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Capillary electrophoresis-based immobilized enzyme reactor using particle-packing technique

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ABSTRACT

A novel method using particle-packing technique to fabricate capillary electrophoresis (CE)-based immobilized enzyme reactor (IMER) was accomplished by utilizing perfusive silica single particles as the frits and large-pore beads as the enzyme supports. The fabrication procedure is rapid and simple; the length and enzyme loading amount of the CE-IMERs could be easily adjusted. Performance and feasibility of the CE-IMERs were investigated using on-line trypsin digestion as the model enzyme reaction. High reproducible on-line enzyme assay was demonstrated with RSD less than 4.1% and 3.8% for peak area and migration time of the substrate and product over 100 consecutive runs, respectively. The enzyme can still maintain the activity for at least 10 days, indicating remarkably stability of the CE-IMERs. The CE-IMERs were successfully applied for accurate analysis of trypsin inhibition as well as on-line digestion of standard proteins (myoglobin and BSA). The present method provides a new interesting alternative to open-tubular and monolithic CE-IMERs, thus expands the application of the CE technique for on-line enzyme assay and analysis and characterization of peptides and proteins.

1. Introduction

Capillary electrophoresis (CE) in combination with immobilized enzyme reactors (IMERs), CE-IMER, has emerged as a powerful platform for on-line enzyme assay [1–5]. In contrast to employing free enzyme in solution, IMERs exhibit many important advantages, such as enhanced stability and activity of enzymes, improved analysis time, reduced experimental cost and manual sample preparation work, etc. [6–10]. In CE-IMER, the products and the unreacted substrates of enzymatic reactions are on-line separated and detected by CE, which is a promising separation technique with properties of simplicity, high efficiency and sensitivity, fast analysis, and low sample consumption. Developing a versatile CE-IMER has been a hot research topic in regarding to a variety of applications in enzyme assay, for example, measurement of enzyme activity, screening of inhibitors, and proteome analysis [11–19].

In practice, it is difficult to fabricate a CE-IMER for on-line integration of enzymatic reaction with CE separation, which requires the capillary IMER to be stable, reproducible, and most importantly, compatible with the small sample volumes used in CE. Two kinds of approaches were applied for fabricating CE-IMERs [2]. One is to use two different capillaries for IMER manufacture and CE separation. The IMER capillary is interfaced with the separation capillary either through a solution gap or a specific multiple-valve design [20–26]. Such configurations allow independent optimization of conditions for on-line enzymatic reaction and CE separation to achieve accurate on-line enzyme assay. However, the detection sensitivity and separation efficiency could be reduced due to the unavoidable sample diffusion in the gap or the complicated multiple-valve design. The other alternative approach, on-line integrated CE-IMER, is to perform the enzymatic reaction and CE separation simultaneously in a single capillary, in which the IMERs are fabricated at the inlet of the capillary [27–33]. Open-tubular and monolithic IMERs are two of the most common used configurations to integrate the enzymatic reaction with the CE separation in the same capillary [e.g. see Refs. [34,35], and references therein]. Open-tubular IMERs are relatively easy to fabricate, but the binding capacity of enzymes and enzymatic catalysis efficiency are limited due to the low phase ratio and sample capacity. On the other hand, monolithic IMERs exhibit micrometersized pores and large surface area thus enhance efficiency of both enzymatic reaction and CE separation, which are

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quite advantageous for enzyme assay. However, the procedure to prepare a monolithic reactor is a time-consuming work, resulting in low batch-to-batch reproducibility.

Nowadays there is a huge library of HPLC grade particulate material in terms of surface chemistry, particle size and porosity, which could be used as the enzyme support. It is therefore expected that the particle-packing technique could have great valuable potential for fabrication of CE-IMERs, and could provide an alternative to open tubular and monolithic CE-IMERs. However, making frits that are compatible with the enzymatic reactions and the followed CE separation is a great challenge. In fact, to the best of our knowledge, particle-packing technique has never been reported for fabricating on-line integrated CE-IMERs so far. On the other hand, Muller and Lytle has reported immobilization of Baker's yeast cells in the capillary using a 50-μm single particle as the mid-capillary frit, showing the advantages of single particles in packing technique for cell profiling [36]. However, their method to make the mid-capillary frit was rather difficult, which required to heat and pull the capillary to make a restriction section and force the single particle into the restriction by pressure. With the application of single particles of larger size (110-μm), recently Zhang et al. developed a much simpler frit-manufacturing method for packing CEC separation, which was accomplished via the keystone effect without making the restriction section [37,38]. Based on such single particle fitting technique, we report here a novel fabrication approach of CE-IMERs by packing enzyme-immobilized particles in the capillary with the 110-μm single particles as the frits. The fabrication procedure is rapid and simple, and the length and enzyme loading amount of the CE-IMER can be easily adjusted. Using trypsin as the model enzyme, the feasibility and accuracy of the present method for on-line enzyme assay were demonstrated. The fabricated CE-IMERs were successfully applied for inhibition study and analysis of on-line trypsin digestion of standard proteins.

2. Materials and methods

2.1. Chemicals

110-μm perfusive spherical silica single particles with 2-μm pore-size were provided by X-tec (Bromborough UK). Trypsin Spin Columns (Proteomics grade, 20 μm spherical silica with 1-μm pore-size as the support), free trypsin (Proteomics grade), and benzamidine hydrochloride hydrate (BH) and myoglobin (equine skeletal muscle, $pI = 7.4$) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N*-*R*-benzoyl-L-arginine ethyl ester (BAEE) and *N*-α-benzoyl-L-arginine (BA) were purchased from Alfa Aesar (Lancs, UK). Poly(diallydimethylammonium chloride) (PDDA) (20%, w/w in water, Mw = 200,000–350,000) was purchased from JingChun Reagent Inc. (Shanghai, China). All other reagents were of analytical grade and were used without further purification. All solvents and solutions were filtered using a 0.22-μm membrane filter prior to use.

2.2. Fabrication of the particle-packed CE-IMERs

Fig. 1 shows schematically the fabrication of the particle-packed CE-IMER and the on-line analysis of trypsin digestion. Briefly, the IMER was fabricated at the inlet of the capillary, by trapping trypsin-immobilized beads between the two single-particles which were used as the frits for packing. After the substrate was injected into the capillary, on-line trypsin digestion was achieved and the resulting peptide products were electrophoretically separated and detected by UV absorption. Fused-silica capillaries (total/effective length of 55/46-cm, Hebei Yong-nian Optical Fiber Factory, China) with 100-μm i.d. and 365-μm o.d. were used for preparation of IMERs as well as CE separation and detection of the substrates and products. Prior to use, the untreated capillary was successively flushed with 0.1 M NaOH, H_2O and buffer for 15 min. For separation and detection of tryptic peptides, PDDA was coated on the surface of the capillary for the reversed EOF.

We employed commercial trypsin beads (Trypsin Spin Columns) which immobilize trypsin on the surface of 20-μm spherical silica particles with 1-μm pore-size, to be packed in the capillary to fabricate the CE-IMER. Two perfusive spherical silica single particles with 110-μm diameter and 2-μm pore-size were used as the outlet and inlet frits which were constructed by the keystone effect as described by Zhang et al. [38,39]. One perfusive spherical silica single particle was first pushed into the capillary as the outlet frit by a 5-cm long capillary (90-μm o.d. 20-μm i.d.). This outlet frit can be placed at any desired position in the capillary, thus the location and the length of the IMER can be easily adjusted. The trypsin beads were then packed into the capillary after being rinsed with the running buffer. Because of the large pore size of the silica single particle, the back pressure is greatly reduced during packing, thus neither high-pressure HPLC pump nor the depressuring step, which is generally needed in the packed capillary technique, is necessary. In fact, just a syringe is needed to pack the trypsin beads into the capillary. After the trypsin beads were loaded in the capillary, another perfusive spherical silica single particle was put into the capillary as the inlet frit. Both the inlet and the outlet frits were manufactured without any heat or pressure, which won't affect the enzyme activity. The size of the trypsin beads are a magnitude order larger than the pore size of the single particles, as indicated in Fig. 1 the SEM images of both trypsin beads and the single particles, thus the trypsin beads are well trapped between the two frits. The fabrication procedure of our packed-capillary IMER is quite rapid and simple, which takes less than 5 min to fabricate a 5-mm long IMER. To regenerate the enzyme reactor, the inlet frit was removed by cutting a small section of the capillary and the trypsin beads were easily pushed out by a syringe (the outlet frit remained in the capillary), then the fabrication procedure was repeated. The whole procedure was monitored and confirmed by observation under a microscope.

In the present work, to control the length of a short IMER (e.g. 1-mm length), the microreactor was fabricated at the inlet end of the capillary. The first frit was placed at a distance of the IMER length from the capillary inlet, then the trypsin beads were packed into the capillary with a syringe. The length of the CE-IMER was thus determined by the position of the first frit, which could be confirmed under a microscope.

2.3. On-line and off-line enzyme assays

All CE experiments were performed in a CE apparatus (CL1020 Beijing Cailu Science Apparatus, China) under 22 °C cooling air with the UV detector. The CE running buffer was 20 mM Tris–HCl buffer at pH 8.0. For analysis of trypsin digestion of proteins, 5 mg model protein was first denatured by 400 μL 20 mM Tris–HCl (pH 8.0) containing 8 M urea and 10 mM dithiothreitol in 56 °C water bath for 1 h, and then alkylated by 20 mM iodoacetic acid at 37 °C in the dark for 30 min. After cooled down to room temperature, the solution was diluted with 20 mM Tris–HCl (pH 8.0) to 4 mL to decrease the urea concentration below 1 M.

On-line enzyme assay was achieved using the fabricated packed CE-IMERs. Prior to analysis, the IMER capillary was filled with the running buffer and was equilibrated at 100 V/cm until a stable current and baseline was achieved. Substrate solutions were then injected into the packed CE-IMER at 280 V/cm for 3 s. For experiments where incubation was necessary, the column was suspended in the buffer. An electric field of 280 V/cm was applied to separate the substrate and products. For the study of trypsin digestion of