Vinyl functionalized silica hybrid monolith-based trypsin microreactor for on line digestion and separation via thiol-ene “click” strategy

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A B S T R A C T
A novel thiol-ene “click” strategy for the preparation of monolithic trypsin microreactor was proposed. The hybrid organic–inorganic monolithic capillary column with enzyme functionality was fabricated by sol–gel process using tetramethoxysilane (TMOS) and γ-methacryloyloxypropyltrimethoxysilane (γ-MAPS) as precursors. The disulfide bonds of trypsin were reduced to form free thiol groups. Then the trypsin containing free thiol groups was attached on the γ-MAPS hybrid monolithic column with ene-functionality via thiol-ene click chemistry to form a trypsin microreactor. The activity of the trypsin microreactor was characterized by detecting the substrate (Nε-p-tosyl-l-arginine methyl ester hydrochloride, TAME) and the product (Nε-p-tosyl-l-arginine, TA) with on-line capillary zone electrophoresis. After investigating various synthesizing conditions, it was found that the microreactor with poly(N,N'-methylenebisacrylamide) as spacer can deliver the highest activity, yielding a rapid reaction rate. After repeatedly sampling and analyzing for 100 times, the monolithic trypsin microreactor still remained 87.5% of its initial activity. It was demonstrated that thiol-ene “click” strategy for the construction of enzyme microreactor is a promising method for the highly selective immobilization of proteins under mild conditions, especially enzymes with free thiol radicals.

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1. Introduction
The reaction of thiols with enes carries many of the attributes of click reactions. These attributes include achieving rapid reaction rates with reactions occurring either in bulk or in environmentally benign solvents over a large concentration range, requiring essentially no clean up, being insensitive to ambient oxygen or water, and yielding a single regioselective product. Its exceptional versatility and propensity for the quantitative conversions under mild conditions makes the thiol–ene chemistry widely applicable in optical, biomedical, sensing, and bioorganic modification fields [1]. The ease of formation and reasonable reaction times under ambient conditions and insensitivity to the presence of water and oxygen have facilitated the application of the reaction in the biomaterials area [2–9].

To immobilize proteins, Waldman et al. coupled biotinylated protein to the thiolated surface via the photoinduced thiol-ene reaction. The density of protein which maintains its activity on the surface could be controlled by the exposure time [10]. Wittrock et al. developed a strategy to modify vaccines via thiol-ene click chemistry. An average of eight glycopolypeptides per BSA molecule was achieved by a “click” reaction of allyl-functionalized BSA core with thiolated glycopolypeptides [11].

Enzyme reactor is an useful tool for the inhibitor screening [12–15] and on-line proteolysis [16–18]. For the construction of enzyme microreactor, many methods and strategies were proposed [19–23]. One of the most useful materials for preparation of microreactor is monolith. They can be easily prepared by in situ polymerization in solutions. The convective mass transfer process can accelerate the diffusion of compounds, especially biopolymers, to access the monolith surface. Covalent immobilization of enzyme on a capillary monolithic column is an useful method to prepare enzyme microreactor, e.g., immobilization by genetic engineering [24], Staudinger ligation reaction [25], or azide-alkyne click reaction [26]. The immobilized trypsin microreactor by coupling with RPLC–MS/MS, CE and CE-MS has been applied for analysis of proteins and peptides [27–30]. Thiol-ene or thiol-acrylate

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polymerization was used in biological researches \([1,4,5,7]\). However, there is no report to prepare microreactor with thiol-ene reaction.

In this paper, with trypsin as a model enzyme, we have developed a new method (Fig. 1) to prepare monolithic enzyme microreactor via thiol-ene click chemistry. It was found that the thio-ene click strategy is feasible and simple. The microreactor can give high catalytic efficiency and stability. In contrast to azide-alkyne click reaction, where both the azides and alkynes groups must be firstly introduced in enzyme, which is complicate and bears risk of decreasing enzyme activity, the proposed immobilization via thiol-ene chemistry is simpler and easier as there are natural thiol groups in enzyme.

2. Experimental

2.1. Materials and chemicals

Water was purified using a Milli-Q synthesis A10 water purification system purchased from Millipore Inc. (Milford, MA, USA), and degassed by ultrasonic for 5 min prior to use. Tetramethoxysilane (TMOS, 99\%) and \(\gamma\)-methacryloxypropyltrimethoxysilane (\(\gamma\)-MAPS, 98\%) were obtained from Sigma-Aldrich Co. (Oakville, Canada). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl), N\(\alpha\)-p-tosyl-\(l\)-arginine methyl ester hydrochloride (TAME), trypsin (1:250), N,N'-methylenebisacrylamide (BAA, 98\%), ammonium persulfate (99\%) and poly(ethylene glycol) (PEG, MW = 10 K, 20 K) were purchased from Aladdin Co. (Shanghai, China). Fused-silica capillary with 100 \(\mu\)m i.d. and 375 \(\mu\)m o.d. was purchased from Yongnian Reafine Chromatography Ltd. (Yongnian, China). All other reagents were of analytical grade.

2.2. Apparatus

The capillary electrophoresis analysis for enzyme activity test was carried out by a P/ACE MDQ (Beckman, CA, USA). Experiments for protein separation were performed on a CBM-20A HPLC System (Shimadzu, Kyoto, Japan). The prepared protein sample was pushed through enzyme microreactors using a precise syringe pump from Longer Pump Company (Baoding, China).

2.3. Preparation of \(\gamma\)-MAPS-TMOS hybrid monolithic column

For the preparation of \(\gamma\)-MAPS-TMOS hybrid monolithic column, the detailed procedures are as follows: To clean and activate the inner surface of capillary, the fused-silica capillary (100 \(\mu\)m × 38 cm, i.d.) was rinsed with 1.0 M HCl for 12 h, water for 30 min, 1.0 M NaOH for 12 h, and water for another 30 min in sequence. Subsequently, it was dried by nitrogen gas at room temperature for further use. The sol solution was prepared by mixing acetic acid (0.01 M, 1.25 mL), PEG (20 K MW, 0.15 g), urea (0.15 g), TMOS (0.4 mL) and \(\gamma\)-MAPS (0.2 mL) in a 10-mL flask. The mixture was then stirred at 0 \(^\circ\)C for 4 h to form a homogeneous mixture solution. 1 mL of this mixture solution was sonicated at 0 \(^\circ\)C for 5 min. Subsequently, the solution was carefully aspirated with a 1.0-mL disposable syringe from the inlet of the capillary until the sol plug was 5 cm long under microscopic observation. After both ends of the capillary were sealed with silicon rubbers, the condensation reaction was carried out at 43 \(^\circ\)C for 12 h. Finally, the obtained \(\gamma\)-MAPS-TMOS hybrid monolithic column was rinsed with water and methanol to remove PEG and other residuals. The capillary monolithic column with both ends sealed was stored at 4 \(^\circ\)C in darkness before use.
2.4. Reduction of disulfide bonds and trypsin immobilization

A 10 mg/mL trypsin solution and 1 mg/mL TCEP solution were prepared in 60 mM KH2PO4/Na2HPO4 buffer (pH 7.0), respectively. Reduction of disulfide bonds was carried out by mixing TCEP and trypsin solution in the ratio of 1:10 (v/v). Subsequently, the mixture was kept at 25 °C for 3 h to react. Then, the mixture was centrifuged (10,000 rpm) at 4 °C for 10 min to remove the insoluble residues. After the solution was returned to room temperature, ammonium persulfate (1 mg/mL) and BAA (2 mg/mL) were dissolved in the reduced trypsin solution to form the trypsin immobilization solution.

Before immobilization, solvent in the γ-MAPS-TMOS hybrid monolithic capillary column was emptied by N2. The trypsin immobilization solution was then carefully aspirated with a 1.0-mL disposable syringe from the inlet of the monolithic column until the solution plug was 5 cm long under microscopic observation. Subsequently, the column was kept at 25 °C for 5 h to immobilize trypsin. After the immobilization reaction was completed, the monolith was rinsed with 60 mM KH2PO4/Na2HPO4 buffer (pH 7.0) containing 10 mM CaCl2, for 1 h to remove excess trypsin. Finally, the monolithic reactor was stored at 4 °C. Before characterizing the enzyme activity by CE, the monolithic reactor was cut to keep a 2-cm length monolithic column at the end of the capillary.

2.5. Morphology characterization of γ-MAPS-TMOS hybrid monolithic column

Optical microscopy and scanning electron microscopy (SEM) were used for the characterization of monoliths in capillary columns. Microscopic pictures were taken with an inverted microscope (Leica, Germany). Scanning electron micrographs were obtained using a JEOL JSM-840A scanning electron microscope (EOL USA, Inc., MA, USA), operated at 15 kV and a filament current of 60 mA. The samples were acquired from sections of γ-MAPS-TMOS hybrid monolithic column, cut into equal lengths (0.5 cm) and then positioned longitudinally within a retractable aluminum stage. These samples were used to depict the surface view of the gel monolith from a longitudinal section of the open tubular column. The sample was then placed into a Balzers SCD 050 sputter coating chamber and coated with a gold/palladium alloy at 40 mA for 60 s.

2.6. Functional group analysis by IR spectrum

Fourier transform infrared spectroscopic (FTIR) studies were accomplished to investigate the methacrylic ester conversion using a Nicolet Magna 750 FTIR spectrometer (Madison, WI, USA). Monolithic material samples with thickness of about 25 μm were placed between two sodium chloride plates while the FTIR sample chamber was continuously purged with dry air. IR spectra (600–4000 cm−1) of the sample were recorded.

2.7. Determination of the grafting density of enzyme on monolithic material

The content of enzyme on monolithic material was estimated with TGA 2050 Thermal Gravimetric Analyzer (TGA) (TA Instruments, USA). The γ-MAPS-TMOS hybrid monolithic material and pure TMOS monolith material were synthesized outside capillary. After immobilizing enzyme, the monolith material were grinded into fine powders, 10 mg of powder sample in the alumina crucible was heated from 20 to 700 °C at 20 °C/min in air.

2.8. Assay of enzymatic activity

The values of Michaelis constant (Km) and maximum velocity (Vmax) were obtained by the analysis of initial velocities with a series of TAME solution of different concentration, the substrate was pumped through the microreactor with 2 cm in length at a 1 μL/min volumetric flow rate at 25 °C. The hydrolysis products were analyzed by CE using 10 mM KH2PO4/Na2HPO4 buffer (pH 6.24) containing 5% MeOH. The reacted TAME was determined by measuring the peak area of the substrate.

2.9. Capillary electrophoresis

The capillary column containing 2 cm enzyme microreactor was installed in a P/ACE MDQ capillary cartridge. The total length of the capillary was 35 cm with the length from the detection window to the outlet being 8.5 cm. The effective length of the column was 23.5 cm. 10 mM KH2PO4/Na2HPO4 buffer (pH 6.24) containing 5% MeOH was used as the running buffer. A voltage of 8 kV for the separation of substrate (TAME) and product (TA) was applied. 2 cm enzyme microreactor region was set at the inlet. Prior to operation, the enzyme monolithic capillary column was conditioned by rinsing with running buffer for 10 min. TAME solution (20 mM) was prepared in 30 mM KH2PO4/Na2HPO4 buffer (pH 7.0) buffer. The substrate solution was injected at 10 kV for 1 sec. After injection, the microreactor was suspended in air for incubation. The running buffer was then applied to separate the substrate and product. The detection wavelength was set at 214 nm.

2.10. Proteins sample digestion and separation

BSA and Hb (10 mg/mL) were dissolved in 1 mL 50 mM Tris–HCl (pH 8.1) buffer containing 8 M urea, respectively. To above solution, 100 μL of 100 mM dithiothreitol (DTT) was added, and the tube was incubated at 50 °C for 20 min. The solution was allowed to cool to room temperature, and then 100 μL of 100 mM iodoacetamide was added. The solution was incubated at room temperature in the dark for 20 min, flowed by the dilution with 50 mM Tris–HCl (pH 8.1) buffer to decrease the urea concentration below 1 M, and then stored in the refrigerator before use [27]. The on-line digestion of proteins in the enzyme microreactor was carried out by pumping the pretreated protein solution at a flow rate of 1 μL/min. Before digestion, the microreactor was balanced with 25 mM NH4HCO3 (pH 8.0) buffer containing 10 wt% MeOH for 20 min. The digest was collected, and separated by HPLC. All HPLC separations were performed using a XB-C18 column (460 μm × 15 cm i.d., 5 μm particle size) from Welch Materials, Inc. (Shanghai, China). Pure water and acetonitrile with 0.1% (v/v) trifluoroacetic acid were used as solvent A and B, respectively. A binary gradient with the following gradient at a flow rate of 1 mL/min was used: 0 min 5% B, then from 5% to 80% B in 30 min, hold for 6 min, and then return from 80% to 5% B in 10 min. The injection volume was 10 μL, the oven temperature was 25 °C, and the detection wavelength was set at 214 nm.

3. Results and discussion

3.1. Preparation of γ-MAPS-TMOS hybrid monolithic column

γ-MAPS-TMOS hybrid monolithic column was prepared via sol–gel process, which involves two major steps: the hydrolysis of TMOS and γ-MAPS, and the polycondensation of hydrolyzed siloxane precursors. The sol was prepared from acid-catalyzed hydrolysis of TMOS and γ-MAPS in ice-bath, as low temperature can decelerate the condensation reaction. The mixture solution of siloxane and porogen was stirred until a homogeneous transparent solution was obtained. It was found that the complete reaction
time depends on the ratio of γ-MAPS vs. TMOS, and the former was harder to hydrolyze than the latter. At the optimum ratio of 1:2 (v/v), 5 h is required to achieve complete hydrolyzation.

For the polycondensation process, the effect of different factors such as the ratio of γ-MAPS vs. TMOS, the contents of PEG and urea, and the reaction temperature were investigated. Several ratios of γ-MAPS vs. TMOS from 1:5 to 5:6 (v/v) were investigated at three different reaction temperatures. The results (Table S-1) show that the skeleton networks of the formed monoliths are different with the different ratio of γ-MAPS vs. TMOS. As seen from their optical microscopic images, good network was obtained at the ratio of 1:3 at 42 °C and 1:2 at 45 °C. Permeability test yielded the same results, good permeability was also obtained with the ratio of 1:3 at 42 °C, and 1:2 at 45 °C.

The content and the molecular weight of PEG in the reaction mixture also affect the morphology of the hybrid structure. Two kinds of PEG with molecular weight of 10 K and 20 K were tested at reaction temperature of 40 °C and 44 °C, respectively. The results are listed in Table S-2. The decrease of PEG content from 0.15 to 0.075 g led to the tendency to crack of the monolithic column at 44 °C. However, at 40 °C, there was no crack in the monolithic column. The results indicated that the effect of PEG on monolith greatly depends on the reaction temperature. And the PEG with higher molecular weight has the higher thermal stability of the formed pores in contrast with the PEG with lower molecular weight. The reason is probably related to the phase separation speed in polycondensation process. Lower temperature and higher molecular weight PEG can speed up the phase separation, which lead to the tendency of forming uniform and small scale porous structure. Furthermore, the effect of urea content was also examined at 40 °C and 44 °C, respectively (Table S-3). Smaller scale skeleton was obtained with the increase of urea content at both reaction temperatures.

3.2. Characterization of monolithic materials

Except characterization by optical microscope, SEM tests were also used to characterize the micro-structure of the γ-MAPS-TMOS hybrid monolithic materials. Fig. 2 illustrates the micro-structure of the optimal hybrid monolithic column prepared in a fused-silica capillary with 75 μm i.d. by using following reaction mixture: 0.4 mL TMOS, 0.2 mL γ-MAPS, 1.25 mL acetic acid (0.01 M), 0.15 g urea, and 0.15 g PEG (20 K). It can be seen that the capillary is fully filled with the homogenous monolithic matrix, which is well attached to inner wall of the capillary. The pore size of the monolithic matrix is about 1–2 μm. Hence, the mass-transfer resistance would be decreased. Nitrogen adsorption analysis was performed to measure the specific surface area and pore size distribution of the monoliths. For the blank monolithic material, the specific surface area is determined as 131.0 m²/g with desorption cumulative volume of 0.401 m³/g, and the pore size distribution is mainly concentrated in the range of 20–40 nm. As for the trypsin immobilized one, the respective data were somewhat decreased to 119.8 m²/g, 0.386 m³/g, and the pore size distribution is mainly concentrated in the range of 35–40 nm (Table S-4, Fig. S-1). TGA analysis was used to investigate the organic component content of the monolithic material. As shown in Table S-5, the mass loss of γ-MAPS-TMOS hybrid monolithic material was more than the mass loss of blank TMOS monolithic material due to successful introduction of organic functional groups into the hybrid monolithic material. The γ-MAPS-TMOS hybrid monolith material was also analyzed by FTIR, the typical C=C stretch bend around 1628 cm⁻¹, and the C=O stretch bend at 1691 cm⁻¹ are observed from the obtained spectra shown in Fig. S-2. The results further confirmed that methacrylic ester groups were successfully incorporated into the monolithic material.

**Fig. 2.** SEM images of γ-MAPS-TMOS hybrid monolithic material.

<table>
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<tr>
<th>TMOS monolith labeled with FITC probe</th>
<th>γ-MAPS-TMOS hybrid monolith labeled with FITC probe</th>
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<tr>
<td>Before washed</td>
<td></td>
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<tr>
<td>After washed with chloroform</td>
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<td>After washed with PBS buffer (pH 8.0, 10 mM)</td>
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**Fig. 3.** Fluorescence microscopic images of γ-MAPS-TMOS hybrid monolith and TOMS monolith labeled with FITC probe.
3.3. Trypsin immobilization via thiol–ene reaction

Prior to enzyme immobilization, the free-radical reaction of ene groups on surface of γ-MAPS-TMOS hybrid monolithic material was investigated by using cysteamine labeled by fluorescein isothiocyanate (FITC) as coupling ligand. The reaction was monitored with probe of FITC. The fluorescence microscopic results are shown in Fig. 3. For the TMOS monolithic column labeled with FITC probe, there was almost no fluorescence after washing with ethanol and further washing with basic buffer. Obviously, the results indicated that the adsorption of fluorescein on TOMS monolith was unstable and easy to be removed.

For the γ-MAPS-TMOS hybrid monolithic column labeled with FITC probes, after washed with ethanol, a significant decrease was observed in fluorescence intensity vs. that before washed with ethanol. Perhaps, the decrease was caused by the fluorescence quenching in ethanol and removing of unbound FITC probe in the monolith. Whereas, a slight increase in fluorescence intensity was observed when further washing was carried out with basic buffer. The results implied that the quenching in ethanol of binding FITC probes on the monolith was incomplete and the FITC probe was strongly bound on the surface of the monolith.

Trypsin is an important protease which can cleave peptide chains mainly at the carboxyl side of the amino acid lysine or arginine. The enzyme has six S–S bonds. The thiol group reduced from disulfide bond located on the surface of enzyme was used as the functional group for the thiol–ene click attachment. After the thiol–ene click immobilization of the enzyme, the monolithic enzyme material was analyzed by TGA (Table S-5). The results indicated that there was a significant difference (0.77%, which corresponds to 462 ng/cm of enzyme bound to the capillary microreactor) of mass loss between enzyme-attached–γ-MAPS-TMOS hybrid monolithic material and none enzyme-attached–γ-MAPS-TMOS hybrid monolithic material. In contrast, there was no difference of mass loss between enzyme-attached-TMOS monolithic material and none enzyme-attached-TMOS monolithic material, which further means that the thiol–ene click immobilization of the enzyme cannot take place if there is no ene functionality on the surface of monoliths. Additionally, the results of nitrogen sorption analysis shown in (Table S-4) implied a drastic decrease of surface area from 131.0 to 119.9 m²/g and a decrease of cumulative volume from 0.401 to 0.386 cm³/g due to the presence of trypsin attached to the surface of γ-MAPS-TMOS hybrid monolithic material.

3.4. Trypsin activity determination

TAME was used as a substrate, which can be hydrolyzed during the catalysis of trypsin to produce TA. It was introduced into the enzyme microreactor and then allowed to react with bound enzyme for different times. The product and undigested substrate were separated from enzyme microreactor by capillary zone electrophoresis (CZE).

For the immobilization of reduced trypsin, we used poly(BAA) as the spacer. To investigate the effect of the spacer on the microreactor activity, three strategies in attaching enzyme procedure were employed. First, no (NH₄)₂S₂O₈ and BAA was present in the trypsin immobilization solution (enzyme microreactor a in Fig. 4). Second, only (NH₄)₂S₂O₈ but no BAA was present in the trypsin immobilization solution (enzyme microreactor b in Fig. 4). Third, both (NH₄)₂S₂O₈ and BAA (enzyme microreactor c in Fig. 4) were present in the trypsin immobilization solution. Fig. 4 illustrates the digestion of 20 mM TAME to TA at different incubation times on these three kinds of enzyme microreactors. It can be seen that the enzymatic reaction on the microreactor c owns a high initial rate and 70% TAME can be hydrolyzed only in 13 s or 95% in 60 s, 100% in 120 s. As for enzyme microreactor a or b, only partial hydrolysis of TAME was achieved, even after the incubation time more than 20 min. This result implied that in both the microreactor a and b, the reaction of mercapto-group with surface methacrylic ester groups was disturbed significantly because of the space obstacle. The advantage of the microreactor c strategy was that the formation of the spacer and the immobilization of the enzyme can be completed in one step.
Double bond at one end of BAA can react easily with mercapto-groups of enzyme, and the double bond at the other end can be bound with other BAA and methacrylic ester groups on monolithic material surface. Thus, the amount of enzyme attached on the surface of the monolith is increased due to the decrease of the space obstacle (Fig. 1). In addition, the critical problem that the polymerization of BAA on the surface of the monolith may decrease the permeability of the enzyme microreactor must also be considered. After investigating, 2 mg/mL BAA and immobilizing at 25 °C were found to be the optimal to prevent enzyme microreactor clogging, and the substrates can access easily to the active site of the enzyme.

The values of Km and Vmax of trypsin were determined for different immobilized microreactors. The values of Km were 52.1, 60.5 and 25.8 mM, whereas the values of Vmax were 6.3, 4.6 and 57.5 μmol/min (mg enzyme, which corresponds to 6.3, 4.6 and 57.5 unit/mg of enzyme) for microreactor a, b and c, respectively, which are significantly higher than those reported in the previous publications, in which similar substrate was used [31,32]. As a reference, mesoporous silica was functionalized via azide-alkyne click reactions, resulting in an enzymatic activity of 65.6 units/mg (enzyme) [33], which was probably caused from much large surface area of porous silica over monolithic material. However, the preparation of the microreactor with our method is much easier and faster.

In order to separate TAME and TA on the microreactor segment by capillary electrophoresis, the pH and concentrations of running buffer were optimized. A 10 mM KH₂PO₄/Na₂HPO₄ buffer (pH 6.24) containing 5% methanol was found to be optimal for CE separation of TAME and TA. Among the three tested enzyme microreactors, better (i.e., narrower and more symmetrical) shapes were obtained on the microreactor c (Fig. 4). It may be that the silanol groups on the monolithic surface were highly covered by the trypsin and spacer reagents, resulting in the decrease of the non-specific interaction of TAME and TA with silanol groups.

The stability of the microreactor was investigated by sampling and analyzing more than 100 times. As shown in Fig. 5a, the activity of the immobilized enzyme decreased slowly with an increase in the assay time. Area TA/area₀ TA in Fig. 5 corresponds to the ratio of area of TA in different analysis time vs. area of 20 mM TA. It can be seen from Fig. 5a that about 45.3%, 43.8% and 87.5% of initial activity of the immobilized enzyme are still remained for the enzyme microreactor a, b and c after 100 assays, respectively. In addition, the lifetime of the enzyme microreactor was also evaluated by assaying five times per day over 15 days. The immobilized enzyme microreactor was kept in the running buffer at 4 °C after test. As shown in Fig. 5b, the activity of the immobilized enzyme decreased slowly with an increase of days, and finally about 59.1%, 75.2% and 88.2% of initial activity remained for enzyme microreactor a, b and c, respectively. However, the free enzyme in solution could keep its activity only for less than 3 days at room temperature. Intraday, interday and batch to batch precision were assessed to evaluate the repeatability of these three kinds of immobilized enzyme microreactor. The relative standard deviation (RSD%) for the peak area of the product TA and its migration time are listed in Table 1. The results imply that the immobilization strategy c was more reliable than strategy a or b for the preparation of the immobilized enzyme microreactor.

### 3.5. Protein digestion

It is more difficult to digest proteins than that of small substrates. Therefore, a protein digestion test was carried out on enzyme microreactor c. BSA and BHb with molecular weight of 67,000 Da and 64,500 Da were selected as standard proteins in this study. Fig. 6 shows the chromatograms under wavelength of 214 nm for digest of 1 mg/mL BSA and BHb. It can be seen that a large number of tryptic peptides from those two proteins were separated and detected, which indicated that the protein with large molecular weight can be effectively hydrolyzed by microreactor in short time (less than 30 s). This result shows that the prepared enzyme microreactor can be used for online digestion of proteins in high-speed analysis and characterization of proteins.

### Table 1

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Fig. 6. Chromatograms for separation of BSA and BHB digests resulted from the microreactor.

4. Conclusion

In this paper, we have proposed a thiol-ene click chemistry strategy to immobilize enzyme onto the hybrid monolith in fabrication of the immobilized enzyme microreactor. In comparison with other “click” strategies such as azide-alkyne click reaction, the immobilization of enzymes via thiol-ene “click” approach is simpler and easier with no risk of decreasing enzyme activity. The microreactor exhibited good stability and high activity. The proposed thiol-ene click chemistry strategy is proved to be a promising method for the highly selective immobilization of proteins under mild conditions, especially enzymes with free thiol radicals.

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Appendix A. Supplementary data


References