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High throughput tryptic digestion via poly (acrylamide-*co*-methylenebisacrylamide) monolith based immobilized enzyme reactor

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ABSTRACT

A poly (acrylamide-*co*-methylenebisacrylamide) (poly (AAm-*co*-MBA)) monolith was prepared by thermal polymerization in the 100 or 250 μm i.d. capillary. The monolithic support was activated by ethylenediamine followed by glutaraldehyde. Trypsin was then introduced to form an immobilized enzyme reactor (IMER). The prepared IMER showed a reliable mechanical stability and permeability (permeability constant $K = 2.65 \times 10^{-13}$ m^2). With BSA as the model protein, efficient digestion was completed within 20 s, yielding the sequence coverage of 57%, better than that obtained from the traditional in-solution digestion (42%), which took about 12 h. Moreover, BSA down to femtomole was efficiently digested by the IMER and positively identified by matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). To test the applicability of IMER for complex sample profiling, proteins extracted from *Escherichia coli* were digested by the IMER and further analyzed by nanoreversed phase liquid chromatography–electrospray ionization-mass spectrometry (nanoRPLC–ESI-MS/MS). In comparison to in-solution digestion, despite slightly fewer proteins were positively identified at a false discovery rate (FDR) of ~1% (333 vs 411), the digestion time used was largely shortened (20 s vs 24 h), implying superior digestion performance for the high throughput analysis of complex samples.

1. Introduction

The "Shot-gun" method is one of the most popular tools in current proteomics study [1,2]. In this method, digestion is the key process for protein profile via mass spectrometry (MS) analysis [3,4]. Traditionally, digestion is often performed by free enzymes in solution, which is time-consuming, tedious and inconvenient for automation [4].

In response to these challenges, the immobilized enzyme reactor (IMER) [5–8] is a remarkable facility to perform digestion in a few minutes or even seconds. It could be also connected with related components directly for on-line digestion [9,10]. Nowadays, a variety of IMERs have been developed with different supporting materials, e.g., membranes [11–13], particles [14–16], monoliths and so on. Among them, the monolith based IMER [17–21] has drawn great attention due to its high capacity for enzymes, fast and simple preparation, low back-pressure, biological inertia and mechanical stability, rendering greatly enhanced digestion efficacy. However, the nonspecific adsorption is its intrinsic problem when substrates were pumped through the monoliths, leading to poor digestion performance [18].

As a post-modification method, the photografting technique [18,22] has been applied to improve hydrophilicity of monoliths for IMERs to reduce nonspecific adsorption of substrates. In contrast, a more straightforward method is to prepare monoliths via hydrophilic monomers [17,23] directly. Acrylamide (AAm) and N,N′-methylenebisacrylamide (MBA), which have been extensively used in polyacrylamide gel electrophoresis [24,25], are regarded as beneficial monomers to form monoliths with excellent hydrophilicity [17,19,20]. However, the traditional poly (AAm-*co*-MBA) monolith, which was formed by free radical polymerization reaction in an aqueous medium [26–30], was cryogels with an elastic feature. With a different approach, heat polymerization, Frechet et al. [31] prepared a poly (AAm-*co*-MBA) monolith which exhibited much better mechanical stability than cryogels.

Herein, a novel poly (AAm-*co*-MBA) monolith was prepared in the capillary by in situ heat polymerization of AAm and MBA via azobisisobutyronitrile (AIBN)-initiated in the presence of

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1,4-butanediol, dodecanol and dimethyl sulfoxide (DMSO) as porogens. After sequentially activated by ethylenediamine [28] and glutaraldehyde, the monolith was ready for trypsin immobilization. The mechanical stability and permeability of prepared poly (AAm-*co*-MBA) monolith were evaluated systematically. Initially, the digestion performance of IMER was tested by standard proteins. As a result, BSA digestion was achieved efficiently down to femtomole. Moreover, proteins extracted from *Escherichia coli* were digested with 20 s, and 333 proteins (2362 peptides) could be identified at ~1% FDR.

2. Experimental

2.1. Materials and chemicals

Fused-silica capillary (100 or 250 μm i.d.) was bought from Sino Sumtech Co., Ltd. (Handan, China). AAm (99+%), MBA (98%), GMA (97%) and EDMA (98%) were purchased from Acros Organics (Geel, Belgium). 1,4-Butanediol, cyclohexanol and dodecanol were from Fluke (Buchs, Switzerland). Trypsin (bovine pancreas), β-lactoglobulin (bovine milk), BSA, myoglobin (equine skeletal muscle), cytochrome *c* (horse heart), γ-methacryloxypropyl trimethoxysilane (γ-MAPS), glutaraldehyde solution (25%), sodium azide, iodoacetamide (IAA) and dithiothreitol (DTT) were all from Sigma–Aldrich (St. Louis, MO). AIBN was obtained from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China), and recrystallized from methanol. DMSO and organic solvents were all of HPLC grade. Water was purified by a Milli-Q system (Millipore, Molsheim, France) with resistance ≥ 18.2 MΩ/cm.

2.2. Instrumentation

Scanning electron micrographic images were acquired using a scanning electron microscope (SEM, JEOL-JSM-6360LV, Tokyo, Japan). A precise syringe pump (Baoding Longer Pump Company, Baoding, China) was used to push samples through IMERs, and a temperature controller (ZW-column oven, Dalian Elite Analytic instruments Co., Ltd.) was utilized to keep the temperature at 37 °C. An Allegra 64R centrifuge (Beckman Coulter. Inc., Miami, FL) was used for sample purification. MALDI-TOF MS experiments were performed on an Ultraflex III MALDI-TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a high rep rate (100 Hz) diodepumped all-solid-state SmartBeam laser (third harmonic at 355 nm) to analyze peptides in the positive reflection mode. A nanoRPLC–ESI-MS/MS system was constructed by combining the nanoRPLC with a Finnigan LTQ XL IT mass spectrometer (Thermo, San Jose, CA).

2.3. Preparation of poly (AAm-co-MBA) monolith

35 mg AAm, 40 mg MBA, 150 mg 1,4-butanediol, 330 mg DMSO, 185 mg dodecanol and 0.8 mg AIBN were mixed homogenously by sonication for 20 min, followed by purging nitrogen gas for 10 min. Then the solution was pumped into a fused-silica capillary activated by γ-MAPS according to a previous procedure [20], and incubated in a 60 °C water bath for 2 h. The resultant monolith was flushed with pure ACN for 30 min to remove porogens.

2.4. Nonspecific adsorption characterization

Cytochrome *c*, β-lactoglobulin and myoglobin were denatured at 90 °C for 20 min to expose hydrophobic parts. Then the solution containing three proteins proportionally was pumped through a poly (AAm-*co*-MBA) monolith (30 cm, 100 μm i.d.) at a flow rate of 250 nL/min for 1 h. Subsequently, 50 mM ammonium bicarbonate solution (pH 8.0) was used to rinse the poly (AAm-*co*-MBA) monolith at a flow rate of 500 nL/min for 30 min. Finally, two 50 mM ammonium bicarbonate solutions containing 5% and 80% ACN respectively were pumped through the poly (AAm-*co*-MBA) monolith in turn to remove possibly adsorbed proteins at a flow rate of 500 nL/min for 30 min. Two effluents were collected, evaporated and reconstituted in 10 μL 50 mM ammonium bicarbonate solution respectively for MALDI-TOF MS analysis.

2.5. Trypsin immobilization

Poly (AAm-*co*-MBA) monolith was activated by ethylenediamine at 90 °C for 3 h. Subsequently, the monolith reacted with 5% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH, 8.0) for 6 h at 25 °C. Trypsin was covalently bonded on the monolithic surface by pumping 2 mg/mL trypsin in 100 mM phosphate buffer containing 50 mM benzamidine and 5 mg/mL sodium cyanoborohydride ($NaCNBH_3$) for 24 h at 4 °C. Finally the IMER was washed continuously with 100 mM phosphate buffer mixed with 20% (v/v) ACN, and stored at 4 °C before use. The amount of trypsin covalently immobilized onto the poly (AAm-*co*-MBA) monolith was estimated by measuring the difference in the UV absorbance at 280 nm of trypsin solutions before and after the immobilization with an UV/Vis spectrophotometer. Specifically, one poly (AAm-*co*-MBA) monolith (100 μm i.d., 25 cm) activated by glutaraldehyde was used for trypsin immobilization. 125 μL of the coupling solution pumped through the column was collected. We obtained the trypsin concentration after immobilization with the BSA standard curve. By calculating the decrease of trypsin after immobilization, the amount of immobilized trypsin onto the column was achieved.

2.6. Protein extraction from Escherichia coli

The proteins of cultured *E. coli* were extracted as follows: *E. coli* (Strain BLT 5403) grown on LB culture medium was cultured at 37 °C for 14 h. Then, cells were centrifuged at 4300 × *g* for 10 min at 4 °C. The precipitated cells were washed with PBS for 3 times. After that, 8 M urea together with 1 mM PMSF and 1 mM protease inhibitor cocktail set I from Merck (Darmstadt, Germany) were added into the precipitate with the ratio of 2:1 (v/w), followed by ultrasonication for 180 s. All those processes were performed in the ice bath. The resulting mixture was centrifuged at 20,000 × *g* for 20 min. As a result, the supernatant was collected and stored at −80 °C. The protein concentration of supernatant was tested by Bradford method at 595 nm using BSA as a standard.

2.7. Protein digestion

Myoglobin, β-lactoglobulin and BSA (1 mg) were respectively dissolved in 50 mM ammonium bicarbonate solution (100 μL) containing 8 M urea, and then reduced via 10 mM DTT for 1 h at 56 °C. Cysteines were alkylated in the dark via 20 mM iodoacetamide for 30 min at 37 °C, followed by dilution with 50 mM ammonium bicarbonate solution to decrease the urea concentration below 1 mol/L. Subsequently, the protein solution was pumped through IMER at 37 °C. Meanwhile, in-solution digestion was carried out by adding trypsin into the same protein solution with an enzyme-to-protein ratio of 1:50 (w/w) at 37 °C for 12 h (standard proteins) or 24 h (*E. coli* proteins). Finally, formic acid was added to terminate digestion.

2.8. MS analysis

External calibration of MALDI-TOF MS spectra was performed with ten commercial standard peptides. Spectra were acquired from an accumulation of 1000 laser shots. Detection scale and energy were all steady in an independent experiment. The voltage

was set as following: acceleration, 21.85 kV; lens, 9.2 kV; reflector 1, 26.39 kV; reflector 2, 14.0 kV. The dried droplet method was employed for sample preparation.

A 16 cm long capillary (75 μm i.d.) with pulled spray tip was packed with C18 particles (5 μm, 300 Å, Sinochrom ODS-AP) at 5000–7000 psi by a high-pressure pump overnight. Meanwhile, a 2 cm long capillary (75 μm i.d.) packed with the same particles was prepared as the pre-column. The ESI voltage was set at 1.8 kV for LTQ, and the spray capillary was heated to 180 °C. Total ion current chromatograms and mass spectra ranging from m/z 400 to 1800 were recorded with the Xcalibur software (v 3.1). The MS was set as one full MS scan followed by seven MS/MS scans. Two kinds of buffer solutions were (A) H_2O with 2% (v/v) ACN containing 0.1% (v/v) formic acid and (B) ACN with 2% (v/v) H_2O and 0.1% (v/v) formic acid, respectively. The gradient was set as follows: 0–10 min, 0% B (v/v); 10–100 min, 10%–40% B (v/v); 100–110 min, 40%–80% B (v/v); 110–120 min, 20% B (v/v).

2.9. Protein identification

MALDI-TOF MS: peptide mass fingerprint (PMF) was searched via MASCOT search engine with SwissProt 57.1 database (462,764 sequences; 163,773,385 residues). Other search parameters were set as follows: enzyme, trypsin; fixed modifications, carbamidomethyl (C); allow up to 2 missed cleavage; peptide tolerance, 100 ppm.

ESI-MS/MS: the tandem mass spectra detection and database searching were operated by Bioworks software version 3.3.1. Peptides searched using fully tryptic cleavage constraints and at most two internal cleavages sites were allowed for tryptic digestion. The mass tolerances were 2 Da for parent masses and 1 Da for fragment masses. Cysteine residues were searched as static modification of 57.0215 Da. Identified peptides were filtered via following standards: Xcorr was higher than 1.9 for singly charged peptide, 2.2 for doubly charged peptide, and 3.75 for triply charged peptides; adjusting ΔC_n to control FDR < 1%. Identified proteins were filtered further via *of top match* = 1.

3. Results and discussion

3.1. Preparation and characterization of poly (AAm-co-MBA) monolith

In this study, a hydrophilic monolith was prepared via polymerizing water soluble AAm and MBA in a capillary (100 or 250 μm i.d.) for further trypsin immobilization. Fig. 1 presents scanning electron microscopic (SEM) images of prepared poly (AAm-*co*-MBA) monolith. The monolithic bed was tightly attached to the capillary inner wall (Fig. 1A); large through-pores and polymerized particles which are characteristic of the monolithic structure were observed obviously from Fig. 1B. The macroporous structure ensures low back-pressure and high permeability. Likewise, the uniform and small particle could offer a high surface area for trypsin immobilization. Such a monolithic structure is helpful to improve mass transfer, rendering a high digestion rate for proteins after trypsin immobilization.

The flow-through properties were evaluated by the permeability constant (K) of monolith [23]. Three different solvents were pumped through the poly (AAm-*co*-MBA) monolith at different flow rates. K was calculated according to the following equation:

$$K = \frac{F\eta L}{\pi\gamma^2 \Delta P}$$

(where F is the flow rate of mobile phase; η is the viscosity of mobile phase; L is the effective length of monolith; γ is the inner radius of monolith; and ΔP is the pressure drop of monolith). Table 1 lists

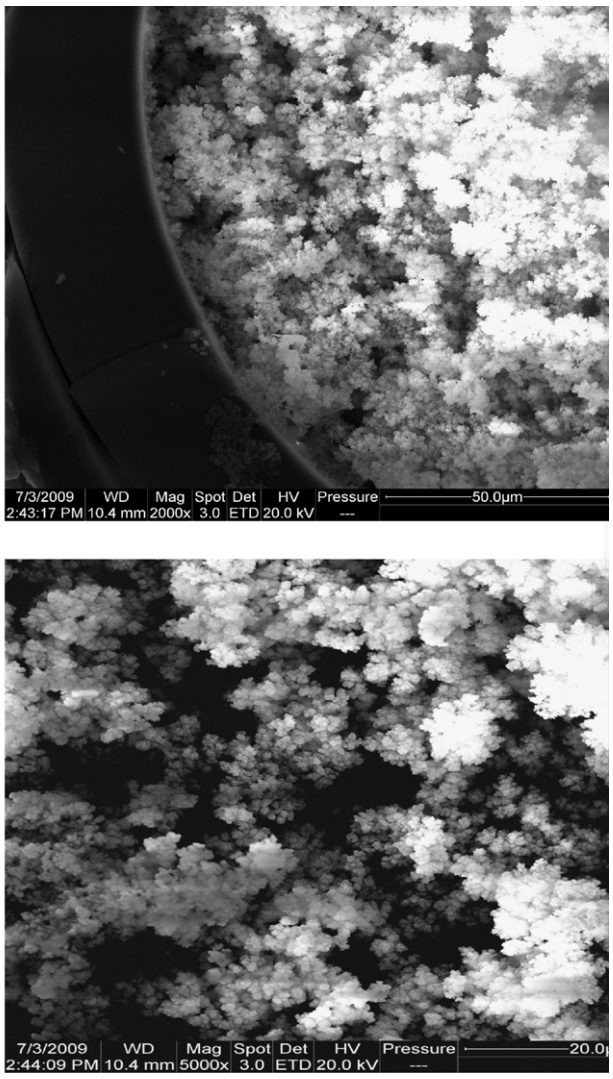

Fig. 1. SEM images of the poly (AAm-*co*-MBA) monolith in capillary. (A) ×2000 and (B) ×5000.

measured K in different polar solvents. Only slightly changed permeability was observed when different solvents passed through the poly (AAm-*co*-MBA) monolith, suggesting the less swelling or shrinkage. Noticeably, in contrast to the reported hydrophilic monolith [23], the K value of poly (AAm-*co*-MBA) monolith was about 10 times up to 2.65×10^{-13} m^2 using methanol as the mobile phase, and was also better than other polymer monolith prepared for trypsin immobilization, such as the polymethacrylate monolith [17,23]. Probably the proper porogen system is the primary reason for its excellent permeability. The relationship between flow rate and back-pressure (Fig. 2) clearly demonstrated that the poly (AAm-*co*-MBA) monolith was mechanically stable even at the

Table 1
Permeability of the poly (AAm-*co*-MBA) monolith.[a]

Mobile phase	Relative polarity	Viscosity η [$\times 10^{-3}$ Pa s]	Permeability K [$\times 10^{-13}$ m^2]
Acetonitrile	0.460	0.35	3.31
Methanol	0.762	0.544	2.65
Water	1	0.89	2.27

[a] Relative polarity data were obtained from http://virtual.yosemite.cc.ca.us/smurov/orgsoltab.htm;the experimental temperature ranged from 24 to 25 °C; poly (AAm-*co*-MBA) monolith: 39 cm, 250 μm. i.d.

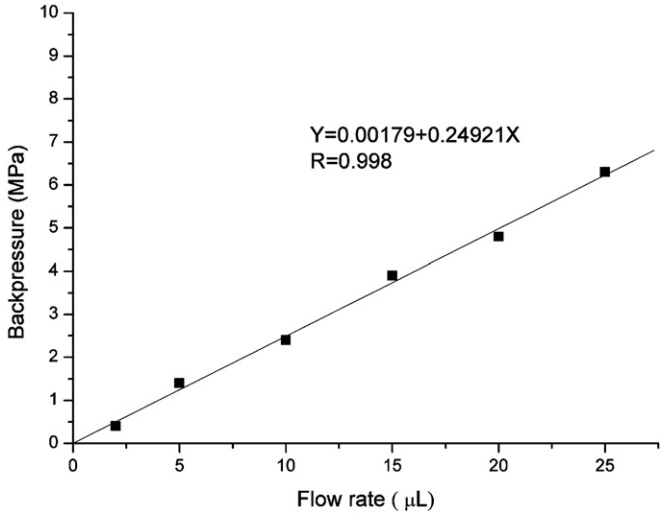

Fig. 2. Effect of flow rate to back-pressure. Condition: mobile phase, methanol. Experimental conditions are the same as described in Table 1.

flow rate of 25 μL/min, corresponding to only 6 MPa back-pressure when pure menthol passed through the column. Moreover, this poly (AAm-*co*-MBA) monolith could withstand up to 25 MPa without any structure damage or shelling off from the capillary wall. Therefore, the mechanical strength of prepared poly (AAm-*co*-MBA) monolith could meet a long term and continuous use after trypsin immobilization.

An adsorption and elution assay was designed to characterize the nonspecific adsorption on the poly (AAm-*co*-MBA) monolith. As presented in Fig. 3, in contrast to the MALDI-TOF MS spectrum of protein mixture, no obvious protein peaks were observed from MALDI-TOF MS spectra corresponding to samples derived from the rinse of poly (AAm-*co*-MBA) monolith via the buffer solution containing 5% or 80% ACN. The water soluble monomers AAm and MBA, which are neutral molecules and thus less interaction might occur between the supporting material and proteins, might be the main reason for this phenomenon.

3.2. Digestion ability

The amount of immobilized trypsin plays an important role in the digestion ability of IMERs. It was found that nearly 29 μg trypsin was immobilized on 1 μL of poly (AAm-*co*-MBA) monolith material, which is comparable to the poly (NAS-*co*-AAm-*co*-EDMA) monolith-based IMER previously reported by our lab [20], rendering high digestion efficacy. Theoretically, the more enzymes immobilized, the higher digestion rate would be anticipated. However, it should be noted that, besides immobilization amount, immobilization methods and the properties of supporting materials (e.g., the permeability and hydrophilicity) might also affect the digestion ability of IMERs.

BSA, a globular protein containing 607 amino acids, 85 tryptic cleavage sites and large number of disulfide bridges is difficult to digest. Fig. 4 demonstrates MALDI-TOF MS spectra of BSA digested by the poly (AAm-*co*-MBA) monolith based IMER and in-solution. As shown, 20 (in-solution) and 36 (poly (AAm-*co*-MBA) monolith based IMER) tryptic peptides were identified confidently via the MASCOT software, corresponding amino acid sequence coverage of 42% and 58%. Meanwhile, BSA digestion time was shortened from 12 h to 20 s. It should be noted that, we also prepared an

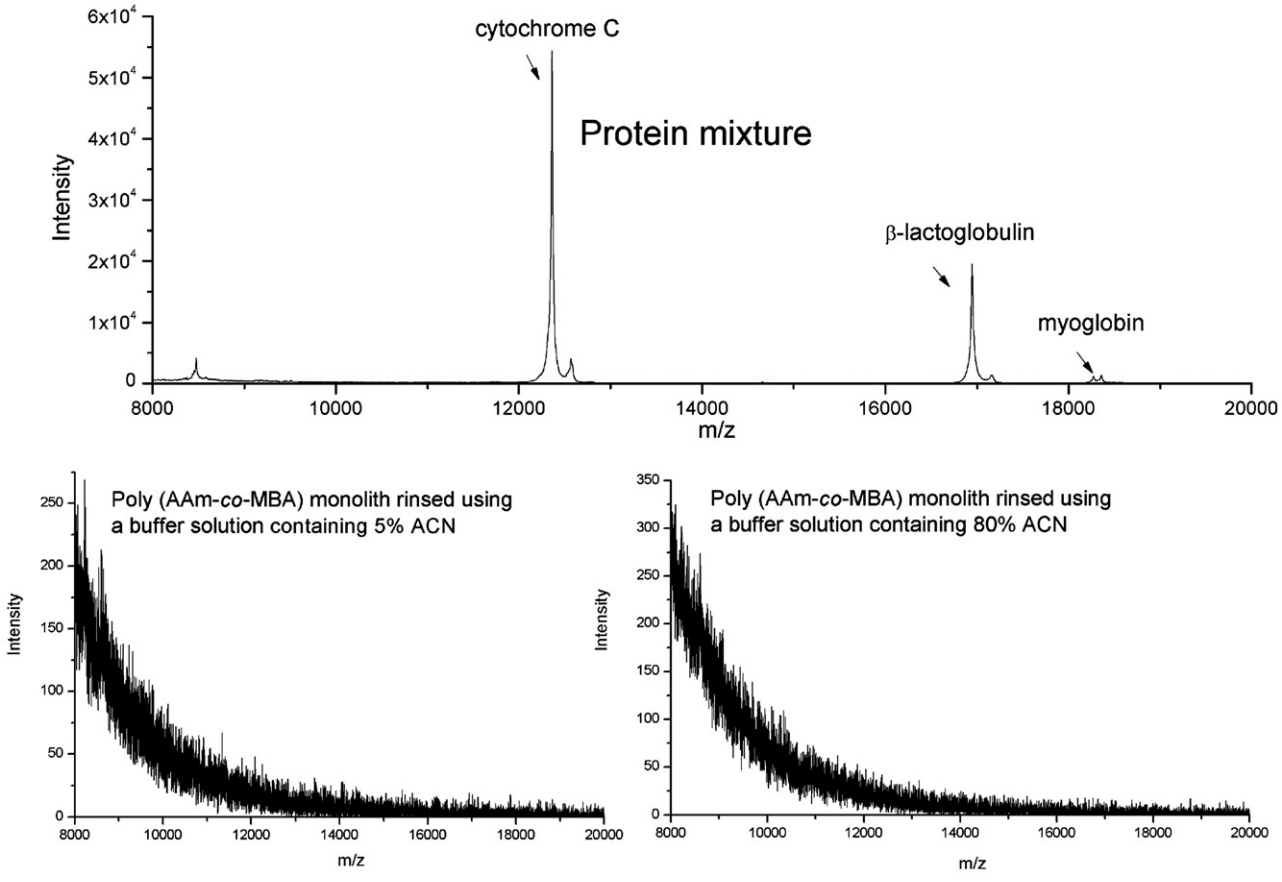

Fig. 3. Assay of protein nonspecific adsorption on the poly (AAm-*co*-MBA) monolith.

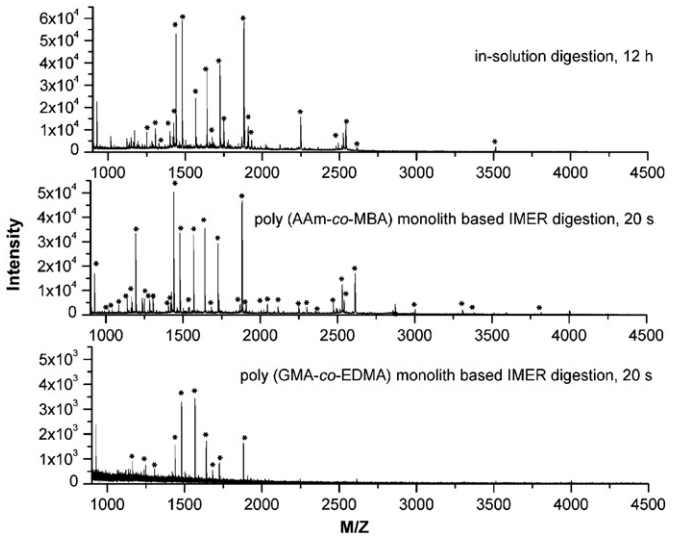

Fig. 4. MALDI-TOF MS spectra of BSA tryptic peptides obtained from three approaches. Sample: 700 fmol BSA; IMER: 100 μm i.d., 5 cm; 37 °C; digestion flow rate: 1.2 μL/min. Specific conditions for in-solution digestion were described in Section 2.

Table 2
Database searching results of proteins digested by IMER and in-solution.[a]

Protein	p*I*	Mw (kDa)	Sequence coverage (%)		Unique peptides	
			IMER	In-solution	IMER	In-solution
BSA	5.82	69	58	42	36	20
β-Lactoglobulin	4.76	18	60	64	7	7
Myoglobin	7.36	17	79	61	14	10

[a] Specific proteolysis conditions were same as described in Fig. 4.

IMER supported on poly (GMA-*co*-EDMA) monolith as a control, according to the preparation procedure suggested by Ref. [18]. In comparison to the poly (AAm-*co*-MBA) monolith based IMER, this IMER demonstrated poor digestion ability with the large protein BSA. As presented in Fig. 4, there were only several peptides which could only be identified via MS/MS analysis, and assigned as tryptic peptides of BSA. It was mainly ascribed to the low efficiency of trypsin immobilization via epoxide groups and existent

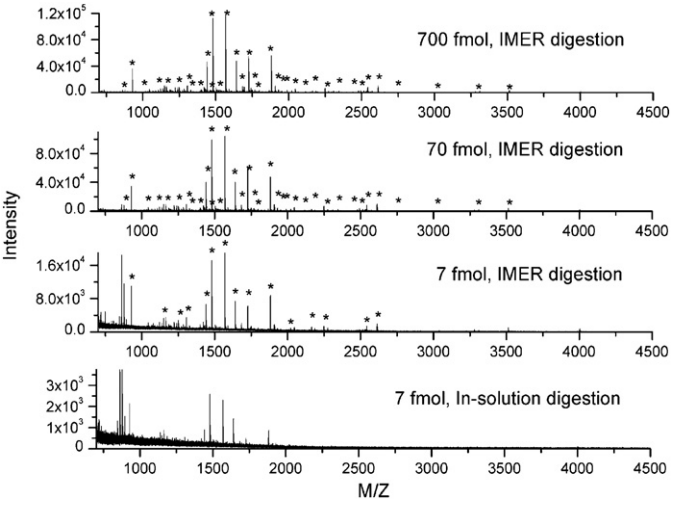

Fig. 5. MALDI-TOF MS spectra of BSA via poly (AAm-*co*-MBA) monolith based IMER and in-solution digestion in different amounts. IMER: 100 μm i.d., 5 cm; 37 °C; digestion flow rate: 1.2 μL/min.

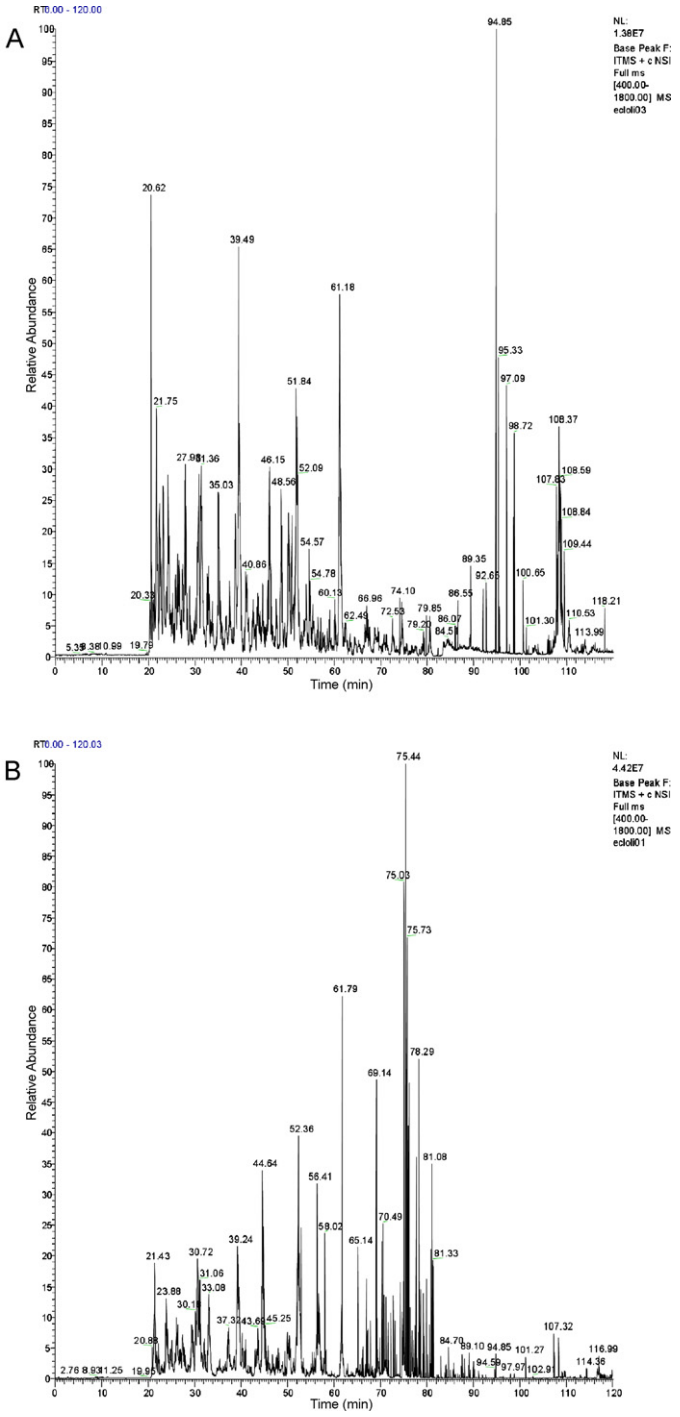

Fig. 6. Base peak chromatograms of 2 μg *E. coli* proteins digested by the poly (AAm-*co*-MBA) monolith based IMER (A) and in-solution digestion (B). IMER: 100 μm i.d., 5 cm; 37 °C; digestion flow rate: 1.2 μL/min.

nonspecific adsorption of proteins and peptides [18]. Moreover, in comparison with some of the previous work [15–18] in which a digestion time of minutes was used, even less digestion time was used herein, better or comparable result concerning BSA digestion was achieved, further indicating superior digestion performance of the poly (AAm-*co*-MBA) based IMER. Considering the column-to-column digestion reproducibility of IMER, the relative standard deviation was 10.1% ($n = 3$) with BSA as the standard, and the average sequence coverage was up to 57%. The digestion efficiency was further evaluated by digesting three proteins (BSA, β-lactoglobulin

and myoglobin) ($n = 3$). As can be seen from Table 2, in comparison to in-solution digestion, equal or even higher sequence coverage were achieved via IMER digestion within 20 s, demonstrating superior digestion performance of the poly (AAm-*co*-MBA) monolith based IMER.

IMER digestion is affected by the concentration of substrates as well. Therefore, BSA samples with different concentrations were digested by the IMER with the digests detected by MALDI-TOF MS (Fig. 5). It was found that even a low amount down to 7 fmol (BSA) was positively identified, yielding the 35% sequence coverage (15 peptides matched). In contrast, there were not peptides identified confidently from 7 fmol BSA via in-solution tryptic digestion which took about 12 h. These results demonstrate that, in comparison to the traditional in-solution digestion, the IMER can provide more effective digestion for low concentration protein samples.

3.3. Real sample analysis

To investigate the digestion performance of this poly (AAm-*co*-MBA) monolith based IMER for complex samples; proteins extracted from *E. coli* were digested. Then tryptic peptides were subjected to a nanoRPLC–ESI-MS/MS system for analysis. Corresponding base peak chromatogram was illustrated in Fig. 6A. After a related database search via the Sequest software, 2362 peptides (1163 unique peptides), corresponding to 333 proteins, were confidently assigned from the poly (AAm-*co*-MBA) monolith based IMER with a digestion time of 20 s. For comparison, the same sample was also digested via in-solution and analyzed by the nanoRPLC–ESI-MS/MS system subsequently (Fig. 6B). A total of 2580 peptides (1251 unique peptides), corresponding to 411 proteins, were obtained from in-solution digestion of 24 h. Although the number of identified proteins via in-solution digestion was slightly higher than that of IMER digestion, there were only 28% proteins identified on the basis of one peptide via IMER digestion, compared to 33% via in-solution digestion. These results show that IMER digestion is comparable to the traditional in-solution digestion, while the digestion time is largely shortened from 24 h to 20 s. In addition, the overlap of identified proteins via both approaches was over 80%, revealing that the poly (AAm-*co*-MBA) monolith based IMER digestion might be a promising alternative even for the digestion of complex samples.

4. Conclusion

A poly (AAm-*co*-MBA) monolith with good mechanical stability, permeability and hydrophilicity was used for trypsin immobilization. Digestion results of BSA and proteins extracted from *E. coli* showed that an efficient protein digestion could be achieved within 20 s. Moreover, the IMER digestion was successful even with a concentration of femtomole. The high efficient protein digestion and confident identification displayed the potential application of this IMER in proteomics research. Such IMER coupled with nanoRPLC–ESI-MS/MS analysis to set up an on-line high throughput platform is underway for proteome profiling study.

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