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# Novel monolithic enzymatic microreactor based on single-enzyme nanoparticles for highly efficient proteolysis and its application in multidimensional liquid chromatography

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ABSTRACT

In this work, a novel and facile monolithic enzymatic microreactor was prepared in the fused-silica capillary via a two-step procedure including surface acryloylation and in situ aqueous polymerization/immobilization to encapsulate a single enzyme, and its application to fast protein digestion through a direct matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) analysis was demonstrated. At first, vinyl groups on the protein surface were generated by a mild acryloylation with *N*-acryloxysuccinimide in alkali buffer. Then, acryloylated enzyme was encapsulated into polyacrylates by free-radical copolymerization with acrylamide as the monomer, *N*,*N*′-methylenebisacrylamide as the cross-linker, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine/ammonium persulfate as the initiator. Finally, polymers were immobilized onto the activated inner wall of capillaries via the reaction of vinyl groups. Capability of the enzyme-immobilized monolithic microreactor was demonstrated by myoglobin and bovine serum albumin as model proteins. The digestion products were characterized using MALDI-TOF-MS with sequence coverage of 94% and 29% observed. This microreactor was also applied to the analysis of fractions through two-dimensional separation of weak anion exchange/reversed-phase liquid chromatography of human liver extract. After a database search, 16 unique peptides corresponding to 3 proteins were identified when two RPLC fractions of human liver extract were digested by the microreactor. This opens a route for its future application in top–down proteomic analysis.



## 1. Introduction

Following genomics, more and more research attention has been paid to proteomics. One of its most important tasks is to develop efficient and rapid approaches to identify various proteins [1]. Proteolysis is the key step for positive sequencing in proteomics research integrated with MALDI-TOF-MS. The conventional techniques of in-solution digestion of proteins offer limited sensitivity and are time-consuming procedures, affecting severely the determination of comprehensive proteomic profiles [2,3].

To solve this problem, immobilized enzyme has been widely utilized owing to their advantage of allowing the use of higher enzyme concentrations that lead to shorter digestion time. Furthermore, the immobilized enzyme could be isolated and removed from the protein digests prior to MS easily, eliminating or reducing the influence of the enzyme fragments on MS results. Additionally, the stability of enzyme toward chemical denaturants and organic solvents could be enhanced when immobilized on the solid supports [4–7].

In recent years, several reports have demonstrated the feasibility of protein digestion using enzyme immobilized on various supports, such as glass [8], membrane [9,10], polymer [11], gel beads [12,13], sol–gel supports [14,15], porous silicon matrix [16–18], and porous monolithic materials [19–21]. These coating materials, however, may hinder the conformational transition of enzyme and the transport of substrate, resulting in low biocatalytic activity.

Porous polymer monolithic supports have been recently introduced as novel materials, in which the diffusion resistance during mass transfer has been proved small [22–25]. Porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith is one of the popular materials that have been used as enzymatic reactor carrier[19,25]. However, the activity of epoxide groups towards the amino groups is relatively poor. Therefore, the further modification of the epoxide groups on the supports is usually necessary, resulting in long time preparation process and poor reproducibility. Recently, synthesized in the work of our group, those trypsin-immobilized magnetic materials were utilized for proteolysis by in-tube digestion [31], on-plate digestion [41] and microwave-assisted digestion

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[42,43]. These proteolysis using functional magnetic microspheres show some advantages such as easy isolation and low cost. However, proteins were digested using off-line mode, which results in a complicated and time-consuming procedure. Monolithic enzymatic microreactor offers the opportunity for on-line protein digestion.

A promising solution is to fabricate a single-enzyme containing capsulate with a thin, permeable coating. Kim and Grate have fabricated enzyme nanoparticles via a multistep procedure including surface modification, lyophilization, polymerization in organic solvent, and shell condensation and obtained enhanced enzyme stability at an insignificant increase in mass transfer resistance [26]. Yan and coworkers have demonstrated a simple procedure to encapsulate enzyme nanoparticles in aqueous polymerization in nanogel [27], which show similar catalytic behavior to the free enzyme.

In this study, we initially demonstrated a simple, effective and versatile procedure to prepare monolithic enzymatic microreactor based on single-enzyme nanoparticles with enhanced stability, high activity, and uniformed size via surface acryloylation on enzyme following in situ polymerization and condensation. To confirm the possibility of applying the monolithic enzyme reactor for effective protein digestion, it was first utilized for peptide mapping analysis of myoglobin and bovine serum albumin with MALDI-TOF-MS and further applied to analyze the fractions from the multidimensional liquid chromatography of human liver extract.

## 2. Experimental

### 2.1. Chemicals

Fused-silica capillaries (250 μm I.D. × 375 μm O.D.) were obtained from Yongnian Optical Fiber Factory (Hebei, China). Acrylamide (99+%), *N*-acryloxysuccinimide (NAS) (99%), benzamidine hydrochloride hydrate (98%), *N*,*N*-methylenebisacrylamide (99%), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), poly(ethylene glycol) (PEG, MW 10 000), γ-methacryloxypropyl trimethoxysilane (γ-MAPS, 98%), Trypsin (bovine pancreas), myoglobin (MYO, horse heart), bovine serum albumin (BSA), phenylmethanesulphonylfluoride (PMSF), dithiothreitol (DTT), and trifluoroacetic acid (TFA) were ordered from Sigma–Aldrich (St. Louis, MO, USA). Sephadex G-25 column was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Acetonitrile was purchased from Mallinckrodt Baker (Phillipsburg, USA). Water was purified by a Milli-Q system (Millipore, Molsheim, France). Other chemicals were analytical-reagent grade, and all solvents were HPLC grade.

### 2.2. Preparation of monolithic enzymatic microreactor

The capillaries were first rinsed, respectively, by methanol, water, 0.1 M HCl, water, 1 M NaOH, 0.1 mol/L sodium hydroxide and methanol. Then, they were dried in an oven at a temperature of 120 °C for 1 h. After that, a 50% solution of γ-MAPS in methanol was poured into the channels and allowed to react at room temperature in the dark for 24 h, followed by washing with methanol and drying with nitrogen gas. With such a procedure, the monolithic supports could be covalently anchored to the activated inner wall of capillaries.

An aliquot of 10 mg of trypsin was freshly dissolved in 5 mL of pH 9.3, 100 mM boric acid buffer containing 0.5 M benzamidine. Then 2.5 mg of NAS dissolved in 0.3 mL of dimethyl sulfoxide (DMSO) was slowly added and the reaction was carried out for 3 h at 30 °C. The acryloylated trypsin was purified by gel filtration on Sephadex G-25 column and the volume was concentrated into 100 μL.

The polymerization mixture consists of 40 mg of acrylamide, 40 mg of *N*,*N*′-methylenebisacrylamide, and 60 mg of PEG per mL of 0.2 M sodium bicarbonate/0.5 M sodium chloride (pH ~ 8.0) buffer. The mixture was vortexed for a few seconds to completely dissolve the monomers. A 50 μL aliquot of this solution was removed and quickly mixed with 3 μL of acryloylated trypsin and vortexed. The radical polymerization from the surface of the acryloylated HRP was started by adding 0.4 μL of 5% APS and 0.1 μL of TEMED. An activated capillary (5 cm in length) was then inserted into the trypsin-monomer vial, whereby the solution filled the tubing by capillary force. Immobilization/polymerization was allowed to proceed for 40 min at room temperature with the capillary ends sealed with silicon rubbers.

### 2.3. Characterization

The scanning electron microscopy characterized the internal structure of monolith. The Fourier transform infrared (FT-IR) spectra of free trypsin, acryloylated trypsin, and polymerization were recorded on a Nexus 470 FT-IR instrument (Nicolet, New York, USA).

### 2.4. Assay of enzymatic activity

Activity of the enzymatic microreactor was studied using BAEE as substrate. BAEE solution (5–100 mM) in a 20 mM Tris buffer (pH 7.5) was pumped through the microreactor using a syringe pump (74900 series, Coleparmer, IL, USA) at a constant volumetric flow rate at 20 °C. The resulting products were analyzed by Capillary Electrophoresis System (Cailu CL3030, Beijing, China). The capillary electrophoresis was run in 20 mM Tris–HCl (pH 7.5) with UV detection at 214 nm. The reacted BAEE was determined by measuring the peak area of the substrate.

### 2.5. Digestion of standard proteins using monolithic enzymatic microreactor

MYO and BSA dissolved in 25 mM ammonium bicarbonate. Then, proteins were passed through the trypsin reactor at a constant flow rate using a syringe pump. The resulting peptide fragments were then pumped out of the reactor and deposited onto 192-well plate for MALDI-TOF-MS identification. For comparison, the digestions of MYO and BSA were also performed by free trypsin in solution according to the conventional procedure (trypsin/protein mass ratio 1:35, 37 °C, 14 h).

### 2.6. Extraction of liver tissue

The normal human liver specimens were offered by Liver Cancer Institute of Zhongshan Hospital, Fudan University. All manipulations in liver specimen collection were approved by the Institutional Bioethics Committee and informed consent was given by all participants. The liver specimen was diced and washed with cold physiological saline solution (0.9% NaCl solution) to remove blood and other possible contaminants. The tissue specimens were suspended in the soluble protein extraction buffer (1 mM PMSF, 50 mM DTT, 0.1% TFA and protease inhibitor cocktail dissolved in the lysis buffer according to the usage specification). Tissue sample was homogenized in an ice bath. The resulting homogenate was swirled for 30 min and centrifuged for 15 min at 18 000 × *g*. The supernatant was collected. Protein concentration of the sample was measured by the Bradford assay, using BSA as standard [28].

### 2.7. WAX/RPLC separation of liver extract

Liquid chromatography separation was performed with a LC-2010A system and fractions were collected using FRC-10A fraction

collector combined with a SCL-10A controller (Shimadzu, Kyoto, Japan). Shimadzu Class-VP station was used to acquire and process data. The separation procedure was fulfilled in 25 °C.

Protein sample of 2 mg was loaded onto a WAX column (75 mm × 7.5 mm, TSK-gel, DEAE-5-PW, Tosoh, Tokyo, Japan) preceded by a TSKguardgel DEAE-5-PW (Tosoh). The buffer solutions used were 10 mM Tris–HCl (pH 7.5, buffer A) and 10 mM Tris–HCl/500 mM sodium chloride (pH 7.5, buffer B). A linear gradient elution had the following profile: 15 min of 100% buffer A, 80 min linear gradient from 0% to 30% buffer B, then 20 min linear gradient from 30% to 100% buffer B, 5 min of 100% buffer B and 5 min back to 0% buffer B. The flow rate is 0.5 mL/min. The chromatograms were monitored at 215 nm. Fractions were collected every 2 min automatically from 3 to 125 min, and 61 fractions were obtained. All fractions were lyophilized and concentrated. Then the dried fractions were dissolved in suitable water, following the second dimensional separation using RPLC (250 mm × 4.6 mm, 5 μm, 300 Å, C18, Hypersil, eliteHPLC, Dalian, China) preceded by a 20 mm × 4.6 mm guard column. The mobile phases consisted of buffer C: 0.1% TFA in water and buffer D: 0.1% TFA in acetonitrile. The gradient contained 5 min of 100% buffer C, 55 min linear gradient from 0% to 30% buffer D, 10 min linear gradient from 30% to 45% buffer D, then 10 min linear gradient from 45% to 80% buffer D, and 5 min of 80% buffer D, after 2 min back to 0% D. The flow rate is 0.8 mL/min. All fractions were collected every 1.5 min automatically, and then lyophilized further. The dried fractions were reconstituted in 25 mM of $NH_4HCO_3$ solution (pH 8.0) and injected into microreactor to carry on tryptic digestion. The resulting peptide fragments were then pumped out of the channel and collected in the reservoir for MALDI-TOF-MS identification.

### 2.8. MALDI-TOF-MS process

Sample solutions were deposited on the MALDI target using dried droplet method. A total of 0.5 μL of sample solution was spotted onto the MALDI plate, and then another 0.5 μL of CHCA matrix solution (5 mg/mL and 0.1% TFA in 50% acetonitrile aqueous solution) was introduced. All MALDI-TOF-MS measurements were performed in positive ion mode using a 4700 proteomics analyzer (Applied Biosystems, Framingham, MA, USA). Sample desorption was achieved using an Nd:YAG laser (355 nm) operated at a repetition rate of 200 Hz and acceleration voltage of 20 kV. GPS Explorer software obtained from Applied Biosystems with MASCOT (Matrix Sciences, London, UK) as a search engine and NCBI as a database was used to identify proteins based on peptide mass spectra. The mass calibration was done externally on the target using a myoglobin digest peptide.

## 3. Results and discussion

### 3.1. The preparation and characterization of monolithic microreactor based on single-enzyme nanoparticles

Epoxy-activated carriers are the most often used enzyme supports, because of their easy fabrication and modification as well as relatively low cost [29]. They can be used directly for enzyme coupling primarily via protein amino groups in buffer, or the epoxy groups can be modified by a variety of different reagents. However, the direct reactions between oxirane groups and amino groups are slower. Therefore, to increase the amount of enzyme immobilized on the supports, high temperature and long reaction time have to be used, leading to the impairment of enzymatic activity. To overcome such problems, glutaraldehyde was often used to couple the support with enzyme [30,31]. Its reactions with protein amino groups could be performed easily with fast speed even under an ambient condition. Reducing the C=N bonds to CN by sodium borohydride improved the stability of immobilized enzyme. However, the reaction between amino groups and aldehyde group is very severe so that enzymatic activity is reduced greatly.

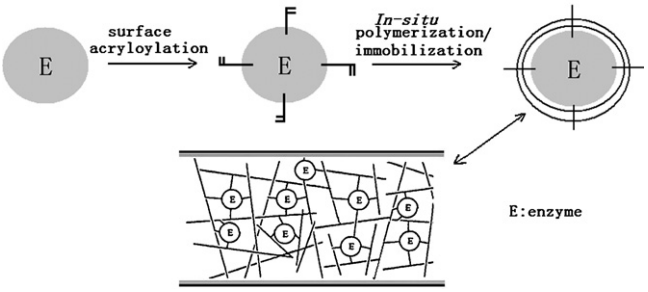


**Fig. 1.** Schematic illustration of encapsulation of single enzyme with a network surface structure and immobilization in the capillary.

To meet the challenges, in this study, NAS was finally selected to immobilize protein by generating vinyl groups on protein surface, followed by in situ polymerization and immobilization that cross-link the attached polymer chains (Fig. 1) into a network around the enzyme.

The reaction between NAS and proteins in alkali is mild and rapid. This kind of monolithic reactor offers high flow permeability as well as biocompatibility. Fig. 2 shows FT-IR spectra of free trypsin, acryloylated trypsin and polymerization of trypsin with monomers, which confirmed the formation of a polymer on trypsin [27]. Fig. 3 shows the scanning electron microscopy images of the internal morphologies of monolith, which demonstrates that the porous polymer was homogeneous across the entire monolith. The macroporous structure showed on the image could result in low backpressure and high throughput.

The reproducibility of fabricating the enzymatic microreactor was determined to be 5.1% relative standard deviation (RSD), as based on the activity of 10 subsequently produced reactors. It is obviously important to have consistent polymerization conditions. The RSD could eventually be further decreased by flushing with $N_2$ gas also during monomer-acryloylated trypsin mixing, and by the use of a controlled temperature environment during polymerization.

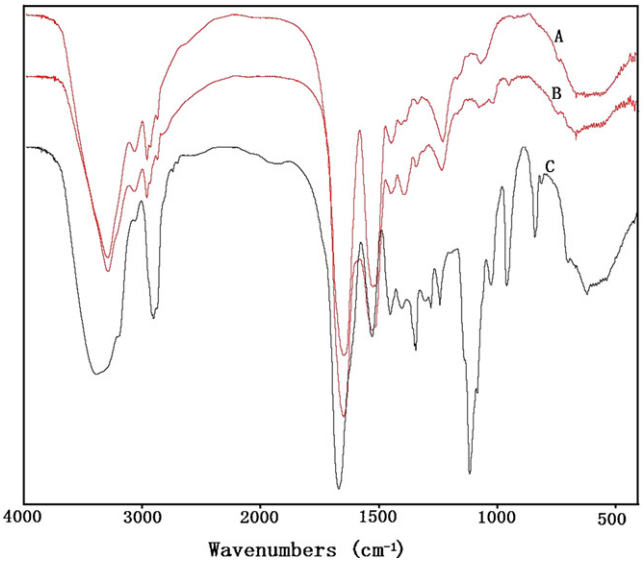


**Fig. 2.** FT-IR spectra of free trypsin (A), acryloylated trypsin (B) and polymerization of trypsin with monomers (C).

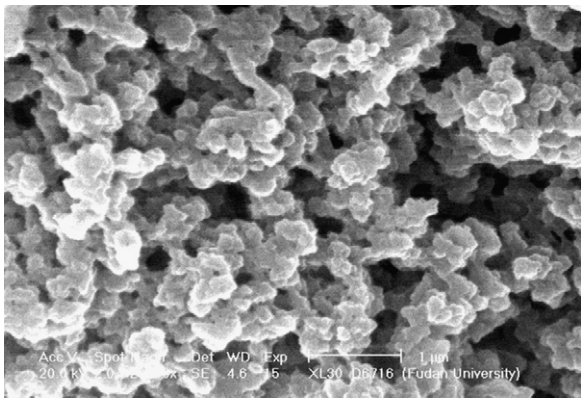


**Fig. 3.** SEM micrographs of internal structure of monolith.

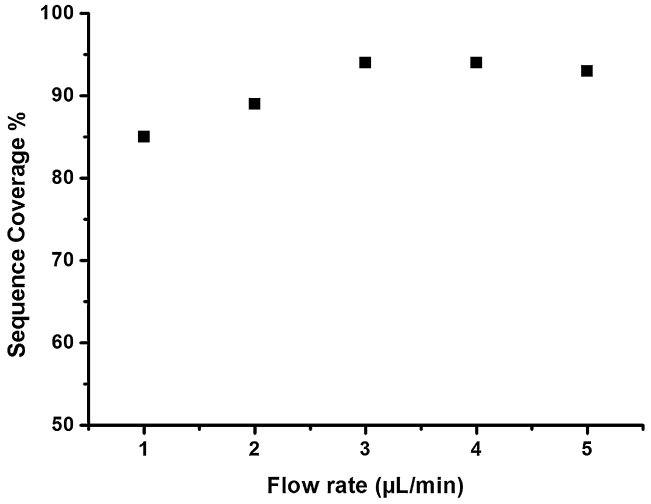


**Fig. 5.** The effect of flow rate on the digestion efficiency. Experimental conditions: MYO, 0.1 mg/mL, flow rates: 1–5 μL/min, MALDI-TOF-MS analysis.

### 3.2. Activities of enzymatic microreactors

The values of the Michaelis constant ($K_m$) and the maximum velocity ($V_{max}$) are investigated to examine the enzymatic bioactivity of trypsin immobilized in the microreactor using BAEE as substrate [44]. Fig. 4 shows the electropherogram of digestion products of BAEE. The most commonly used transformation is the doublereciprocal or Lineweaver–Burke plot. The Lineweaver–Burk equation is

$$\frac{1}{\upsilon_0} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}.$$

The values of $K_m$ reflect the enzymatic affinities, and $V_{max}$ reflect the activity of the encapsulated trypsin in the microreactor. The calculation gives a corresponding $K_m$ as 81.3 mM with $V_{max}$ as 50.5 μmol/min, similar to those values reported previously [3]. The high proteolytic velocity might be attributed to the following reasons. The first reason is the high amount of trypsin immobilized in the monolithic microreactor, which reaches about 75 μg/mg. The aqueous prepared supports have the good hydrophilicity and the good biocompatibility of gel matrix provides milder environmental conditions to minimize denaturation of the encapsulated biomolecules.

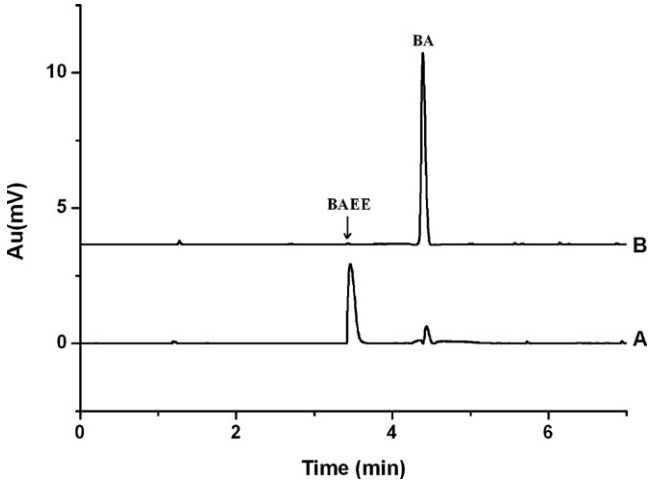


**Fig. 4.** Electropherograms of digestion products of BAEE. Experimental conditions: 10 mM BAEE (20 mM Tris–HCl, pH 7.5); 20 mM Tris–HCl (pH 7.5), 15 kV, 0.5 psi, 10 s, 20 °C, 214 nm.

### 3.3. Application of the monolithic enzymatic microreactor for digestion

Two standard proteins, MYO (a protein known to be rather resistant to proteolysis, MW 16 900) and BSA (MW 66 000) (0.1 mg/mL for each protein) were used to test the performance of trypsin-immobilized monolithic microreactor. For comparison, the digestions of MYO and BSA were also performed by free trypsin in solution for 14 h.

In this kind of monolithic enzymatic microreactor, individual enzyme molecules are stabilized within a hydrophilic network by using acrylamide and *N*,*N*-methylenebisacrylamide as co-monomer and PEG as porogenic reagent. The structure is sufficiently porous and hydrophilic to allow substrates to access the active site. A key result is that stabilization of the activity was achieved with minimal substrate mass-transfer limitation. Fig. 5 shows the effect of flow rate on the proteolytic efficiency of tryptic monolithic microreactor. The volumetric flow rates were 1, 2, 3, 4, 5 μL/min, corresponding to residence times of 147, 74, 49, 37, 30 s, respectively. As demonstrated, the protein digestion efficiency increases with the flow rate of up to 3 μL/min, which is presumably attributed to the increase of the mass transfer of the substrate towards the fixed enzyme molecules and the reduce of the stagnant diffusive solvent layer at the monolithic materials.

The stability toward organic solvents was one of the advantages for immobilized enzyme monolithic reactor [4–7]. Additionally, it is known that organic solvents affect the overall structure of proteins [32,33], making them more accessible to tryptic proteolysis. While we have not optimized the type and amount of the organic solvent, we found 20% acetonitrile to be more effective than buffer alone, and the digestion efficiency had no obvious impairment with the ratio of acetonitrile close to 80%. The stability may be due to, on one hand, the hydrophilic groups in the polyacrylamide nanogels that retain a hydrophilic environment, that is, resembling the "essential water" that enables trypsin to display its biological function. The stripping of the essential water by the polar solvents leads to the denaturation of the free enzyme [34,35]. On the other hand, the multiple covalent attachments to the polyacrylamide the shell strengthen the structure of the encapsulated trypsin.

Fig. 6A and B displays the mass spectra of tryptic fragments using monolithic enzymatic microreactor. Many digest fragments were observed from the MS spectra. Detailed identification results were listed in Table 1. The observation corresponded to the detection of fragments containing 148 out of the 153 possible amino

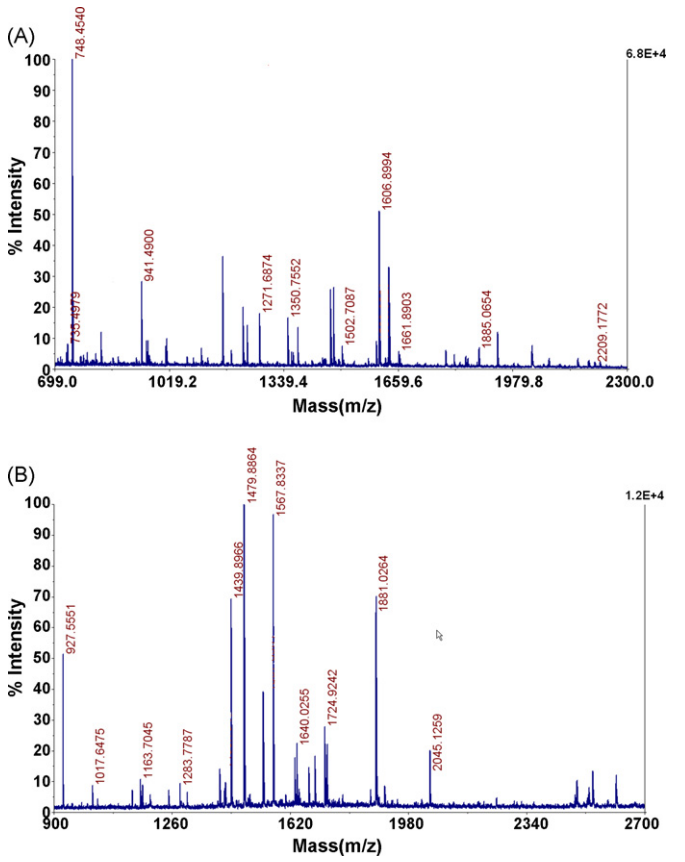


**Fig. 6.** MALDI-TOF-MS spectra of tryptic peptides originated from (A) MYO, (B) BSA digested with monolithic enzymatic microreactor. Experimental conditions: 0.1 mg/mL MYO and BSA, 25 mM $NH_4HCO_3$, pH 8.0.

acids of MYO and 202 out of the 583 possible amino acids of BSA. The sequence coverages of 94% for MYO and 29% for BSA from the database were obtained. The identification results are comparable with or even better than those by in-solution digestion that required a complicated denaturing procedure and a reaction time of 14 h (Table 2). Meanwhile, the applicable protein concentration range for the microreactor was tested by hydrolyzing MYO, which can be digested well from 1 ng/μL to10 μg/μL. This kind of preparation procedure of monolithic enzymatic reactor with mild surface acryloylation and in situ aqueous polymerization offered high flow permeability as well as biocompatibility, which results in satisfying proteolytic efficiency.

The trypsin-immobilized monolithic microreactors were stored at 4 °C to test the stability of the immobilized enzyme. The proteolytic reaction was done repeatedly with the microreactor during 2 weeks' storage. Similar identification results witnessed by MALDI-TOF-MS demonstrated that the stability of enzyme molecules and their bioactivity were preserved well. To further test the protein residue on matrix, the trypsin-immobilized monolithic microreactor was washed with 25 mM $NH_4HCO_3$ mixed with 50% acetonitrile buffer solution after MYO digestion. The resulted products were thereafter analyzed by MALDI-TOF-MS. It can be found that there are no obvious signal peaks of tryptic peptides after 98 s residence times at the flow rate of 3 μL/min (about two times of column volume). These results show that the trypsin-immobilized monolithic microreactor has good stability and low residue.

**Table 1**
MALDI-TOF-MS results obtained from the digestion with monolithic enzymatic microreactor compared with 14-h in-solution digestion.

| | MYO | | BSA | |
|---|---|---|---|---|
| | Microreactor | In-solution | Microreactor | In-solution |
| Amino acids identified | 148 | 115 | 202 | 253 |
| Sequence coverage (%) | 94 | 75 | 29 | 41 |
| Peptide matched | 16 | 11 | 19 | 24 |

**Table 2**
Identified peptide fragments digested by monolithic enzymatic microreactor.

| Myo | | BSA | |
|---|---|---|---|
| Position | Peptide resides | Position | Peptide resides |
| 51–56 | TEAEMK | 212–218 | VLTSSAR |
| 97–102 | HKIPIK | 161–167 | YLYEIAR |
| 134–139 | ALELFR | 212–220 | VLTSSARQR |
| 57–63 | ASEDLKK | 223–232 | CASIQKFGER |
| 146–153 | YKELGFQG | 66–75 | LVNELTEFAK |
| 32–42 | LFTGHPETLEK | 25–34 | DTHKSEIAHR |
| 134–145 | ALELFRNDIAAK | 35–44 | FKDLGEEHFK |
| 64–77 | HGTVVLTALGGILK | 361–371 | HPEYAVSVLLR |
| 119–133 | HPGDFGADAQGAMTK | 402–412 | HLVDEPQNLIK |
| 64–78 | HGTVVLTALGGILKK | 360–371 | RHPEYAVSVLLR |
| 17–31 | VEADIAGHGQEVLIR | 421–433 | LGEYGFQNALIVR |
| 32–45 | LFTGHPETLEKFDK | 347–359 | DAFLGSFLYEYSR |
| 1–16 | GLSDGEWQQVLNVWGK | 184–197 | YNGVFQECCQAEDK |
| 80–96 | GHHEAELKPLAQSHATK | 437–451 | KVPQVSTPTLVEVSR |
| 103–118 | YLEFISDAIIHVLHSK | 281–297 | ADLAKYICDNQDTISSK |
| 79–96 | KGHHEAELKPLAQSHATK | 168–183 | RHPYFYAPELLYYANK |
| | | 300–309 | ECCDKPLLEK |
| | | 452–459 | SLGKVGTR |
| | | 460–468 | CCTKPESER |

### 3.4. Digestion of human liver extract

Studies show that top–down strategy using intact protein based multidimensional chromatographic separation followed with mass spectrometric identification offered an optional strategy to analyze complex protein mixtures [36–40]. The top–down methods provide purified protein for subsequent identification steps. Protein fragmentation should be encompassed in order to generate sequence information and ultimately identification by mass spectrometry. Thus, our monolithic enzymatic microreactor based on single-enzyme encapsulation provides a facile way to solve the problem of proteolysis.

Herein, to demonstrate the applicability of the monolithic microreactor in top–down proteome analysis, it was applied to WAX/RPLC fractions of liver extract, and the resulted digests were analyzed by MALDI-TOF-MS. The WAX chromatogram of the liver extract was shown in Fig. 7A. The fractions through WAX were collected in 2.0 min intervals from 3 to 125 min during the run. Fraction 21 (45–47 min, indicated with arrow in Fig. 7A) was selected randomly and separated further by second dimensional RPLC. The sub-fractions through RPLC were collected in 1.5 min intervals and then lyophilized. The dried fractions were reconstituted in 25 mM of $NH_4HCO_3$ solution (pH 8.0) and injected into microreactor to carry on tryptic digestion. The resulting peptide fragments were then collected and analyzed by MALDI-TOF-MS/MS. The RPLC fractions collected from the interval of 66–67.5 and 70.5–72 min (Nos. 43 and 46 indicated with arrow in, Fig. 7B, respectively) were analyzed as a model. Numbers of peaks were observed from the mass spectrum of the resulted digests (data not shown), which indicates that the protein mixture was efficiently digested in the monolithic enzymatic microreactor. The searches were performed against the NCBI database using combined PMF and MS/MS method, allowing for one missed cleavage. Peptide mass spectra were collected automatically with an acquisition mass range of 700–3500 *m*/*z*. External standards were used to achieve a mass accuracy of 100 ppm. Table 3 lists the proteins identified from the RPLC fraction Nos. 43 and 46. According to the results, three unique proteins with protein score more than 59 were identified. The results demonstrated the high separa-

**Table 3**
Proteins identified from the WAX/RPLC fraction Nos. 43 and 46 with tryptic digestion by monolithic enzymatic microreactor followed by MALDI-TOF-MS/MS.

| No | Protein name | Accession no | Protein MW | Protein PI | Peptide count | Protein score | Sequence coverage |
|---|---|---|---|---|---|---|---|
| 43 | Glutathione S-transferase A3 | gi\|951352 | 25883.8 | 9.2 | 3 | 76 | 10% |
| | Alpha-class glutathione S-transferase omega 2 subunit | gi\|265545 | 19624.2 | 5.5 | 1 | 68 | 7% |
| 46 | Keratin 10 | gi\|186629 | 39718.0 | 4.72 | 12 | 138 | 23% |

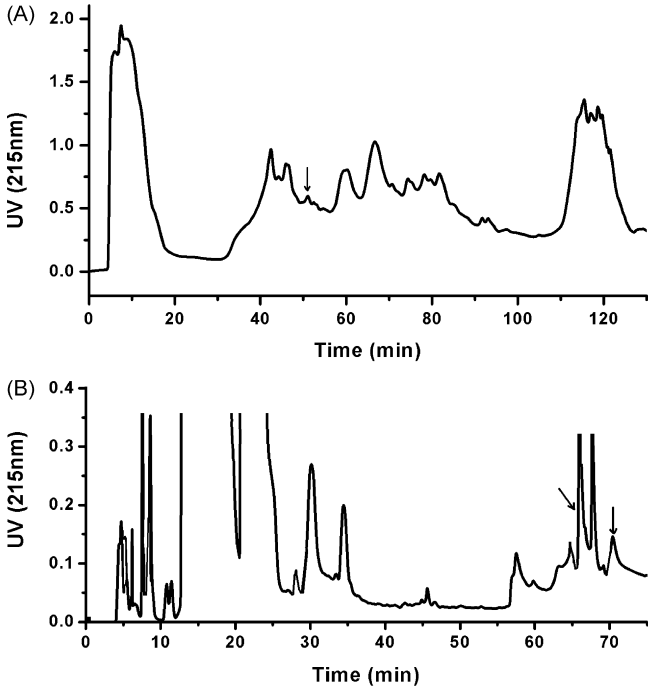


**Fig. 7.** (A) Separation chromatogram of human liver tissue extract by 1D WAX. (B) Panel B shows the RPLC elution profile of WAX Fraction 21. Experimental conditions: 1D WAX conditions: 75 mm × 7.5 mm, flow rate: 0.5 mL/min, mobile phase: (A)10 mM Tris–HCl (pH 7.5), (B) 10 mM Tris–HCl/500 mM sodium chloride (pH 7.5). gradient: 15 min, 100% A; 80 min, 30% B; 20 min, 100% B; 5 min, 100% B. 2D RPLC conditions: 250 mm × 4.6 mm, flow rate: 0.8 mL/min, mobile phases: (C) 0.1% TFA in water, (D) 0.1% TFA in acetonitrile, gradient: 5 min, 100% C; 55 min, 30% D; 10 min 45% D; 10 min, 80% D; 5 min, 80% D; 2 min, 0% D.

tion capacity of two-dimensional WAX/RPLC system and admirable digestion efficiency of our monolithic enzymatic microreactor. This proved the method's promising potential for analysis and application in proteomics.

## 4. Conclusion

We demonstrated a simple and efficient method to prepare monolithic enzymatic microreactor by modifying individual enzymes with networked surface structures, following in situ aqueous polymerization/immobilization to encapsulate a single enzyme into capillary. Compared with the free trypsin, our monolithic enzymatic microreactor has demonstrated the desirable characteristics of flow permeability, biocompatibility, stability toward organic solvents and rapid digestion efficiency with the decrease of mass transfer limitation. The proteolytic efficiency was further demonstrated in off-line mode by the digestion of WAX/RPLC fractions of a real proteome sample, the human liver extract. Further investigation of our monolithic enzymatic reactor coupled with micro- or nano-HPLC for on-line protein digestion, separation and identification is being carried out in our laboratory.

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