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An injectable alginate-based hydrogel for microfluidic applications

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ABSTRACT

The objective of this study was to develop an injectable alginate based formulation for immobilizing enzymes into microfluidic systems. The gelation was induced upon lowering the pH by addition of D-glucono-δ-lactone (GDL) and release of Ca^+ ions from solid $CaCO_3$. The effects of GDL concentration on enzymatic activity and gelation time were investigated. The results indicated that increasing the GDL concentration increased both surface area and enzymatic activity. Also, chitosan was added to the formulation at different ratios to enhance the stability of enzyme during immobilization. For microfluidic application, 100 μl spiral coil single channel microchip was fabricated and alginate GDL mixture containing β-glucosidase was injected to the microchannel prior to gelation. Enzymatic conversion was performed by pumping substrate (pNPG) through the microchannel. The results indicated that the entire substrate was converted continuously during 24 h without any leakage or deactivation of immobilized enzyme.

1. Introduction

Microfluidic systems have recently attracted considerable attention because of the possibility to provide an incorporated environment using small amounts of reagents and often providing rapid reactions and measurements. The reactions take place in a very small space with volumes in the nano- to milliliter range which provides an advantage of high efficiency and repeatability (Mross, Pierrat, Zimmermann, & Kraft, 2015). Therefore, microreactor technology has also been applied to the field of biocatalysis since 90s where enzymes have been employed for many processes including synthesis and diagnostics (Wirth, 2008). They have several advantages over traditional technologies especially in performing enzymatic reactions. The most remarkable benefits of microsystems are fast heat and mass transfer that cannot be reached in the conventional batch systems as well as reduced amounts of substrates and enzymes. Likewise flow of solutions in the microchannel systems is generally in laminar region which allows strict control of reaction conditions and time. Moreover, microchannel reaction systems offer high surface areas, which are valuable for many chemical processes such as catalytic reactions (Yesil-Celiktas, 2014).

Enzymatic biotransformations offer attractive solutions in many divisions of industry due to their high specificity, selectivity and environmentally-friendly nature (Meller, Pomastowski, Grzywiński, Szumski, & Buszewski, 2016). Enzymatic catalysis results in cleaner reactions and easier product recovery in comparison to the whole-cell catalysis. Since the enzyme stability can be enhanced while reducing the enzyme cost via recycling, the immobilization of enzymes has become increasingly essential for applied biocatalytic synthesis (Huang, Wu, Goldman, & Li, 2015; Park et al., 2015). Nevertheless, improvement of the complete enzymatic process is still an important demand for many fields (Yesil-Celiktas, Cumana, & Smirnova, 2013). Usually, enzymes can be immobilized onto a solid support by physical adsorption, entrapment in polymeric gels or covalent bonding. Different carrier materials such as inorganic pore glass, microporous silica, nanoporous aluminium oxide or organic polymers such as alginate and chitosan have been frequently used for immobilization (Dräger, Kiss, Kunz, & Kirschning, 2007). Although, several methods have been employed for preparation of microfluidic reactors, including immobilization of enzymes in microchannels by crosslinking, polymeric membrane or adsorption (Bolivar, Tribulato, Petrasek, & Nidetzky, 2016; Hickey et al., 2009; Honda, Miyazaki, Nakamura, & Maeda, 2006), the lack of practical methods for immobilized microflu-

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idic enzyme reactors require novel techniques for such processes. Immobilization on monolithic support is a simple method among various immobilization procedures (Meller et al., 2016). The polymeric matrix prevents aggregation and denaturation of enzymes by unfolding and can be easily loaded in microsystems (Cumana, Simons, Liese, Hilterhaus, & Smirnova, 2013).

Many inorganic and organic polymers are used as supports for immobilizing enzymes. The route used for specific enzymes depends on the selected enzyme and should be chosen cautiously. Therefore the support and the method of immobilization are fundamental aspects for a successful biocatalytic application (De Matteis, Germani, Mancini, Di Renzo, & Spreti, 2015). Immobilization of biocatalysts in Ca-Alginate matrices is a well-known technique amongst the various immobilization methods (Chai, Mei, Wu, Lin, & Yao, 2004). Alginate is a naturally derived linear polysaccharide copolymer containing β-D-mannuronic acid and α-L-guluronic acid, which can be obtained both from algal and bacterial sources. Alginates have various applications such as restraining dehydration, thickening, gel-forming and colloidal stabilizing in food industry and various applications in biomedical and pharmaceutical industries (Tavassoli-Kafrani, Shekarchizadeh, & Masoudpour-Behabadi, 2016). A significant property of alginate chains is that the guluronic residues can bind to adjacent alginate chains to form an egg-box structure in the presence of divalent metal ions such as Ca^{+2}. This ability causes the gelation of aqueous alginate solutions (Li, He, Huang, Li, & Chen, 2015; Wilson, Najia, Saeed, & Mcdevitt., 2014). Two fundamental techniques have been developed for alginate gelation; the diffusion method and the internal setting method. In the diffusion method, gelation starts at the outside layer and proceeds towards the center while divalent cations diffuse from bulk solution into an alginate droplet or layer. Depending on the gel thickness, this method might result in inhomogeneous gels (Draget, Skjåk-Bræk, & Smidsrød, 1997). In the internal setting method, cross-linking cations are dispersed into the alginate solution in insoluble form and gelation is initiated with releasing of the cations mostly by changing of pH, e.g. in presence of D-glucono-δ-lactone (GDL) or acetic acid. Alginate monoliths, beads and microspheres can be produced by both methods (Draget, Østgaard, & Smidsrød, 1990; Gurikov, Raman, Weinrich, Fricke, & Smirnova, 2015; Wan et al., 2008). However homogenous distribution of slowly hydrolyzing GDL gradually decreases the pH and might produce the alginate gels which can provide a homogeneous and mild immobilization matrix for enzymes. Particularly, for injection in complex and/or larger dimensions of microsystems, fast and uncontrollable gelation procedures are the major difficulties.

Therefore, we hypothesized that if GDL is added at an optimum concentration to the alginate solution than an injectable alginate hydrogel under controllable gelation conditions can be developed to immobilize enzymes into microsystems. The effects of GDL on gelation time and structural properties of the hydrogels were investigated. β-glucosidase was immobilized in the formulated hydrogel and enzymatic activities were measured to demonstrate immobilization performance of resulting gels. Furthermore, a single microchannel reactor was fabricated for testing efficiency and suitability of optimized alginate monolith for microfluidic applications. To the best of our knowledge, alginate gels have never been applied as immobilization matrix for enzymes in microsystems.

2. Materials and methods

2.1. Materials

Sodium alginate (ALG) (suitable for immobilization of microorganisms grade, G/M ratio 1.56), Chitosan (CHT) (degree of deacetylation: 75–85%, viscosity: 200–800 cps, medium molecular weight), Citric Acid Monohydrate, Tri-Sodium acetate anhydrous, Sodium Carbonate (Na2CO3) 4 nitrophenyl β-D-glucopyranoside (pNPG), 4 nitrophenol (pNP), β-D-*B*-glucosidase (from almonds, activity ≥ 6 U/mg) were purchased from Sigma Aldrich (Germany). D-Glucono-δ-lactone (GDL) and Calcium Carbonate (CaCO3) were purchased from Merck. β-glucosidase was obtained in liquid form from Novozyme (N188, 20–30% beta-glucosidase).

2.2. Microfabrication

Fabrication of microchannel reactor was carried out at the Institute of Microsystems Technology, TUHH, Hamburg. Pyrex glass with a thickness of 500 μm and silicon wafers were used for fabrication. Basic lithography techniques were applied to achieve the desired patterns on the wafers. Microchannels were structured in Si wafer by Deep Reactive Ion Etching (DRIE) while Pyrex wafer was etched chemically for inlet and outlet openings and two wafers were connected to each other by anodic bonding. The microreactor was designed as a single spiral coil with 5 turns (Fig. 1).

The inner and outer diameter of coil was 10 mm and 23 mm respectively, whereby the dimensions of the microchannel were $0.4 \times 0.92 \times 260$ mm (D × W × L) making up a total volume of approximately 100 μl.

2.3. Gelation procedures

Alginate solution (ALG) at a concentration of 2% (w/w) was prepared by mixing sodium alginate powder with an appropriate amount of water overnight. The lower ALG concentration could lead to enzyme leaching, while the higher might cause additional back pressure inside the microchannel. $CaCO_3$ was dispersed in the sodium alginate solution (2% w/w) by vigorous mixing (Ultra-Turrax). An ALG (dry basis)/$CaCO_3$ ratio of 1: 0.183 (w/w) was used as previously described (Gurikov et al., 2015). The enzyme was prepared in liquid form solution at the required concentration (0.8 mg enzyme/g gel), added to ALG/$CaCO_3$ mixture and homogenized. Freshly made aqueous GDL solution at various concentrations (25–200 mM) was added to the mixture (0.6 g GDL/g gel) and vortexed for a short time (<1 min). The homogenized mixture was left overnight for gelation. Optimized GDL-alginate gels were also combined with chitosan at different alginate-chitosan ratios. Gels were prepared with and without chitosan addition to determine the effect of chitosan addition on enzymatic stability. Both formulations were kept at 37 °C for 6 days and tested every day to determine loss in the enzymatic activity during the time period.

2.4. Enzymatic assay

Enzymatic assay was performed with 100 mg of ALG gel pieces containing 0.8 mg enzyme/g gel and 487.5 μl 20 mM pNPG solution in Na/Ca Acetate buffer (50 mM, pH 5.7). Samples were mixed with 9× volumes of 0.1 M Na_2CO_3 to stop the reaction. The absorbance was read at 420 nm. The temperature and assay time for N188 were 50 °C and 30 min, which were 37 °C and 15 min for pure β-glucosidase. The activities were expressed as moles of substrate (pNPG) converted per unit time and enzyme. The calibration curve was prepared with different concentrations of pNP.

2.5. Characterization of the gel matrices

Shrinkage experiment was performed by placing alginate samples in Na/Ca Acetate buffer and by measuring the weight and diameter of each sample periodically. Shrinkage (**S**) was quantified by measuring diameter of the hydrogel D_t after a given time t