and others pass through the micropores into the inside of the frits. As the packing process proceeded, the first immobilized nanoparticles inside ravines and on the surface of the frit acted as keystones for blocking subsequent nanoparticles and allowing the packed segment to increase in length [40,41]. The "keystone effect" ensures the success of the packing process. Finally, after packing, we obtained the TiO_2 nanoparticle packed channel array glass SPME microchip with a ~2.0 cm effective length of extraction phase.

3.5. Enrichment of phosphopeptides by TMA-microchip-SPME

TiO_2 has a strong affinity for phosphopeptides resulting from Lewis acid–base action, resulting in the formation of bridging bidentate bonds with phosphate anions; TiO_2 binding is one of the most prevalent approaches for the enrichment of phosphopeptides. The enrichment performance of SPME for phosphopeptides using the TiO_2 nanoparticle packed channel array microchip was initially investigated using a mono-phosphopeptide (FQ-pS-EEQQQTEDELQDK) and a tetra-phosphopeptide (RELEELNVPGEIVEpSLpSpSpSEESITR); details are provided in the Experimental section. Phosphopeptides were retained using 0.1% formic acid (pH 3) and were eluted with 1% aqueous NH_4OH (pH 10). The procedure for phosphopeptide analysis using the SPME-MALDI-MS method is schematically shown in Fig. 4. Briefly, the sample was placed into a solution vial, then the loading solution was driven through the SPME microchannels by applied a pressure of 100 psi sequentially. We collected the effluent solution from the outlet after extraction and desorption for further analysis. Experiments confirmed that, even after nanoparticles were added as a packing material, the

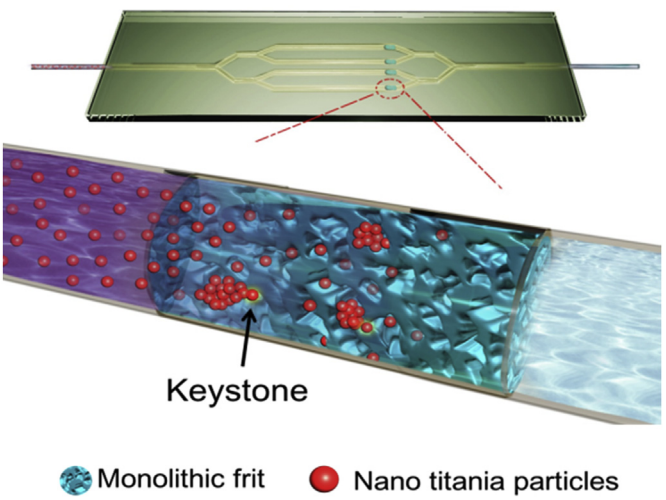


Fig. 3. Schematic of the principle by which the monolithic frit holds the nanoparticles.

Please cite this article in press as: S. Wang, et al., Plasma-assisted alignment in the fabrication of microchannel-array-based in-tube solid-phase microextraction microchips packed with TiO_2 nanoparticles for phosphopeptide analysis, Analytica Chimica Acta (2018), https://doi.org/10.1016/j.aca.2018.02.018

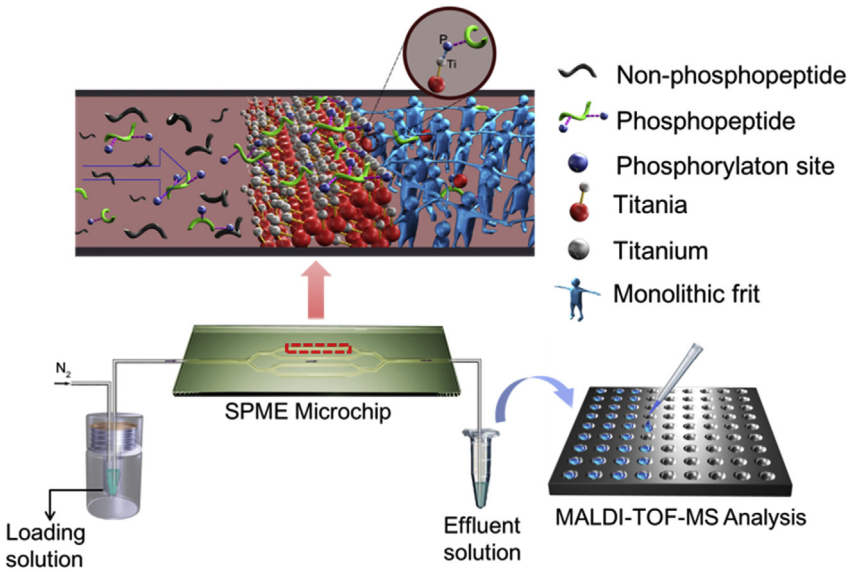


Fig. 4. Schematic of the procedure for phosphopeptide analysis using TMA-microchip-SPME.

channel array design ensured the smooth progress of the experiment at pressures as low as 100 psi. Fig. S7 and Fig. S8 show chromatograms and a series of concentration and calibration curves collected with UV detection of the mono- and tetra-phosphopeptides before and after enrichment. These results reveal that both the mono- and multi-phosphopeptides could be extracted with TiO_2 nanoparticles. These results demonstrate that TiO_2 nanoparticles with large specific surface areas exhibit a high enrichment capacity.

As for the characterization of the monolithic frit with packed TiO_2 nanoparticles before and after enrichment, capillaries which prepared with monolithic frit and packed with TiO_2 nanoparticles were used to simulate microchips for obtaining SEM images. The Fig. S11 implies that TiO_2 nanoparticles packed monolithic frit had a stable structure before and after enrichment.

In practical applications, the enrichment of low abundance phosphopeptides in complicated biological systems becomes an inevitable obstacle. To further evaluate the performance of TMA-microchip-SPME under complex circumstances, the TMA-microchip-SPME was used to enrich phosphopeptides from digestion mixtures of α-casein, β-casein and BSA with different molar ratios of 1:1:1, 1:1:10 and 1:1:100. The concentrations in the tryptic digest of α-casein and β-casein were 3.8×10^{-7} mol/L and 4.0×10^{-7} mol/L, respectively. The processes of tryptic digestion are described elsewhere [16], and the extraction process is described in the Experimental section. Four parallel TiO_2 nanoparticle packed channels in the microchip had a total effective length of ~2.0 cm. The results of the direct analysis of the digested proteins without extraction are shown in Fig. 5(A, C). Only three phosphopeptides could be detected in the mixture with an α-casein, β-casein and BSA molar ratio of 1:1:1. When the complexity of the biological environment was increased, with an α-casein, β-casein and BSA molar ratio of 1:1:10, only two phosphopeptides could be identified, indicating that identification of phosphopeptides in more complex systems was impossible without extraction.

After enrichment with TMA-microchip-SPME, the signals of phosphopeptides dramatically increased, as shown in Fig. 5(B, D). More than fourteen phosphopeptides were detected; the details for the identified phosphopeptides are listed in the Supporting Information, Tables S–1. The signals at $m/z = 1944.6$, 2721.5, 3008.1, 2352.1, 2966.4, 3123.0 correspond to the multi-phosphopeptides; the others are unambiguously identified as mono-phosphopeptides. These results indicate that the TiO_2 nanoparticles used in the experiments were specifically selective toward both mono- and multi-phosphopeptides. Because of their stronger Lewis acid-base action, the multi-phosphopeptides have a more stable binding affinity. However, almost all of the multi-phosphopeptides could be detected by one step elution, despite the fact that the S/N ratio was lower than that of the mono-phosphorylated peptide as a consequence of poorer ionization efficiency. In addition, as reported in the literature [42], extraction with an acidic mobile phase containing substituted aromatic carboxylic acids can avoid nonspecific binding of acidic non-phosphopeptides. We demonstrated this effect by using a trypsin digestion mixture with a molar ratio of 1:1:100 and including a certain amount of DHB (Fig. S9). The signals, which exhibited a high signal-to-noise ratio after extraction, were clearly identified. We also extracted a small amount of β-casein to evaluate the detection limit of this enrichment method (see the Experimental section). As shown in Fig. S10, both the mono- and multi-phosphopeptides were clearly recognized, with a high signal to noise ratio, at 40 fmol. These results indicate that SPME using a TiO_2 nanoparticle packed channel array microchip demonstrated excellent selectivity toward phosphopeptides, with high detection sensitivity.

The control experiment of using the microchips without packing the TiO_2 nanoparticles for enrichment is presented in Fig. S12. It shows clearly that phosphopeptides couldn't be identified after enrichment with the microchips without packing TiO_2 nanoparticles. The length of monolithic frits is about 1 mm, compared with TiO_2 nanoparticles, the monolithic frits have little influence on the extraction, and the TiO_2 nanoparticles have high specific surface area and obvious Lewis acid-base effect with the phosphopeptide, which is far greater than that of monolithic frits adsorption.

3.6. Application in actual egg samples analysis using TMA-microchip-SPME

Further, actual egg samples were applied for demonstration. Before enrichment, the sample solution of egg white was diluted 10 times with ultrapure water and the enrichment procedures for egg white were similar with the digestion samples of α-casein, β-casein and BSA. Briefly, 20 μL 1% TFA/ACN 50/50 (v/v) loading buffer was

Please cite this article in press as: S. Wang, et al., Plasma-assisted alignment in the fabrication of microchannel-array-based in-tube solid-phase microextraction microchips packed with TiO_2 nanoparticles for phosphopeptide analysis, Analytica Chimica Acta (2018), https://doi.org/10.1016/j.aca.2018.02.018

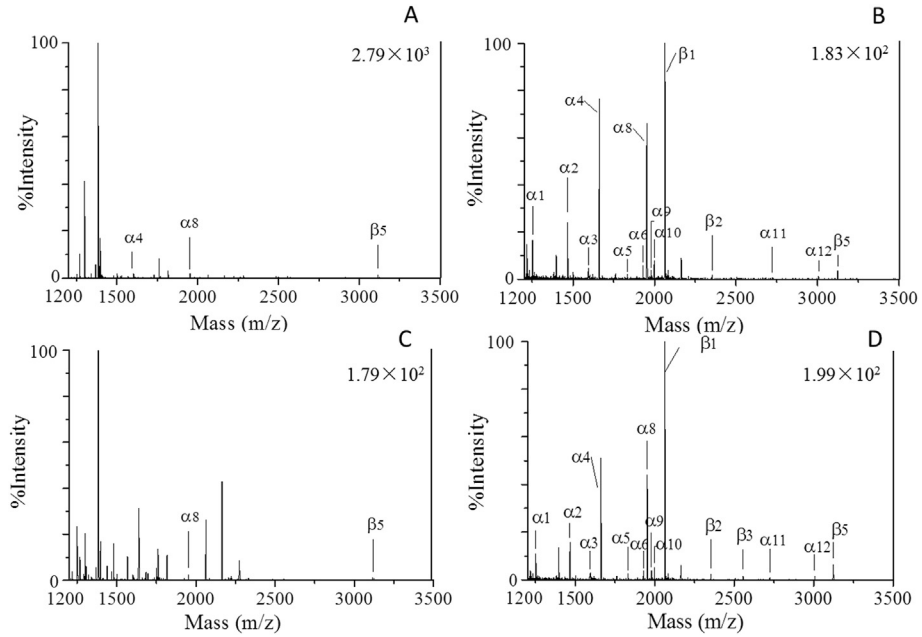


Fig. 5. MALDI mass spectra obtained from a tryptic digest of α-casein, β-casein and BSA without (A, C) and with (B, D) SPME extraction using TMA-microchip. The molar ratios of α-casein, β-casein and BSA are 1:1:1 (A, B) and 1:1:10 (C, D). The concentrations in the tryptic digest of α-casein and β-casein were 3.8×10^{-7} mol/L and 4.0×10^{-7} mol/L, respectively. Phosphopeptides are labeled as α and β.

pumped through the SPME channels in the microchip for conditioning. Then the mixture containing 10 μL diluted egg white sample and 10 μL loading solution was placed into a nitrogen-pressurized chamber and driven through the microchip. After washing with the same acidic loading buffer (20 μL), the extracted phosphopeptides in egg white was desorbed by 10 μL 1% NH_4OH. The driving pressure was set to 200 psi at all times. The effluent solution was collected from the outlet for MALDI-TOF-MS analysis.

The Fig. 6 illustrates the significant changes before and after TMA-microchip-SPME from the tryptic digest of egg white. There is no detectable phosphopeptide from the egg white solution without extraction. Gratifyingly, there are three distinct peaks for phosphopeptides, 2090.98(EVVGpSAEAGVDAASVSEEFR), 2904.41 (FDKLPGFGDpSIEAQCGTSVNVHSSLR), 3846.47 (ISQAVHAAHAEINEAGREVVGpSAEAGVDAASVSEEFR), which derived from ovalbumin in egg white. The extraction effect of the TMA-microchip is better than that of pure TiO_2 packed monolith column. These results indicate again that TMA-microchip-SPME demonstrated excellent selectivity toward phosphopeptides, with high detection sensitivity.

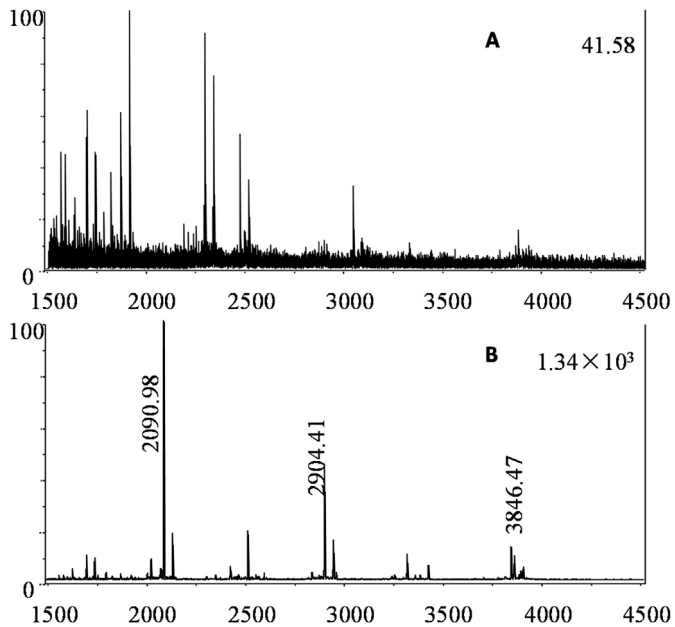


Fig. 6. MALDI mass spectra obtained from a tryptic digest of egg white without (A) and with (B) SPME extraction using a TMA-microchip.

4. Conclusions

In summary, we have developed an SPME method involving a TiO_2 nanoparticle packed channel array glass microchip coupled with MALDI-TOF-MS for the analysis of phosphopeptides. Four parallel TiO_2 nanoparticle-packed array channels with a total effective length of ~2.0 cm in the microchip achieved the extraction of phosphopeptides from a complex protein system. SPME using a TiO_2 nanoparticle packed microchip exhibits ideal properties for the enrichment of phosphopeptides, including clear selectivity and high capacity. Meanwhile, the TMA-microchip-SPME strategy has a high sensitivity and is amenable to automation and miniaturization. The array-oriented design contributes to the full use of the advantages of the enormous surface area and variety of nanomaterials and the miniaturization of microchips. This approach eliminates the limitations of high pressures, and this technique is expected to be extended to more complex biological or environmental analyses and to more integrated total microanalysis systems. Because, faced with intricate samples, in general, a series of processing steps is indispensable for accurate analysis the target analyte, the array design narrows down the space and increases the effective area also can integrate a variety of pretreatment and analysis channels in a single microchip. In addition, we proposed a plasma-assisted glass microchip fabrication method that provides an easy technique for the precise alignment of complex microstructures meeting the demands of production for glass microchip

Please cite this article in press as: S. Wang, et al., Plasma-assisted alignment in the fabrication of microchannel-array-based in-tube solid-phase microextraction microchips packed with TiO_2 nanoparticles for phosphopeptide analysis, Analytica Chimica Acta (2018), https://doi.org/10.1016/j.aca.2018.02.018

with more and more complicated design. Thus, the methods demonstrated in this article are expected to advance the integration of total analysis systems using microchips in combination with high functional nanomaterials.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Nos. 21375005, 21527808), the National Science Fund for Distinguished Young Scholars (No. 21625501). Special thanks are extended to the Mass Spectrometry Lab in the Analysis Center, Tsinghua University, Beijing, China, for its kind support of detection by mass spectrometry.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.02.018.

References

[1] C.L. Arthur, J. Pawliszyn, Solid phase microextraction with thermal desorption using fused silica optical fibers, Anal. Chem. 62 (1990) 2145–2148.
[2] M. Fernández-Amado, M.C. Prieto-Blanco, P. López-Mahía, S. Muniategui-Lorenzo, D. Prada-Rodríguez, Strengths and weaknesses of in-tube solid-phase microextraction: a scoping review, Anal. Chim. Acta 906 (2016) 41–57.
[3] G.F. Ouyang, D. Vuckovic, J. Pawliszyn, Nondestructive sampling of living systems using *in vivo* solid-phase microextraction, Chem. Rev. 111 (2011) 2784–2814.
[4] É.A. Souza-Silva, R.F. Jiang, A. Rodriguez-Lafuente, E. Gionfriddo, J. Pawliszyn, A critical review of the state of the art of solid-phase microextraction of complex matrices I. environmental analysis, TrAC Trends Anal. Chem. 71 (2015) 224–235.
[5] É.A. Souza-Silva, E. Gionfriddo, J. Pawliszyn, A critical review of the state of the art of solid-phase microextraction of complex matrices II. food analysis, TrAC Trends Anal. Chem. 71 (2015) 236–248.
[6] É.A. Souza-Silva, N. Reyes-Garcés, G.A. Gómez-Ríos, E. Boyacı, B. Bojko, J. Pawliszyn, A critical review of the state of the art of solid-phase microextraction of complex matrices III. bioanalytical and clinical applications, TrAC Trends Anal. Chem. 71 (2015) 249–264.
[7] S. Ansari, M. Karimi, Novel developments and trends of analytical methods for drug analysis in biological and environmental samples by molecularly imprinted polymers, TrAC Trends Anal. Chem. 89 (2017) 146–162.
[8] M. Sajid, Porous membrane protected micro-solid-phase extraction: a review of features, advancements and applications, Anal. Chim. Acta 965 (2017) 36–53.
[9] A. Speltinie, A. Scalabrini, F. Maraschi, M. Sturini, A. Profumo, Newest applications of molecularly imprinted polymers for extraction of contaminants from environmental and food matrices: a review, Anal. Chim. Acta 974 (2017) 1–26.
[10] H. Kataoka, Automated sample preparation using in-tube solid-phase microextraction and its application – a review, Anal. Bioanal. Chem. 373 (2002) 31–45.
[11] J. Qiu, G. Chen, S. Liu, T. Zhang, J. Wu, F. Wang, J. Xu, Y. Liu, F. Zhu, G. Ouyang, Bioinspired polyelectrolyte-assembled graphene-oxide-coated C18 composite solid-phase microextraction fibers for in vivo monitoring of acidic pharmaceuticals in fish, Anal. Chem. 88 (2016) 5841–5848.
[12] S. Wei, W. Lin, J. Xu, Y. Wang, S. Liu, F. Zhu, Y. Liu, G. Ouyang, Fabrication of a polymeric composite incorporating metal-organic framework nanosheets for solid-phase microextraction of polycyclic aromatic hydrocarbons from water samples, Anal. Chim. Acta 971 (2017) 48–54.
[13] I.D. Souza, L.P. Melo, I.C. Jardim, J.C. Monteiro, A.M. Nakano, M.E. Queiroz, Selective molecularly imprinted polymer combined with restricted access material for in-tube SPME/UHPLC-MS/MS of parabens in breast milk samples, Anal. Chim. Acta 932 (2016) 49–59.
[14] D. Vuckovic, I. de Lannoy, B. Gien, R.E. Shirey, L.M. Sidisky, S. Dutta, J. Pawliszyn, In vivo solid-Phase microextraction: capturing the elusive portion of metabolome, Angew. Chem. Int. Ed. 50 (2011) 5344–5348.
[15] M. Sajid, C. Basheer, M. Daud, A. Alsharaa, Evaluation of layered double hydroxide/graphene hybrid as a sorbent in membrane-protected stir-bar supported micro-solid-phase extraction for determination of organochlorine pesticides in urine samples, J. Chromatogr. A 1489 (2017) 1–8.
[16] S.T. Wang, M.Y. Wang, X. Su, B.F. Yuan, Y.Q. Feng, Facile preparation of SiO_2/TiO_2 composite monolithic capillary column and its application in enrichment of phosphopeptides, Anal. Chem. 84 (2012) 7763–7770.
[17] J.J. Grandy, E. Boyacı, J. Pawliszyn, Development of a carbon mesh supported thin film microextraction membrane as a means to lower the detection limits of benchtop and portable GC/MS instrumentation, Anal. Chem. 88 (2016) 1760–1767.
[18] Z.D. Lu, M.M. Ye, N. Li, W.W. Zhong, Y.D. Yin, Self-assembled TiO_2 nanocrystal clusters for selective enrichment of intact phosphorylated proteins, Angew. Chem. Int. Ed. 49 (2010) 1862–1866.
[19] H.Y. Zhong, X. Xiao, S. Zheng, W.Y. Zhang, M.J. Ding, H.Y. Jiang, L.L. Huang, J. Kang, Mass spectrometric analysis of mono- and multi-phosphopeptides by selective binding with NiZnFe2O4 magnetic nanoparticles, Nat. Commun. 4 (2013) 1656.
[20] L.P. Li, T. Zheng, L.N. Xu, Z. Li, L.D. Sun, Z.X. Nie, Y. Bai, H.W. Liu, SnO_2–$Zn(OH)_6$: a novel binary affinity probe for global phosphopeptide detection, Chem. Commun. 49 (2013) 1762–1764.
[21] C. Silva-Sanchez, H.Y. Li, S.X. Chen, Recent advances and challenges in plant phosphoproteomics, Proteomics 15 (2015) 1127–1141.
[22] E. Kanshin, S. Michnick, P. Thibault, Sample preparation and analytical strategies for large-scale phosphoproteomics experiments, Semin. Cell Dev. Biol. 23 (2012) 843–853.
[23] M. Mazanek, G. Mituloviae, F. Herzog, C. Stingl, J.R.A. Hutchins, J.M. Peters, K. Mechtler, Titanium dioxide as a chemo-affinity solid phase in offline phosphopeptide chromatography prior to HPLC-MS/MS analysis, Nat. Protoc. 2 (2007) 1059–1069.
[24] J.J. Duan, X.M. He, L.N. Zhang, Magnetic cellulose–TiO_2 nanocomposite microspheres for highly selective enrichment of phosphopeptides, Chem. Commun. 51 (2015) 338–341.
[25] G.M. Whitesides, The origins and the future of microfluidics, Nature 442 (2006) 368–373.
[26] A.F. Sarioglu, N. Aceto, N. Kojic, M.C. Donaldson, M. Zeinali, B. Hamza, A. Engstrom, H. Zhu, T.K. Sundaresan, D.T. Miyamoto, X. Luo, A. Bardia, B.S. Wittner, S. Ramaswamy, T. Shioda, D.T. Ting, S.L. Stott, R. Kapur, S. Maheswaran, D.A. Haber, M. Toner, A microfluidic device for label-free, physical capture of circulating tumor cell clusters, Nat. Methods 12 (2015) 685–691.
[27] Z.F. Zhu, H. Chen, W. Wang, A. Morgan, C.Y. Gu, C.Y. He, J.J. Lu, S.R. Liu, Integrated bare narrow capillary–hydrodynamic chromatographic system for free-solution DNA separation at the single-molecule level, Angew. Chem. Int. Ed. 52 (2013) 5612–5616.
[28] H.R. Ma, E.A. Gibson, P.J. Dittmer, R. Jimenez, A.E. Palmer, High-throughput examination of fluorescence resonance energy transfer-detected metal-ion response in mammalian cells, J. Am. Chem. Soc. 134 (2012) 2488–2491.
[29] B. Hu, J. Li, L. Mou, Y. Liu, J. Deng, W. Qian, J. Sun, R. Cha, X. Jiang, An automated and portable microfluidic chemiluminescence immunoassay for quantitative detection of biomarkers, Lab Chip 17 (2017) 2225–2234.
[30] X. Liu, Q.L. Yi, Y.Z. Han, Z.N. Liang, C.H. Shen, Z.Y. Zhou, J.L. Sun, Y.Z. Li, W.B. Du, R. Cao, A robust microfluidic device for the synthesis and crystal growth of organometallic polymers with highly organized structures, Angew. Chem. Int. Ed. 54 (2015) 1846–1850. Angew. Chem. 127(2015) 1866–1870.
[31] B. Renberg, K. Sato, T. Tsukahara, K. Mawatari, T. Kitamori, Hands on: thermal bonding of nano- and microfluidic chips, Microchim. Acta 166 (2009) 177–181.
[32] B.R. Fonslow, M.T. Bowser, Free-flow electrophoresis on an anodic bonded glass microchip, Anal. Chem. 77 (2005) 5706–5710.
[33] W. Wang, C. Gu, K.B. Lynch, J.J. Lu, Z. Zhang, Q. Pu, S. Liu, High-pressure open-channel on-chip electroosmotic pump for nanoflow high performance liquid chromatography, Anal. Chem. 86 (2014) 1958–1964.
[34] M. Callewaert, J.O.D. Beeck, K. Maeno, S. Sukas, H. Thienpont, H. Ottevaere, H. Gardeniers, G. Desmet, W.D. Malsche, Integration of uniform porous shell layers in very long pillar array columns using electrochemical anodization for liquid chromatography, Analyst 139 (2014) 618–625.
[35] N. Chiem, L. Lockyear-Shultz, P. Andersson, C. Skinner, D.J. Harrison, Room temperature bonding of micromachined glass devices for capillary electrophoresis, Sens. Actuators B 63 (2000) 147–152.
[36] S.C. Jacobson, R. Hergenrder, L.B. Koutny, R.J. Warmack, J.M. Ramsey, Effects of injection schemes and column geometry on the performance of microchip electrophoresis devices, Anal. Chem. 66 (1994) 1107–1113.
[37] K. Tsougeni, D. Papageorgiou, A. Tserepi, E. Gogolides, "Smart" polymeric microfluidics fabricated by plasma processing: controlled wetting, capillary filling and hydrophobic valving, Lab Chip 10 (2010) 462–469.
[38] D.P. Papageorgiou, K. Tsougeni, A. Tserepi, E. Gogolides, Superhydrophobic, hierarchical, plasma-nanotextured polymeric microchannels sustaining high-pressure flows, Microfluid. Nanofluidics 14 (2013) 247–255.
[39] Z.J. Jia, Q. Fang, Z.L. Fang, Bonding of glass microfluidic chips at room temperatures, Anal. Chem. 76 (2004) 5597–5602.
[40] A. Gaspar, M.E. Piyasena, F.A. Gomez, Fabrication of fritless chromatographic microchips packed with conventional reversed-phase silica particles, Anal. Chem. 79 (2007) 7906–7909.
[41] M.R. Larsen, T.E. Thingholm, O.N. Jensen, P. Roepstorff, T.J.D. Jorgensen, Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns, Mol. Cell. Proteomics 4 (2005) 873–886.
[42] L. Ceriotti, N.F. de Rooij, E. Verpoorte, An integrated fritless column for on-chip capillary electrochromatography with conventional stationary phases, Anal. Chem. 74 (2002) 639–647.

Please cite this article in press as: S. Wang, et al., Plasma-assisted alignment in the fabrication of microchannel-array-based in-tube solid-phase microextraction microchips packed with TiO_2 nanoparticles for phosphopeptide analysis, Analytica Chimica Acta (2018), https://doi.org/10.1016/j.aca.2018.02.018