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The development of a capillary microreactor for transesterification reactions using lipase immobilized onto a silica monolith

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ABSTRACT

The use of lipase immobilized on a silica monolith as a microreactor for performing lipid transformations is reported. The microreactor consists of a monolithic network formed within a 320 μm internal diameter fused silica capillary that provides a very large surface area for enzyme immobilization. *Candida antarctica* lipase was covalently bound onto the silica monolith using glutaraldehyde as the cross linking reagent. Successful immobilization was demonstrated by FTIR measurements on monolith fragments. The effectiveness of flow-through microreactors was tested for the ethanolysis of triolein (TO) at room temperature. TO was quantitatively transformed into ethyl oleate when using flow rates of < 1 μL/min. Non-aqueous reversed phased-high performance liquid chromatography (NARP-HPLC) with an evaporative light scattering detector (ELSD) and gas chromatography/mass spectrometry with electron impact ionization (GC/MS-EI) were both used for characterization and quantification of the products. The microreactors were used to perform lipid transformations directly online with atmospheric pressure photoionization (APPI) ionization mass spectrometry. They were also shown to be reusable without loss of activity for ~15 runs when operated at room temperature and flow rates of < 1 μL/min.

1. Introduction

A microreactor is a device that consists of microstructured components in which chemical reactions can take place in continuous flow [1]. New technologies for producing microreactors have been employed both in organic synthesis and more recently in biotechnology [2]. Microreaction devices were initially designed primarily for integrating different analytical or chemical processes including sample preparation, derivatization, separation and detection into a single platform. Most of the microreactors currently made enable the use of micro- and sometimes nano-liter process volumes, hence the terms 'microfluidic' and 'nanofluidic' are often applied [3]. Microreactors can be used as an efficient and sustainable way of doing chemical synthesis [1–4] that achieve high separation efficiencies and use small reagent volumes, thereby reducing waste. Other reported applications of microreactor technologies include their integration on-line with mass spectrometry for real-time proteomic analysis [[5,6], as emitters in electrospray [7] and atmospheric pressure photoionization [8] mass spectrometry and in chromatography and electrophoresis [9,10].

Commercially available microreactors can be expensive due to the complex methods that are required for their manufacture [11]. These fabrication methods are varied based on the end purpose of the microreactor, choice of the support (e.g. silica, quartz, metal, glass or polymer supports) [12–16] and the catalyst that is immobilized [13–18].

The objective of this work is to create and test a flow-through lipase immobilized silica monolithic microreactor for small-scale lipid transformations. The use of a lipase-mediated approach for lipid transformations is of major interest in biodiesel, structural lipids synthesis and in lipidomics [16,19–24]. A commonly used enzyme in lipid catalysis is the commercially available lipase isolated from *Candida antarctica* [19]. Immobilized *C. antarctica* lipase has been used by others for the conversion of triacylglycerides (TAG) to fatty acid alkyl esters, diacylglycerides and monoacylglycerides [13,23,24]. It has also been employed for synthesis of flavor compounds such as butyl laurate [14] or isoamyl acetate [25] and

Abbreviations: APPI, atmospheric pressure photoionization; APTES, 3-(aminopropyl)triethoxysilane; *C. antarctica*, *Candida antarctica* lipase enzyme; DO, diolein; ELSD, evaporative light scattering detector; FTIR, Fourier transform infrared spectrometry; GC/MS-EI, gas chromatography/mass spectrometry – electron impact ionization; EO, ethyl oleate/C18:1 ethyl ester; NARP-HPLC, non-aqueous reversed-phased-high performance liquid chromatography; MAG, monoglyceride/monoacylglycerol; MO, monoolein; ID, inside diameter; *p*NP, *p*-nitrophenol; *p*NPB, *p*-nitrophenyl butyrate; PEG, polyethylene glycol; SEM, scanning electron microscope; TAG, triglyceride/triacylglycerol; TEOS, tetraethyl orthosilicate; TO, triolein; U, unit of lipase activity.

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in hydrolysis of nitrophenyl butyrate [26]. Since enzymes are generally expensive, their immobilization is desirable to promote its recovery and reusability [1–3,13–17,26–28].

In the present study we investigate the use of a silica monolith support for lipase immobilization via silanol chemistry. A silica monolith should be a favorable substrate for enzyme immobilization since it can provide a large surface area while maintaining a good flow-through characteristic with minimal back-pressure [7,9,17,27–29]. The structure of the silica scaffold also allows a high loading capacity for the immobilized lipase.

The fabricated silica monolith microreactor has been employed for small-scale (microlitre volumes) transesterification targeted to analytical lipid applications. Previously, the product was collected off-line and then injected into a mass spectrometer for analysis [7,8]. Here, we demonstrate how transesterification products eluting from the microreactor can flow directly into an atmospheric pressure photoionization-mass spectrometer (APPI-MS) ion source, allowing real-time monitoring of the reaction. Although there are some reports of in-line coupling mass spectrometry (especially using ESI and MALDI) with enzymatic microfluidic devices [2,5–7] the technology is still in its infancy. Furthermore, while ESI is the ionization method of choice in proteomics, it is not suitable for the analysis of lipids such as fatty acid alkyl esters due to its poor response toward these low polarity compounds. For this reason, in this work we have used a photoionization source which is better suited to the analysis of many lipids compared to ESI. To the best of our knowledge, the integration of APPI-MS with enzymatic microreactor has not been reported before.

2. Material and methods

2.1. Materials

Fused silica capillary (ID: 320 μm) was obtained from Polymicro Technologies (Pheonix, AZ, USA). All the PEEK tubing (ID: 0.005 in), PTFE fittings (F-110), sleeves and ferrules were obtained from Upchurch Scientific (WA, USA). Tetraethyl orthosilicate (TEOS, 98%), 3-(aminopropyl)triethoxysilane (APTES, 99%), polyethylene glycol (PEG, mw: 10 000), sodium hydroxide powder (reagent grade, 97%), sodium phosphate monobasic monohydrate, disodium hydrogen phosphate (BioUltra, Fluka, 99%), glutaraldehyde solution (BioChemika, 50% in H_2O), sodium cyanoborohydride ($NaCNBH_3$, reagent grade, 95%), lipase from *C. antarctica* (EC 3.1.1.3, BioChemika), potassium sodium tartrate, copper sulfate, and Folin phenol reagent all were obtained from Sigma–Aldrich Ltd (ON, Canada). Acetic acid (glacial, HPLC) was purchased from Fisher Scientific (New Jersey, USA). Pure triolein (TO) together with ethyl oleate (EO) and TLC 18-1-A (contains by weight 25% of each of triolein (TO), diolein (DO), monoolein (MO), methyl oleate (MeO)) standards were purchased from Nu-Chek (Elysian, MN, USA). Acetonitrile, ethanol, dichloromethane, hexane, isopropanol and methanol were all HPLC analytical grade from Sigma–Aldrich (ON, Canada).

2.2. Preparation of the silica monolith capillary

The silica monolith capillary was prepared following the procedure reported elsewhere [7,9], but with some modification. Briefly, fused silica capillary (15 cm) was prepared by activating the surface with 1 M NaOH solution for 2 h at a flow rate of 10 μL/min. The residual alkali was washed by 0.1 M HCl for 2 h at a flow rate of 10 μL/min, and then the silica capillary was dried by a flow of air. The prepolymer sol–gel was prepared by mixing 0.1 g PEG with 0.45 mL TEOS and 1 mL 0.01 M acetic acid in ice for 45 minutes. The silica capillary was the filled with the prepolymer sol–gel at a flow rate of 5 μL/min for 2 h. Both ends of the capillary were then sealed before it was immersed into a water bath at 40 °C for 24 h. Finally, the resulting monolith was calcined to degrade all organic moieties by heating the capillary in an oven at 200 °C for a further 24 h. After this, the monolith was ready for lipase immobilization.

2.3. Lipase Immobilization onto the monolithic silica support

The lipase from *C. antarctica* was immobilized onto the monolithic supports using the glutaraldehyde as a cross linking agent, as previously reported [13,14]. To prepare the silica monolith capillary for immobilization, it was flushed with 0.1 mM sodium phosphate buffer (at pH 7.2). The capillary was filled with APTES solution containing 2:3:5 (v/v/v) parts of APTES: CH_3CH_2COOH: H_2O and left overnight at room temperature in order to ensure that the APTES had been fully grafted onto the silica monolith. This results in a surface containing free primary amines available for reaction with the cross-linker. The capillary was then washed with the 0.1 mM phosphate buffer for 1 h at 5 μL/min, followed by 5% glutaraldehyde solution in 0.1 mM sodium phosphate buffer for 4 h at the same flow rate. The capillary was again washed with a flow of phosphate buffer followed by loading with an 8 mg/mL lipase solution in sodium phosphate buffer containing 0.1% $NaCNBH_3$ for 24 h at room temperature and a flow rate of 1 μL/min. The lipase-immobilized microreactor was ready to use after a final flush with sodium phosphate buffer.

2.4. Evaluation of the lipase-immobilized mcroreactor

2.4.1. Characterization of mcroreactor

A scanning electron microscope (SEM) (JEOL 6301F; JEOL Ltd., Akishima-Tokyo, Japan) was used for imaging the silica monolith. Attenuated-total reflection (ATR) Fourier transform infrared (FTIR) spectroscopy (TENSOR 27 FTIR Spectroscopy with Diamond ATR A225/Q, Bruker Optics GmbH, Ettlingen Germany) equipped with OPUS 6.5 spectroscopy software (Bruker Optics GmbH, Ettlingen Germany) was used to characterize the changes in the surface functional groups during the multiple steps of lipase immobilization. Before the analysis, the diamond ATR crystal was solvent washed, dried and an infrared background was collected. The sample was loaded onto the ATR crystal and scanned in the mid infra-red region of 4000–400 cm^{-1}; 8 scan were averaged over 20 sec for each spectrum. For each step of microreactor fabrication (i.e. (a) silica monolith formation, (b) APTES treatment, (c) glutaraldehyde crosslinking, and (d) enzyme immobilization), 3 cm of the capillary was cut and crushed using a small grinder so the outer layer of capillary could be removed. Then, a small amount of each sample of monolith was taken out from the capillary respectively, and IR spectra acquired.

The porosity of the fabricated silica monolith capillary is described as a volume fraction [28]:

$$\text{Porosity} = \frac{(M_{mw} - M_M)/\rho}{(M_{mw} - M_M)/\rho}$$

where M_{MW} is the weight of the microreactor with water loaded, M_M is the weight of the microreactor alone; M_{TW} is the weight of empty capillary (i.e. without the silica monolith) with water loaded; M_T is the weight of empty capillary and ρ is the density of water.

2.4.2. Immobilized lipase assay

The concentration of the lipase solution introduced into the capillary and the concentration of lipase eluting from the capillary during the immobilization process were determined. Then from the mass balance, the amount of immobilized lipase on the silica monolithic based microreactor could then be determined. The