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MsAcT in siliceous monolithic microreactors enables quantitative ester synthesis in water†

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Acyltransferase from *Mycobacterium smegmatis* (MsAcT) immobilised in continuous-flow microchannel (30–50 μm dia.) reactors with hierarchical pore structure (4 cm^3 g^{-1} total pore volume) enabled quantitative, full and rapid transesterification of neopentylglycol (NPG) with ethyl acetate in a biphasic 50/50% system in less than one minute. MsAcT was attached either covalently *via* amino groups or by a specific His-tag-mediated adsorption on Ni or Co sites. Both methods gave similar results for enzyme loading (*ca.* 3 mg g^{-1} carrier, 60–70% immobilisation yield) and specific activity. The experiments revealed that the rate of mono-ester formation in the microreactor was exceedingly fast compared to that of diester synthesis and also the native enzyme behaviour in a batch reactor. The studies show that the course of transesterification was fully controlled by the biocatalytic properties of MsAcT confined in the mesoporous environment. These findings may be of significant interest from both fundamental and practical perspectives.

Introduction

Acyltransferase from *Mycobacterium smegmatis* (MsAcT) catalyses ester synthesis in water (Scheme 1).[1,2] Its unique active site is rather apolar, thus creating a local situation in which the course of a reaction is not dominated by the thermodynamics of the aqueous medium but by the hydrophobicity of the active site. This asset makes MsAcT an excellent candidate for application in ester syntheses and transesterifications (such as biodiesel production) that are typically performed in aqueous or biphasic media.[3] To date, only a few hydrolytic enzymes are known for their ability to catalyse synthesis reactions in aqueous environments, namely the very specialised polyketide thioesterases[4–6] and a very limited number of lipases.[7–9] To enable reaction engineering with MsAcT, the aim was to immobilise the enzyme. The octameric MsAcT (72 × 72 × 60 Å, Fig. 1) needs to be tightly bound to ensure its long-term use,[2] and hydrophobic carriers (*e.g.* CNT) should be

avoided since they may result in unfavourable partition effects and non-specific interactions of the enzyme or the products, which are detrimental for its catalytic properties and overall performance.[10,11]

The most recent study of MsAcT's synthetic properties shed light on its application prospects to catalyse reactions in aqueous media.[1] However, the reported experiments made use of cell-free extracts with low enzyme concentrations and were carried out in a batch system. Thus, while indicating the potential of MsAcT, the yields were modest and useful application was limited to primary alcohols. From an application perspective, continuous-flow systems with very high performance are desirable, in particular since they offer a new handle on reaction engineering.[12]

For this reason we deemed it important to undertake studies of the MsAcT behaviour in a continuous-flow transesterification of neopentylglycol (NPG) in a microchannel (micro)reactor. NPG is the substrate employed for the activity assay of MsAcT. It is particularly suitable for this study as it is a symmetric diol with two primary hydroxyl groups. This allows us to follow two conversion steps, mono- and diesterification. Additionally, it is a good model for other primary diols and even for glycerol that also has two primary hydroxyl groups.[13] Thus, under these conditions the enzyme and the reactor should demonstrate their full capabilities, while at the same time offering an effective processing system. To preclude non-specific hydrophobic interactions between the enzyme and its support the microreactor was made of (hydrophilic) silica monolith with a hierarchical pore structure in micro- and nanometric size scales and meandering flow-

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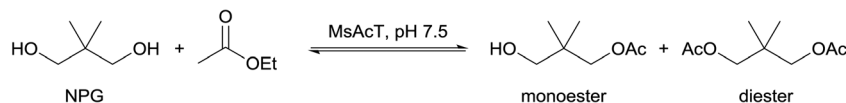[b] *Gebouw voor Scheikunde, Biokatalyse, Afdeling Biotechnologie, Technische Universiteit Delft, Julianalaan 136, 2628BL Delft, The Netherlands.*
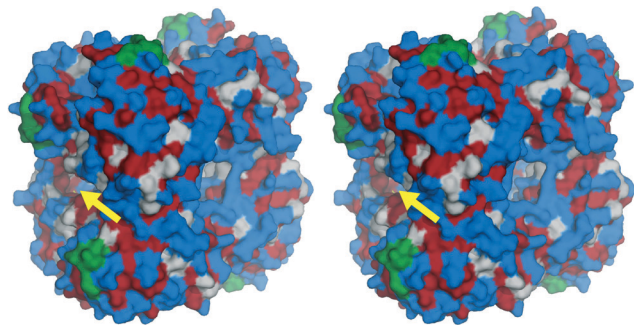*E-mail: u.hanefeld@tudelft.nl*
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Scheme 1 Esterification of a primary diol, NPG, with ethyl acetate, catalysed by MsAcT in aqueous buffer.

Fig. 1 The octameric MsAcT (stereoview) displays an overall mixed hydrophilic surface: hydrophilic residues are highlighted in blue; grey residues are neither hydrophobic nor hydrophilic; hydrophobic residues are highlighted in red. The His-tag is located at the C-terminal (highlighted in green; please note that the His-tag is not included in the structure); the arrow indicates the entrance of the active site. Structural data from PDB: 2Q0S. The picture was generated using the PyMOL Molecular Graphics System.

through channels. This enables a facile flow of reactants and results in intensive mixing and mass transport to the enzyme. The latter was immobilised in very large mesopores using two distinctly different immobilisation concepts not only to improve operational stability but also to make use of the protein confinement effect by increasing the surface-to-volume ratio in microreactor technology.[12]

Experimental

Materials and methods

2,2-Dimethyl-1,3-propanediol (NPG), cethyltrimethylammonium bromide (CTAB), and tetraethoxysilane (TEOS) were from Acros Organics. Polyethylene glycol 35000 (PEG), ethyl acetate, cobalt(II) chloride, nickel(II) chloride, 1,4,8,11-tetraazacyclotetradecane (cyclam), 3-iodopropyl trimethoxysilane, and 3-aminopropyltrimethoxysilane were from Sigma-Aldrich. The other chemicals used in support synthesis, immobilisation and activity assay were from Avantor Poland.

Cloning and overexpression of MsAcT. A synthetic gene encoding for MsAcT (GenBank accession: ABK70783) from *Mycobacterium smegmatis* str. MC2 155, including a C-terminal His-tag, was ordered from BaseClear. The gene was cloned into vector pET16b (Novagen). The resulting vector pET16b-MsAcT was afterwards transformed into chemically competent *E. coli* BL21 (DE3) for heterologous expression. *E. coli* BL21 (DE3) pET16b-MsAcT was grown at 30 °C in lysogeny broth (LB) medium containing ampicillin (0.1 mg L^{-1}) to an optical density of 0.4 (OD$_{600}$). The expression of MsAcT was induced by the addition of isopropyl β-D-1-
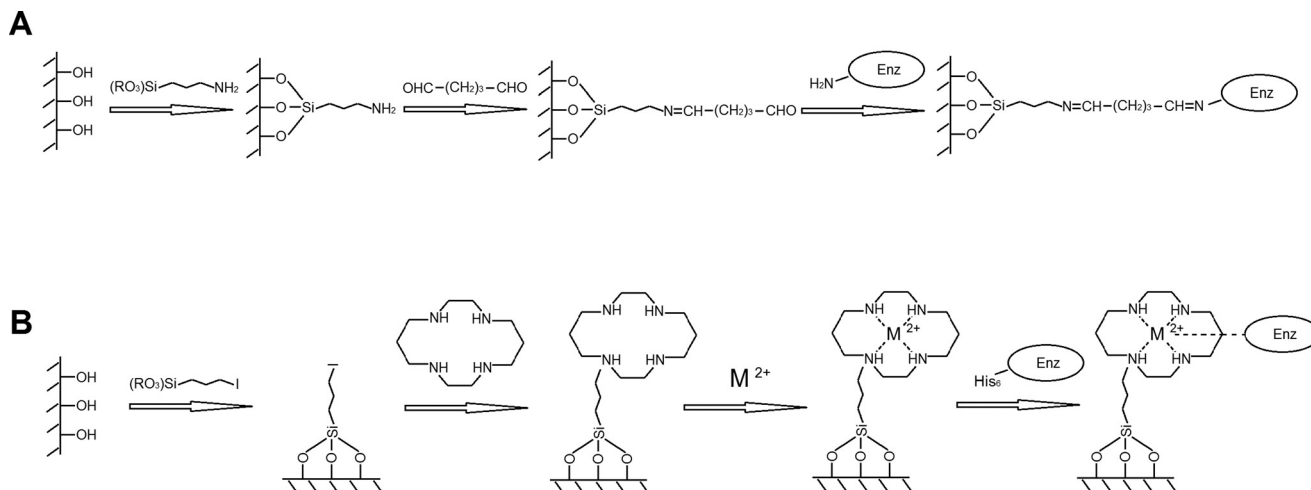
thiogalactopyranoside (IPTG; 1 mM), and the culture was further incubated for 24 h at 30 °C. The cells were harvested by centrifugation for 20 min at 8000 rpm at 4 °C. Pelleted cells were resuspended in ice-cold 20 mM potassium phosphate buffer (pH 7.5) and disrupted using ultrasound for 6 minutes (duty cycle 30%, output 10%). The cell debris was removed by centrifugation for 5 min at 15 000 rpm and 4 °C. The resulting cell-free extract (CFE) was lyophilised overnight, and the crude enzyme powder was stored at 4 °C. Activity assays were performed according to ref. 2 using neopentylglycol (NPG) as substrate and equal to 230 μmol min^{-1} (mg CFE)$^{-1}$.

Synthesis of silica monoliths (MH). The general procedure was as follows:[14,15] first polyethylene glycol 35000 (PEG, 9.09 g) was dissolved in 1 M HNO$_3$ (104.6 mL), after which tetraethoxysilane (TEOS, 87.1 mL) was added slowly under stirring (500 rpm) to the PEG solution in an ice bath followed by the addition of cethyltrimethylammonium bromide (CTAB, 4.016 g). The solution was mixed and then left to gel in polypropylene tubes at 40 °C and aged for 10 days. Next the alcogels obtained were impregnated with 1 M ammonia solution for 9 h at 90 °C. The samples were washed with water, dried at room temperature and then calcined at 550 °C for 8 h (ramp of 1 °C min^{-1}) to obtain silica rods 50 mm in length and 6 mm in diameter. The monoliths were functionalised and clad with polymer resin (L285MGS-H285MGS type) to obtain single-rod continuous-flow microreactors.

Functionalisation of silica monoliths (MH) – amino-functionalised carrier. Pristine silica monoliths were modified to attach the enzyme non-specifically by means of amino-glutaraldehyde linkage[16–18] (Scheme 2A). In short, oven-dried (overnight, 150 °C) silica monoliths (1 g) were gently stirred and refluxed with 3-aminopropyltrimethoxysilane (0.27 mL) in 35 mL of dry toluene for 72 h. After taking out the monoliths were washed with toluene and dried.

Functionalisation of silica monoliths (MH) – metal-functionalised carrier. In an alternative approach for immobilisation *via* adsorption by the His linkage Ni and Co were incorporated[19,20] (Scheme 2B). Silica monoliths (1 g, oven-dried at 150 °C overnight) were gently stirred and refluxed with 3-iodopropyl trimethoxysilane (0.41 mL) in 30 mL of dry toluene for 10 h under Ar atmosphere in the dark. After 10 h the toluene was removed and the monoliths were washed in MeCN (acetonitrile). Next cyclam (1,4,8,11-tetraazacyclotetradecane, 0.2 g) and excess potassium carbonate (0.37 g) were added and the mixture was refluxed in MeCN (30 mL) for a further 21 h. After separating the monoliths, they were refluxed with an excess of nickel(II) chloride or cobalt(II) chloride (0.35 g) in H$_2$O (80 mL) for 3 h. Finally, the monoliths were filtered off and washed with H$_2$O to remove any unattached nickel/cobalt.

A

B

Scheme 2 Functionalisation of silica carriers and immobilisation of MsAcT by covalent bonding (A) and specific His-tag-mediated adsorption on Ni/Co sites (B).

Enzyme immobilisation – specific adsorption. Prior to MsAcT adsorption, the Ni/Co functionalised monolith (6 mm × 40 mm) was washed with ethanol and then distilled water for 45 min each (flow rate of 0.5 mL min^{-1}) and then with 0.1 M phosphate buffer (Na$_2$HPO$_4$/KH$_2$PO$_4$, pH 7.5). A solution of MsAcT (1.1 mg of MsAcT (CEF) per mL, 5.5 mL) in buffer was pumped (0.2 mL min^{-1}, 2.5 h) through the reactor under recycling to immobilise the protein. Excess protein was removed by washing the columns with a 0.05 M phosphate buffer (pH 7.5). The amount of the attached enzyme was determined using the Lowry method.[21,22]

Enzyme immobilisation – nonspecific covalent method. Before the attachment of MsAcT to the amino-functionalised silica monolith (6 mm × 40 mm), the carrier was washed with ethanol and then distilled water for 45 min each (0.5 mL min^{-1}), followed by 0.1 M phosphate buffer (Na$_2$HPO$_4$/KH$_2$PO$_4$, pH 7.0). To attach aldehyde groups onto the monolith's surface a 2.5 vol% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.0) was circulated (0.5 mL min^{-1}) through the reactor for 45 min, whereupon it was washed with water and 0.1 M phosphate buffer (pH 7.5). Finally, 5.5 mL of MsAcT (1.1 mg MsAcT (CEF) per mL) solution in the phosphate buffer was passed (0.2 mL min^{-1}, 2.5 h) through the reactor as described above. Excess protein was removed by washing as described in ref. 15–18. Prior to activity assays, the monolith was washed with 0.05 M phosphate buffer (pH 7.5). The amount of immobilised enzyme was determined using the Lowry method.[21,22]

Activity assays/ester synthesis. The activity of the monolith-bound MsAcT was determined in a continuous (single-pass) flow of biphasic solution, 50/50% v/v of 0.05 M phosphate buffer (Na$_2$HPO$_4$/KH$_2$PO$_4$, pH 7.5) and 2,2-dimethyl-1,3-propanediol (NPG) solution (10 mg mL^{-1}, 96 mM) in ethyl acetate at room temperature. Two pumps (one for the buffer and one for the organic phase) and a mixing unit, located immediately before the microreactor inlet, were applied. For experiments with organic solvent (Fig. 5) only

one pump was employed. The reaction mixture was separated into water and organic phase, and products in the organic phase were determined by GC (Scheme 1). The concentrations of the substrate and products were determined by GC analysis on an Agilent Technologies model 6890N Network GC System gas chromatograph equipped with a flame ionisation detector (FID) and an Agilent Technologies HP-5 capillary column (diameter 0.32 mm, length 30 m, thickness 0.25 μm). The following temperature program was applied: initial oven temperature 70 °C held for 9 min, next to 135 °C (45 °C min^{-1}) and held in this temperature for 1 min, then from 135 °C to 140 °C (45 °C min^{-1}) and held at 140 °C for 1 min and finally to 300 °C (50 °C min^{-1}). The injector and detector temperatures were 320 °C and 350 °C, respectively. The carrier gas was helium with a flow rate of 2.5 mL min^{-1}. The retention times of the NPG and its esters under these conditions were equal to 4.5 min (NPG), 8.1 min (monoester) and 11.5 min (diester). The concentrations were calculated from the peak areas using calibration curves. All the samples were analysed twice.

Results and discussion

Characterisation of enzyme carriers

To date, MsAcT was immobilised only on carbon nanotubes (CNTs) and used for its perhydrolase activity in latex paint.[10,23,24] Here we propose the application of hydrophilic macro/mesoporous monoliths with meandering/twisting flow-through channels, stimulating a chaotic movement (perturbations) of fluids and additionally enhancing mass transport.[25] Employing a nonhydrophobic carrier would help in avoiding nonspecific interactions between the surface of the supports and the MsAcT active site.[10]

Microscopic (SEM) and low-temperature nitrogen adsorption studies of silica monoliths revealed the prevalent presence of through-pores of 30–50 μm sizes and also mesopores of about 20 nm, and a total pore volume of over 4 cm^3 g^{-1},

determined by Hg porosimetry measurements as reported earlier[15] (see the ESI†). This very open structure can also be seen from the images displayed in Fig. 2. Equally important, the modification of the monoliths appeared to have no effect on porosity in the macroscale. The specific surface area (S_{BET})[26] of silica monolith measured by nitrogen adsorption was *ca.* 300 m^2 g^{-1}; after surface modification a slight decrease in this parameter was observed. Thus, not surprisingly, the measurements of backpressure *vs.* flow rate of water through the monoliths in the flow rate range of 0.03–3 mL min^{-1} gave a backpressure drop of only 0.028–2.8 kPa, which is significantly less than that in capillary reactors or packed bed columns filled with beads of micrometric sizes.[27,28] These experiments also corroborated the very good stability of the monolith structure during at least two weeks of continuous flow operation, as observed in our earlier studies.[18]

Metal ions can be introduced onto a solid support by chelators such as iminodiacetic acid (IDA), nitriloacetic acid (NTA) or cyclam.[19,20,29–33] As cyclam is known to be a stable ligand for several metals, MH-cyclam-Ni and MH-cyclam-Co were prepared as described previously[20] (after minor modification). ICP mass spectrometry measurements gave the metal loading values of 8.96 mg Co (g silica)$^{-1}$ ± 16% and 7.81 mg Ni (g silica)$^{-1}$ ± 10%. After metal incorporation, the originally white silica monolith turned to yellow or blue for Ni and Co, respectively (Fig. 2). The same colour intensity, despite the considerable size (6 mm × 4 mm) of the monolith, indicated a homogenous distribution of the metal over its volume. To the best of our knowledge it is the first time that metal ions were homogenously incorporated into a monolithic column of that size, as to date only capillary or packed bed columns with metals were used.[29–33]

For non-specific MsAcT immobilisation the same silica monolith but functionalised with amino groups was used, and the presence of the latter was confirmed by FT-IR, as shown earlier,[15] and elemental analysis (LECO CHN analyser). The amino group loading was 1.32 mmol (g silica)$^{-1}$ (1.85 wt% of nitrogen). Before enzyme immobilisation the amino groups were activated with glutaraldehyde, after

which the column changed colour uniformly to brown over the whole length of the monolith.

Performance of the monolithic microreactor

The recent extensive report on MsAcT-catalysed transesterification of NPG in buffer/ethyl acetate aqueous solutions in a batch reactor using the native enzyme[1] provides an excellent platform to compare its behaviour with that of continuous-flow monolithic microreactors with the immobilised enzyme (Scheme 1). While effective immobilisation of the enzyme in the silica monolith's mesopores remained a critical factor, our studies primarily centered on the synthetic potential of the immobilised enzyme/microchannel reaction system. Two immobilisation methods were applied: (i) conventional covalent bonding tends to give more stable catalysts, but could reduce enzyme activity,[11,18] and (ii) specific adsorption onto the Ni/Co-modified support *via* the His-tag,[19,20,29–33] since the His-tag is remote from the active site (Fig. 1). At first, the activity, stability and enzyme loadings were studied using porous silica monoliths as enzyme support.

Monolithic silica supports were functionalised with amino groups activated with glutaraldehyde (GLA) to produce a Schiff's base for covalent attachment and with Ni or Co for specific adsorption. However, we started with the evaluation of enzyme loadings obtained from different immobilisation methods, which appeared to be fairly similar: 2.96 mg g^{-1} for covalent, 3.15 and 3.07 mg g^{-1} carrier for adsorption on Ni and Co, respectively. That gives 60–70% of immobilisation yield. As it is not possible to predict whether the octameric MsAcT will be immobilised *via* one metal or one lysine amino group or rather by a multipoint attachment it was considered important to ensure that an excess of both metal and aldehyde groups was available for binding. This clearly was the case (Table 1). The performance of the biocatalysts in 50/50% v/v biphasic conditions of 0.05 M phosphate buffer (Na$_2$HPO$_4$/KH$_2$PO$_4$, pH 7.5) and ethyl acetate also appeared to be much the same (Fig. 3). We believe that this similarity in the enzyme loading and activity indicates a similar orientation of the enzyme attached by means of different immobilisation procedures and a free access to its active site. Bearing in mind that silica is hydrophilic by nature, it is likely that in a nonspecific covalent immobilisation, hydrophilic regions of the protein were involved, and hence its active center located in a very hydrophobic region (Fig. 1) was free and not distorted by immobilisation. As shown in Fig. 1, the His-tag residues are surrounded by hydrophilic amino acids, implying that the same region of protein was involved in both immobilisation procedures.
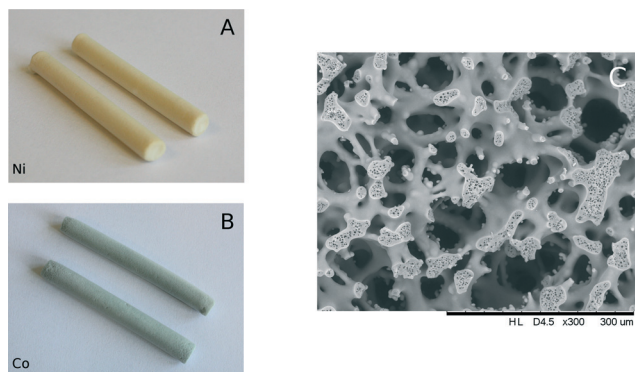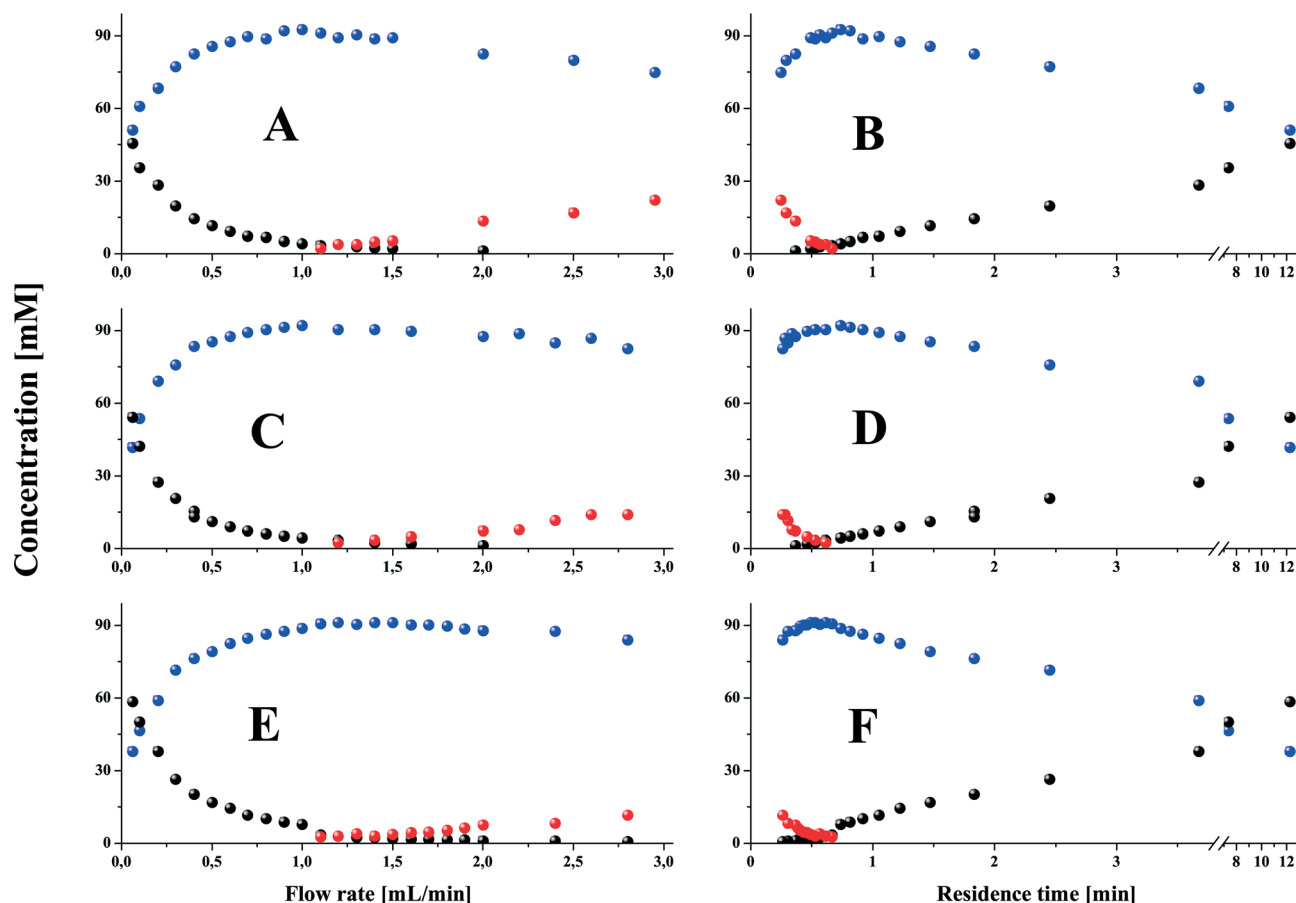

Fig. 2 Silica monoliths with Ni (A) and Co (B). SEM image of monolith structure (C).

Table 1 Loading per microreactor (6 mm × 40 mm, 0.26 g silica)

Carrier	Functional group loading	Protein loading
MH-amino	0.343 mmol	0.77 mg
MH-Ni	2.031 mg ± 10%	0.82 mg
MH-Co	2.329 mg ± 16%	0.80 mg

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Fig. 3 Transesterification of neopentylglycol in a biphasic (50/50% v/v) system of buffer/ethyl acetate by MsAcT covalently immobilised on amino-modified monolith (A, B), adsorbed by His-tag on Ni-modified monolith (C, D) and on Co-modified monolith (E, F). Conditions: 0.05 M phosphate buffer pH 7.5; 96 mM neopentylglycol in ethyl acetate; room temperature. Red, substrate; black, diester; blue, monoester.

A closer inspection of the data given in Fig. 3 indicates that the NPG transesterification in the microchannel reactor qualitatively portrays all trends observed earlier in the batch reactor studies.[1] However, it also reveals a huge quantitative discrepancy between the operating characteristics of the batch reactor and the performance of the continuous-flow microchannel reactor with immobilised enzymes. The results displayed in Fig. 3B, D, and F demonstrate that the reaction of monoester formation in a biphasic system was exceedingly fast, in strong contrast to the following reaction of diester formation, the concentration of which grew steadily but slowly with time. Already after about 30 s of mean residence time the concentration of monoester reached 90 mM, whereas those of the substrate and diester were very low. Thus, very shortly after the reaction start-up (reactants' entry into the microreactor) substrate conversion exceeded 90%. After a further 15 s (longer residence time owing to the application of a lower flow rate) the substrate was fully converted and the concentration of the monoester reached a maximum of over 90 mM. For longer reaction/residence times (lower flow rates) the concentration of the diester at exit continued to grow as before, at the expense of the monoester. For the longest residence time of *ca.* 12 min, the diester yield was

50–60% for a single-pass flow. Thus, the rate of the enzyme-catalysed reaction appeared to be significantly enhanced in the proposed microchannel reactor compared to that in the batch reactor with the native enzyme,[1] in which full substrate conversion was not achieved even during 7 hours irrespective of the enzyme loading (*cf.* Fig. 1 and 2 in ref. 1), and the mono-to-diester molar ratio was also much lower in the latter system. These results show not only the exceptional performance of the proposed monolithic microchannel reactor in boosting the enzyme-catalyzed reaction, which may be of major practical interest, but also that the course of trans-esterification was fully controlled by the catalytic properties of MsAcT confined in a well-defined, functionalised meso-porous environment. That again may be a major asset of the proposed solution, seen from both a fundamental and the most practical point of view.

We believe that this performance can be explained by two factors: (i) the flow-induced reaction effects typically observed in microfluidic/microchannel reactors, which in the case under study boosted monoester formation, additionally enhanced by (ii) the large concentration of the enzyme embedded in the mesoporous environment most favourable for expressing its activity. Similar effects were recently reported

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for the consecutive reactions carried out in microchannel reactors but without heterogeneous catalysts.[12] Asano *et al.* showed that the application of microreactor technology could increase the yield of the monosubstitution product from 58.6% to 98.6% in the case of bromination, from 77% to 86.3% (nitration) and from 25.2% to 38.1% (ester reduction) in comparison with the batch method.[12] A very high activity of enzymes embedded in large mesopores of silica monoliths and in particular mesoporous materials was also observed by other groups and us before.[17,18,28,34]

Remarkably, at least 50% conversion to the diester is achieved under these aqueous conditions, independent of the carrier. Equally remarkably, up to a flow rate of 1 mL min^{-1} no diol was observed. A residence time of 1 min was thus sufficient to achieve complete esterification of at least one alcohol group of NPG. This implies that this catalytic system has a great potential for rapid ester synthesis (including bio-diesel) under aqueous conditions.

For practical applications the stability of immobilised biocatalysts is a factor of major importance. In this respect, both the enzymes coupled by His-tag adsorption and by covalent binding showed a very good performance during almost 50 h of continuous operation (Fig. 4), and only a small decrease in activity was observed after 100 h (*cf.* ESI†). Also, intermediate washing of the reactor and storage of the reactor in buffer did not change the activity.

Another factor of significance for this reaction system is the water phase present. Typically this contains buffers leading to a significant salt load that has to be separated after the reaction and that increases corrosion. Therefore, the stability and the catalyst performance at different flow rates were studied for MsAcT immobilised on Ni-modified silica in the absence of a buffer (Fig. 5).
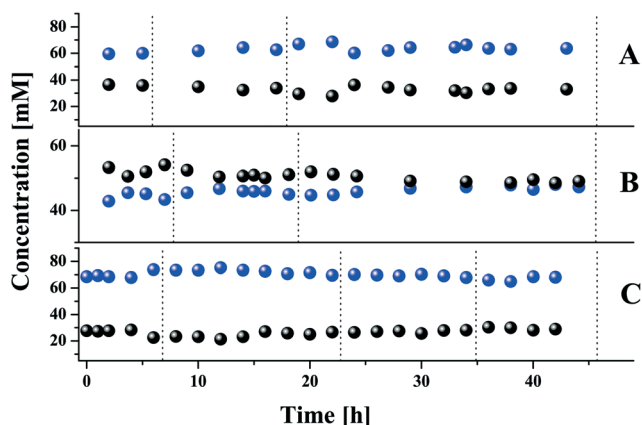

Fig. 4 Catalytic stability of MsAcT covalently immobilised on amino-modified monolith (A), adsorbed by His-tag on Ni-modified monolith (B) and on Co-modified monolith (C). For covalent immobilisation and His-tag adsorption on Co the cumulative flow rate was 0.1 mL min^{-1} (buffer/ethyl acetate 50/50% v/v); for His-tag adsorption on Ni the cumulative flow rate was 0.06 mL min^{-1} (buffer/96 mM neopentylglycol in ethyl acetate 50/50% v/v). Vertical lines show the interruptions in measurements; the immobilised enzyme was washed and stored in buffer: black, diester; blue, monoester.
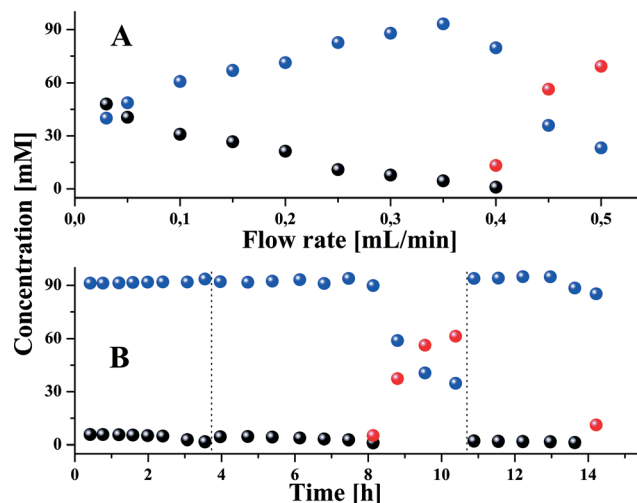

Fig. 5 Transesterification of neopentylglycol in the absence of buffer by MsAcT adsorbed by His-tag on Ni-modified monolith (A) and process stability (flow rate 0.1 mL min^{-1}) (B). Conditions: 96 mM neopentylglycol in ethyl acetate; room temperature. Red, substrate; black, diester; blue, monoester. Vertical lines show the interruptions in measurements; the immobilised enzyme was washed and stored in buffer.

Also in this case the increase in monoester and decrease in diester formation were observed when the flow rate increased (Fig. 5A). However, while in a biphasic system the largest concentration of monoester, at full substrate conversion, was observed for the flow rates in the range of 0.7–1.0 mL min^{-1} (Fig. 3), in the non-aqueous situation (Fig. 5A) it was only about 0.35 mL min^{-1}. This is partly due not only to the higher NPG loading (no dilution by buffer) but also to a significant activity drop. Moreover, the non-aqueous system also detrimentally affected enzyme stability, as seen from a rapid decline in the enzyme activity after 4 h of continuous steady operation. MsAcT catalyses not only the formation of the esters but also some hydrolysis of ethyl acetate, leading to the formation of acetic acid and consequently a pH drop. After washing the enzyme with the buffer initial activity could be restored (Fig. 5B), indicating that the deactivation was reversible.

A destructive effect of the absence of a water phase was also observed by Mathews *et al.*,[2] who found that below 2.5% water content MsAcT activity significantly decreased. The relatively high activity of the immobilised enzyme in the organic solvent system (Fig. 5) might be ascribed to water adsorbed to the silica carrier. Acetic acid formation and loss of water slowly led to a deactivation that could be reversed by treatment with a buffer (Fig. 5B). This demonstrates the great gain in enzyme stability achieved by immobilisation.

Conclusions

Our studies demonstrated that the metal ions and amino groups can be uniformly introduced onto the silica monolith surface and that post synthesis treatment has no detrimental effect on their macroporous morphology. Functionalised

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silica monoliths were successfully used to immobilise MsAcT and demonstrate its exceptional properties in a continuous-flow reaction. Complete ester synthesis could be achieved in aqueous media within very short reaction times. No differences in activity were observed for the enzyme attached using nonspecific covalent immobilisation and a very specific chelation procedure. This highlights the importance of a judicious selection of the carrier. The microreactor demonstrated very high activity and stability of catalytic and structural properties in the continuous esterification of NPG with ethyl acetate at room temperature and in biphasic conditions.

Acknowledgements

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