Enzyme-functionalized polymer brush films on the inner wall of silicon–glass microreactors with tunable biocatalytic activity†

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The lipase from *Candida Rugosa* was immobilized to a poly(methacrylic acid) polymer brush layer, grown on the inner wall of silicon–glass microreactors. The hydrolysis of 4-nitrophenyl acetate was used as a model reaction to study the activity of this biocatalytic system. The amount of bound lipase could be tuned by changing the polymerization time of the brush formation. The Michaelis–Menten constants and V_{max} values, determined for immobilized and free lipase, are similar, demonstrating that the lipase's substrate affinity and its activity remain unchanged upon immobilization to the microchannel wall.

Introduction

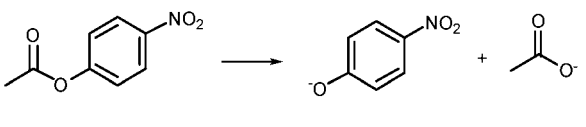
Microreactors for performing enzymatic reactions exhibit numerous advantages compared with conventional batch systems in terms of *e.g.* laminar flow, reduced reaction times, and high conversion efficiencies.[1–3] Microreactors have been used both for homogeneous[4,5] and heterogeneous biocatalysis, using free and immobilized enzymes, respectively. Several publications describe the development of new methodologies to conduct heterogeneous biocatalytic reactions, since they allow easy separation and recycling of enzymes, however, they still contain disadvantages.

Packed-bed and slurry microreactors with silica[6,7] and magnetic nanoparticles,[8] agarose beads[9] and zeolites,[10] all functionalized with enzymes, have been reported. These examples and monolithic microreactors, in which an enzyme is absorbed within a polymeric[11] or macroporous silica[12,13] matrix, comprise simple and efficient ways to perform biocatalysis. However, these methods are inconvenient for large scale processing, because they may exhibit increased pressure drops along the microchannel.[1] In order to solve this issue, other approaches were investigated. Enzyme immobilization on a nylon membrane in a microchip, formed by interfacial polycondensation, was reported.[14] Silica spheres were deposited on micro-capillary inner walls as support for anchoring lipase.[15,16] However, chemical modification of the microreactor surface interior was the most exploited method. A mesoporous silica layer[17] was implemented on the channel interior for enzyme immobilization, but it resulted in low substrate association with the active enzyme sites. Recently, covalent immobilization of an enzyme to a coated macroporous γ-aluminum oxide layer was reported.[18,19]

A cross-linking enzyme membrane on a microchannel surface[20] for biocatalysis was developed. However, this technique is limited to enzymes with low isoelectric points. Other examples of wall coatings are based on silane nanostructures,[21,22] which provide the formation of an enzyme multilayer, offering a good biocatalytic activity. On the other hand, these methods do not display control over the amount of anchored enzymes. Moreover, it is known that silanized surfaces may cause nonspecific absorption and enzyme denaturation.[23]

Polymer brushes have been widely applied for anchoring enzymes and proteins onto flat gold or silicon surfaces,[23–25] since they offer mechanical and chemical stability, providing a high number of reactive functional groups controllable by the selected monomer type. We demonstrated that thin layers based on polymer brushes, grown on a microchannel wall, can be efficiently used for anchoring an organic catalyst[26] and metal nanoparticles[27] in microreactors. In this paper the fabrication of a poly(methacrylic acid) (PMAA) polymer brush layer grown by iniferter-mediated photopolymerization[28] on the interior of silicon–glass microreactors is described, in addition to the covalent attachment of an enzyme to the carboxylic acid groups of the polymer and a study of its activity. *The advantage of this approach is that, varying the polymer thickness, the number of biocatalytic sites can be easily tuned.*

The lipase C*andida Rugosa* type VII was selected, and the hydrolysis of 4-nitrophenyl acetate (**1**) to give 4-nitrophenolate (**2**) and acetate (**3**) (Scheme 1) was performed as a model reaction.



Scheme 1 Lipase-catalyzed hydrolysis of 4-nitrophenyl acetate (**1**).

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† Electronic supplementary information (ESI) available: Figure with a comparison of the thickness of the PMAA brush *versus* the polymerization time measured both by AFM and ellipsometry. See DOI: 10.1039/c0lc00187b

Results and discussion

Synthesis of the poly(methacrylic acid) brush film and immobilization of the lipase on a flat surface

The fabrication of the biocatalytic polymer brush layer was first performed on a flat silicon oxide surface in order to fine-tune the reaction conditions.

Polymethacrylic (PMAA) polymer brushes[29] were synthesized according to the procedure summarized in Scheme 2 (top). First a monolayer of [(chloromethyl)phenylethyl]trichlorosilane (CTS)[30] was grown on a silicon wafer. This layer was subsequently functionalized with sodium diethyldithiocarbamate trihydrate (DTC), in order to obtain the initiator-bearing monolayer. The samples were then placed in a glass flask containing an aqueous solution of methacrylic acid (MAA) and irradiated using a UV-lamp for 5–40 min, to give PMAA polymer brushes. The PMAA layer thickness values were analyzed with Atomic Force Microscopy (AFM) by mechanically removing the polymer brush film and subsequently measuring the step height between the bare underlying substrate and the brush surface in five different positions on each sample. The thickening rate was also analyzed by ellipsometry and the thickness values were in very good agreement with those of the AFM measurements (see ESI†). A plot of the brush layer thickness *versus* polymerization time (Fig. 1) shows the typical profile for iniferter-based surface-initiated photopolymerization.[29,31] The PMAA layer thickness values were also confirmed by ellipsometry.

The PMAA brush layers, formed on the silicon oxide surface, were subsequently functionalized with *N*-hydroxysuccinimide (NHS) and then immersed in a buffered solution of the lipase (Scheme 2, bottom). The enzyme-functionalized polymer films were analyzed by AFM, showing about a doubling of the layer thickness value compared to that measured for the precursor PMAA film (Fig. 1), confirming the immobilization of the lipase and the correlation between the amount of immobilized lipase

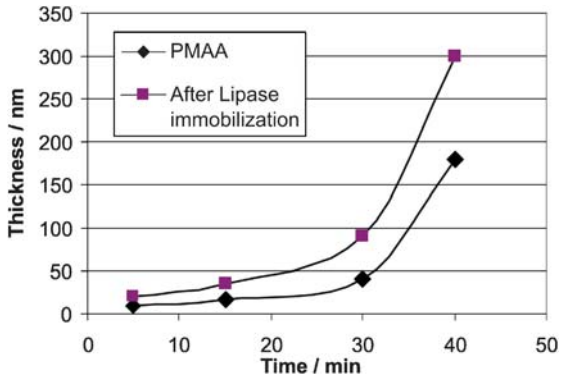


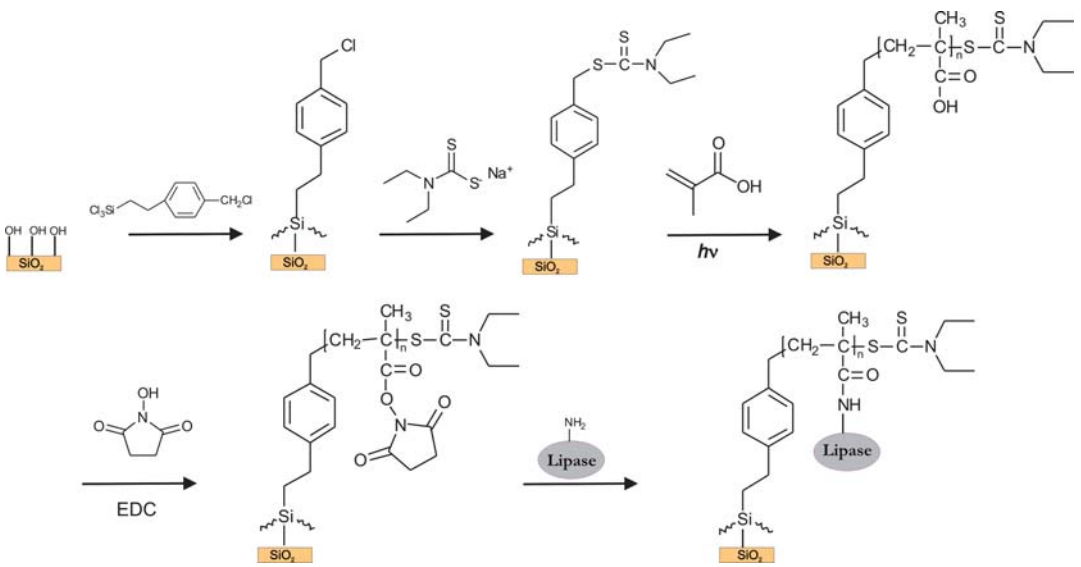
Fig. 1 Plot of PMAA polymer thickness *versus* polymerization time on a flat surface measured by AFM: (◆) = PMAA and (■) = PMAA–lipase. A line was added to guide the eye.

and the layer thickness. Additional proof for the functionalization was obtained by FT-IR spectroscopy, which revealed the presence of the typical NHS-ester peaks at 1744 and 1780 cm^{-1} and subsequently the amide peaks at 1550 and 1650 cm^{-1}, upon treatment with the NHS/EDC and enzyme solutions, respectively (Fig. 2).

Synthesis of the poly(methacrylic acid) brush film and immobilization of the lipase on the microreactor channel wall

The same procedure, as developed to immobilize the enzyme on the silicon oxide surface, was applied to the channel wall of silicon–glass microreactors[26] (Fig. 3), in order to obtain the biocatalytic brush layer.

Successively, solutions of CTS and DTC were flowed through the microchannel at a flow rate of 0.1 μL min^{-1}. Afterwards a 1 M aqueous solution of MAA was pumped through the microreactor at 1 μL min^{-1}, while irradiating by a UV lamp. The polymerization time was varied between 5 and 20 min, in order to



Scheme 2 General scheme for monolayer formation of the photo-initiator, followed by surface-initiated photopolymerization of MAA, NHS ester formation, and lipase immobilization.

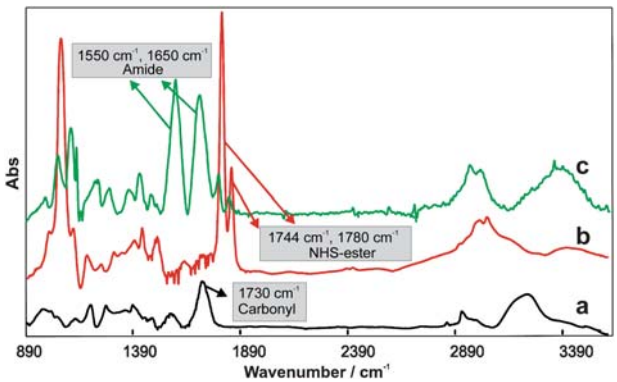


Fig. 2 FT-IR spectra of: (a) PMAA, (b) PMAA functionalized with NHS/EDC, and (c) PMAA–lipase.

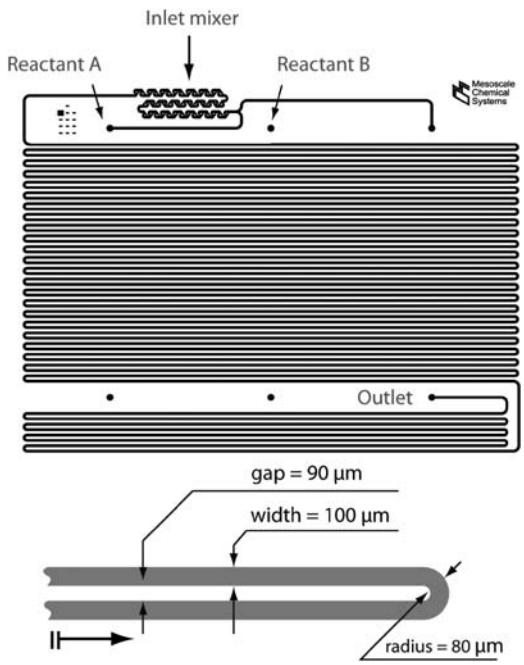


Fig. 3 Schematic representation of the silicon–glass microreactor used in this study.

achieve different thicknesses, and consequently different numbers of functional groups.

Once the PMAA polymer brushes were formed, the carboxylic acid groups were reacted with NHS. Afterwards a buffered lipase solution was flowed through the microreactor, to give the PMAA–lipase brush layer (Scheme 2).

The thickness values of the polymeric coatings were determined by high resolution scanning electron microscopy (HR-SEM), after 5, 10, and 20 min polymerization time, resulting in values of 13.5, 23, and 30 nm, respectively. These analyses were carried out on the channel cross-section after breaking the device (Fig. 4).

The catalytic devices exhibited no leaching and could be re-used for at least 40 days when stored in a buffer solution at 4 °C.

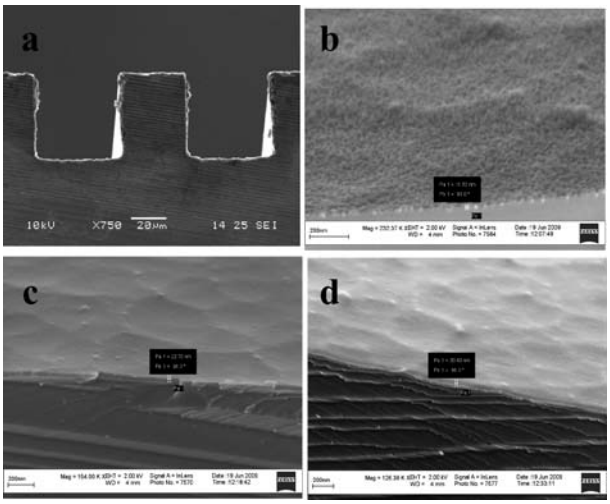


Fig. 4 (a) HR-SEM image of the channel cross-section before PMMA brush growth. The channel surface shows some roughness as a consequence of the etching procedure. Channel cross-sections of devices bearing PMAA–lipase brushes formed with polymerization times of 5 min (b), 10 min (c), and 20 min (d).

Hydrolysis of 4-nitrophenol acetate in the biocatalytic microreactor

Microreactors, bearing a PMAA–lipase brush layer, were used to study the hydrolysis of 4-nitrophenyl acetate (**1**) (Scheme 1) in PBS buffer solution (pH = 7.4) at room temperature. The brush layer turned out to be very efficient in the biocatalysis; 4-nitrophenolate (**2**) was fully formed after a few minutes. No product could be detected when a solution of **1** was flowed through microreactors having only PMAA polymer brushes, proving that the lipase was the catalytically active species.

A kinetic study of the hydrolysis of 4-nitrophenyl acetate (**1**) was performed in microreactors bearing a PMAA–lipase brush layer obtained after 5 min polymerization time. The reaction was followed measuring the formation of the 4-nitrophenolate (**1**) UV-vis absorption peak at 405 nm, using an on-line UV-vis detection set-up.[26] The hydrolysis was carried out with different concentrations of **1** (70–210 µM). The data were fitted to a first-order rate equation (Fig. 5). The rate constants for different

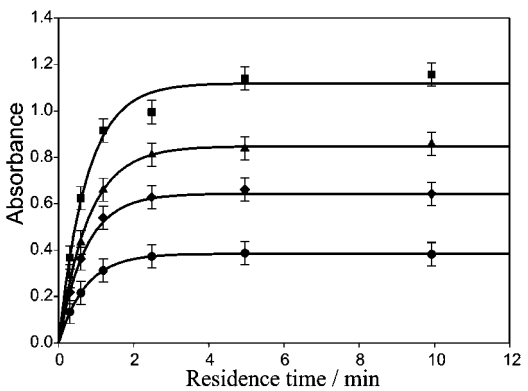


Fig. 5 Formation of **2** catalyzed by lipase immobilized on PMAA brushes at different concentrations of **1**: (■) = 210 µM, (▲) = 140 µM, (◆) = 105 µM, (●) = 75 µM.

concentrations of 4-nitrophenyl acetate (**1**) were the same [(22 ± 1) × 10^{-3} s^{-1}], within experimental error, as expected for first-order conditions.

The reaction was also performed with microreactors having PMAA–lipase layers fabricated applying 10 and 20 min polymerization time; the rate constants were (26.0 ± 1.3) × 10^{-3} s^{-1} and (30.0 ± 1.5) × 10^{-3} s^{-1}, respectively.

The enzyme coverages in the microreactors were measured using the BCA assay, being 3.25, 3.75, and 4.25 × 10^{-8} μg μm^{-2}, for microreactors with PMAA–lipase brush layers having thickness values of 13.5, 23, and 30 nm, respectively. A linear dependence was found between these enzyme coverages and the corresponding rate constants (Fig. 6). This infers that, using PMAA brushes, it is possible to control the amount of immobilized enzyme changing the polymerization time. In addition, it shows that all enzyme molecules are involved in the biocatalysis and consequently that the whole layer is accessible for reagents.

In order to investigate the activity of the anchored lipase, the Michaelis–Menten constant (K_M) and the maximum velocity (V_{max}) were determined both for lipase immobilized in the microreactor having a PMAA layer thickness of 13.5 nm, and for free lipase in solution, at the same enzyme concentration. The initial rates (r_{in}) of the hydrolysis of **1**, at different concentrations, were derived from the kinetic data and plotted *versus* substrate concentration (Fig. 7). Fitting to the Michaelis–Menten equation: $V = V_{max} \times [\mathbf{1}]/(K_M + [\mathbf{2}])$, yielded values for the Michaelis–Menten constant and V_{max}. The K_M values were similar, being (5.6 ± 0.2) × 10^{-4} M and (6.7 ± 0.3) × 10^{-4} M, for immobilized and free enzyme, respectively. Also the V_{max} value of (10.0 ± 0.5) × 10^{-4} M s^{-1} is the same for both the immobilized and the free lipase. This experiment clearly shows that the covalent immobilization of lipase on PMAA does not affect the enzyme's affinity for the substrate, nor the activity. The brush layer does apparently not limit the substrate diffusion to the enzyme active site.

Conclusions

In this paper we demonstrated the usefulness of a tunable biocatalytic coating in glass microreactors consisting of PMAA brushes to which the lipase *C. Rugosa* type VII was anchored.

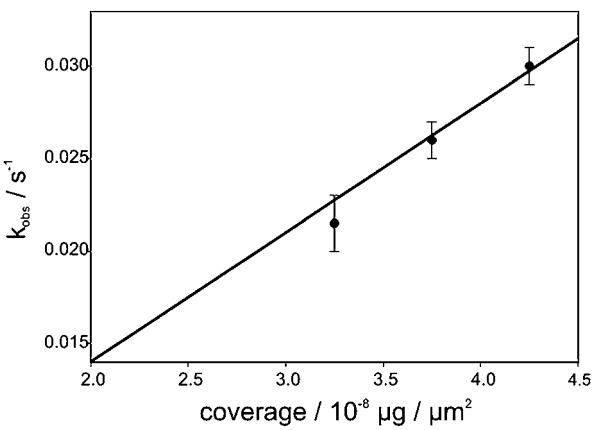


Fig. 6 Dependence of rate constant *versus* lipase coverage in microreactors having a PMAA–lipase brush layer thickness of 13.5, 23, and 30 nm. It is assumed that the line should go through the origin.

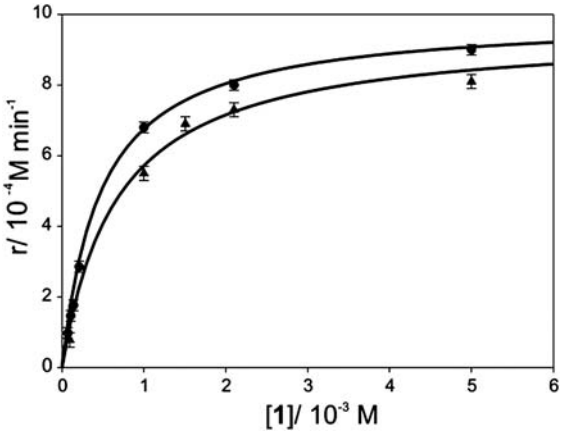


Fig. 7 Michaelis–Menten plots for the hydrolysis of 4-nitrophenyl acetate (**1**) mediated by: (●) = lipase anchored on PMAA–lipase nanostructure with a thickness of 13.5 nm, (▲) = free enzyme in solution.

Very importantly, all enzyme molecules in the brush layer are involved in catalysis and exhibit the same activity as in solution.

We feel that our method could be used as a general approach for anchoring enzymes on a microreactor interior, providing a new platform for studying the activity of enzymes and for performing biocatalysis.

Experimental

Materials and equipment

All commercial reagents were purchased from Aldrich Chemicals unless specified. All chemicals were used without purification unless specified.

[(Chloromethyl)phenylethyl]trichlorosilane was purchased from ABCR. Toluene (VWR, analytical reagent grade) was distilled over sodium. Methacrylic acid (MAA) was purified from inhibitors by condensation under high-vacuum. Water was purified with the Milli-Q plus (MILLIPORE, $R = 18.2$ MΩ cm) ultra pure water system. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride was purchased from Across Organics. PBS buffer, pH = 7.4, was purchased from Braun. 4-Nitrophenyl acetate (**1**) was purchased from Fluka. Acetonitrile was purchased from Across. Ethanol, methanol, and acetone (analytical reagent grade) were purchased from VWR. Photopolymerization was performed with a UV spot light source xenon lamp, with a 300 nm cut-off filter, equipped with an optical fiber (Hamamatsu, Japan).

Ellipsometry measurements were performed with a plasmon ellipsometer ($\lambda = 632.8$ nm) assuming a refractive index of 1.5 for the polymer. Samples were measured using an atomic force microscope (AFM) NanoScope III (Veeco Digital Instrument, USA) in the tapping mode, equipped with a Si_3N_4 tip with a J scanner at a scan rate of 0.6 Hz. FT-IR spectra were recorded using a BioRad FTS-60A spectrometer in transmission mode using a bare silicon wafer as background.

High resolution scanning electron microscopy (HR-SEM)

Polymer brushes in microreactors were analyzed by HR-SEM. All HR-SEM images were taken with an HR-LEO 1550 FEF SEM.

Microreactor fabrication

The silicon–glass microreactor[32] was fabricated by a standard microfabrication process. Channel dimensions are 100 μm × 100 μm (width × depth), length 98.96 cm.

Photopolymerization

Microchannels and silicon wafers were first cleaned with a Piranha solution (H_2SO_4 : H_2O_2 3 : 1) and then copiously rinsed with Milli-Q water and dried with a stream of nitrogen. *Caution: Piranha solution is a very strong oxidant and reacts violently with many organic materials.* The cleaned silicon wafers were socked in a 5% solution of [(chloromethyl)phenylethyl]trichlorosilane in dry toluene for 18 h.[30] For the synthesis in the device the same solution was flowed for 18 h at a flow rate of 0.1 μL min^{-1}. The silicon wafer and the microchannel were rinsed with dried toluene, acetone, and ethanol and finally dried with a stream of nitrogen. The so prepared surfaces were then immersed in a 0.1 M methanol solution of sodium diethyldithiocarbamate trihydrate overnight. For the device the same solution was flowed overnight at a flow rate of 0.1 μL min^{-1}. The surfaces and the microchannel were rinsed with methanol and dried with a stream of nitrogen.

Silicon wafers were placed in a glass flask containing a 1 M aqueous solution of MAA. They were extensively purged with argon (30 min) and finally irradiated with a UV xenon lamp (150 watt, emission wavelength 300–400 nm) at 18 cm distance, for 5–40 min. For the microreactors, a 1 M solution of MAA in water was purged with argon (30 min) and then flowed at 1 μL min^{-1} through the microchannel, and meanwhile irradiated with a UV lamp towards the glass side of the microreactor for 5–20 min at 18 cm distance. After photopolymerization the silicon wafer and microchannel bearing the polymer film were rinsed abundantly with Milli-Q water. PMAA polymer brushes grown on the silicon oxide surface were reacted with a PBS buffer solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) (38 mg mL^{-1}: 6 mg mL^{-1}) for 2 h. For the microreactor the same solution was flowed at 0.1 μL min^{-1} for 2 h. Subsequently, the so prepared surfaces were soaked in a buffer solution containing 250 μg mL^{-1} of lipase overnight. The enzyme solution was flowed through the microchannel at 0.1 μL min^{-1} for the same time. Surfaces and microchannel were rinsed with buffer in order to remove all the excess of unbound enzyme.

Kinetic study

The hydrolysis of 4-nitrophenyl acetate (**1**) (0.7–2.1 × 10^{-4} M) to 4-nitrophenolate (**2**) was performed in PBS buffer pH = 7.4 at room temperature.

Since **1** is not very soluble in water, initially a 0.14 M stock solution of **1** was prepared in acetonitrile/buffer 1 : 1. The formation of the product was followed using UV-vis detection,[26] measuring the increase of the **2** peak at 405 nm. The molar extinction coefficient of **2** in PBS buffer is ε_{405} = 10 166 cm^{-1} M^{-1}. The k_{obs} values were calculated by fitting (least-squares method) the experimental data with the following equation: $[\mathbf{2}] = [\mathbf{1}]_0 \times (1 - e^{(-k_{obs} \times t)})$. The experimental error in these measurements was ±5%.

Quantification of lipase immobilized in the microreactors

The lipase concentration in the microchannel was quantified using the Bovine serum albumin (BCA) protein assay kit (Thermo Scientific). Several standard samples were prepared with a known concentration of BSA standard protein, from which a calibration curve was determined, plotting the BSA concentrations *versus* the absorbance at 562 nm measured for each individual standard sample. A working reagent solution[33,34] was pumped through the microchannels and kept inside for 30 min at 37 °C. Subsequently, the microchannels were rinsed with buffer and the eluted solution collected in a volumetric flask. From the absorbance at 562 nm the enzyme concentration was determined.

Acknowledgements

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