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Lipase immobilized microstructured fiber based flow-through microreactor for facile lipid transformations

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ABSTRACT

A facile continuous flow-through *Candida antarctica* lipase B immobilized silica microstructured optical fiber (SMOF) microreactor for application in lipid transformations has been demonstrated herewith. The lipase was immobilized on the amino activated silica fiber using glutaraldehyde as a bifunctional reagent. The immobilized lipase activity in the SMOF was tested calorimetrically by determination of *p*-nitrophenyl butyrate hydrolysis products. The specific activity of the immobilized lipase was calculated to be 0.91 U/mg. The SMOF microreactor performance was evaluated by using it as a platform for synthesis of butyl laurate from lauric acid and *n*-butanol in *n*-hexane and *n*-heptane at 50 °C, with products identified by gas chromatography–mass spectrometry (GC–MS). Different substrate mole ratios were evaluated, with 1:3, lauric acid:*n*-butanol showing best performance. Remarkably, percentage yields of up to 99% were realized with less than ~38 s microreactor residence time. In addition, the SMOF microreactor could be reused many times (at least 7 runs) with minimal reduction in the activity of the enzyme. The enzyme stability did not change even with storage of the microreactor in ambient conditions over one month.

1. Introduction

Microreactor technology (reagents and starting materials typically driven through channels on the order of 10–1000 μm) has seen exponential growth over the last decade with applications in the fields of analytical chemistry, chemical synthesis, chemical engineering and biotechnology [1–3]. The growth of microreactor technology could be attributed to their merits and unique operating characteristics compared to conventional batch reactors. Decreased channel dimensions of microreactors afford increased reaction efficiency due to their characteristic high surface area to volume ratio, faster diffusion dominated mass transport, enhanced heat transfer and thus reduced energy demands. In addition, microreactors enable good spatial and temporal controllability of system variables, high degree of chemical selectivity and use minute (microliters) reagent volumes thus environmentally friendly processes. Microreactors are also distinguished for their high through-put, potential for continuous processing and on demand scalability via parallelization. Moreover microreactors are amenable for synthetic and derivatization methodologies all integrated with online instrumental analytics [1,3].

Microreactors are typically fabricated on the planer surface of substrates (e.g. quartz, glass, polymers, ceramics, stainless steel, etc.) using microfabrication and precision engineering techniques such as photolithography, wet-etching, powder blasting, hot embossing, injection molding and laser micromachining, etc. [4–15]. For example, using these techniques microliter scale systems in multi-well plates and microfluidic chip formats have been reported extensively. On this reported platforms, the catalysts were either used in solution or immobilized [5–7]. A number of approaches have been investigated with regard to enzyme immobilization in microfabricated microreactors; these include immobilization on a microchannel surface (e.g. on polydimethylsiloxane, polyvinylidene fluoride, poly(methyl methacrylate) microchips), the use of porous polymer and silica monoliths prepared in the microchannels, cross-linked enzyme beads, agarose beads packed in the microchannel [8–14]. The walls of the microchannels could also be coated with a thin layer of γ-aluminum oxide, on which the enzyme is immobilized as reported by Thomsen and Nidetzky [15]. However, capillary microreactor format is probably the easiest and more facile to use for they do not demand specialized fabrication methods. Several authors have published on the use of capillary microreactors for continuous flow chemical synthesis, employing chemical catalysts and in some cases enzymes (in solution or immobilized either on a monolith or in continuous packed bed reactor) [3,16–23].

Enzyme catalyzed reactions are more desired for they are more efficient, highly selective, energy efficient, and require mild reaction conditions (temperature and pressure), thus meeting the target set by green chemistry [3]. In addition, the use of enzymes for catalysis compared to classical chemical catalysts is highly desired in food industrial processes because resultant products are classi-

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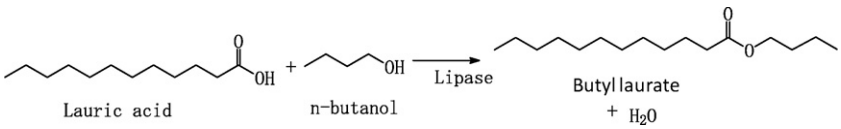

Fig. 1. Schematic of lipase catalyzed lauric acid and *n*-butanol reaction to form butyl laurate.

fied as 'natural' by food regulatory agencies, a feature that increases their public acceptance as ingredients for foods [24–28]. The commercial application of enzymes is however being still explored due to associated problems with enzyme stability, cost, efficiency of reactions and narrow substrate ranges for a given enzyme. Deployment of enzymatic catalysis in tandem with microreaction devices synergistically blends the many advantages of each and is thus a highly coveted development approach.

In our study, lipase (triacylglycerol acylhydrolases, E.C. 3.1.1.3) was selected as enzyme of choice for it is an extensively studied enzyme, relatively inexpensive and retains activity in organic solvents. Lipases have both hydrolytic and synthetic activity, catalyzing a plethora of reactions, including alcoholysis, transesterification, hydrolysis and aminolysis, with high chemo-, regio- and/or enantioselectivity. As such, lipases are highly promising enzymes with many applications in fine chemical synthesis, hydrolysis of fats and oils, modification of fats, flavor enhancement in food processing, resolution of racemic mixtures and many other applications in biotechnology [24–28].

We report herewith the use of a silica microstructured optical fiber (SMOF) containing highly ordered pore structure, often referred to as photonic crystal fiber (PCF). Since its development by Russell [29], the primary use of PCF has been in guiding light and has found utility in telecommunication, spectroscopy and biomedicine [30]. Other emerging but still scanty applications of the optical fiber include biosensor fabrication and their use as nanoelectrospray emitters [31,32]. We herewith demonstrate optical fiber revolutionary application for lipase immobilization and its use as a microreactor for facile lipid transformations. In our research we shall employ these fibers as enzyme supports. The immobilization of lipase is requisite for enzyme operational stability, ease of separation of desired products and recyclability. It has also been reported immobilization could enhance enzyme activity. Some of the different immobilization approaches that have been hitherto developed include: physical adsorption, cross-linking and covalent immobilization on a support (e.g. silica, polyacrylamide, PDMS, epoxy resins, agarose, etc.), with the latter method beneficial due to reduction in enzyme leaching [3,22,23]. In our work, lipase was covalently immobilized on SMOF using the bifunctional reagent glutaraldehyde, a well-known approach [23,33–35].

The performance of the microreactor was evaluated by its efficiency to mediate a simple reaction involving synthesis of butyl laurate (an important flavor compound) from lauric acid and *n*-butanol; synthetic scheme shown in Fig. 1.

2. Experimental

2.1. Materials

All aqueous solutions were prepared using >18 MΩ Milli-Q water (Millipore, Bedford, MA, USA). Sodium cyanoborohydride ($NaCNBH_3$), (3-aminopropyl)triethoxysilane (APTES), *Candida antartica* lipase B, HCl, sodium hydroxide, glutaraldehyde (50%), *p*-nitrophenyl butyrate, potassium sodium tartrate, *p*-nitrophenol, *n*-butanol and lauric acid were obtained from Sigma Aldrich. Glacial acetic acid, ACS grade *n*-hexane and ethanol (99%) were obtained from Fisher Scientific. The silica optical fiber, with product number, F-SM 20 (holes at each 4–5 μm), was purchased from Newport Corporation (Irvine, CA, USA).

2.2. Immobilization of lipase on the SMOF

Lipase was immobilized on the SMOF using a well-known glutaraldehyde method used by many other authors [23,33–35]. Fig. 2 illustrates the lipase immobilization schematic procedure on the SMOF. Briefly, the silica microstructured fiber (20 cm long) was treated with 1 M NaOH, and then flushed with 0.1 M HCl to neutralize the base. Due to the backpressure issues especially with the syringe pump used, which had a linear force of only 71 N, the length immobilized with lipase in the SMOF was limited to 20 cm, each time. In all cases a 3 mL BDS syringe and a Harvard Apparatus, 11 plus syringe pump were used. The fiber was then flushed with a 20% APTES solution (200 μL APTES, 300 μL glacial acetic acid and 500 μL H_2O) at a flow rate of 2 μL/min for 4 h, then capped at the distal ends and left overnight. Next, the fiber was flushed with a 0.1 mM phosphate buffer (always at pH 7.0) for 1 h at 3 μL/min, followed by 5% glutaraldehyde activation in 0.1 mM phosphate buffer at the same flow rate for 4 h. After being flushed with the 0.1 mM phosphate buffer, 8 mg/mL lipase solution in 0.1 mM phosphate buffer, with 1% $NaCNBH_3$, was flushed through the fiber for 24 h at 2 μL/min. The SMOF was finally flushed with the phosphate buffer and was ready for use as a microreactor.

2.3. Reaction experiments

A typical reaction mixture consisted of 0.015 M lauric acid and 0.045 M *n*-butanol reaction dissolved in *n*-hexane or *n*-heptane. The reactant solution was infused through the SMOF microreactor at a flow rate of 1 μL/min in a thermostated oven, 50 °C. A schematic showing the reaction set-up is shown in Fig. 3a. The reaction products were analyzed by gas chromatography–mass spectrometry (GC–MS). The reactant solution was also injected into the GC–MS as a reference to calculate % conversion.

2.4. Measurement of lipase activity

Activity of the immobilized lipase was determined by spectrophotometric determination of the hydrolysis products *p*-nitrophenyl butyrate (*p*NPB), a well-known procedure [36]. Briefly, a 3.4 mM solution of *p*NPB was prepared (in a 1:1 mixture of acetonitrile and 0.1 mM phosphate buffer) and flushed through a 20 cm SMOF microreactor at 1 μL/min. The hydrolysis products were collected, the volume measured, and the sample was diluted to 2.5 mL. Absorbance was measured at 410 nm on a Jenway 3600 spectrophotometer. Standard solutions of *p*-nitrophenol (*p*NP) were prepared at 7.0, 5.0, 3.0, and 1.0 mM to prepare a calibration curve. The solvent mixture was used to calibrate the spectrophotometer for the standards, while the 3.4 mM *p*NPB was used to calibrate the spectrophotometer for the sample. The concentration of *p*NP hydrolyzed was determined and lipase enzyme activity calculated. One enzyme unit (U) was the amount of protein liberating 1 μmol of *p*NP per minute.

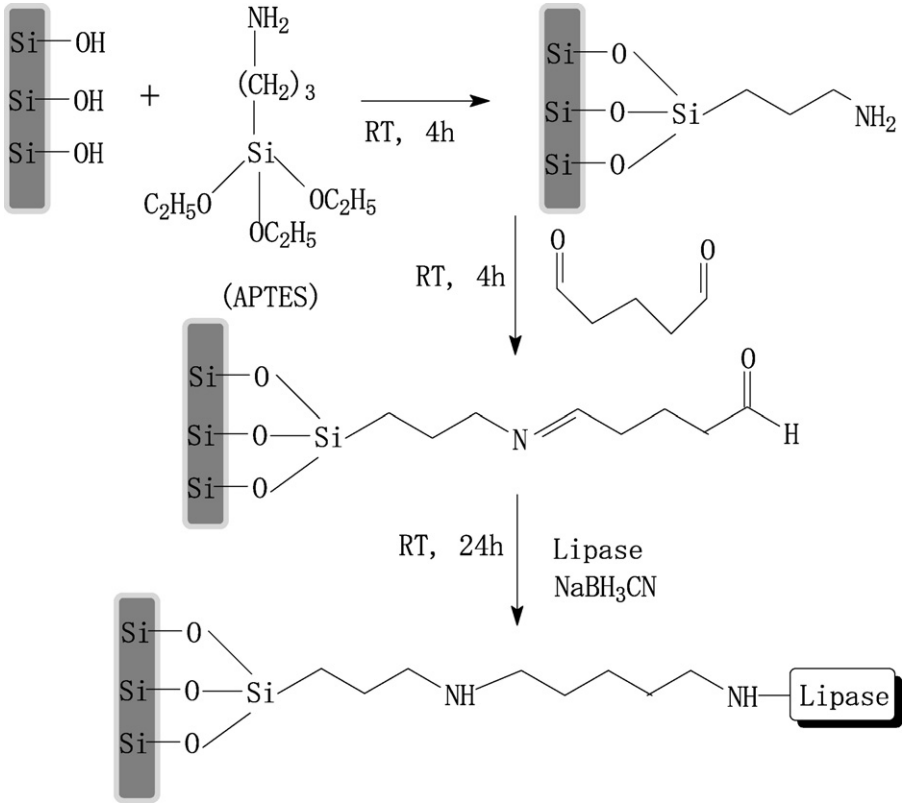

Fig. 2. Schematic diagram showing the immobilization of lipase on the silica microstructured optical fiber.

2.5. Measurement of amount of lipase loaded

The *C. antartica* lipase B concentration immobilized on the SMOF was determined using the standard Lowry's protein assay [37]. Using starting lipase solution as a standard, different concentrations were prepared and a standard curve was generated. The enzyme loaded was determined indirectly from the difference between the amount of enzyme introduced into the SMOF and the amount of enzyme collected from the effluent emerging from the SMOF.

2.6. Instrumentation

An Agilent 6890N GC system coupled to a 5975C MSD series was employed for the analysis of the reactants and the products. The products were confirmed by comparison of mass spectral fragmentation patterns with those stored in the MS data bank (Wiley 7, NIST 2002). The temperature program was relatively simple: from 40 °C to 220 °C at 8 °C/min; the final oven temperature was maintained for 3 min. The injection port was maintained at 270 °C. A capillary column, HP-5MS (30 m, I.D. 0.250 mm, film, 0.25 μm) was employed.

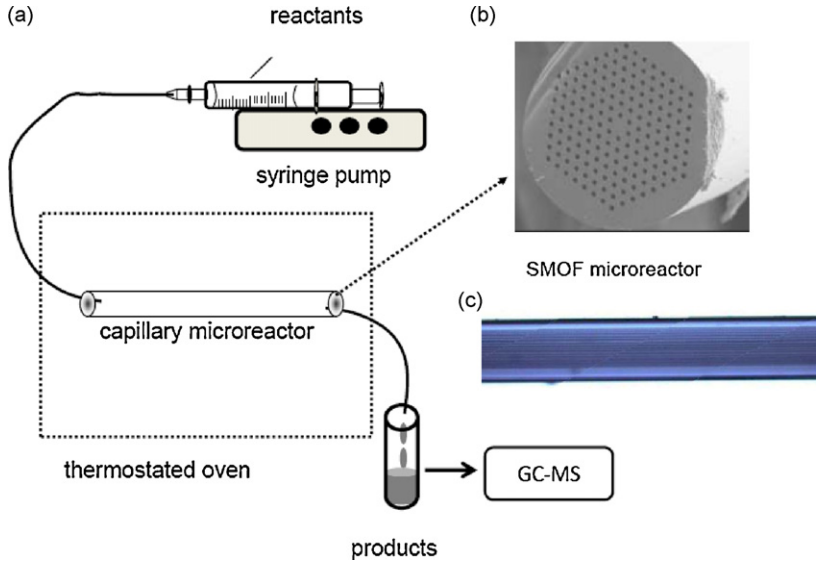

Fig. 3. (a) Schematic set-up of the flow-through SMOF microreactor; (b) SEM image of a cross-section of the SMOF microreactor; (c) micrograph of the SMOF microreactor.

2.7. Scanning electron microscopy

The SMOF was cleaved using a ceramic cutter and mounted normal to the aluminum stub using a tape to facilitate imaging of the cross-section. Scanning electron microscopy (SEM) images were obtained using Zeiss EVO MA 15 LaB_6 filament scanning electron microscope. The specimens were coated with gold using a Nanotech SEM Prep 2 DC sputter coater.

3. Results and discussion

Prior to immobilization the SMOF morphology was visually evaluated by SEM and a microscope and the images are shown in Fig. 3b and c. Clearly the SMOF has got a highly ordered and numerous (154 holes) pore structured morphology, which lends itself to good porosity, high surface area for enzyme loading, and as such ideal as a flow-through enzymatic microreactor platform. The pores in the SMOF were about 4–5 μm, with a jacket made from acrylate, which improves rigidity and robustness of the fiber. The fact that the fiber was made of silica made it amenable for straightforward enzyme immobilization using well-known silica chemistry, schematic delineated in Fig. 2 [3,23,33–35].

The enzyme immobilization procedure entailed reaction of the silanol groups present on the silica fiber with APTES introducing amino groups on the silica surface. The surface amino group provides an active site to react with a bifunctional reagent glutaraldehyde via an imine bond, leaving a reactive pendant aldehyde functional group. Consequently, the primary amino groups of the lipase react with the aldehyde group potentially forming a Schiff base (C=N bond), which is further reduced to C–N bonds by a reducing agent ($NaCNBH_3$) to enhance the stability of the immobilized lipase. Before employing the lipase loaded SMOF microrector it was essential to determine the success of the immobilization method. The total free protein concentration was determined using the well-documented Lowry protein assay. The amount of protein bound to the SMOF microreactor support was estimated by the difference between amount of protein in lipase solution infused in the SMOF and the effluent emanating from the microreactor. Using this method, the amount of protein loaded on the SMOF was determined to be 5.62 μg/μL. It is apparent that not all lipase loaded in the microreactor is active, due to underlying physicochemical factors associated with the immobilization. The activity of the *C. antartica* lipase B was determined as described in the methods section. The lipase activity was calculated to be 5.1 μg *p*NP/min (U) for the 20 cm SMOF microreactor. In most cases, enzyme activity is typically reported in U/mg-support [36]. As such the specific activity of the lipase loaded in the SMOF microreactor was calculated to be 0.91 U/mg. The *C. antartica* lipase B specific activity used as reported by the manufacture was 1.16 U/mg. Therefore, only about 22% lipase activity was lost due to the immobilization process. The lipase activities data reported were confirmed by repeating the assays on four SMOF microreactors.

The SMOF microreactor was then tested for its lipid transformation performance. Esterification of lauric acid with *n*-butanol to form butyl laurate was employed in evaluating the effectiveness of the immobilized SMOF microreactor. Butyl laurate could be classified as a short chain fatty acid ester commonly used as flavor agents. Other flavor compounds that fit in this category could include ethyl caproate (apple, banana, and pineapple flavor), ethyl butyrate (banana and pineapple), and ethyl valerate (apple flavor), etc. [26,38–41]. The choice of the reaction was based on the incessant current market trend for use of natural products as flavoring agents (e.g. those stated above) in food applications. Compounds synthesized enzymatically fit the definition of 'natural' and are highly desired. The reactants mole ratio evaluated were

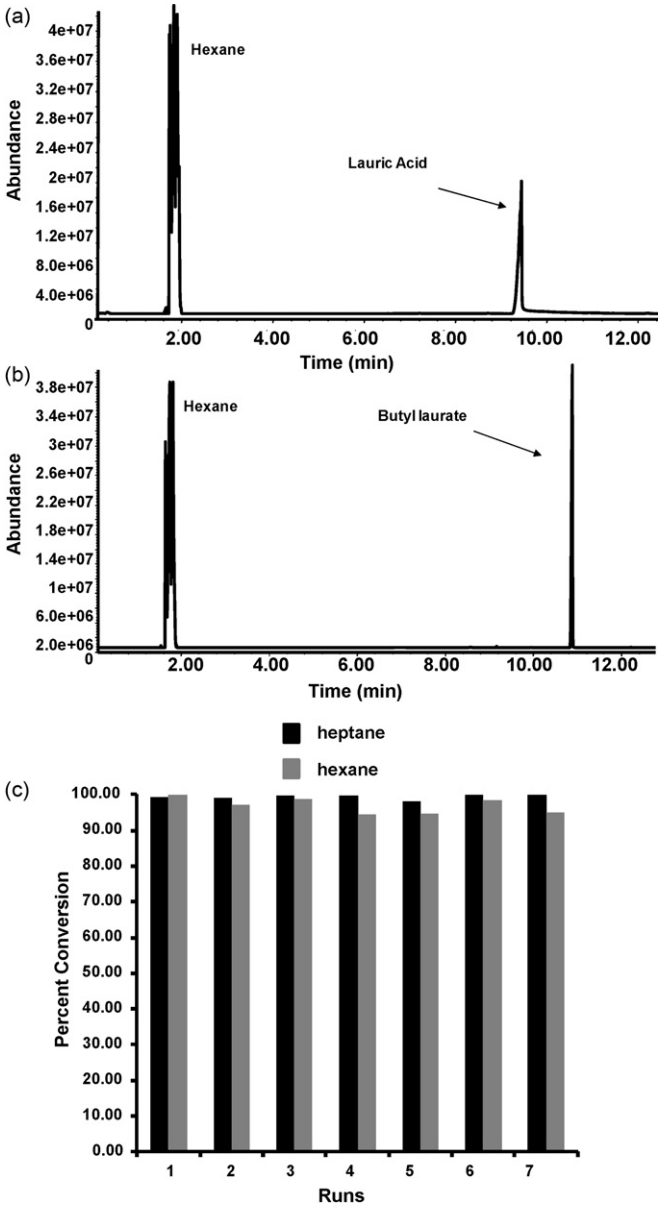

Fig. 4. (a) Lauric acid and *n*-butanol reactants mixture obtained chromatogram; (b) chromatogram of the reaction products (butyl laurate) with hexane as solvent; (c) graph showing comparison of percentage conversions of lauric acid and *n*-butanol (1:3) using *n*-hexane and *n*-heptane as solvent at 50 °C and run-to-run stability on the same SMOF microreactor.

1:1, 1:2, and 1:3, lauric acid to *n*-butanol (dissolved in *n*-hexane or *n*-heptane), which is similar to what has been used by others doing related transformations [38–41]. The reactants were infused through the SMOF microreactor at a flow rate of 1 μL/min, and therefore the reactants had a very low residence time, calculated to be ~38 s in the microreactor. The reaction temperature was maintained at 50 °C using an oven as shown in the reaction schematic set-up (Fig. 3a). This optimal temperature was chosen based on other researchers' findings working on similar reactions [40,41].

To evaluate the efficiency of the SMOF microreactor, the reactants were passed though a blank SMOF (fiber gone through all the treatments other than lipase immobilization) and the eluent injected into a GC–MS. Consequently, the area of the lauric acid was determined; a representative chromatogram for the reactants is shown in Fig. 4a. Clearly, no product peak was obtained in this case. Similarly, the eluted products from the SMOF microreactor

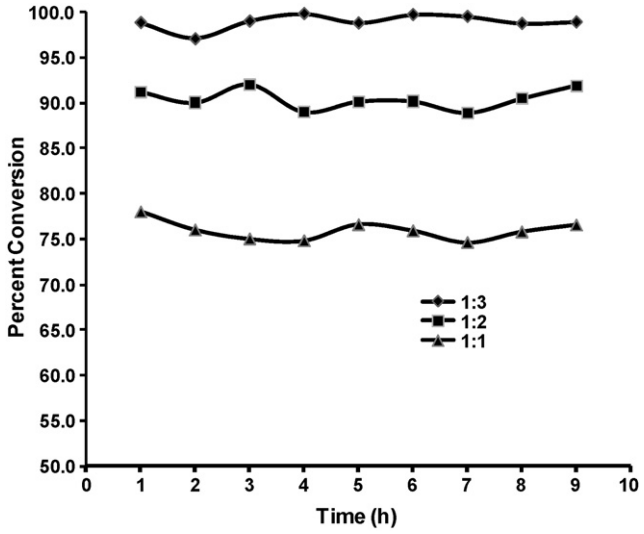

Fig. 5. Comparison of the performance of SMOF microreactor at different lauric acid and *n*-butanol ratios (1:1, 1:2, 1:3) over a 9 h run at 50 °C, with analysis of the products by GC–MS after every hour.

were subsequently injected into the GC–MS and as clearly shown in Fig. 4b, the product consisted of a huge product (butyl laurate) peak and a small remnant lauric acid (reactant) peak. Clearly based on the products chromatogram, almost complete conversion was obtained. The percent conversion was calculated by evaluating the reduction in the area of the lauric acid based on the following equation:

$$\%\,\text{Conversion} = \left[1 - \left(\frac{\text{Lauric acid peak (products)}}{\text{Lauric acid peak (reactants)}}\right)\right] \times 100$$

Fig. 4c shows graphically the percentage conversions obtained for several runs using *n*-hexane and *n*-heptane as solvents, temperature was maintained at 50 °C and the lauric acid to *n*-butanol ratio was 1:3. No significant difference in percent conversion between the two solvents was apparent. However, *n*-heptane may be preferred for it is known to be less toxic. The conversions for the 7 runs are consistent at an average of about 99%. Clearly, the percentage conversion is impressive especially when the short contact time of the reactants in the microreactor is considered.

Important to note though, is the reusability of the lipase immobilized microreactor in biotransformation. No loss in conversion (and as such in activity of lipase) was observed after carrying 7 runs all done over a period of one month. The immobilized microreactor was stored at room temperature with no special care taken in terms of keeping it in storage buffers. All that was required was to flush the microreactor before and after the reaction with 0.1 mM phosphate buffer (pH 7.0). The achieved stability of the immobilized lipase on the SMOF microreactor sharply contrasts to the 20% loss of activity of free lipase in 0.025 M phosphate buffer (pH 7.0) at room temperature in only 4 h reported by Sun et al. [42]. As such, it is evidently possible to use the SMOF-lipase immobilized microreactor for rapid biotransformations with reuse of the same microreactor multiple times without any loss in enzyme activity. This would be highly attractive and cost effective, because one of the prohibitive reasons why enzymes have not been overly exploited in industry for biotransformations is the cost involved. The substrate ratio is known to be a parameter that would affect the product yields. An experiment (Fig. 5) was carried out where different substrate ratios were tested. The reactants were allowed to infuse through the microreactor incubated at 50 °C for 9 h continuously; the products ere analyzed every 1 h. The reduction of the lauric acid to *n*-butanol ratio from 1:3 to 1:2 and then to 1:1 was found to reduce the percentage conversion from ~99% to ~90% to ~75% respectively. As such, it quite obvious to conclude the lauric acid to *n*-butanol (1:3) ratio would be the optimum with the near complete conversion observed.

While it is arguable how scalable SMOF microreactors would be for industry applications, the high conversions obtained with such short microreactors (20 cm) show with longer fibers even much better conversions would be achievable, resulting into near complete conversions. This is highly desirable because of the pure products alleviates the need for downstream purification strategies which can be expensive. Clearly, no other side products were observed due to the mild conditions employed for the synthesis, which alleviates waste and lends itself for good atom economical synthesis. In addition, it is possible to prepare parallel (multiplexed) synthesis where numerous microreactors are employed for synthesis; this could go a long way in scaling production. It is also envisioned it is possible to tailor make microstructured fibers with slightly bigger pores and probably a bigger cross-sectional area, which would increase the surface area for immobilization of the enzyme and as such increase the possible amounts of reactants that can be infused into the microreactor. We are also working on possible use of the SMOF microreactors carrying out biotransformations requiring multi-step and possibly tandem multi-catalytic conversion, e.g. compounds of pharmaceutical applications. This can be done by hyphenating SMOF microreactors with different enzymes immobilized. The long length of the combined SMOF microreactor would require a high performance syringe pump with higher linear force.

One immediate feasible application for the microstructured platforms as microreactors would be in analytical derivatizations especially in lipid analysis. For example, we are currently demonstrating the microreactor application to derivatization of blood lipids to make them amenable to analysis by mass spectrometry. In addition the design of the SMOF microreactor is such that it can be online coupled to the mass spectrometry for use as a derivatizing platform and also as a multinoozle emitter in nanoelectrospray mass spectrometry [32]. This will be particularly attractive and valuable for the lipidomics community.

4. Conclusion

There is a general consensus the current chemical synthesis paradigm in which metal catalysts (most of which are toxic) in tandem with batch synthesis is wasteful (due to poor mass transfer) and results in many unwanted byproducts which have to be separated from the product of interest. Sustainable (green) synthesis demands use of benign catalysts (enzymes) and a careful choice of reactants to get highly atom economical reactions with preferably large yields of the product of interest and no side products. In this project, we have clearly demonstrated it is possible to develop facile enzyme loaded microreactors that can be used as continuous flow systems for important chemical transformations. Since one of the limitations of the use enzymes is their initial cost, we have demonstrated the immobilized enzymes can be reused numerous times without loss of activity, making the synthesis economical.

Whilst the project has proved very promising, more rigorous research is underway to fully optimize enzyme loading and evaluate other methods of enzyme immobilization. Different custom made SMOF morphologies with much higher surface area will also be investigated. In addition, we are evaluating the scope of applicability of the SMOF microreactor in synthesis of interesting class of compounds such functional foods, biodiesel, structured lipid, lipid antioxidants, loading other types of bio and organocatalysts. Indeed the microreactor could be used for laboratory pilot (reaction opti-

mization and enzyme screening tool) study of potential product synthesis of industrial interest.

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