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# Organic–Inorganic Hybrid Silica Monolith Based Immobilized Trypsin Reactor with High Enzymatic Activity

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**A novel kind of immobilized trypsin reactor based on organic–inorganic hybrid silica monoliths has been developed. With the presence of cetyltrimethyl ammonium bromide (CTAB) in the polymerization mixture, the hybrid silica monolithic support was prepared in a 100 μm i.d. capillary by the sol–gel method with tetraethoxysilane (TEOS) and 3-aminopropyltriethoxysilane (APTES) as precursors. Subsequently, the monolith was activated by glutaraldehyde, and trypsin was covalently immobilized. By monitoring the reaction of a decapeptide, C-myc (EQKLISEEDL), the enzymatic activity of the immobilized trypsin was calculated, and the results showed that the digestion speed was about 6600 times faster than that performed in free solution. The performance of such a microreactor was further demonstrated by digesting myoglobin, with the digested products analyzed by microflow reversed-phase liquid chromatography coupled with tandem mass spectrometry (μRPLC–MS/MS). With a stringent threshold for the unambiguous identification of the digests, the yielding sequence coverage for on-column digestion was 92%, the same as that obtained by in-solution digestion, whereas the residence time of myoglobin in the former case was only 30 s, about $^{1}/_{1440}$ of that performed in the latter case (12 h). Moreover, such an immobilized trypsin reactor was also successfully applied to the digestion of a mixture of model proteins and proteins extracted from *E. coli*.**

Up to date, two-dimensional gel electrophoresis (2-DE) and the gel-free shotgun strategy are the most popular tools for proteome study.[1–3] No matter by which means, protein digestion is indispensable. In comparison to chemical digestion, enzymatic digestion is popular due to its versatility. The routine proteolysis of proteins is performed in solution, but it suffers from drawbacks, such as long incubation time, enzyme autodigestion, and manual manipulation. As a promising alternative, recently the immobilized enzyme reactor has drawn much attention.[4–6]

Among the various proteases, trypsin, which could provide peptides in the mass range compatible with mass spectrometry (MS) for amino acid sequence determination, is the most favorable one for protein digestion. To prepare enzymatic reactors, trypsin could be covalently bound to, trapped into, or physically adsorbed on different supports, such as nanoparticles,[7–9] membranes,[10–12] plates,[13] fused-silica capillaries,[14,15] commercially available beads,[16–18] and monolithic materials. Among them, the monoliths, composed of porous solid with small-sized skeletons and relatively large through-pores, could offer fast mass transfer and high enzyme binding capacity, making them superior supports for enzyme immobilization.

Generally, monolithic supports could be divided into organic polymers-[19–26] and silica-based monoliths.[27–31] Though the former ones have good biocompatibility and excellent pH stability, the

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swelling in organic solvents might lead to the change of pore structure and the decrease of mechanical stability. In comparison, the latter ones could be prepared with independent control of silica skeletons and through-pores via a sol–gel process, offering high permeability, high mechanical strength, and good organic solvent tolerance. However, the preparation of conventional silica-based monoliths is time-consuming, and difficult to control, leading to poor reproducibility.[32] Furthermore, the nonspecific adsorption of silica is obvious due to the residual silanols on the surface.

As an attractive alternative, the organic–inorganic hybrid silica monoliths, which combine the merits of silica with organic polymer monoliths, have been recently employed in separation fields.[33–36] To our knowledge, immobilized trypsin reactors with hybrid silica monolith as the support have not been reported yet. Followed by our recent success in the preparation of the hybrid silica monoliths for separation,[35] immobilized trypsin reactors based on such materials have been prepared, and extremely high enzymatic activity is obtained compared to that performed in free solution digestion and other previously reported enzymatic reactors.

## EXPERIMENTAL SECTION

**Materials and Chemicals.** Fused-silica capillaries (100 μm i.d. × 375 μm o.d.) were obtained from Sino Optical Fiber Factory (Handan, China). Tetraethoxysilane (TEOS, 95%) and 3-aminopropyltriethoxysilane (APTES, 99%) were purchased from Acros Organics (Geel, Belgium) and dried by SPE columns packed with anhydrous silica (Sipore Chrom Corp., Dalian, China). *N*-α-Benzoyl-L-arginine ethyl ester (BAEE) was purchased from Fluka (Buchs, Switzerland). A decapeptide, C-myc (EQKLISEEDL, 97%), was ordered from Dalian Biotech Company Ltd. (Dalian, China). Trypsin (bovine pancreas), cytochrome *c* (horse heart), myoglobin (equine skeletal muscle), carbonic anhydrase (bovine erythrocytes), and bovine serum albumin (bovine serum) were obtained from Sigma (St. Louis, MO). Organic solvents were all of HPLC grade. Water was purified by a Milli-Q system (Millipore, Molsheim, France).


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**Apparatus.** The scanning electron micrographic images were obtained using a JEOL-JSM-6360LV SEM scanning electron microscope (JEOL, Tokyo, Japan). A precise syringe pump (Baoding Longer Pump Company, Baoding, China) was used to push the substrates through microreactors. All experiments for quantitative analysis were performed on a high-performance liquid chromatograph (HPLC) system equipped with a UV detector (Jasco, Tokyo, Japan).

A paradigm GM4 μHPLC system (Michrom Bioresources Inc., Auburn, CA) coupled with an LCQ$^{DUO}$ quadrupole ion trap mass spectrometer (LCQ-IT MS, Thermo Fisher, San Jose, CA) was used for protein identification.

**Preparation of Organic–Inorganic Hybrid Silica Monolith.** The monolith was prepared according to our previous procedure,[35] but with some modifications. The polymerization solution, consisting of 112 μL of TEOS, 118 μL of APTES, 215 μL of anhydrous ethanol, 8 mg of cetyltrimethyl ammonium bromide (CTAB), and 32 μL of water, was vortexed at room temperature for 1 min before being introduced into a capillary. With both ends sealed by silicon rubbers, the capillary was placed at 40 °C for 24 h and subsequently rinsed with ethanol and water for 30 min, respectively.

**Glutaraldehyde Activation and Trypsin Immobilization.** A solution of 10% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH 8.0) was flushed through the monolithic capillary column for 6 h at room temperature. Then trypsin was immobilized on the activated support by continuously pumping 2 mg/mL trypsin in 100 mM phosphate buffer (pH 8.0) containing 50 mM benzamidine and 5 mg/mL sodium cyanoborohydride ($NaCNBH_3$) for 24 h at 4 °C. Subsequently, the nonspecifically adsorbed trypsin was removed by purging with 100 mM phosphate buffer (pH 8.0) containing 20% acetonitrile (ACN) (v/v) for 4 h, and the residual aldehyde groups on the surface of the support were depleted by pumping with 1 M Tris–HCl (pH 8.0) for 2 h. When not in use, the immobilized trypsin reactor is stored in 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ and 0.02% $NaN_3$ at 4 °C.

**Determination of Immobilized Trypsin.** The amount of immobilized trypsin on microreactors was determined by Bradford assay, which is similar to the previously described ones[15,30] but with minor modifications. Briefly, the microreactor was first chopped into small pieces with a length of 1 cm and then immersed into 100 μL of 100 mM NaOH for 2 h at room temperature to cleave trypsin completely. Trypsin standard solutions were prepared in 100 mM NaOH in the concentration range of 1–50 μg/mL. A volume of 100 μL of each trypsin standard and the cleaved trypsin solution was mixed with 900 μL of Bradford reagent, respectively. After each mixture was incubated at room temperature for 5 min, the absorbance was measured with a spectrophotometer at 595 nm, and the immobilized trypsin content was calculated.

**Enzymatic Activity of Immobilized Trypsin.** A synthetic decapeptide, C-myc (EQKLISEEDL, 97%), was utilized as the substrate. After being dissolved in 50 mM Tris–HCl (pH 8.0), a series of C-myc solutions with different concentrations was pumped through the immobilized trypsin reactor at a flow rate of 1 μL/min (corresponding to a linear rate of ~2 mm/s) at 25 °C. The eluants were collected and analyzed by HPLC. By measuring the decrease of the peak area at 214 nm, the amount of reacted

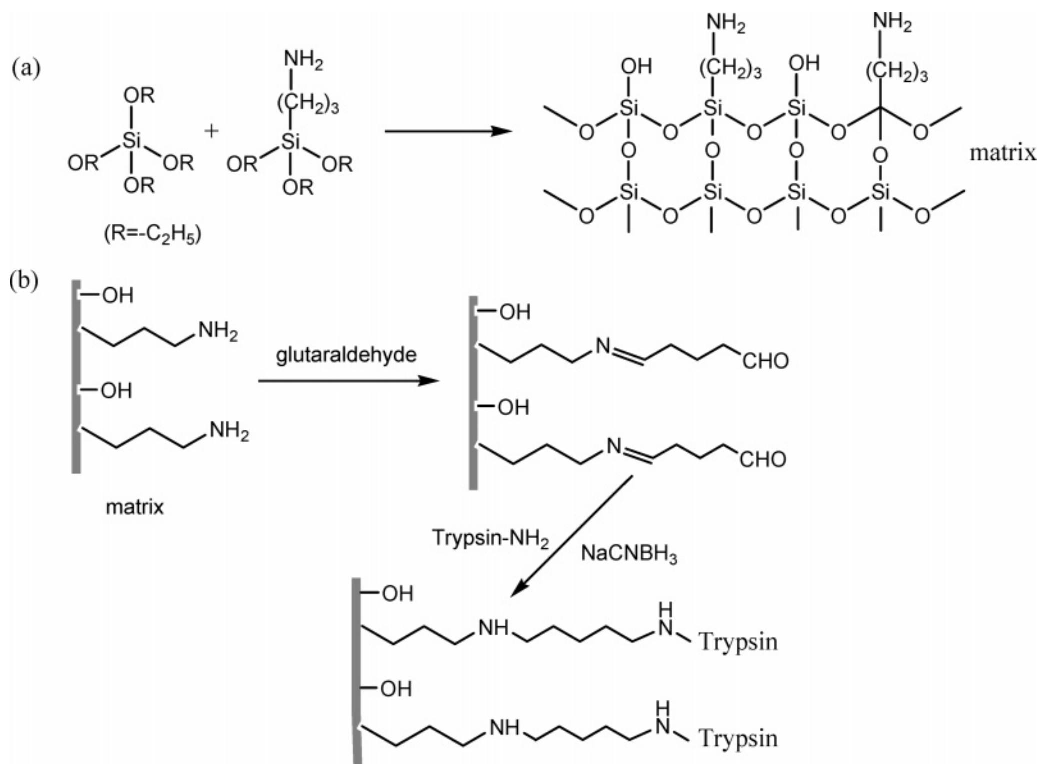


**Figure 1.** Scheme of the organic−inorganic hybrid silica monolithic matrix formation (a) and trypsin immobilization (b).

substrate was determined, and the velocity of enzymatic reaction was calculated.

For comparison, the enzymatic activity of free trypsin was also measured. The reaction solution containing trypsin (0.125 mg/mL) and substrates was incubated at 25 °C for 5 min. After 2 $\mu$L of formic acid was added, the product was analyzed by HPLC, and the enzymatic activity was determined by the same procedure as mentioned above.

**Sample Preparation.** The sample was dissolved in 50 mM $NH_4HCO_3$ (pH 8.0) containing 8 M urea and then reduced in 10 mM dithiothreitol for 1 h at 56 °C. When cooled to room temperature, cysteines were alkylated in the dark in 20 mM iodoacetic acid for 30 min at 37 °C, followed by the dilution with 50 mM $NH_4HCO_3$ (pH 8.0) to decrease the urea concentration below 1 M. The sample was desalted on an SPE-C8 column (Sipore Chrom. Corp., Dalian, China) with 2% and 90% ACN as the loading and eluting buffer, respectively. The eluants were lyophilized in a SpeedVac (Thermo Fisher, San Jose, CA) and then resuspended in 50 mM $NH_4HCO_3$ (pH 8.0) to desired concentration.

The in-solution digestion was performed by adding trypsin into the pretreated protein sample with a substrate-to-enzyme ratio of 50:1 (w/w). Then the solution was incubated at 37 °C for 12 h. Finally, 2 $\mu$L of formic acid was added into the solution to terminate the reaction.

The on-column digestion using immobilized trypsin reactor was carried out by pumping the pretreated protein sample at a constant flow rate. The digest was collected, separated, and identified by $\mu$HPLC−ESI-MS/MS. Between each operation, the microreactor was rinsed with 50 mM $NH_4HCO_3$ (pH 8.0) containing 20% ACN for 20 min.

**$\mu$HPLC−ESI-MS/MS Analysis.** A $\mu$HPLC−ESI-MS/MS system with a homemade C18 column (300 $\mu$m i.d. × 15 cm) was used for protein identification and operated at the flow rate of 5 $\mu$L/min. 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 algorism for further analysis.

## RESULTS AND DISCUSSION

**Preparation and Characterization of Immobilized Trypsin Reactors.** In our experiments, a supramolecular template-based approach together with sol−gel chemistry was used for the preparation of organic−inorganic hybrid silica monoliths, by which the pore structure of silica monoliths could be well tailored.[37,38]

The whole procedure of enzyme immobilization is illustrated in Figure 1. TEOS and APTES were utilized as precursors, and CTAB was chosen as the template. The electrostatic interaction between them played an important role to form a suitable network. Furthermore, ethanol was added into the reaction solution, not only to dissolve all sol−gel ingredients homogeneously, but also to retard the hydrolysis rate of precursors. With an appropriate amount of water added, the cooperative effect between sol−gel reaction and template self-assembling occurred. From the kinetic point of view, it is just the delicate balance of two competitive processes that determines the structure of organic−inorganic hybrid silica monoliths. Under the optimized conditions, the cocondensation of TEOS and APTES (with a molar ratio of 1:1)

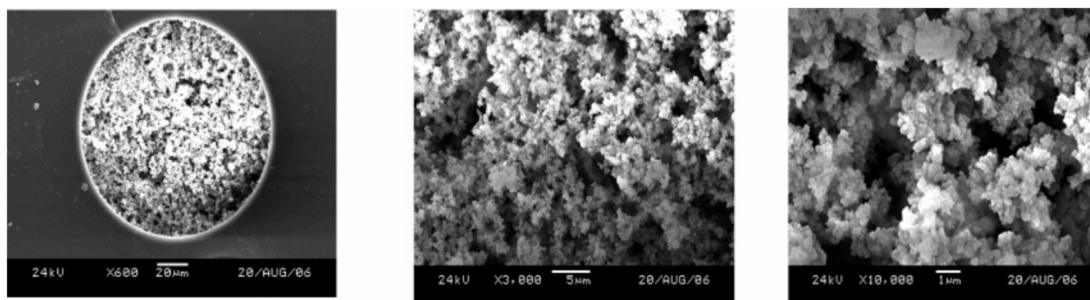


**Figure 2.** Scanning electron micrographs (×600, ×3000, ×10 000) of the cross section of the hybrid silica monolith based immobilized trypsin reactor.

would occur in the presence of CTAB, generating hybrid silica monolithic supports with active amine groups.

In comparison to inorganic silica monoliths, the amount of residual silanols on hybrid silica monoliths is quite small.[39,40] Therefore, the end-capping treatment is avoided. Glutaraldehyde, a commonly used bifunctional reagent, was adopted to couple the monolithic support with trypsin. In the immobilization solution, benzamidine was added to minimize the autolysis of trypsin, and sodium cyanoborohydride was added to reduce C═N to C−N, thus enhancing the stability of immobilized trypsin. By such a procedure, spacer-arms with suitable length between trypsin and the support backbone were formed, allowing the improved enzymatic flexibility.

Scanning electron microscopic (SEM) images of immobilized trypsin reactors are presented in Figure 2. It could be seen that the organic−inorganic hybrid silica monolith is not only homogeneous but also tightly attached to the inner wall of the capillary. The cross-sectional images reveal porous structures consisting of interconnecting spheres with the diameter of ca. 1 μm. The uniform and small sizes of the particles could offer high surface area for the immobilization of trypsin, and the macroporous structure would result in low back-pressure and high permeability. Noticeably, such a monolithic structure could render improved mass transfer, leading to high enzymatic digestion rate.

In comparison to the grafting of organosilanes onto a silica surface,[8,30] the one-step in situ polymerization strategy herein is of advantage, especially for the preparation of immobilized enzyme reactors. With the addition of APTES, the amine group is presented on the support surface, and distributed uniformly throughout the monoliths, leading to high loading capacity of trypsin.[38,39] Moreover, the aminolysis of epoxy rings, required by the previously reported methods to immobilize trypsin,[19,21,26] could be avoided due to the existing active amine functional groups, thus facilitating the preparation of enzymatic reactors. In addition, the pH stability of hybrid silica monoliths could also be enhanced with silicon−carbon bonds external to the surface. All these benefits render the hybrid silica monoliths good candidates for enzyme immobilization.

**Kinetic Characterization of the Immobilized Trypsin.** A micro-flow-rate pump was used to deliver substrates through the monolithic support, and the flow rate was measured by weighing the effluent from the monolithic capillary column. The results showed that the flow rate as high as 5 μL/min could be achieved at a pressure of 5.4 MPa, indicating the low back-pressure and the good permeability. Furthermore, no obvious shrinkage and blockage of the monolith were observed even when the pressure was increased to 20 MPa, demonstrating the excellent mechanical stability.

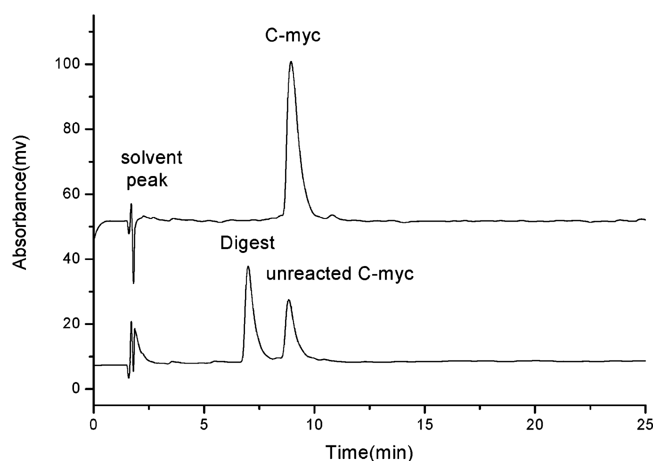


**Figure 3.** Chromatograms of C-myc and its on-column digest. Conditions: substrate, 1 mM C-myc; immobilized trypsin reactor, 100 μm i.d. × 1 cm; 25 °C; 1 μL/min; HPLC column, 4.6 mm i.d. × 150 mm; packing material, ODS; 5 μm; 300 Å; mobile phase, (A) water with 0.1% trifluoroacetic acid; (B) 95% ACN with 0.1% trifluoroacetic acid; a linear gradient of B% from 18% to 26% at 1.0 mL/min in 40 min; injection, 10 μL; detection wavelength, 214 nm. Another resultant small peptide with three amino acids was coeluted with the solvent.

Initially the enzymatic activity of the immobilized trypsin reactor was evaluated with BAEE as the substrate.[12,25,29,30] Surprisingly, almost all of 200 mM BAEE was converted into its product BA when passed through a 1 cm length microreactor at 1 μL/min. However, with the further increase of the substrate concentration, BAEE became insoluble in 50 mM Tris−HCl buffer (pH 8.0), which meant that BAEE was not suitable to assess the enzymatic activity of our microreactor. Therefore, a synthetic decapeptide, C-myc (EQKLISEEDL, 97%), was chosen as the substrate, and the chromatograms of C-myc and its on-column digest are shown in Figure 3.

Through the analysis of the initial digestion velocity with the substrate concentration varying from 0.5 to 2.5 mM, the double-reciprocal plots (Lineweaver−Burk plots) of enzymatic activity were acquired with good linearity ($R^2$ = 0.994 for the immobilized

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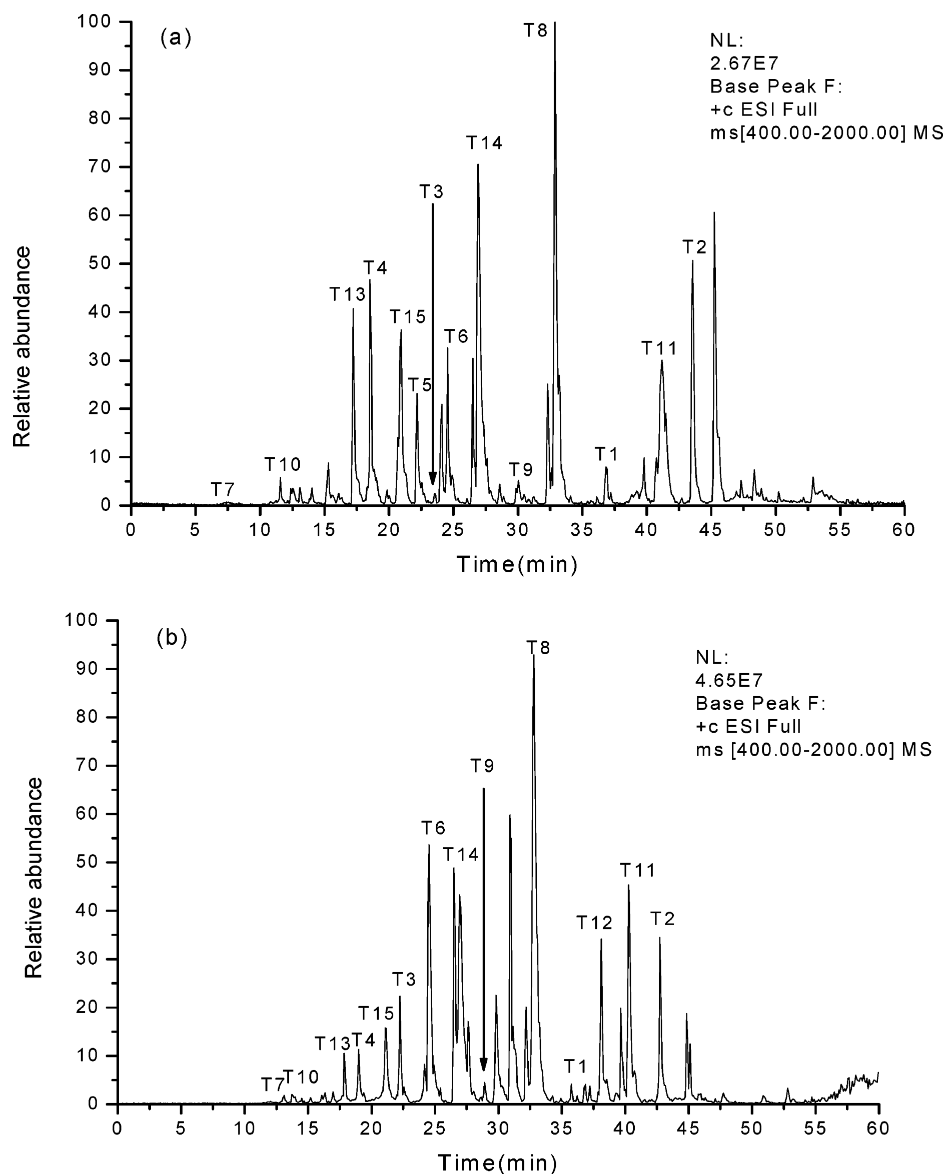


**Figure 4.** Base peak chromatograms for myoglobin digested by the immobilized trypsin reactor (a) and in solution (b). Enzymatic conditions: substrate, 0.1 mg/mL myoglobin in 50 mM $NH_4HCO_3$ (pH 8.0); immobilized trypsin reactor, 100 $\mu$m i.d. × 2 cm; 300 nL/min, 37 °C; in-solution digestion, enzyme/protein = 1/50 (w/w); 12 h; 37 °C. HPLC conditions: separation column, 300 $\mu$m i.d. × 15 cm; packing material, ODS; 5 $\mu$m; 300 Å; mobile phase, (A) 2% ACN (containing 0.1% formic acid); (B) 98% ACN (containing 0.1% formic acid); flow rate, 5 $\mu$L/min; gradient, 0 min, 2% B; 5 min, 10% B; 55 min, 40% B; 60 min, 80% B; 65 min, 80% B; injection, 2 $\mu$L; MS conditions are as in the text. (Assignments of myoglobin peptides are summarized in Table 1. Peaks are marked with their position numbers from Table 1.)

trypsin, and $R^2 = 0.997$ for the free trypsin in solution). The values of Michaelis–Menten constant ($K_m$) and maximum velocity ($V_{max}$) could be obtained from the plots according to the following equation,

$$1/v = (K_m/V_{max})(1/[S]) + 1/V_{max}$$

where $v$ is the velocity of the enzymatic activity, $K_m$ is the Michaelis constant, $V_{max}$ is the maximum velocity, and [S] is the concentration of substrate.

It is calculated that the $K_m$ value of C-myc for the immobilized trypsin is 1.54 mM, quite similar to that of the free trypsin (1.49 mM), whereas the $V_{max}$ value of the immobilized trypsin is 1.69 $\times 10^4$ mM min$^{-1}$ (mg of trypsin)$^{-1}$, nearly 6600 times higher than that of the free trypsin (2.56 mM min$^{-1}$ (mg of trypsin)$^{-1}$). It is well-known that the $K_m$ value represents the binding strength between enzyme and substrate. The lower the value, the stronger the binding force. The fairly small discrepancy between the $K_m$ values herein means similar affinity of the substrate toward trypsin in both procedures, indicating that the diffusion limitation in the immobilized trypsin reactor is quite small. In addition, at a given substrate concentration, $v$ is in proportion to $V_{max}$. When the sample concentration is equal to the $K_m$ value, $v$ reaches one-half of $V_{max}$. As mentioned above, since the $V_{max}$ value of enzymatic reactor was about 6600 times higher than obtained from the in-solution digestion, the enzymatic activity of the former case should be 6600 times higher than that of the latter case, which is significantly higher than those reported in the previous publica-

**Table 1. Database Searching Results of Myoglobin Digested In Solution and by the Immobilized Trypsin Reactor[a]**

| peak | position | peptide sequence | mass (Da) | charge | in-solution | on-column |
|---|---|---|---|---|---|---|
| T1 | 1−16 | −.GLSDGEWQQVLNVWGK.V | 1817.00 | 2 | √ | √ |
| T2 | 1−31 | −.GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR.L | 3405.77 | 3 | √ | √ |
| T3 | 17−31 | K.VEADIAGHGQEVLIR.L | 1607.79 | 2 | √ | √ |
| T4 | 32−42 | R.LFTGHPETLEK.F | 1272.43 | 2 | √ | √ |
| T5 | 32−45 | R.LFTGHPETLEKFDK.F | 1662.87 | 2 | | √ |
| T6 | 32−47 | R.LFTGHPETLEKFDKFK.H | 1938.22 | 2 | √ | √ |
| T7 | 48−56 | K.HLKTEAEMK.A | 1087.28 | 2 | √ | √ |
| T8 | 64−77 | K.HGTVVLTALGGILK.K | 1379.67 | 2 | √ | √ |
| T9 | 64−78 | K.HGTVVLTALGGILKK.K | 1507.85 | 2 | √ | √ |
| T10 | 79−96 | K.KGHHEAELKPLAQSHATK.H | 1983.22 | 3 | √ | √ |
| T11 | 103−118 | K.YNEFISDAIIHVLHSK.H | 1887.13 | 2 | √ | √ |
| T12 | 103−133 | K.YNEFISDAIIHVLHSKHPGDFGADAQGAMTK.A | 3371.73 | 3 | √ | |
| T13 | 119−133 | K.HPGDFGADAQGAMTK.A | 1503.62 | 2 | √ | √ |
| T14 | 134−145 | K.ALELFRNDIAAK.Y | 1361.57 | 2 | √ | √ |
| T15 | 146−153 | K.YKELGFQG.− | 942.05 | 1 | √ | √ |
| digestion time | | | | | 12 h | 30 s |
| peptides matched | | | | | 14 | 14 |
| sequence coverage, % | | | | | 92 | 92 |

[a] Threshold: Xcorr(±1, 2, 3) = 1.90, 2.20, 3.75; ΔCn ≥ 0.1. Database: *equine*.fasta.

tions.[26,29,30] The remarkable increase is mainly attributed to the high concentration of trypsin in the limited microenzymatic reactor space and the partitioning or microenvironmental effects surrounding the immobilized trypsin. The enhanced mass transfer induced by the unique structure of the monolithic support might be another cause. Moreover, the covalent attachment of trypsin could effectively avoid the autodigestion and increase the stability of enzymatic reactors.[30]

**Characterization of Immobilized Trypsin Reactor.** To determine the amount of immobilized trypsin on hybrid silica monoliths, NaOH solution was used to cleave trypsin from the support, and then the supernatant was analyzed by the Bradford assay. The result showed that 1.3 μg of trypsin was immobilized on a 1 cm length microreactor, comparable to a trypsin-encapsulated enzymatic reactor,[29] and about 10-fold higher than that trypsin−PSG−PEG monolith.[30] The homogeneous distribution of the amine groups on the surface of the support could largely reduce the stereochemical hindrance, thus increase the amount of immobilized trypsin. In addition, the hybrid silica monolithic material could offer high surface area and high permeability, also leading to increased trypsin immobilization capacity.

The batch-to-batch reproducibility of microreactors was studied with respect to the enzymatic activity of immobilized trypsin. With C-myc as the substrate the relative standard deviation (RSD) is 13.4% ($n = 3$), similar to that performed in free solution (RSD = 12.0%, $n = 3$).[29] The RSD value for the column-to-column analysis is 7.2% ($n = 4$), which could be further improved by the fine control of the preparation process. These results show that the preparatory reproducibility of the organic−inorganic hybrid silica monolith based microreactor is acceptable for the routine analysis.

The longevity of the immobilized trypsin reactor was investigated by storing it in 50 mM Tris−HCl buffer (pH 7.5) at 4 °C and ambient temperature (25 °C), respectively. After 30 days, the remaining enzymatic activity under the former condition was 93.2%, while that under the latter case was 84.6% of its initial value. Both were much better than that of free trypsin in solution, with the activity almost completely lost within 1 day when stored in the same buffer at 25 °C,[29] which suggests that the longevity of the microreactors can be obviously improved after trypsin is immobilized on hybrid silica monoliths.

**Single Protein Analysis.** The digestion of proteins is more difficult than that of small substrates. Therefore, in this study, an immobilized trypsin reactor with a length of 2 cm was used, and the flow rate applied for protein digestion was reduced to 300 nL/min to ensure enough sample residence time in the microreactors. The digest with an aliquot of 2 μL was collected before being analyzed by μHPLC−MS/MS.

The sequence coverage of myoglobin, a typical globular protein which contains 153 amino acids and 21 cleavage sites, was used to evaluate the performance of the hybrid silica monolith based microreactor. Figure 4a illustrates the base peak chromatogram of the on-column digest of 0.1 mg/mL myoglobin. With four consecutive runs, an average sequence coverage of ~90% (RSD = 3.8%) was obtained, indicating good reproducibility of the digestion, separation, and identification of myoglobin.

For comparison, the in-solution digestion of myoglobin with an incubation time of 12 h was also performed, as shown in Figure 4b. It could be seen that the obtained chromatographic profiling was similar to Figure 4a. After database searching with a strict threshold of Xcorr(±1, 2, 3) = 1.90, 2.20, 3.75 and ΔCn ≥ 0.1, as shown in Table 1, a sequence coverage of 92% was achieved in both cases, and 13 of the identified 14 peptides were identical, indicating that good cleavage specificity of trypsin is remained after immobilization. However, the reaction time was shortened to $^{1}/_{1440}$ (30 s vs 12 h), demonstrating that the hybrid silica monolith based microreactor has superior characteristics toward single-protein digestion. In comparison to other reported immobilized trypsin,[8,13,22,23,26,27] with hybrid monoliths as the supports, the enzymatic reactors could offer high enzymatic activity and fast digestion speed, decreasing the digestion of single protein from minutes to seconds, which further demonstrates the supe-

**Table 2. Digestion Results of Myoglobin with Different Concentrations[a]**

| | in-solution | on-column | | | | |
|---|---|---|---|---|---|---|
| protein concn (mg/mL) | 0.0001 (10 fmol) | 0.0001 (10 fmol) | 0.001 (100 fmol) | 0.01 (1 pmol) | 0.1 (10 pmol) | 1 (100 pmol) |
| peptides matched | 1 | 2 | 11 | 10 | 14 | 15 |
| sequence coverage, % | 20 | 31 | 74 | 79 | 92 | 86 |

[a] The injection volume was 2 $\mu$L for each.

**Table 3. Database Searching Results of Digests of the Protein Mixture[a,b]**

| | | | in-solution digestion (12 h) | | on-column digestion (30 s) | |
|---|---|---|---|---|---|---|
| protein | MW (Da) | pI | peptides matched | sequence coverage, % | peptides matched | sequence coverage, % |
| cytochrome *c* | 11 702 | 9.6 | 8 | 51 | 8 | 47 |
| myoglobin | 16 953 | 7.4 | 5 | 42 | 8 | 52 |
| carbonic anhydrase | 28 981 | 7.9 | 4 | 21 | 2 | 17 |
| bovine serum albumin | 69 324 | 5.8 | 16 | 34 | 21 | 42 |

[a] Conditions: Xcorr(±1, 2, 3) = 1.90, 2.20, 3.75; ΔCn ≥ 0.1. The database searched for cytochrome *c*, myoglobin, carbonic anhydrase, and bovine serum albumin is the *horse*.fasta, *equine*.fasta, *bovine*.fasta and *bovine*.fasta, respectively. [b] Conditions are as in Figure 4.

riority of the support. In addition, after 15 consecutive runs, a sequence coverage of 85% was obtained for 0.1 mg/mL myoglobin, which shows the good stability of such microreactors.

In our experiments, with the flow rate increased from 300 to 500 nL/min, the sequence coverage was decreased to 74%, and 11 peptides were identified from 0.1 mg/mL myoglobin, which indicates that the faster the flow rate, the shorter residence time in the microreactor, and thus lower sequence coverage of proteins. Therefore, either low flow rate or a longer microreactor is necessary to improve the digestion capacity of microreactors.

Protein concentration is another important factor that affects the digestion by microreactors. The digestion of myoglobin with the concentration ranging from 0.1 $\mu$g/mL to 1 mg/mL was carried out, and the corresponding sequence coverage is listed in Table 2. It could be seen that for 10 fmol of myoglobin, a sequence coverage of 31% could be obtained within 30 s, higher than that performed in solution digestion for 12 h (20%). Although the limit of detection is not as low as that obtained by Slysz et al.,[18] it could be further improved with high sensitive nano-HPLC–MS/MS. On the other hand, it was found that myoglobin solution with concentration up to 1 mg/mL (ca. 100 pmol) could be almost completely digested with sequence coverage as 86%. All these results imply that such a microreactor is suitable for high-throughput digestion of proteins with a wide dynamic range.

**Protein Mixture Analysis.** A standard protein mixture including cytochrome *c*, myoglobin, carbonic anhydrase, and bovine serum albumin, dissolved in 50 mM $NH_4HCO_3$ (pH 8.0) with a total concentration of 0.08 mg/mL (0.02 mg/mL for each protein), was used to further examine the performance of the immobilized trypsin reactors. From Table 3, it could be seen that four proteins ranging in molecular mass from 11.7 to 69.3 kDa were all identified, no matter by which digestion protocol. In comparison with the in-solution digestion, more peptides and higher sequence coverage were obtained for most of the proteins when digested by the microreactor, while the digestion time was shortened from 12 h to 30 s. It is also noteworthy that the larger the molecular weight, the lower sequence coverage of proteins. However, this

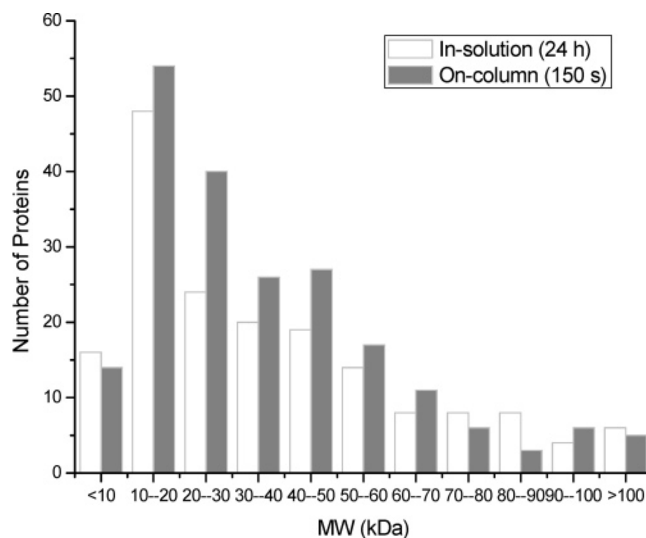


**Figure 5.** Distribution of molecular weights of identified proteins extracted from *E. coli* digested in solution and by the immobilized trypsin reactor. Enzymatic conditions: substrate, 1 mg/mL proteins extracted from *E. coli* in 50 mM $NH_4HCO_3$ (pH 8.0); immobilized trypsin reactor, 100 $\mu$m i.d. × 10 cm; 300 nL/min, 37 °C; in-solution digestion, enzyme/protein = 1/50 (w/w); 24 h; 37 °C. HPLC conditions: 0 min, 0% B; 10 min, 5% B; 220 min, 40% B; 230 min, 80% B; 240 min, 80% B; injection, 20 $\mu$L; other conditions are as in Figure 4.

trend seems not suitable to all proteins (i.e., carbonic anhydrase), as observed by Russell et al.[41] The content of arginine and lysine residues in a protein and the possible interactions between the resulting peptides in the digest mixture might be the main causes for this abnormality. As a whole, these results demonstrate that the hybrid silica monolith based microreactors could be used for the analysis of proteins with different molecular masses and isoelectric points, and the sequence coverage could be improved further with the increased enzymatic column length and decreased flow rate.

(41) Russell, W. K.; Park, Z. Y.; Russell, D. H. *Anal. Chem.* **2001**, *73*, 2682–2685.

To evaluate the applicability of the microreactor for proteome analysis, 20 μg of proteins extracted from *E. coli* were digested by a 10 cm in length microreactor, and then analyzed by μHPLC−MS/MS. Totally, 208 proteins were identified after a 150 s digestion by the enzymatic reactor, while only 176 proteins were recognized by the in-solution digestion for 24 h. From Figure 5, it could be seen that proteins with molecular weight ranging from below 10 kDa (the lowest as 2.56 kDa) to over 100 kDa (the highest as 155 kDa) could be digested and identified, demonstrating the capacity for the digestion of various proteins by the microreactor. To achieve better proteome profiling, an integrated platform involving protein separation, on-line digestion, peptide separation, and protein identification is being constructed in our group.

## CONCLUSIONS

A novel immobilized trypsin reactor based on the organic−inorganic hybrid silica monolith formed via a one-step reaction was developed. And the microreactor rendered high enzymatic activity and long-term stability. For the digestion of standard protein, high sequence coverage was obtained within a short digestion time. Furthermore, such a microreactor was also successfully applied to the digestion of complex protein mixtures, suggesting the potential of the immobilized trypsin reactor in proteome analysis. In addition, the microreactor showed good permeability and high pressure resistance, allowing it to be coupled with micro/nano-HPLC−MS/MS to set up a high-throughput platform for proteome profiling study.

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