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Hydrophilic monolith based immobilized enzyme reactors in capillary and on microchip for high-throughput proteomic analysis

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ABSTRACT

A novel kind of hydrophilic monolith based immobilized enzyme reactors (IMERs) was prepared both in UV-transparent capillaries and on glass microchips by the photopolymerization of N-acryloxysuccinimide and poly(ethylene glycol)diacrylate, followed by trypsin immobilization. The performance of capillary IMERs for protein digestion was evaluated by the digestion of myoglobin with the residential time from 12 s to 71 s. With μRPLC–ESI-MS/MS analysis, the obtained sequence coverages were all over 80%, comparable to that obtained by in-solution digestion for 12 h. The nonspecific absorption of BSA on monolithic support was evaluated, and no obvious protein residue was observed by a fluorescence assay. Moreover, no carry-over of the digests on the capillary IMER was found after the digestion of myoglobin (24 μg) and BSA (9 μg), which further demonstrated the good hydrophilicity of such matrix. In addition, an integrated microchip-based system involving on-line protein digestion by microchip-based IMER, peptides separation by nanoRPLC and identification by ESI-MS/MS was established, by which a mixture of standard proteins and one RPLC fraction of *Escherichia coli* extract were successfully identified, indicating that the hydrophilic monolith based IMER might provide a promising tool for high-throughput proteomic analysis.

1. Introduction

Proteomics, as a new subject to understand various biological problems, is directed toward the identification of all proteins in cells, tissues or body fluids. At present, the "bottom-up" approach is widely applied for proteome study [1–3], by which protein digestion is an indispensable step. However, the traditional in-solution based protein digestion has several drawbacks, such as long digestion time, unavoidable enzyme autodigestion and off-line operation [4]. To solve these problems, recently much attention has been paid to the development of immobilized enzyme reactors (IMERs), which not only have high digestion efficiency, but also can be easily coupled with separation and detection systems to achieve automated and high-throughput protein analysis [5–7]. Furthermore, with the development of nanoscale reversed phase liquid chromatography coupled with mass spectrometry (nanoRPLC–MS) and chip-MS, IMERs prepared in capillaries and on microchips are highly desirable for the construction of integrated platforms involving online protein digestion, peptides separation and identification [8–12].

For IMERs, enzymes can be immobilized on different supports, such as membranes [8], particles [13], the inner walls of capillaries or microchannels [14–17], and monolithic materials. As a large category of favorable supports for enzyme immobilization, monolithic supports have attracted significant attention due to facile preparation, fast mass transfer and low backpressure [18,19]. Generally, monolithic supports could be prepared by thermal polymerization [9–11], photopolymerization [20,21] and sol–gel technique [22–24], among which, photopolymerization has advantages of fast polymerization rate, controllable location and convenient operation both in UV-transparent capillaries and on microchips.

However, the nonspecific adsorption of proteins and peptides on supporting materials is still of challenging for IMERs, especially for the analysis of minute proteomic samples (e.g. some clinical samples). Thus the reduction or elimination of undesired nonspecific adsorption on IMERs is urgently required, making the development of hydrophilic supports attractive. To this end, two approaches have been exploited recently. One is to modify supporting materials with hydrophilic groups. For example, Svec et al. [21] grafted poly(ethylene glycol)methacrylate onto poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith for enzyme immobilization. Another straightforward approach is to form hydrophilic supports by choosing hydrophilic reagents for polymerization. Liu et al. [14] used natural polysaccharides (i.e. chitosan

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and hyaluronic acid) to form biocompatible network on the surface of poly(ethylene terephthalate) microfluidic chip for trypsin immobilization. With acrylamide as the hydrophilic additive, Palm and Novotny [25] and Zhang et al. [9] prepared polyacrylamide-based monolith and polymethacrylate-based monolith for trypsin covalent bonding, respectively. Zare et al. [23] incorporated poly(ethylene glycol) to prepare hydrophilic sol–gel monolith as the support.

In this study, with poly(ethylene glycol)diacrylate (PEGDA) as hydrophilic cross-linker and N-acryloxysuccinimide (NAS) as active monomer, a novel kind of hydrophilic monolithic supports was prepared both in UV-transparent capillaries and on microchips by photopolymerization. With trypsin immobilized, the hydrophilic monolith based IMERs showed high digestion efficiency and low non-specific adsorption. Furthermore, such a microchip-based IMER was further integrated with nanoRPLC–ESI-MS/MS to achieve high-throughput analysis of proteins by on-line protein digestion, peptides separation and protein identification.

2. Experimental

2.1. Materials and chemicals

Microchips were fabricated with Type SG2506 glass plates (Shaoguang Microelectronics Corp, Changsha, China), by photolithography, wet chemical etching and room-temperature bonding, as described by our previous method [26]. UV-transparent capillaries (100 μm i.d. × 375 μm o.d.) were purchased from Polymicro Technologies Corp (Phoenix, Arizona, USA). NAS, 2,2-dimethoxy-2-phenylacetophenone (DMPA), dithiothreitol (DTT), and iodoacetic acid (IAA) were purchased from Acros Organics (Morris Plains, NJ, USA). PEGDA (Mn ~ 258), γ-MAPS (98%), trypsin (bovine pancreas), cytochrome c (from horse heart), and myoglobin (from horse heart) were ordered from Sigma–Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA, bovine serum) was obtained from Sino-American Biotec (Luoyang, China). *N*-α-Benzoyl-L-arginine ethyl ester (BAEE) was purchased from Fluka (Buchs, Switzerland). Methanol (CH_3OH) and acetonitrile (ACN) were obtained from Merck (Darmstadt, Germany). C18 particles (5 μm, 200 Å) and C8 particles (5 μm, 300 Å) were purchased from Sipore Chrom (Dalian, China).

2.2. Apparatus

Photopolymerization was performed in an XL-1500 UV crosslinker (Spectronics, NY, USA) with an overall intensity of 4500 μW/cm^2. Scanning electron micrographic images were obtained using a JEOL-JSM-6360LV SEM scanning electron microscope (JEOL, Tokyo, Japan). The fluorescence images were obtained by a fluorescence microscope (Nikon, Tokyo, Japan) with a CCD camera (Ottawa, Canada) installed. A precise syringe pump (Baoding Longer Pump Company, Baoding, China) was used to push the liquid through monoliths. SpeedVac (Thermo Fisher, San Jose, CA, USA) was used to lyophilize samples. Desalting of standard proteins and the proteins extracted from *Escherichia coli* was performed on an HPLC-UV system (Jasco, Tokyo, Japan). A paradigm GM4 μHPLC system (Michrom Bioresources Inc., Auburn, CA, USA) and an LCQ^{DUO} quadrupole ion trap mass spectrometer (LCQ-IT MS, Thermo Fisher, San Jose, CA, USA) were used for peptide separation and protein identification.

2.3. Monolithic supports preparation

The internal wall surface of UV-transparent fused-silica capillaries and the channel of microchips were first vinylized to enable the covalent attachment of the monolith to the wall, as described in our previous research [26]. For photopolymerization in capillaries, the polymerization mixture containing 15 wt% NAS, 10 wt% PEGDA, 75 wt% CH_3OH and 1 wt% DMPA with respect to monomers, was purged with N_2 for 30 s to remove dissolved O_2, and then filled into the capillaries. With both ends sealed by silicon rubbers, the capillaries were exposed to UV light at 365 nm for 15 min in the XL-1500 UV-crosslinker. For photopolymerization on microchips, with the reservoirs sealed by sealing tapes, the microchannels were filled with the polymerization mixture containing 12 wt% NAS, 8 wt% PEGDA, 80 wt% CH_3OH and 1 wt% DMPA with respect to monomers. With the certain section masked, the microchannels were exposed to UV light at 365 nm for 5 min in the XL-1500 UV-crosslinker. Finally, the monoliths were washed with methanol for 2 h using a syringe pump.

2.4. Trypsin immobilization

Trypsin was immobilized on the monolithic support by continuously pumping 5 mg/mL trypsin in an aqueous solution containing 0.2 mol/L $NaHCO_3$, 0.5 mol/L NaCl (pH 8.0) and 0.05 mol/L benzamidine for 4 h. Subsequently, the monolith was washed with 1 M Tris–HCl (pH 8.0) for 2 h to quench the unreacted succimide functionalities, followed by equilibration with 50 mM NH_4HCO_3 buffer (pH 8.0) for 2 h.

2.5. Investigation of protein adsorption on supports

The adsorption of proteins on poly(NAS-co-PEGDA) monoliths was evaluated by a fluorescence assay [21], and compared to that on poly(GMA-co-EDMA) monoliths prepared by the following steps. The polymerization mixture, consisting of 25.5 wt% GMA, 17.5 wt% EDMA, 40 wt% 1-dodecanol, 17 wt% cyclohexanol, and 1 wt% AIBN with respect to monomers, was purged with nitrogen for 10 min, and then filled into the UV-transparent fused-silica capillary. With both ends sealed by silicon rubbers, the polymerization was performed at 70 °C for 24 h. Finally, the monoliths were flushed with methanol.

First of all, to quench the succimide functionalities of poly(NAS-co-PEGDA) monoliths and the epoxide groups of poly(GMA-co-EDMA) monoliths, 1 M Tris (pH 10.3) was pumped through the columns for 6 h and 12 h, respectively. Then the monoliths were flushed with 0.45 mg/mL fluorescence-labeled BSA for 1 h at a flow rate of 500 nL/min. Finally, the monolith was flushed with 50 mM CH_3COONH_4 for 30 min at a flow rate of 500 nL/min. Before and after the last step, images of monoliths were recorded by the fluorescence microscope.

2.6. Sample preparation

Each standard protein was dissolved in 50 mM NH_4HCO_3 (pH 8.0) containing 8 M urea, reduced by DTT and alkylated by iodoacetamide, and diluted with 50 mM NH_4HCO_3 (pH 8.0) to decrease the urea concentration below 1 M. Then the protein was desalted on a home-made SPE-C8 column with 2% and 80% ACN as the loading and eluting buffers, respectively. The eluates were lyophilized, and then dissolved in 50 mM NH_4HCO_3 (pH 8.0) to desired concentration. Without specific statement, all percentages represent volume ratio.

Proteins extracted from *Escherichia coli* containing 8 M urea were reduced by DTT, alkylated by iodoacetamide, and diluted with 50 mM NH_4HCO_3 (pH 8.0) to decrease the urea concentration below 1 M. Then proteins were desalted and separated by RPLC using buffer A (2% ACN containing 0.1% TFA) and buffer B (95% ACN containing 0.1% TFA) on a home-packed C8 column (4.6 mm i.d. × 250 mm). The gradient was set as followed: 0–10 min, B% maintained at 20%; 10–50 min, B% from 20% to 80%; B% maintained

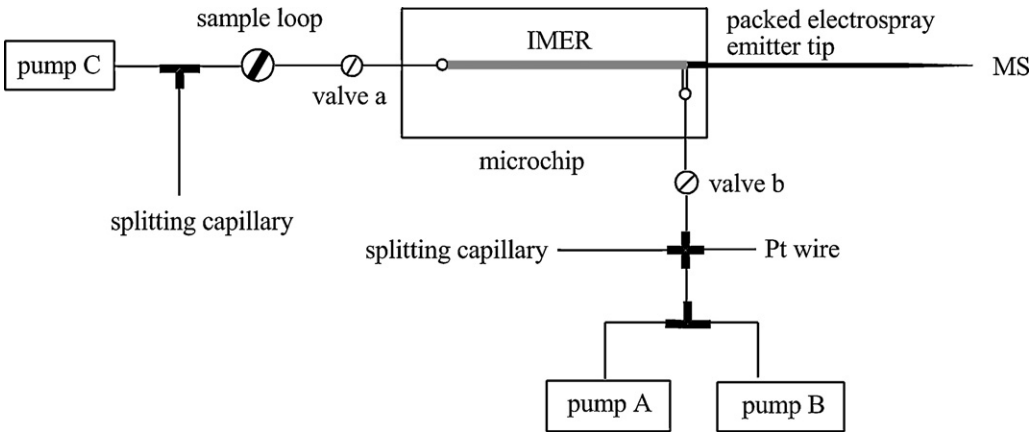

Fig. 1. Schematic diagram of integrated microchip-based IMER-nanoRPLC–ESI-MS/MS system.

at 80% for 5 min. The eluates from 26 min to 30 min were collected, lyophilized, and reconstituted in 20 μL of 50 mM NH_4HCO_3 (pH 8.0).

The in-solution digestion was performed by adding trypsin into the pretreated protein sample with a substrate-to-enzyme ratio of 25:1 (w/w). Then the solution was incubated at 37 °C for 12 h. Finally, 1 μL formic acid was added into the solution to terminate the reaction.

The off-line digestion by IMERs was carried out by pumping the pretreated protein sample through the column at a constant flow rate. The digests were collected, separated, and identified by μRPLC–ESI-MS/MS.

2.7. μRPLC–ESI-MS/MS analysis

The paradigm GM4 μHPLC system was online coupled with LCQ^{DUO} quadrupole ion trap mass spectrometer using a homemade C18 column (300 μm i.d., 15 cm length). μHPLC conditions were as follows: mobile phase, (A) 2% ACN (containing 0.1% formic acid); (B) 98% ACN (containing 0.1% formic acid); flow rate, 5 μL/min; gradient: 0–5 min, B% from 0% to 10%; 5–45 min, B% from 10% to 40%; 45–55 min, B% from 40% to 80%; 55–60 min, B% maintained at 80%; injection, 4 μL. The LCQ instrument was operated at positive ion mode. The spray voltage was 3 kV, and the heated capillary temperature was 150 °C. Total ion current chromatograms and mass spectra covering the mass range from m/z 400 to 2000 were recorded with Xcalibur software (version 1.4). MS/MS spectra were acquired by data-dependent acquisition mode with two precursor ions selected from one MS scan. Precursor selection was based on parent ions intensity, and the normalized collision energy for MS/MS scanning was 35%. Besides the manual inspection of mass spectra, data were also submitted to the SEQUEST algorithm for further analysis.

2.8. Online microchip-based IMER-nano RPLC–ESI-MS/MS analysis

As shown in Fig. 1, the integrated microchip-based system was established by attaching a home-made capillary electrospray emitter tip (6-cm length, 75-μm i.d., 190-μm o.d.) packed with C18 particles (5 μm, 200 Å) to the outlet of the microchip-based IMER (206 μm width, 90 μm depth, 4 cm length). The packed electrospray emitter tip was coupled with ESI-MS/MS directly, used as both separation column and chip-MS interface. The paradigm GM4 μHPLC system was used to pump the buffer and the flow rate was adjusted by splitting capillaries. The ESI voltage was 2–3 kV, supplied through the platinum wire plugged in the microcross. First of all, with valve A open and valve B blocked, proteins in the sample loop were introduced into IMER and on-line digested, followed by trapping on the packed electrospray emitter tip. Then, with valve A blocked and valve B open, the digests were separated by nano-RPLC and identified by ESI-MS/MS. Analytical conditions were as follows: injection, 2 μL; mobile phase: (A) 2% ACN (0.1% formic acid), (B) 98% ACN (0.1% formic acid), (C) 50 mM CH_3COONH_4 (pH 8.0); digestion condition: room temperature, 270 nL/min; sample loading time, 25 min; Separation condition: flow rate, 240 nL/min; gradient: 0–10 min, 0–10% B; 10–50 min, 10–40% B; 50–55 min, 40–80% B; 55–60 min, 80% B. The MS conditions were the same to those applied in μRPLC–ESI-MS/MS analysis.

2.9. MS data analysis

The acquired MS/MS spectra were searched against the corresponding database in fast format using SEQUEST (BioWorks 3.1). Reversed sequences were appended to the database for the evaluation of the false positive rate (FDR). Cysteine carbamidomethylation was set as a static modification of +57.02 Da, while methionine oxidation as a variable modification of +15.99 Da. Other search parameters included full tryptic cleavage, up to two missed cleavages allowed, 2 Da parent mass tolerance and 1 Da fragment mass tolerance. The widely accepted SEQUEST thresholds were used, as Xcorr (+1, 2, 3) = 1.90, 2.20, 3.75 and $\Delta Cn \geq 0.1$ for standard proteins (FDR not calculated) while a slight increase of ΔCn to ≥ 0.2 for *Escherichia coli* (FDR $\leq$ 3%).

3. Results and discussion

3.1. Preparation of IMERs

IMERs with hydrophilic monoliths as supports were prepared by the photopolymerization of NAS and PEGDA with CH_3OH as the porogen and DMPA the photoinitiator, followed by trypsin immobilization (Fig. 2). NAS was the active monomer, offering extremely high activity to react with the amino group of enzymes [9,25]. To develop a hydrophilic IMER, PEGDA that could evidently improve the hydrophilicity of monoliths [27,28], was adopted as the cross-linker. CH_3OH was chosen as porogenic solvent, since it can dissolve all monomers and the initiator, to form a homogeneous solution.

The effect of CH_3OH content and NAS/PEGDA ratio on the monolith structure and digestion efficiency of capillary IMERs was investigated. With the increase of CH_3OH content, the penetrability of the monolith was improved, but the digestion efficiency was decreased; the increase of NAS/PEGDA ratio benefited the digestion efficiency, but led to the decrease of the rigidity of monoliths.

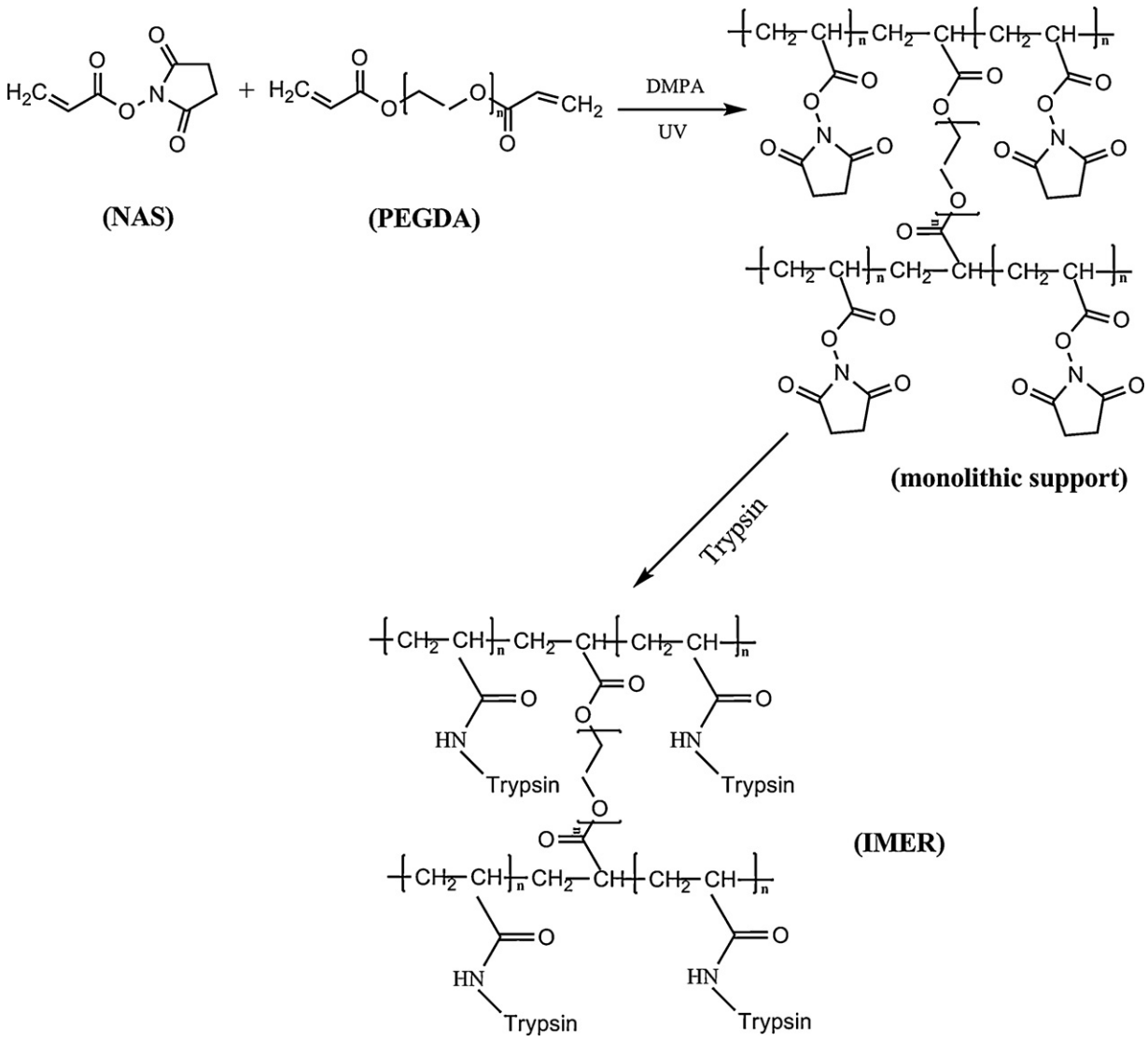

Fig. 2. Procedures for the preparation of monolithic supports and trypsin immobilization.

Under the systematic optimization, the optimal percentages of NAS, PEGDA, and CH_3OH for 15-min photopolymerization to prepare monolithic supports in capillaries were 15 wt%, 10 wt%, and 75 wt%, respectively.

Due to the minor differences between glass microchips and UV-transparent capillaries on light transparency at 365 nm, the photopolymerization conditions in microchannels were slightly modified, with the polymerization solution composed of 12 wt% NAS, 8 wt% PEGDA, 80 wt% CH_3OH and 1 wt% DMPA with respect to mononers, and the polymeration time decreased to 5 min.

The SEM images of the monolithic supports in capillaries and microchannels are shown in Fig. 3. It can be seen that the supports are of homogeneous and porous structure with both macro- and micropores, enabling fast mass transfer, low back pressure and enough enzyme immobilization sites. Furthermore, no obvious shrinkage and blockage of the supports were observed even when 20 MPa pressure was applied, demonstrating the excellent mechanical stability of the monolithic matrices.

Since succimide functionalities had extremely high activity to react with the amino group of proteins [9,25], trypsin immobilization via the reaction of the amino group of trypsin with the succimide functionalities on the support could be completed within 4 h at the room temperature.

3.2. Evaluation of capillary IMERs for protein digestion

The performance of hydrophilic monolithic IMERs for protein digestion was evaluated in the format of capillaries. Myoglobin, a typical globular protein which contains 153 amino acids and 21 cleavage sites, was used as the sample. 400 ng myoglobin was digested by a 2 cm-length IMER at 37 °C under different flow rates with the residential time ranging from 12 s to 71 s. By μRPLC–ESI-MS/MS analysis, the obtained sequence coverages of myoglobin were all over 80%, comparable to that obtained by in-solution digestion for 12 h (shown in Table 1). More than 10 peptides identified by IMER digestion were identical to those obtained by in-solution digestion, indicating that good cleavage specificity of trypsin is remained after immobilization (shown in Table S1 of supplementary data).

A mixture of cytochrome c (200 ng), myoglobin (200 ng), and BSA (200 ng) was further used to evaluate the performance of capillary IMERs. From Table 2, it could be seen that three proteins with molecular weight (Mw) ranging from 11.7 to 69.3 kDa and isoelectric point (pI) ranging from 5.8 to 9.6 were all identified. Compared with the in-solution digestion, more peptides and higher sequence coverage were obtained for proteins digested by IMERs, while the

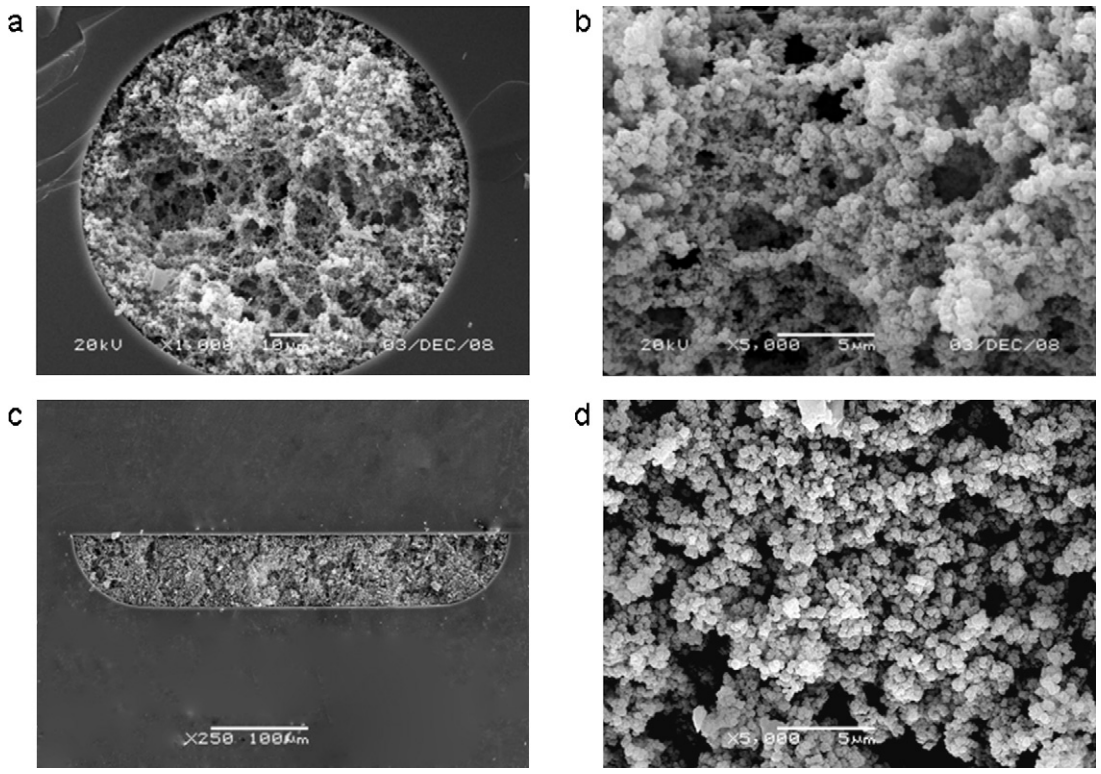

Fig. 3. SEM images of poly(NAS-co-PEGDA) monoliths in capillaries (a × 1000, b × 5000) and microchannels (c × 250, d × 5000).

Table 1
Database searching results for myoglobin digested by capillary IMER and in-solution protocol,[a] followed by μRPLC–ESI-MS/MS analysis.

	In-solution digestion	Capillary IMER digestion Flow rate (nL/min)					
		100	200	300	400	500	600
Residence time	12 h	71 *s*	35 s	24 s	18 s	14 s	12 s
Number of peptides matched	12	13	13	13	14	15	11
Sequence coverage (%)	81.70	81.70	81.70	81.70	86.27	86.27	81.70

[a] Conditions for capillary IMER digestion: capillary IMER, 100 μm i.d. × 2 cm length; temperature, 37 °C; conditions for in-solution digestion: trypsin/myoglobin = 1/25 (w/w); temperature, 37 °C.

Table 2
Database searching results for a three-protein mixture digested by capillary IMER and in-solution protocol[a], followed by μRPLC–ESI-MS/MS analysis.

Protein	Mw (Da)	pI	In-solution digestion (12 h)		Capillary IMER digestion (59 s)	
			Number of matched peptides	Sequence coverage (%)	Number of matched peptides	Sequence coverage (%)
Cytochrome c	11,702	9.6	5	38.46	7	40.38
Myoglobin	16,953	7.4	7	64.71	9	73.20
BSA	69,324	5.8	18	37.23	16	37.73

[a] Conditions for capillary IMER digestion: IMER, 100 μm i.d. × 5 cm; flow rate, 300 nL/min; temperature, 37 °C; conditions for in-solution digestion: enzyme/protein = 1/25 (w/w); temperature, 37 °C.

digestion time was shortened from 12 h to 59 s, further demonstrating that capillary IMERs could be used for high efficiency and high throughput protein digestion.

The batch-to-batch ($n = 3$) reproducibility of capillary IMERs was evaluated through the digestion of myoglobin at 37 °C at the flow rate of 300 nL/min. The obtained sequence coverages of myoglobin were 81.05%, 81.70%, and 85.62%, respectively, with the relative standard deviation (RSD) as 2.99%, and 11 peptides identified were identical (shown in Table S2 of supplementary data), indicating the good preparation reproducibility of IMERs.

3.3. Hydrophilicity of capillary IMERs

The development of hydrophilic supports should be one effective means to avoid the nonspecific adsorption of proteins and the resulting peptides on IMERs. Since molecules that contain more than three ethylene glycol units can effectively resist the adsorption of proteins, as suggested by Ostuni et al. [29], the monolithic support prepared with NAS (with an acrylate group and a succimide group, but no hydrophobic group or carbon chains) and PEGDA (with an acrylate group at each end of the

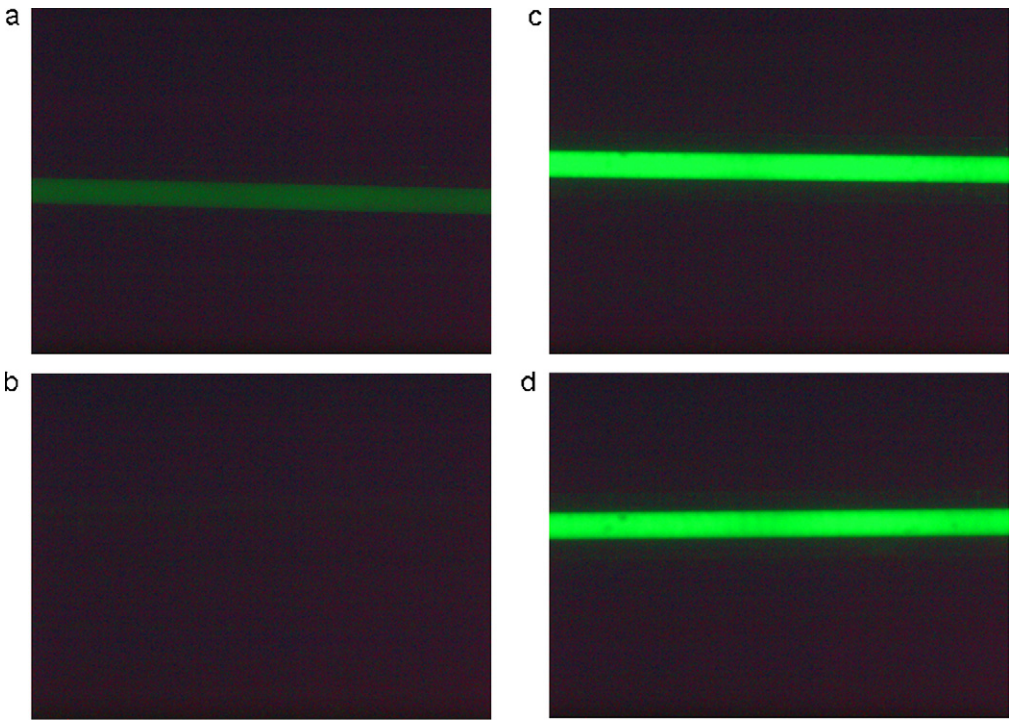

Fig. 4. CCD images of fluorescent-labeled BSA adsorbed on poly(NAS-co-PEGDA) (a, b) and poly(GMA-co-EDMA) monoliths (c, d). (a) and (c), Monoliths were flushed with 0.45 mg/mL fluorescence-labeled BSA for 1 h at a flow rate of 500 nL/min; (b) and (d), Monoliths were further flushed with 50 mM CH_3COOH_4 for 30 min at a flow rate of 500 nL/min.

molecule, and a PEG chain between) should be of good hydrophilicity.

By using a fluorescence assay, slightly modified from Svec et al. [21], the hydrophilicity of poly(NAS-co-PEGDA) monoliths was compared with that of poly(GMA-co-EDMA) monoliths which were commonly used as supports for IMERs [10,21]. To ensure the fluorescence observed by CCD was generated by the non-specific adsorption rather than the covalent bonding of fluorescence-labeled BSA on the supports, the succimide functionalities of poly(NAS-co-PEGDA) monoliths and the epoxide groups of poly(GMA-co-EDMA) monoliths were quenched with the amino group of Tris. As shown in Fig. 4a and b, most of the fluorescence-labeled BSA loaded on poly(NAS-co-PEGDA) monoliths (with succimide functionalities quenched) could be easily removed by

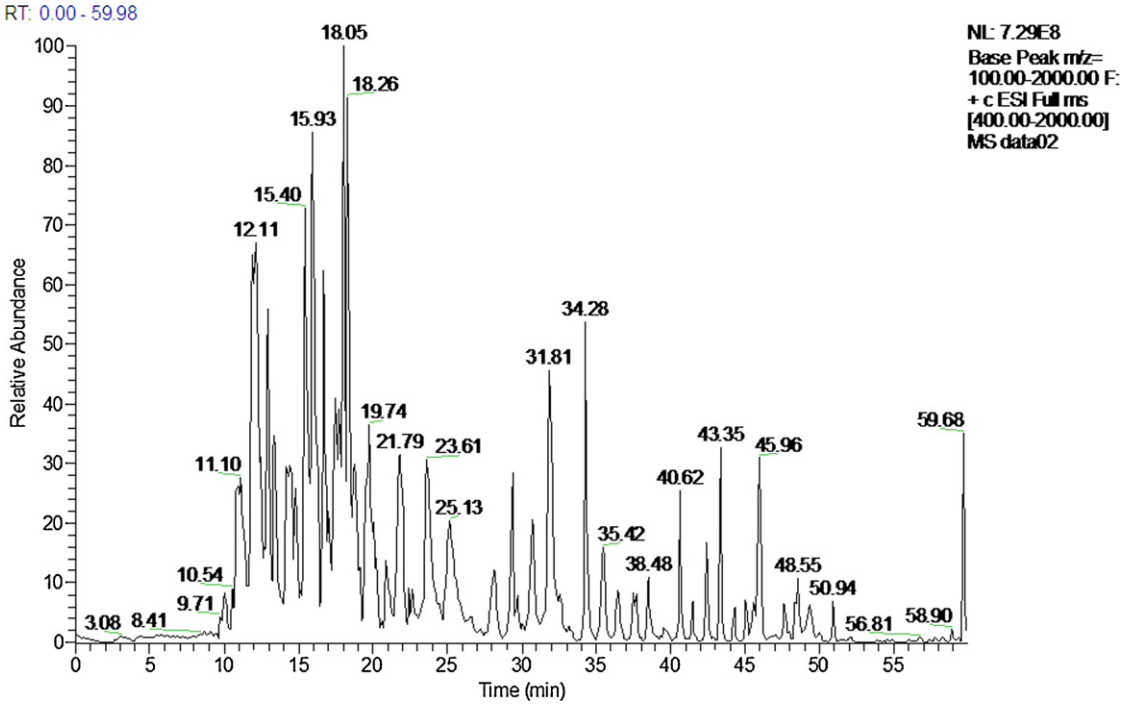

Fig. 5. Base peak chromatogram for BSA, myoglobin and cytochrome c mixture (200 ng for each protein) analyzed by integrated microchip-based IMER-nanoRPLC–ESI-MS/MS system.

Table 3
Database searching results for a three-protein mixture analyzed by integrated microchip-based IMER-nano-RPLC–ESI-MS/MS system in triplicate runs.

	First run		Second run		Third run	
	Number of matched peptides	Sequence coverage (%)	Number of matched peptides	Sequence coverage (%)	Number of matched peptides	Sequence coverage (%)
Cytochrome c	9	51.92	11	63.46	12	63.46
Myoglobin	10	60.78	10	60.78	10	60.78
BSA	28	49.75	27	49.26	27	54.86

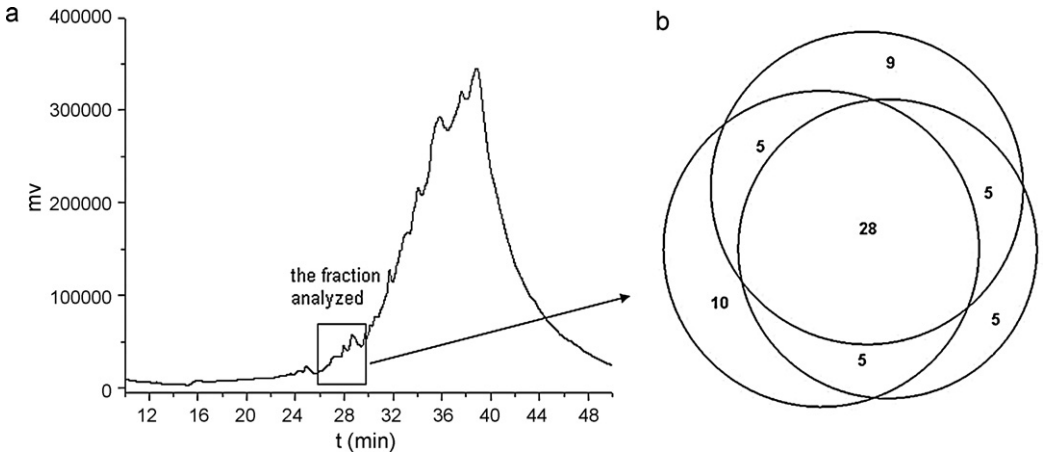

Fig. 6. Separation of proteins extracted from *Escherichia coli* by RPLC (a) and the overlap of identified proteins from one RPLC fraction (as indicated) analyzed by integrated microchip-based IMER-nanoRPLC–ESI-MS/MS system in triplicate runs (b).

50 mM CH_3COONH_4 solution, while a considerable amount of fluorescence-labeled BSA was absorbed on poly(GMA-co-EDMA) monoliths (with epoxide groups quenched) after washing, with strong fluorescence observed (Fig. 4c and d), which indicated that the hydrophilicity of poly(NAS-co-PEGDA) monoliths was much better than that of poly(GMA-co-EDMA) monoliths and the IMERs based on poly(NAS-co-PEGDA) monoliths had better hydrophilicity.

The hydrophilicity of IMERs was further evaluated by checking the carry-over of proteins and peptides on capillary IMERs. After the digestion of myoglobin (24 μg) and BSA (9 μg), the IMERs were washed with 50 mM NH_4HCO_3 (pH 8.0) buffer for a few minutes. Subsequently, 80% ACN was pumped through the IMERs to elute possibly adsorbed proteins and peptides. Finally, the eluates were collected, lyophilized, resuspended, and then analyzed by μRPLC–ESI-MS/MS. Our experimental results showed that no digests of myoglobin and BSA were found, further demonstrating the excellent hydrophilicity of the IMERs.

3.4. Integrated microchip-based IMER-nanoRPLC–ESI-MS/MS system for high-throughput proteomic analysis

Microchip-based IMERs are very important to the development of fully integrated, high-throughput microfluidic systems containing protein digestion, peptides separation and identification [5,12,30]. Based on the hydrophilic monolith that can be easily and quickly prepared in the specified position of microchannels, microchip-based IMERs were prepared and integrated with nanoRPLC–ESI-MS/MS via a packed electrospray emitter tip. Proteins were on-line digested by the microchip-based IMER, meanwhile the digests were trapped by the packed electrospray emitter tip, and then the digests were separated by nanoRPLC and identified by ESI-MS/MS. With such an integrated system, a mixture of BSA, myoglobin and cytochrome c (200 ng for each protein), was analyzed (Fig. 5). In triplicate runs, the sequence coverages for such three proteins were 51.29 ± 3.10%, 60.78 ± 0%, and 59.61 ± 6.66%, respectively, as shown in Table 3, demonstrating the excellent digestion performance of microchip-based IMERs and the good identification reproducibility of such integrated system.

To further demonstrate the applicability of such integrated platform for complex sample analysis, a randomly selected RPLC fraction of *Escherichia coli* extract (collected from 26 to 30 min, as indicated in Fig. 6a) was analyzed. In three consecutive runs, the number of identified proteins was 48, 43 and 47, respectively (as illustrated in Fig. 6b). In total, 67 proteins were successfully identified, among which 28 proteins were all identified in three runs. All these results showed that the integrated microchip-based IMER-nanoRPLC–ESI-MS/MS system had great potential for high-throughput proteomic analysis. Base on such system, the preparation of integrated and even array IMER and RP column on the microchip to achieve high throughput proteome study is our undergoing work.

4. Conclusions

A novel kind of hydrophilic monolith based IMERs was prepared not only in the UV-transparent capillaries but also on microchips by photopolymerization and trypsin covalent boding. Besides high digestion efficiency and good preparation reproducibility, IMERs showed good hydrophilicity, beneficial to eliminate undesired nonspecific adsorption of proteins and peptides, lower the overlap of identified peptides and decrease the cross contamination of samples. Furthermore, an integrated system involving online protein digestion by microchip-based IMER, peptide separation by nanoRPLC and identification by ESI-MS/MS was established. Through the successful application for the analysis of standard proteins and complex samples, such a platform showed great potential for high-throughput proteome profiling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.02.073.

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