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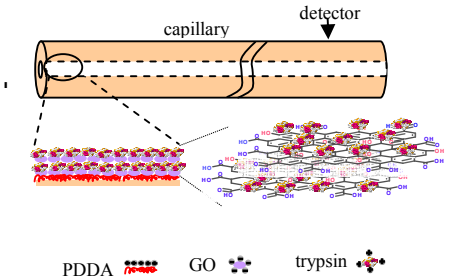

Using graphene oxide as enzyme support, we developed a novel CE-based microreactor via layer-by-layer electrostatic assemble, which can be used for accurate on-line analysis and characterization of peptides and proteins.

Capillary electrophoresis-based immobilized enzyme reactor using graphene oxide as support via layer by layer electrostatic assembly

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Abstract

A novel capillary electrophoresis (CE) -based immobilized enzyme reactor (IMER) using graphene oxide (GO) as support was developed by a simple and reliable immobilization procedure based on layer by layer electrostatic assemble. Using trypsin as model enzyme, performance of the fabricated CE-based IMERs was evaluated. Various conditions, including trypsin concentration, trypsin coating time, numbers of trypsin layers and buffer pH were investigated and optimized. The Michaelis constant K_m (0.24 $\pm$ 0.02 mM) and the maximum velocity V_{max} (0.32 $\pm$ 0.04 mM/s) were determined using the CE-based IMERs, and the values are consistent with those obtained using free trypsin, indicating that enzyme immobilized via the proposed approach does not cause significant structural change of the enzyme or any reduction of enzyme activity. The presented CE-based IMERs exhibit excellent reproducibility with RSD less than 2.8% over 20 runs, and still remain 79.5% of the initial activity after five days with more than 100 runs. Using the proposed CE-based IMERs, the digestion of angiotensin was completed within 3 min, while quite a number of trypstic peptides were observed for BSA on-line digestion with incubation of 30 min. As identified by MS analysis, the online digestion products of BSA using the present CE-based IMER is comparable with those obtained using free trypsin digestion for 12 h incubation. It is indicated that the present immobilization strategy using GO as support is reliable and practicable for accurate on-line analysis and characterization of peptides and proteins.

1. Introduction

Since enzyme immobilization has been revealed as a powerful tool to improve almost all enzyme properties, such as stability, activity, specificity, selectivity and reusability, immobilized enzyme reactors (IMERs) have been applied widely in chemical and biological assays [1-7]. Usually IMERs are integrated with separation and identification system for on-line separation and detection of substrates and products of enzyme reactions, thus fast, efficient, high-throughput and automated enzymatic analysis can be achieved [8-10]. Among a variety of separation techniques, capillary electrophoresis (CE) offers several advantages, such as high efficiency, sensitivity, fast analysis, low sample volume requirement and so on [11-16]. By combining with IMERs, CE can be applied not only as a separation tool with high performance but also as a versatile platform for on-line enzyme studies. During the past decades, CE-based IMERs, in which IMERs are fabricated on capillaries (or microfluidic chips), have attracted intense research interest, representing a promising miniature approach over a wide range of application of enzyme assay including enzyme activity, peptide mapping in proteomics, inhibition screening and diagnostics [17-23].

Efforts have been made to prepare CE-based IMERs, which can be assigned to three different approaches: (i) immobilizing enzymes on the surface of a capillary leading to an open tubular enzyme reactor; (ii) immobilizing enzymes on beads or membranes that are entrapped in a defined area of a capillary network; (iii) immobilizing enzymes on monoliths formed in situ in a capillary. For either of the approaches, developing new enzyme support in CE-based IMERs remains an important research aspect and a challenging work. Several factors have to be considered for searching suitable material of enzyme support, such as binding capacity of enzymes to the capillary, improvement of activity and stability of enzymes, ease-to-operate immobilization procedure, high-efficient separation and sensitive detection of substrates and products [24-26].

Recently, along with the rapid development in nano-science, nano-structured materials have emerged as support for enzyme immobilization [27-31]. It has been demonstrated that the enzymes immobilized on the nano-structured materials have some advantages over the bulk solid substrates due to their large surface areas and good biocompatibility. Generally, surface modification or

functionalization is required in order to efficiently immobilize enzymes onto the nano-structured materials, which could be a labored work and could reduce reproducibility and accuracy of enzyme assay. As one of the most studied sheet-based materials, graphene oxide (GO) has shown several advantages such as ease of synthesis, large surface area to mass ratio, surface functionalities for induced-fit interactions for enzyme binding [32-38], thus making it potential synthetic support for enzyme immobilization. In particular, since GO sheet is enriched with oxygen-containing groups, it is possible to immobilize enzymes without any surface modification or any coupling reagents. To date, there is no application of GO in CE-based IMERs, however, few recent significant advances, which have made in the GO-based nanobiocatalystic systems [32, 33, 36, 37], show the promise to use GO as support for efficient immobilization of various enzymes, such as lipases, esterase, protease, etc.

In this work, we reported a novel CE-based IMER using GO as enzyme immobilization support, which was fabricated with a simple and reliable immobilization procedure based on layer by layer (LBL) electrostatic assembly. Using trypsin as model enzyme, performance of the activity of the CE-based IMERs was investigated to demonstrate the feasibility and accuracy of the present method for on-line enzyme assay. Analysis of on-line trypsin digestion of peptide (angiotensin) and protein (BSA) was also studied using the fabricated CE-based IMERs.

2. Experimental

2.1 Chemicals

Poly(diallydimethylammonium chloride (PDDA) (20%, w/w in water, Mw = 200,000 – 350,000) was purchased from Jing Chun Reagent Inc. (Shanghai, China). GO dispersion (1 mg/ml) was purchased from XF NANO Inc (Nan Jing, China). N-α-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) and N-α-Benzoyl-L-arginine (BA) was purchase from Alfa Aesar (Lancs, UK). Angiotensin (HPLC purity > 98%) (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) was synthesized by Shanghai Science Peptide Biological Technology Co. (Shanghai, China). Trypsin TPCK treated from bovine pancreas and bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (Mt. Louis, MO). Other reagents were analytical grade and used without further purification. All the solutions were filtered

using a 0.22-μm membrane filter prior to use.

2.2 Preparation of the CE-based IMERs

GO, which was immobilized on the inner surface of the capillary, has been proved as a stable stationary phase for open-tubular capillary electrochromatography[39-41]. The CE-based IMERs using GO as enzyme support were developed using LBL electrostatic assembly, as shown schematically in Figure 1. Prior to modification, an untreated capillary was successively rinsed with 0.1 M NaOH for 30 min and deionized water for 10 min. Once preconditioned, PDDA solution was injected into the capillary by pressure at 50 mbar for 20 s, resulting in an about 2 cm-long plug of PDDA solution. The plug was then stayed in the capillary for 1 h to create positive-charged coating on the inner wall of the 2 cm-long capillary. The charge polarity was reversed after adsorption of a layer of negative-charged GO, which was achieved by injection of 1 mg/mL GO dispersion solution (50 mbar for 20 s) and remained in the capillary for 30 min. A single-layer IMER was then developed by injecting the trypsin enzyme solution (1 mg/mL in 50 mM Tris-HCl buffer at pH 8.5) into the capillary and remained in the capillary for 30 min. Because the pI value of trypsin is about 10.5, trypsin should be positively charged at pH 8.5 and can be absorbed on the negative-charged GO layer by electrostatic assembly coating. Between the steps, the capillary was flushed with deionized water for 5 min to wash out any unreacted reagent. To fabricate multi-layer IMERs, the procedure for coating GO and trypsin was repeated. The modification of the capillary at each step was characterized by scanning electron microscope (SEM) images, which were recorded using an XL30ESEM-FEG SEM Microscope (SEI).

2.3 Enzyme assay using the CE-based IMERs

Enzyme assay using the fabricated IMER in a capillary column (25 μm i.d., 365 μm o.d.) was performed in a CE apparatus (CL1020, Beijing Cailu Science Apparatus, China) with UV detector. The total length of the capillary was 40 cm and the length between the detection window and the outlet was 8 cm. The CE running buffer was 20 mM Tris-HCl buffer at pH 8.5. As shown in Figure 1, the IMER with the length of 2 cm was set at the inlet of the capillary. Prior to analysis, the IMER capillary was filled with the running buffer and was equilibrated at 200 V/cm until a stable current and baseline was achieved. Substrate solutions were injected into the IMER capillary at 240 V/cm for 3 s.

After incubation by suspending the column in buffer, an electric potential of 240 V/cm was applied to separate the substrate and products. The reacted substrate was determined by measuring the peak height of the product, which was detected by UV absorption at wavelength of 214 nm.

2.4 Digestion of angiotensin and BSA

1 mg/mL angiotensin and 10 mg/mL BSA was digested using the CE-based IMERs. Angotensin solution was directly injected into the IMER capillary at 240 V/cm for 3 s. BSA was first denatured into 50 mM Tris-HCl buffer containing 8 M urea for 1 h at 37 °C, then the sample was diluted with same buffer to the concentration of urea less than 1 M and was stored at 4 °C prior to use. On-line digestion of Angiotensin or BSA was then analyzed using the CE-based IMERs. After incubation by suspending the colunn in buffer,, digested product were separated by applying 240 V/cm electric potential and detected by UV absorption at wavelength of 214 nm.

For comparison, the digestion of BSA was also carried out using free trypsin. The in-solution digestion was performed by adding 0.5 mg free trypsin into the denatured BSA solution and the mixed solution was incubated at 37 °C for 12 h. After adding 20 μl of formic acid to stop the reaction, the digested solution was then ready for CE analysis.

2.5 ESI-MS conditions and data analysis

Digestion of standard BSA sample using either free trypsin or the CE-based IMER was identified with peptide fingerprint mass spectra. To collect the eluent from the CE-based IMERs for MS analysis, the cathode end of the capillary was placed inside a stainless steel needle using a coaxial liquid-sheath-flow configuration (three-way connection). The sheath flow buffer was 50 mM Tris buffer (pH 8.0) with a flow rate of 2 μL/min controlled by a digital syringe pump (Jiashan Ruichueng Electronic Tec. Co., Ltd., China). The eluent was collected after 5 min CE running for 10 min, and then was subsequently 1:1 (v/v) diluted with a 0.3 wt% TFA in water. After desalting on Milipore ZipTip C18 tips with 10 μL 0.1 wt %FA / 50 wt ACN as the eluting buffer, the sample was introduced directly to the MS. For MS analysis of the offline trypsin digestion, the digested sample was directly desalted and sent to MS spectrometer. The LTQ XL linear ion trap mass spectrometer (Thermo, USA) was operated in positive ionization mode. The ESI(+) source parameters are: capillary voltage 3.0 kV,

sample cone voltage 35 V, extraction cone voltage 3 V, radio frequency lens voltage 450 V, desolvation temperature 120°C and desolvation gas 250 L/h. Signals were recorded in a m/z range of 600-2000 at 1.0 s scan time.

The peak list from MS spectrum was exported to peptide mass fingerprint for protein identification using the MASCOT search engine (www.matrixscience.com) with the SwissProt database. Up to 1 missed cleavage in trypsin digestion was allowed. Peptide tolerance was set to ±2.0 Da. Peptide masses searched were monoisotopic. Entries with a MASCOT MOWSE score correlative to $p<0.05$ were identified as significant hits.

3. Results and discussion

3.1 Performance of the CE-based IMERs

To show the modification process on the inner surface of the capillary, we present in Figure 2 the SEM images of (a) a PDDA coated capillary end, (b) a PDDA-GO coated capillary end, (c) a single-layer IMER and (d) a double-layer IMER. The surface of a bare capillary was very smooth after rinsed with 0.1 M NaOH for 30 min. (SEM image not shown). After the bare capillary was modified with PDDA, a series of small hills or mounds of less varying depth, width, and shape on the inner surface of the capillary were observed, as shown in Figure 2a. Obviously, the surface area of the inner wall was greatly increased after coating with PDDA, and the new surface was quite uniform and well defined. When GO was coated onto the PDDA-column, it was observed that the surface of the PDDA-column was then covered by a layer of GO sheet (Figure 2b). Such sheet-layer-like structure was quite identical to the TEM image of GO dispersion, which was shown in the inset figure of Figure 2b. The SEM image clearly indicates successful modification of GO onto the capillary wall. In addition, our results showed that GO can maintain sheet-layer-like structure when coated onto the capillary via electrostatic assembly. After immobilization of trypsin, the surface of either the single-layer or the double-layer IMER is also uniform and well-defined and present the similar sheet-layer-like structure as the PDDA-GO column (Figure 2 c and d).

Several key factors were studied to optimize the performance of the CE-based IMERs. The conditions for fabrication of CE-based IMERs are essential to the enzyme loading capacity and IMER

activity. Shown in Figure 3 (a), (b) and (c) are the effects of trypsin concentration, trypsin coating time and trypsin layer numbers on the enzymatic activity, respectively. Each data point is an averaged result of three replicated analysis. For those experiments, BAEE with concentration of 0.5 mM was used as the substrate. The CE running buffer was 20 mM Tris-HCl buffer at pH 8.5, the electric field strength was set at 240 V/cm. As presented in Figure 3 (a), the peak height of the product BA first increases sharply as the trypsin concentration is increased to 1 mg/mL, then remains almost constant as the concentration is further increased, indicating that the enzyme loading capacity as well as the activity of the fabricated CE-based IMER reaches the maximum. As the trypsin coating time is increased, the amount of enzyme that can interact with the GO layer and can immobilize on the inner surface of the capillary increases, resulting in the sharply increased peak height of the product BA in the coating time of 10 min – 30 min (Figure 3 (b)). For the coating time larger than 30 min, the peak height of BA only increases slowly which could be attributed to saturation of trypsin that covers the GO layer. Similarly, increasing the trypsin layers also could increase the enzyme loading amount thus increase the activity of the IMER, as shown in Figure 3 (c). Considering the reactor activity as well as the fabrication time, a two-layer-trypsin format, a trypsin concentration of 1 mg/mL and coating time of 30 min for each layer, were chosen for fabrication of the CE-based IMERs.

Figure 4 shows the effect of buffer pH on the trypsin cleavage reaction of BAEE in the pH range of 6.5-9.5 using the CE-based IMER. For comparison, we also present in the figure the result using free trypsin. 1 mM BAEE was used as the substrate for the tests and 20 mM Tris-HCl buffer at different pH values was used as the running buffer. For either of the plots in Figure 4, relative activity of trypsin was presented, corresponding to the BA peak height normalized to its maximum value. The maximum activity of the CE-based IMER was observed at pH 8.5, which was shifted by 1 unit towards the alkaline comparing to that of the free enzyme. Such shift may be attributed to the alteration of the microenvironment of the enzyme after immobilization on the inner surface of the capillary. In this study, the buffer pH was kept at the optimal value of 8.5 for experiments using the IMER, and 7.5 for experiments using free trypsin.

The digestion of BAEE by trypsin using the CE-based IMER was determined by measuring the

BA peak height as a function of the concentration of the substrate BAEE, as shown in Figure 5. Each data point in the figure was carried out using the same IMER and under the same experimental conditions. By nonlinear regression of the Michaelis−Menten diagrams, the Michaelis constant (*Km*) and the maximum velocity (V_{max}) were determined to be 0.24 ± 0.02 mM and 0.32 ± 0.04 mM/s, respectively. For comparison, the K_m and V_{max} values were also measured using free trypsin, and the values were determined to be 0.18 ± 0.03 mM and 0.39 ± 0. 03 mM/s, respectively. It can be seen that the K_m and V_{max} values using the CE-based IMER and free trypsin are close, indicating immobilization of trypsin via the proposed approach does not cause significant structural change of the enzyme or any reduction of accessibility of the substrate to the active sites of the immobilized trypsin.

3.2 Reproducibility and Stability of the CE-based IMERs

The reproducibility and stability of the CE-based IMERs was investigated by sampling and analyzing more than 20 times per day in consecutive 5 days. After each day's test, the same IMER was kept in the running buffer at 4 °C for the next day's test. The activity of the IMER keeps relatively constant in 20 runs during the same day. As shown in Figure 6 the results of the first three days, the RSD for 20 runs in each day is less than 2.8%. The activity of the IMER decreases slightly day by day, but still remains 79.5% of initial activity after five days with more than 100 runs, as shown in the insert figure of Figure 6. The decrease of enzyme activity of IMER might be caused by the small amount of enzyme release from the IMER during rinse and separation procedure. In the insert figure of Figure 6, we also present the results using free trypsin. The results show that the activity of free trypsin decreases rapidly and can only maintain 21.4% of initial activity after five days, which could be due to autolysis of trypsin in solution. Regarding the batch-to-batch reproducibility, five freshly prepared IMERs were test under the optimized conditions, and an average value of three runs for each IMER was provided. The results give a good batch-to-batch reproducibility with RSD of 6.8%. Our results show excellent intraday and interday stability and batch-to-batch reproducibility of the fabricated CE-based IMERs, implying the present immobilization strategy using GO as enzyme support is reliable and practicable for accurate on-line enzyme assay.

3.3 Angiotensin and BSA digestion and analysis on the CE-based IMERs

To show the feasibility of the fabricated CE-based IMERs for on-line analysis of trypsin digestion of peptides and proteins, angiotensin and BSA were used as the substrate for enzyme assay. Figure 7 a shows the electropherograms for digestion of 1 mg/mL angiotensin on the IMER with different incubation time. Peak 1 and peak 2 in each electropherogram refer to UV absorption of angiotensin (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) and the digested product (Val-Tyr-Ile-His-Pro-Phe), respectively. It can be seen that, as increasing incubation time from 0 - 3 min, the peak of substrate gradually decreases while that of the corresponding product gradually increases. The peak of angiotensin almost disappears and only the product peak remains in the electropherogram with 3 min incubation, indicating the digestion of angiotensin is completed.

Figure 7 b presents electropherograms for digestion of 1 mg/mL BSA on the CE-based IMER, with incubation time of 10 min and 30 min. The result recorded using free trypsin with 12 h incubation time was also shown in Figure 7b for comparison. A bunch of peaks appearing within the migration time of 3 – 8 min correspond to various peptide products from BSA digestion. With incubation time of only 10 min, the electropherogram for digestion of BSA using the CE-based IMER show fewer peptide peaks than that recorded using free trypsin with 12 h incubation time. On the other hand, as the incubation time is increased to 30 min, the number and shapes of digestion products are comparable with the results obtained using free trypsin digestion for 12 h. To further demonstrate the feasibility of the fabricated CE-based IMER for enzyme assay, we performed MS analysis of the eluent from the IMER column (with 30 min incubation time) and compared with that from free trypsin digestion (with 12 h incubation time). The identified peptides from trypsin digestion were listed in Table 1. For digestion using the CE-based IMER, 20 peptides were identified with 31% coverage of the BSA sequence, which is very comparable with that obtained using free trypsin (23 peptides, 34% coverage of BSA sequence). The results indicate that the present CE-based IMERs can be used for online digestion of peptides and proteins for efficient analysis and characterization of proteins.

4. Summary

In this work, a novel CE-based IMER using GO as enzyme support was developed using a simple

and reliable immobilization procedure based on layer-by-layer electrostatic assemble, which can easily load multiple layers of enzyme thus increase the capacity of the IEMR. SEM images clearly show successful modification of the capillary surface, and indicate that the surface of either single-layer or double-layer IMER is uniform and well-defined and present the similar sheet-layer-like structure as the PDDA-GO coating column. Using trypsin as model enzyme and BAEE as substrate, the performance of the fabricated CE-based IMERs was evaluated. Various conditions which are essential for fabrication of the IMERs, including trypsin concentration, trypsin coating time and numbers of trypsin layers, were investigated to optimize the enzyme activity. The maximum activity of the IMER was observed at the buffer pH of 8.5, which was shifted by 1 unit towards the alkaline comparing to free enzyme; however, the tendency of dependence of the enzyme activity on the buffer pH is identical for the IMER and free trypsin. Michaelis constant and maximum velocity of BAEE determined using the CE-based IMER (0.24 $\pm$ 0.02 mM and 0.32 $\pm$ 0.04 mM/s) were close to those obtained using free trypsin, indicating that enzyme immobilized via the proposed approach does not cause significant structural change of the enzyme or any reduction of enzyme activity. Run-to-run and batch-to-batch reproducibility as well as stability of the IMERs were investigated. The RSD over 20 reduplicate runs is less than 2.8%, and that of five batches is 6.8%. The IMER can still remain 79.5% of the initial activity after five days with more than 100 runs. Such good reproducibility and stability ensure accurate on-line enzyme assay using the present method. Finally, analysis of on-line trypsin digestion of peptide or protein was investigated using the CE-based IMERs. Both the CE assay and the MS analysis give comparable results of BSA digestion using the CE-based IMER and free trypsin, indicating the potential valuable application of our approach using GO as enzyme support to fabricate IMERs for efficient on-line analysis and characterization of proteins.

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References

[1] Y. Asanomi, H. Yamaguchi, M. Miyazaki and H. Maeda, *Molecules*, 2011, **16**, 6041-6059.

[2] J. Ma, L. Zhang, Z. Liang, Y. Shan and Y. Zhang, *Trac-Trends Anal. Chem.*, 2011, **30**, 691-702.

[3] A. Monzo, E. Sperling and A. Guttman, *Trac-Trends Anal. Chem.*, 2009, **28**, 854-864.

[4] R. A. Sheldon, *Adv. Synth. Catal.*, 2007, **349**, 1289-1307.

[5] G. Ghafourifar, A. Fleitz and K. C. Waldron, *Electrophoresis*, 2013, **34**, 1804-1811.

[6] T. Wang, J. Ma, G. Zhu, Y. Shan, Z. Liang, L. Zhang and Y. Zhang, *J. Sep. Sci.*, 2010, **33**, 3194-3200.

[7] X. Wang, K. Li, E. Adams and A. V. Schepdael, *Electrophoresis*, 2014, **35**, 119-127.

[8] G. Massolini and E. Calleri, *J. Sep. Sci.*, 2005, **28**, 7-21.

[9] Y.-l. Nie and W.-h. Wang, *Chromatographia*, 2009, **69**, S5-S12.

[10]. C. I. C. Silvestre, P. C. A. G. Pinto, M. A. Segundo, M. L. M. F. S. Saraiva and J. L. F. C. Lima, *Anal. Chim. Acta*, 2011, **689**, 160-177.

[11] C. Cao, W. Zhang, L. Fan, J. Shao and S. Li, *Talanta*, 2011, **84**, 651-658.

[12] M. Shanmuganathan and P. Britz-McKibbin, *Anal. Chim. Acta*, 2013, **773**, 24-36.

[13] C. Chang, G. Xu, Y. Bai, C. Zhang, X. Li, M. Li, Y. Liu and H. Liu, *Anal. Chem.*, 2013, **85**, 170-176.

[14] M. Geiger, A. L. Hogerton and M. T. Bowser, *Anal. Chem.*, 2012, **84**, 577-596.

[15] Y. Fan and G. K. E. Scriba, *J. Pharmaceut. Biomed. Anal.*, 2010, **53**, 1076-1090.

[16] X. Hai, B.-f. Yang and A. Van Schepdael, *Electrophoresis*, 2012, **33**, 211-227.

[17] L. Blanes, M. F. Mora, C. L. Do Lago, A. Ayon and C. D. Garcia, *Electroanalysis*, 2007, **19**, 2451-2456.

[18] S.-S. Park, S. I. Cho, M.-S. Kim, Y.-K. Kim and B.-G. Kim, *Electrophoresis*, 2003, **24**, 200-206.

[19] J. Qiao, L. Qi, H. Yan, Y. Li and X. Mu, *Electrophoresis*, 2013, **34**, 409-416.

[20] B. M. Simonet, A. Rios and M. Valcarcel, *Electrophoresis*, 2004, **25**, 50-56.

[21] Z. Tang, T. Wang and J. Kang, *Electrophoresis*, 2007, **28**, 2981-2987.

[22] J. Iqbal, *Anal. Biochem.*, 2011, **414**, 226-231.

[23]. Y. Li, R. Wojcik and N. J. Dovichi, *J. Chromatogr. A*, 2011, **1218**, 2007-2011.

[24] Y. Fang, X. Huang, P. Chen and Z. Xu, *BMB REPORTS* 2011, **44**, 87-95.

[25] P. Jochems, Y. Satyawali, L. Diels and W. Dejonghe, *Green Chem.*, 2011, **13**, 1609-1623.

[26] T. Xie, A. Wang, L. Huang, H. Li, Z. Chen, Q. Wang and X. Yin, *Afr. J. Biotech.*, 2009, **8**, 4724-4733.

[27] H.-G. Hicke, M. Becker, B.-R. Paulke, M. Ulbricht, *J. Membrane Sci.* 2006, **282**, 413-422.

[28] Y. Zhang, F. Gao, S.-P. Zhang, Z.-G. Su, G.-H. Ma, P. Wang, *Bioresource Technol.* 2011, **102**, 1837-1843.

[29] M. Soleimani, A. Khani, K. Najafzadeh, *J. Mol. Catal. B-Enzym.* 2012, **74**, 1-5.

[30] M. L. Verma, C. J. Barrow, M. Puri, *Appl. Microbiol. Biot.* 2013, **97**, 23-39.

[31] S. Zhao, X. Ji, P. Lin, Y.-M. Liu, *Anal. Biochem.* 2011, **411**, 88-93.

[32] H. Bao, Q. Chen, L. Zhang and G. Chen, *Analyst*, 2011, **136**, 5190-5196.

[33] B. Jiang, K. Yang, Q. Zhao, Q. Wu, Z. Liang, L. Zhang, X. Peng and Y. Zhang, *J. Chromatogr. A*, 2012, **1254**, 8-13.

[34] L. Jin, K. Yang, K. Yao, S. Zhang, H. Tao, S.-T. Lee, Z. Liu and R. Peng, *Acs Nano*, 2012, **6**, 4864-4875.

[35] H. Razmi and R. Mohammad-Rezaei, *Biosen. Bioelectron.*, 2013, **41**, 498-504.

[36] M. Sakata, A. Funatsu, S. Sonoda, T. Ogata, T. Taniguchi and Y. Matsumoto, *Chem. Lett.*, 2012, **41**, 1625-1627.

[37]. G. Xu, X. Chen, J. Hu, P. Yang, D. Yang and L. Wei, *Analyst*, 2012, **137**, 2757-2761.

[38] Y. Zhang, J. Zhang, X. Huang, X. Zhou, H. Wu and S. Guo, *Small*, 2012, **8**, 154-159.

[39] C. Wang, S. de Rooy, C.-F. Lu, V. Fernand, L. Moore, Jr., P. Berton and I. M. Warner, *Electrophoresis*, 2013, 34, 1197-1202.

[40] X. Liu, X. Liu, M. Li, L. Guo and L. Yang, *Journal of Chromatography A*, 2013, 1277, 93-97.

[41] X. Liu, X. Liu, X. Liu, L. Guo, L. Yang and S. Wang, *Electrophoresis*, 2013, 34, 1869-1876.

Figure Captions.

Figure 1 (color online) Schematic drawing of the strategy to fabricate CE-based IMERs using GO as enzyme support.

Figure 2 SEM images of (a) a PDDA-coated capillary, (b) a PDDA-GO capillary, (c) a single-trypsin-layer IMER and (d) a double-trypsin-layer IMER. The insert image in figure (b) is the TEM image of GO dispersion.

Figure 3 Effect of (a) trypsin concentration, (b) trypsin coating time and (c) trypsin layer numbers on the enzymatic activity of the IMER. BAEE with concentration of 0.5 mM was used as the substrate. The buffer pH value was kept at 8.5. Conditions for fabrication of the IMER in each figure are (a) double-layer IMER and 30 min trypsin coating time for each layer, (b) double-layer IMER and 1 mg/mL trypsin, (c) 1 mg/mL trypsin and 30 min trypsin coating time for each layer. Without incubation after injection of BAEE, the product was separated and detected by UV absorption at wavelength of 214 nm. The CE running buffer was 20 mM Tris-HCl buffer at pH 8.5, the electric field strength was set at 240 V/cm.

Figure 4 (color online) Effect of buffer pH on the relative activity using a CE-based IMER and free trypsin. The conditions for fabrication of the CE-based IMER were double-trypsin-layer, 1 mg/mL trypsin and 30 min trypsin coating time for each layer. Other conditions were same as those in Figure 3.

Figure 5 Michaelis−Menten diagram of trypsin on IMER. The buffer pH value was kept at 8.5. Other conditions were same as those in Figure 4.

Figure 6 (color online) Reproducibility over 20 runs per day in three days using the same CE-based IMER. Inserted Figures show the change of enzyme activity after 5 days using the CE-based IMER

(blue) and free trypsin (red). The measured BA peak height in the first day was set as 100% enzyme activity. For the results of free trypsin, 1 mM BAEE was used as a substrate, the trypsin solution (1 mg/mL) was placed in 20 mM Tris-HCl buffer (pH 7.5) at 4 °C for storage and the enzyme assay was carried out at 25 °C. Enzyme activity was assayed at regular intervals. Other conditions were same as those in Figure 4.

Figure 7 (color online) (a) Electropherograms for digestion of 1 mg/mL angiotensin on the CE-based IMER with different incubation time. Analyte peaks in order of elution: peak 1, angiotensin and peak 2, digested product. (b) Electropherograms for digestion of 1 mg/mL BSA on the CE-based IMER with incubation time of 10 min and 30 min, and that recorded using free trypsin with 12 h incubation time.

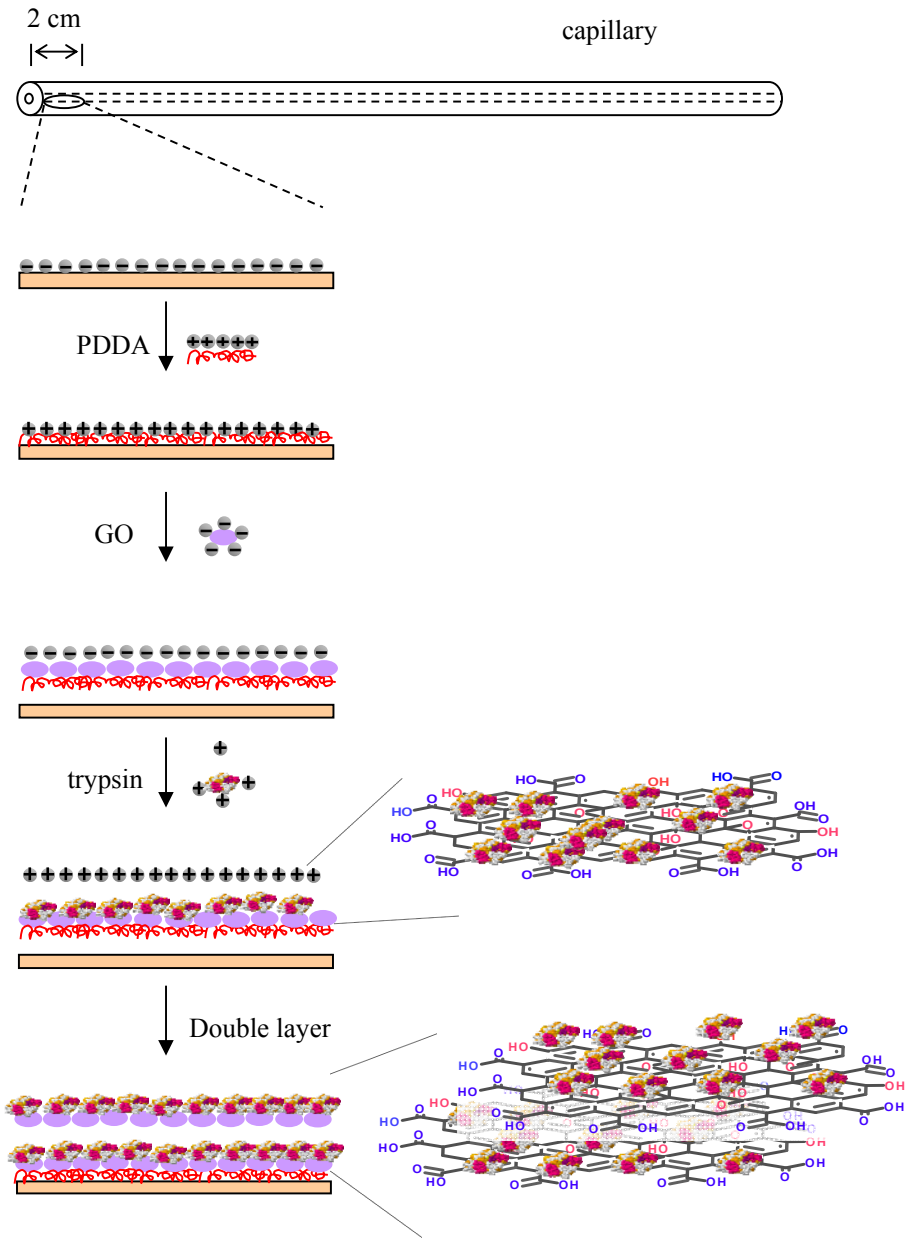

Figure 1

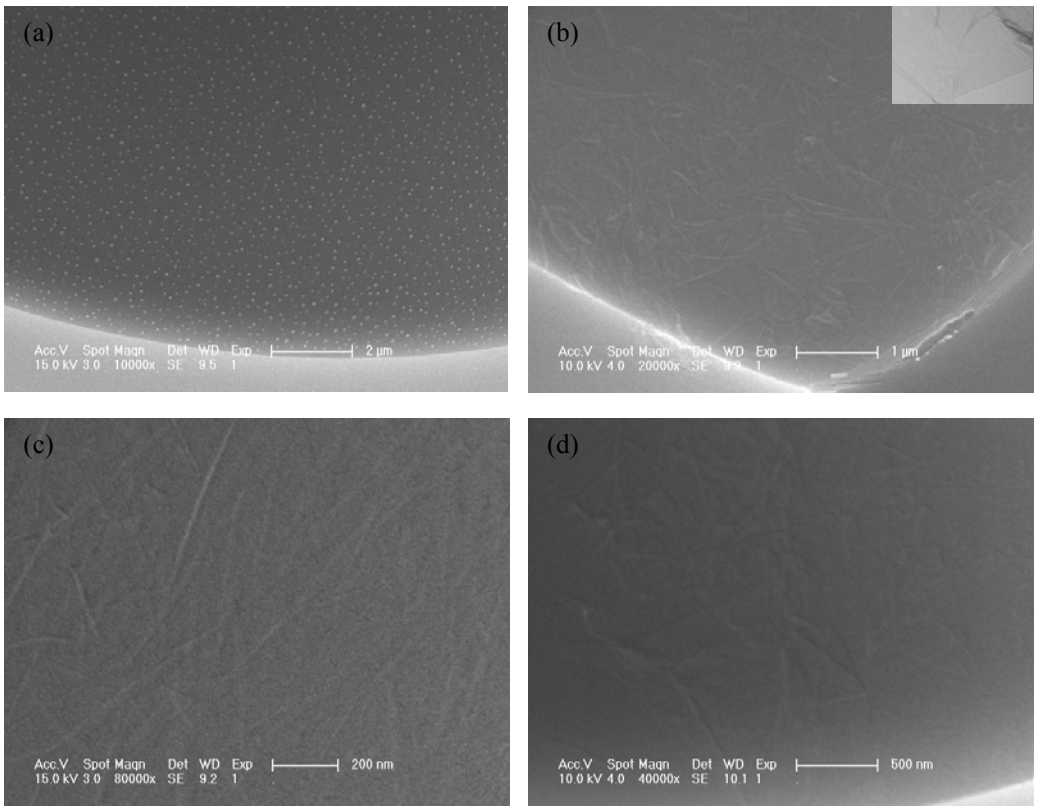

Figure 2

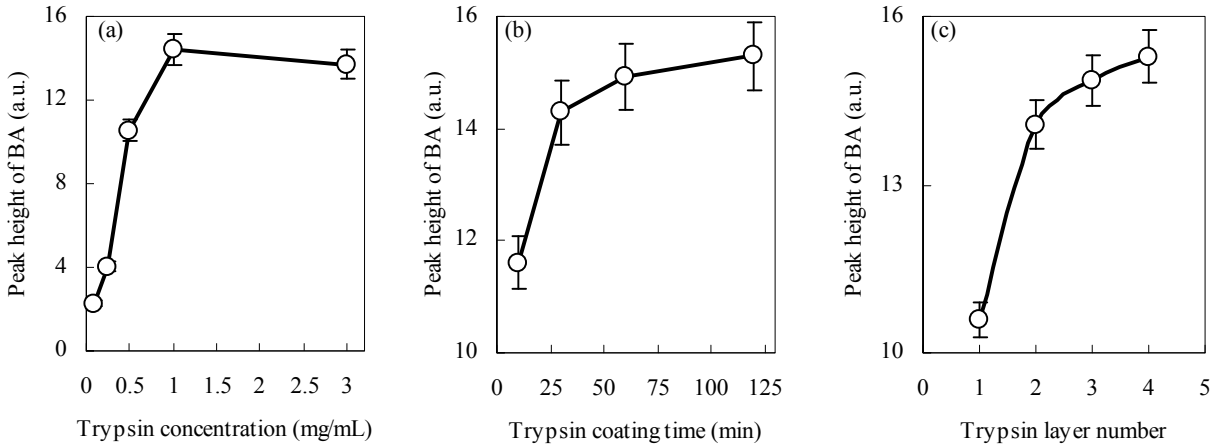

Figure 3

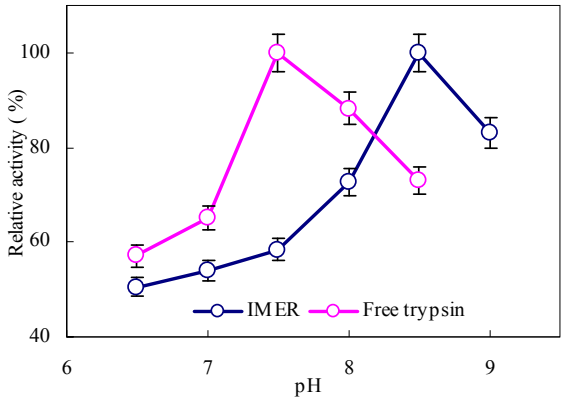

Figure 4

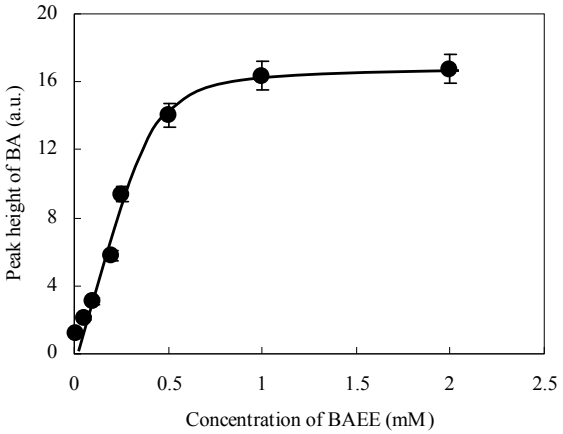

Figure 5

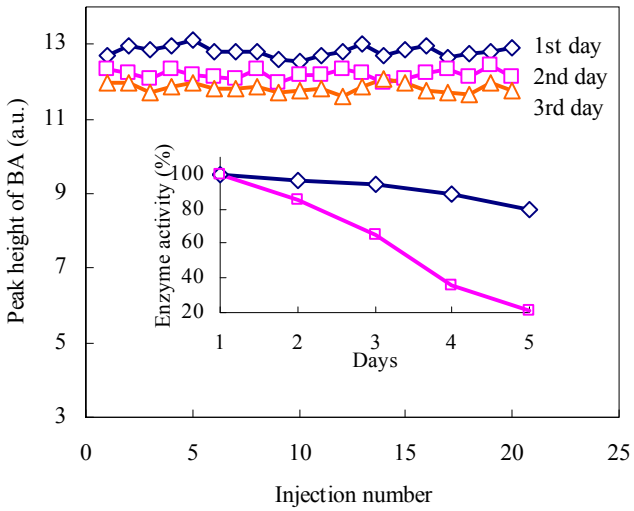

Figure 6

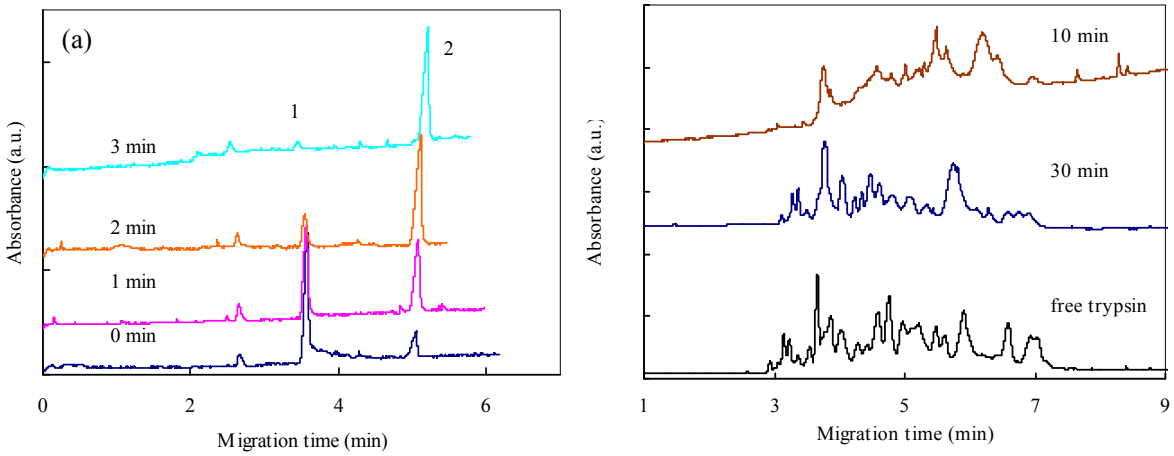

Figure 7

Table 1 Identified peptide masses and sequence from trypsin digestion of BSA using the fabricated CE-based IMER and the free trypsin.

Mass (Da)	position	Missed cleavage	Peptide sequence	CE-based IMER	Free trypsin
657	24-28	1	R.RDTHK.S	√	
711	29-34	0	K.SEIAHR.F		√
2434	45-65	0	K.GLVLIAFSQYLQQCPFDEHVK.L	√	√
3578	45-75	1	K.GLVLIAFSQYLQQCPFDEHVKLVNELTEFAK.T		√
1361	89-100	0	K.SLHTLFGDELCK.V		√
976	123-130	0	R.NECFLSHK.D	√	√
926	161-167	0	K.YLYEIAR.R	√	√
2044	168-183	1	R.RHPYFYAPELLYYANK.Y	√	√
1632	184-197	0	K.YNGVFQECCQAEDK.G	√	√
2315	184-204	1	K.YNGVFQECCQAEDKGACLLPK.I	√	√
700	198-204	0	K.GACLLPK.I	√	√
648	223-228	0	R.CASIQK.F	√	√
688	236-241	0	K.AWSVAR.L	√	√
846	242-248	1	R.LSQKFPK.A	√	
921	249-256	0	K.AEFVEVTK.L	√	√
1577	267-280	0	K.ECCHGDLLECADDR.A		√
1385	286-297	0	K.YICDNQDTISSK.L	√	√
1566	347-359	0	K.DAFLGSFLYEYSR.R	√	√
1478	421-433	0	K.LGEYGFQNALIVR.Y	√	√
816	452-459	1	R.SLGKVGTR.C	√	√
2700	460-482	1	R.CCTKPESERMPCTEDYLSLILNR.L	√	√
1666	469-482	0	R.MPCTEDYLSLILNR.L		√
840	483-489	0	R.LCVLHEK.T	√	√
659	490-495	0	K.TPVSEK.V	√	√
724	581-587	0	K.CCAADDK.E	√	√