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Comparison of activity behaviors of particle based and monolithic immobilized enzyme reactors operated in semi-micro-liquid chromatography system

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ABSTRACT

The activity behaviors of particle based and monolithic immobilized enzyme reactors (IMERs) developed for proteomics applications in semi-micro-liquid chromatography were comparatively investigated. For monolithic IMER, the plain monolith was synthesized by in situ thermal copolymerization of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDM) in fused silica capillary. For particle based IMER, monodisperse-porous poly(GMA-co-EDM) beads 6.8 μm in size were synthesized by staged-shape template polymerization and used as support in a stainless steel microbore column. α-Chymotrypsin (CT) was covalently attached onto the particle based and monolithic supports via amination and glutaraldehyde coupling stages. In the case of monolithic IMER, higher final substrate conversions were achieved particularly with high substrate feed rates with respect to particle based IMER probably due to the convective diffusion in the macropores of continuous monolithic block. The final substrate conversion increased with increasing substrate feed concentration for monolithic IMER while a decrease was observed for particle based IMER. Hence monolithic IMER was superior when high substrate feed concentration was used with sufficiently high substrate feed flow rates.

1. Introduction

Immobilized enzyme reactors (IMERs) have been improved so as to identify proteins for the use of enantioselective analyses, to detect inhibitors, in drug metabolism research [7–14], when connected in separation devices [1–17]. The assembling of an IMER with the high performance liquid chromatography (HPLC) combines the specificity and sensitivity of an enzymatic reaction with the rapidity, selectivity, reproducibility of the chromatographic system. However in such systems, when microparticulate supports are employed, mass transfer is controlled exclusively by molecular diffusion that brings about some drawbacks such as band broadening and the conversion efficiency [17,18]. At the same time, widely used in modern chromatography, macroporous monolithic sorbents are unique in point of lack of diffusion limitations, associated with the low back pressure, high permeability for a liquid flow and high binding capacity of substrate to the active site of the immobilized enzyme. Since monoliths are devoid of diffusion resistance during mass transfer, they provide fast separation and rapid enzymatic conversion of substrates. Fast transfer of macromolecules along with the monolithic media is occurred by the existence of large mesopores. Many monolithic supports equipped with multifunctionality are present for protein immobilization [19–21]. The studies on the IMERs obtained via immobilization of trypsin on GMA–EDMA or silica monoliths for particularly protein digestion were published [22–25]. The enzymatic activity behaviors of methacrylate-based ultra-short monolithic minicolumns were also investigated [18]. Chymotrypsin (CT) has been widely preferred for the synthesis of monolithic IMERs particularly for proteomics studies. In most of these studies, silica based monoliths were utilized as the support matrix [26,27].

In present study, IMERs for micro-liquid chromatography were synthesized based on the monolithic and particulate stationary media. For particle based IMER, monosized-macroporous poly(GMA-EDM) particles were synthesized and aminated. The enzyme α-chymotrypsin (CT) was covalently attached to the amine carrying monosized-macroporous particles via glutaraldehyde activation. CT immobilized particles were slurry packed to 1000 μm i.d. stainless steel column. The IMER in the monolithic form was obtained by following the same chemical route for CT immobilization with poly(GMA-EDM) monolithic rod prepared in a 530 μm i.d. fused silica capillary tube. The chromatographic performances of particle and monolith based capillary IMERs were

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comparatively investigated in a micro-liquid chromatography system.

2. Materials and methods

2.1. Materials

Styrene (Aldrich Chem. Co., USA) was distilled under vacuum for the removal of inhibitor and stored in refrigerator. The other monomers, glycidyl methacrylate (GMA), ethylene dimethacrylate (EDM) were supplied from Aldrich Chem. Co., USA and used without further purification. Cyclohexanol (Cyc-OH), dibutylphthalate (DBP), 1,4-butanediol (BD), ethanol (EtOH), n-propanol (n-Pr-OH), and tetrahydrofuran (THF) were obtained from Merk A.G., Darmstad, Germany. Benzoyl peroxide (BPO), 2,2′-azobisizobutyronitrile (AIBN) recrystallized from methanol were supplied from Merck. Sodium lauryl sulfate (SLS) and polyvinylalcohol (PVA, 87–89% hydrolyzed, molecular weight: 85.000–146.000) and polyvinylpyrrolidone (PVP K-30, average molecular weight: 40.000 Da) were obtained from Sigma Chemical Co., USA. Fused silica capillary tube (OD: 850 μm, ID: 530 μm) was supplied from Polymicrotechnologies Inc., USA. 3-trimethoxysilylpropyl methacrylate (TMSPM) was supplied from Aldrich. Chem. Co. WI, USA. and used as received. The reagent, glutaraldehyde (GA, 25% w/w solution), α-chymotrypsin (CT) and benzoyl-L-tyrosine ethyl ester (BTEE) were supplied from Sigma Chem. Co.

2.2. Synthesis and characterization of monodisperse-porous poly(GMA-co-EDM) particles

Polystyrene (PS) seed latex 2.4 μm in size was obtained by dispersion polymerization [28]. The monodisperse poly(GMA-co-EDM) particles were obtained by a multi-stage polymerization protocol and their characterization were performed as described elsewhere [29]. Briefly, the organic phase including Cyc-OH (7 mL), DBP (2.7 mL), GMA (3.2 mL), EDM (2.1 mL) and AIBN (0.16 g) were emulsified in water (180 mL) including SLS (0.3 g) and PVA (1.4 g) by ultrasonication for 30 min. The dispersion containing PS seed particles (0.35 g) was added to the emulsion. The dispersion was magnetically stirred at room temperature for 24 h. Polymerization in the swollen seed particles was performed at 70 °C and 120 cpm shaking rate for 24 h. Monodisperse-porous poly(GMA-co-EDM) particles were washed with ethanol several times by successive centrifugation and decantation and extracted with THF.

2.3. Amination of poly(GMA-co-EDM) particles

The amination of poly(GMA-co-EDM) particles was performed by treating the particles (1.5 g) with aqueous NH_4OH solution (20 mL, 25% w/w) at 50 °C for 24 h with a shaking rate of 100 cpm in a temperature-controlled shaking water bath. The particles were extensively washed with DDI water and 0.05 M borate buffer at pH 7.8 and dispersed in borate buffer.

2.4. Glutaraldehyde activation of aminated monodisperse-porous particles

GA solution (3 mL, 25% w/w) was added into the aqueous dispersion containing aminated monodisperse-porous particles (ca 1.5 g) and borate buffer (20 mL). GA activation was performed at room temperature with magnetic stirring at 100 rpm for 30 min. GA activated particles were extensively washed with borate buffer at pH 7.8 by a centrifugation-decantation procedure.

2.5. Enzyme immobilization and slurry-packing of particles into fused-silica capillary tube

GA activated monodisperse-porous particles (1.5 g) were treated with CT (3 mg) in a borate buffer (50 mL) at pH 7.8 at + 4 °C for 24 h. CT immobilized particles were washed with borate buffer and dispersed in the same solution. CT immobilized particles were slurry packed to the fused silica tube (OD: 850 mm, ID: 530 μm, 100 mm in length) by a micro-LC pump. Finally, the column was extensively washed with aqueous monoethanolamine and borate buffer at pH 7.8 at a flow rate of 25 μL/min at room temperature for 24 h.

2.6. Monolith synthesis

The inner wall of the fused silica capillary was silanized according to the procedure given elsewhere [30]. The polymerization mixture containing 10% w/w GMA, 10% w/w EDM, 30% w/w n-PrOH, 20% w/w BD and 1% w/w AIBN was passed through the capillary with a flow rate of 100 mL/min then both ends of the capillary tube were sealed with GC septa. The sealed capillary tube was put in a thermostated water bath at 50 °C. The polymerization was performed at 50 °C for 24 h. Poly(GMA-co-EDM) monolith was washed with ethanol with a flow rate of 10 μL/min for 6 h for the removal of porogen.

2.7. Amination of monolith

NH_4OH solution (25% w/w) was passed through the poly(GMA-co-EDM) monolith for 1 h. Both ends of the capillary were then sealed by GC septa and the capillary tube was put into a thermostated water bath and kept 50 °C for 6 h. DDI water and borate buffer (pH: 7.8) were passed through the capillary monolith at a flow rate of 50 μL/min.

2.8. Glutaraldehyde activation

The activation solution (0.50 mL, 25% w/w GA in 2.0 mL, pH 8 borate buffer) was passed through the monolith at a flow rate of 10 μL/min at room temperature for 2 h. Both ends of the capillary monolith were sealed and the monolith was kept at 25 °C for 24 h. The capillary monolith was extensively washed with borate buffer at pH: 7.8.

2.9. Enzyme immobilization

The enzyme solution prepared by dissolving CT (5 mg) in borate buffer at pH 7.8 (2 mL) was passed through the aminated monolith with a flow rate of 10 μL/min for 2 h at +4 °C. Capillary monolith was washed with aqueous monoethanolamine and borate buffer for 2 h.

2.10. Determination of enzymatic activities of particulate or monolithic IMERs

A micro-liquid chromatography system (Dionex Ultimate 3000, USA) containing an oven, an injection unit, a gradient pump, and a diode array-detector (DAD) was used. All enzymatic activity experiments were done in isocratic mode. The monolithic or particle based micro-IMER was put in the oven thermostated at 25 °C. For conditioning of micro-IMER, the solution including 5% (v/v) ethanol and 95% borate buffer (pH 7.8) at 25 °C was fed for 1 h. The substrate feed solution (5% (v/v) ethanol, 95% borate buffer (pH 7.8) and 400 μM BTEE) was passed through the micro-IMER at 25 °C at a prescribed flow rate. The time when the flow of substrate feed solution started, was defined as zero-time in chromatographic