Development of a microfluidic immobilised enzyme reactor†

Malene S. Thomsen,[a] Peter Pölt[b] and Bernd Nidetzky*[a]

Received (in Cambridge, UK) 12th February 2007, Accepted 6th March 2007
First published as an Advance Article on the web 20th March 2007
DOI: 10.1039/b702115a

A microfluidic immobilised enzyme reactor consisting of a catalytically functionalised microstructure fabricated from silicone rubber material was used for steady-state kinetic characterisation of a thermophilic β-glycosidase under pressure-driven flow conditions and continuous conversion of lactose by this enzyme at 80 °C.

Recent developments in the field of microfluidics have provided organic chemists with a powerful platform of systems for performing simple and complex (*e.g.* multi-step)[1a] transformations in precision manufactured, microstructured elements under flow conditions.[1] Microstructured reactors minimally integrate the functional microfluidic element in a suitable and appropriately interfaced housing.[2] They have been used successfully for various catalytic and non-catalytic synthetic transformations.[1b,f] Although enzyme-catalysed conversions in microstructured reactors have also been reported recently, biocatalytic organic synthesis in microfluidic systems is only slowly forthcoming.[3] Difficulties in recycling the enzyme *via* its immobilisation onto the walls of the microchannels and limited availability of ready-to-use, practical equipment may have hampered widespread application of enzyme catalysis for preparative synthesis in microstructured reactors. However, the microscale flow reactor could be an excellent system for biocatalytic process analysis and optimisation. In addition, it could also facilitate screening of candidate enzymes from natural biodiversity or mutant libraries.

Here, we describe design and fabrication by micro-injection molding[4a,b] of a multichannel microfluidic element made from vinyl group-containing poly(dimethylsiloxane) (PDMS)[4c–e] supplemented with pyrogenic silicic acid as a filler, which should also provide hydroxy groups for surface chemistry. Enzyme was covalently immobilised on silanised walls of the microchannels using coupling with glutardialdehyde. The microstructure thus obtained was used for in-flow studies of enzyme kinetics as well as for continuous conversion experiments. We have chosen β-glycosidase-catalysed hydrolysis of lactose as a model system.

Fig. 1 shows through a photo and scanning electron micrographs the configuration of the microstructured element used, which contained nine linear microchannels, inlet and outlet zones. Each of the microchannels had a length of 64 mm and was characterised by an aspect ratio of 1.4 (width: 350 μm; height: 250 μm). To improve mass transfer to and from the microchannel surface, we incorporated passive mixing elements[1b,5] every 2.5 mm along the microchannel, alternating between the left and right wall. The top of each channel was equipped with sealing lips ensuring that the microchannel seals optimally to a flat hard surface and remains watertight under pressure. Details of micromould fabrication are given in the ESI.† The microfluidic element was integrated with housing (see ESI†) that enabled temperature control from an external water-bath and interfacing to a Knauer Smartline Pump 1000 and online detection.

To make the microchannel surface hydrophilic and reactive for covalent protein immobilisation,[6] we used in-flow treatment (0.15 mL min^{-1}) of the microfluidic element with an aqueous solution (10% w/v; pH 3.5–4.0) of 3-aminopropyl triethoxysilane at 80 °C for 3 h. Comparison of silanised and untreated microchannels in Fig. 2 reveals surface modification with a layered structure whose formation is readily explained by and most likely requires reaction of the surface silanol groups. Note that conventional PDMS is unreactive towards 3-aminopropyl triethoxysilane and needs pretreatment, with air plasma for example, to oxidise the surface prior to silanisation.[6a] Here, the silanised microstructure was then derivatised with glutardialdehyde solution (2.5% w/v; pH 8.4) for 2 h at room temperature. Finally, protein solution (95 μg mL^{-1}) was recirculated over the washed microstructure for 10–15 h, yielding the ready-to-use immobilised enzyme reaction plate. The amount of protein attached to the microstructure was determined from the difference in the protein concentration before (95 μg mL^{-1}) and after immobilisation (87 μg mL^{-1}) as well as the total volume of the enzyme solution (7 mL). Suitable controls showed that virtually no protein (and activity; see later) was bound to untreated plates. Likewise, protein binding to interfacing Teflon tubes was not significant. Scanning electron microscopic analysis of the entire immobilised enzyme plate showed that protein binding in the microchannels was not uniform, and Fig. 2 reveals formation of protein aggregates preferentially around the mixing elements. The binding capacity of the microstructured plate was about 56 μg protein under the conditions used.

CelB is a broadly specific β-glycosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*.[7] It is optimally active at 80 °C or greater, and is very stable at the high temperatures.[7a,b] The recombinant enzyme was produced *via* heterologous gene expression in *Escherichia coli*. Prior to immobilisation, it was purified efficiently by a single step of heat treatment (80 °C, 20 min) in which most of the mesophilic proteins precipitate.[7c] The specific activity of the enzyme preparation used in all experiments was

[a]*Research Centre Applied Biocatalysis, Petersgasse 14, A-8010 Graz, Austria c/o Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Petersgasse 12/I, A-8010, Graz, Austria. E-mail: bernd.nidetzky@tugraz.at; Fax: +43 316 873 8434; Tel: +43 316 873 8400*
[b]*Institute for Electron Microscopy, Graz University of Technology, Steyrergasse 17, A-8010, Graz, Austria*

† Electronic supplementary information (ESI) available: Details of micromold fabrication; housing and set up of the enzyme microreactor; details of the immobilisation procedure; recombinant enzyme preparation; assays and other analytical procedures; electron microscopy. See DOI: 10.1039/b702115a

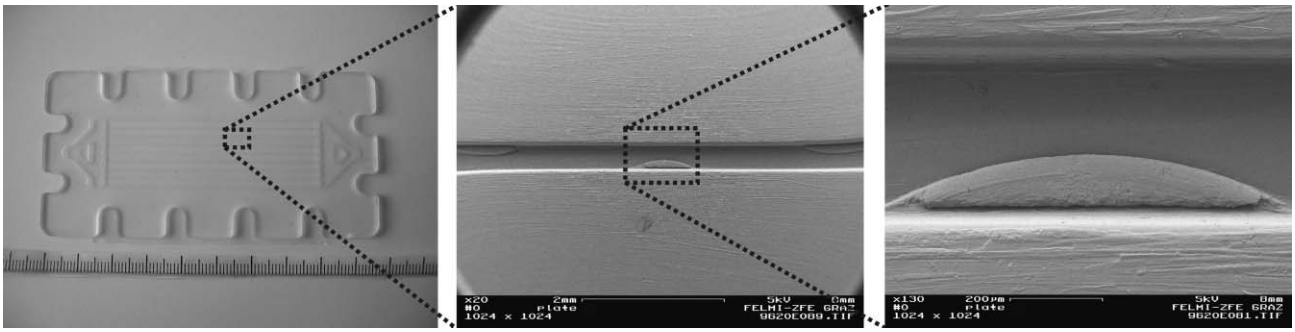

Fig. 1 Microstructured multichannel plate (left) and close-up electron micrographs of the microchannel structure (middle and right).

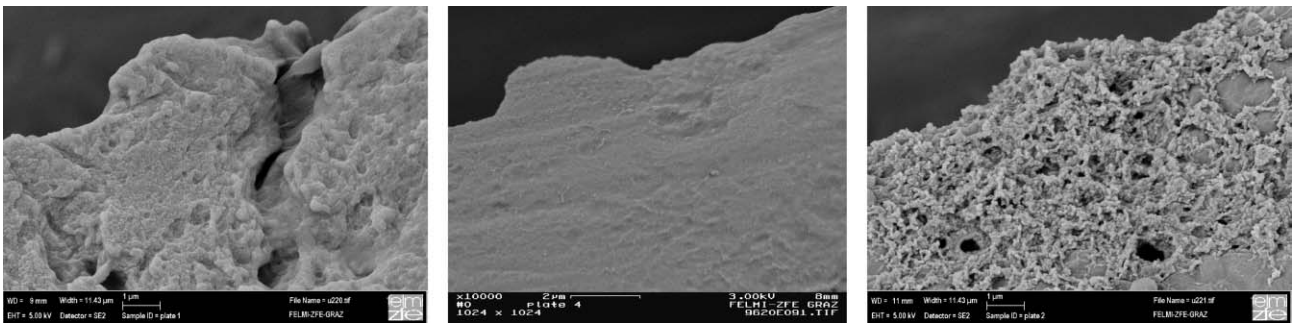

Fig. 2 Electron micrographs of identical sections of microplates without treatment (left), after silanisation and activation with glutardialdehyde (middle), and with immobilised enzyme (right).

1120 and 820 μmol (min mg)$^{-1}$, measured at 80 °C with 2-nitrophenyl-β-D-galactopyranoside (oNPGal) and lactose as the substrate, respectively. The activity of the immobilised enzyme was determined at the same temperature under continuous flow conditions, initially by feeding oNPGal solution (13 mM; pH 5.5) and measuring on-line the outlet concentration of the released 2-nitrophenol with a UV/Vis detector at 405 nm. The flow rate was varied in the range 0.05–1.00 mL min^{-1} and always such that the level of substrate conversion was 20% or smaller. The dependence of the product concentration formed on the residence time (τ), calculated as the ratio of total reactor volume (167 μL) and flow rate, was linear under these conditions. However, when the reactor was in use for longer periods of time, reversible absorption of 2-nitrophenol by the polymeric material caused significant alterations in the steady-state concentration of product. To minimise solute interactions which for PDMS materials are known to be strong for hydrophobic substances,[8] we used the polar disaccharide lactose (600 mM; pH 5.5) for measuring the enzyme activity. Hydrolysis of lactose yields glucose and galactose. The outlet concentration of glucose was determined off-line using an enzymatic test kit. Results of continuous flow assays using oNPGal and lactose as substrate are, however, consistent in showing that immobilised CelB retained only about 3% of the activity of the free enzyme. By way of comparison, CelB attached onto silanised macroporous glass beads (see ESI†), using conditions otherwise exactly identical to those employed for immobilisation into microchannels, showed about 35% of the original activity. Tethering of the enzyme to the surface is random *via* exposed protein amino groups and needs not be identical in the two systems. Differences in bound specific activity could indicate the impact of tethering. However, inactivation of immobilised enzymes could also be apparent due to mass transfer resistances. Considering that flow conditions in the microchannels will probably be laminar, kinetic studies were performed to determine the extent to which the observed activity loss depends on diffusion.

We evaluated the flow dependence of the apparent Michaelis constant of CelB for lactose (K_m). If mass transfer resistances altered kinetics of the immobilised enzyme, one would expect according to previous studies[9] (for work on enzyme kinetics in microfluidic systems, see refs. 9*b–h*) that the K_m increases in response to a decrease in flow rate. Substrate solutions (seven concentrations in the range 10–600 mM) were fed at four different values of τ in the range 0.08–0.33 min, and for each pair ([lactose], τ) the steady-state level of glucose was measured. Non-linear fits of the Michaelis–Menten equation to data obtained at constant τ revealed a K_m of about 71 ($\pm$9) mM which was flow-independent and agreed with a value of 61 mM for the free enzyme. We also compared temperature dependences of free and immobilised CelB in the range 50–80 °C considering that random tethering and mass-transfer effects often cause a decrease in the apparent activation free energy (E_a). The value of E_a was obtained from the slope of the respective Arrhenius plot. The values were 72 kJ mol^{-1},[7b] and 38 ($\pm$4.6) kJ mol^{-1} (this work) for free and immobilised CelB, respectively. The E_a value of surface-bound enzyme was not dependent on flow rate. We introduced a dendrimeric spacer between the enzyme and the microchannel surface (see ESI†) and found that E_a for the immobilised enzyme was now 68 kJ mol^{-1}. Therefore, the kinetic properties of immobilised CelB seem not to be altered by mass transfer resistances but are affected by the mode of attachment of the protein. Kerby *et al.* give a detailed discussion of mass-transfer effects on K_m in microfluidic systems.[9d]

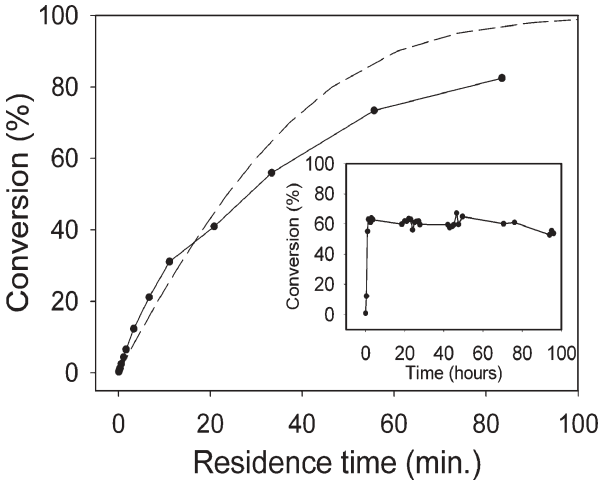

Fig. 3 Analysis of the performance of the microfluidic immobilised enzyme reactor during continuous conversion of 100 mM lactose at 80 °C and pH 5.5. Full circles show experimental data and the broken line shows model predictions. The inset shows the operational stability of the reactor using a residence time of 33 min.

To demonstrate the utility of the microstructured enzyme reactor for continuous production, we examined the flow rate dependence of lactose hydrolysis at high substrate conversion and performance stability at constant operational conditions. Fig. 3 summarises the results and shows that yields of $\geqslant$60% require τ to be 33 min or greater and can be maintained for a minimum of 5 days of continuous processing. The relationship between substrate conversion and τ could be predicted reasonably up to a conversion of about 60% using the mass balance for a continuous tubular reactor[10] and Michaelis–Menten kinetics, whereby the immobilised enzyme activity and K_m are known (Fig. 3). At conversion $\geqslant$60%, product inhibition by glucose is probably important.[7b]

Summarising, the paper reports design of a multichannel microfluidic reactor for continuous biotransformations with immobilised enzymes. The material used for microstructure fabrication was selected for enzyme attachment *via* the common procedure of silanisation and protein tethering by crosslinking with glutardialdehyde. Solute–material interactions and control of the immobilisation with regard to uniformity of surface coverage and retention of enzyme activity require attention for further improvement.

We gratefully acknowledge the contributions of A. P. Zurk (Daily Business Support) in the fabrication of the microstructured reactor. Many thanks go to C. Elis for the assistance at scanning electron microscopy. Financial support was received from the Austrian Federal Ministry of Transport, Innovation, and Technology (Program "Factory of Tomorrow", Project 807947). Dr D. Kirschneck (Microinnova), Dr M. Koncar (VTU Engineering), and Mag. H. Reichl (Hämosan) are thanked for their support.

Notes and references

1 (*a*) I. R. Baxendale, J. Deeley, C. M. Griffiths-Jones, S. V. Ley, S. Saaby and G. K. Tranmer, *Chem. Commun.*, 2006, 2566–2568; (*b*) K. Jähnisch, V. Hessel, H. Löwe and M. Baerns, *Angew. Chem., Int. Ed.*, 2004, **43**, 406–446; (*c*) A. J. deMello, *Nature*, 2006, **442**, 394–402; (*d*) P. Watts and S. J. Haswell, *Chem. Eng. Technol.*, 2005, **28**, 290–301; (*e*) K. F. Jensen, *Chem. Eng. Sci.*, 2001, **56**, 293–303; (*f*) P. Watts and C. Wiles, *Chem. Commun.*, 2007, 443–467.

2 V. Hessel, S. Hardt, H. Löwe, A. Müller and G. Kolb, *Chemical Micro Process Engineering*, Wiley-VCH, Weinheim, 2005, vol. 1 and 2.

3 (*a*) M. Miyazaki and H. Maeda, *Trends Biotechnol.*, 2006, **24**, 463–470; (*b*) P. L. Urban, D. M. Goodall and N. C. Bruce, *Biotechnol. Adv.*, 2006, **24**, 42–57; (*c*) K. Kanno, H. Maeda, S. Izumo, M. Ikuno, K. Takeshita, A. Tashiro and M. Fujii, *Lab Chip*, 2002, **2**, 14–18; (*d*) T. Honda, M. Miyazaki, H. Nakamura and H. Maeda, *Adv. Synth. Catal.*, 2006, **348**, 2163–2171; (*e*) B. Ku, J. Cha, A. Srinivasan, S. J. Kwon, J.-C. Jeong, D. H. Sherman and J. S. Dordick, *Biotechnol. Prog.*, 2006, **22**, 1102–1107.

4 (*a*) H. Becker and C. Gärtner, *Electrophoresis*, 2000, **21**, 12–26; (*b*) Ö. Rötting, W. Röpke, H. Becker and C. Gärtner, *Microsyst. Technol.*, 2002, **8**, 32–36; (*c*) J. C. McDonald, D. C. Duffy, J. R. Anderson, D. T. Chiu, H. Wu, O. J. A. Schueller and G. M. Whitesides, *Electrophoresis*, 2000, **21**, 27–40; (*d*) J. M. K. Ng, I. Gitlin, A. D. Stroock and G. M. Whitesides, *Electrophoresis*, 2002, **23**, 3461–3473; (*e*) A. D. Stroock and G. M. Whitesides, *Acc. Chem. Res.*, 2003, **36**, 597–604.

5 J. M. Ottino and S. Wiggins, *Philos. Trans. R. Soc. London, Ser. A*, 2004, **362**, 923–935.

6 (*a*) H. Makamba, J. H. Kim, K. Lim, N. Park and J. H. Hahn, *Electrophoresis*, 2003, **24**, 3607–3619; (*b*) S. Hu, X. Ren, M. Bachman, C. E. Sims, G. P. Li and N. L. Allbritton, *Langmuir*, 2004, **20**, 5569–5574; (*c*) H. Qu, H. Wang, Y. Huang, W. Zhong, H. Lu, J. Kong, P. Yang and B. Liu, *Anal. Chem.*, 2004, **76**, 6426–6433.

7 (*a*) S. W. Kengen, E. J. Luesink, A. J. Stams and A. J. Zehnder, *Eur. J. Biochem.*, 1993, **213**, 305–312; (*b*) I. Petzelbauer, B. Nidetzky, D. Haltrich and K. D. Kulbe, *Biotechnol. Bioeng.*, 1999, **64**, 322–332; (*c*) B. Splechtna, I. Petzelbauer, B. Kuhn, K. D. Kulbe and B. Nidetzky, *Appl. Biochem. Biotechnol.*, 2002, **99**, 473–488.

8 M. W. Toepke and D. J. Beebe, *Lab Chip*, 2006, **6**, 1484–1486.

9 (*a*) P. M. Doran, *Bioprocess Engineering Principles*, Academic Press, San Diego, 1995; (*b*) N. J. Gleason and J. D. Carbeck, *Langmuir*, 2004, **20**, 6374–6381; (*c*) H. Mao, T. Yang and P. S. Cremer, *Anal. Chem.*, 2002, **74**, 379–385; (*d*) M. B. Kerby, R. S. Legge and A. Tripathi, *Anal. Chem.*, 2006, **78**, 8273–8280; (*e*) W.-G. Koh and M. Pishko, *Sens. Actuators, B*, 2005, **106**, 335–342; (*f*) J. H. Kang and J.-K. Park, *Sens. Actuators, B*, 2005, **107**, 980–985; (*g*) H. Wu, Y. Tian, B. Liu, H. Lu, X. Wang, J. Zhai, H. Jin, P. Yang, Y. Xu and H. Wang, *J. Proteome Res.*, 2004, **3**, 1201–1209; (*h*) G. H. Seong, J. Heo and R. M. Crooks, *Anal. Chem.*, 2003, **75**, 3161–3167; (*i*) L. A. DeLouise and B. L. Miller, *Anal. Chem.*, 2005, **77**, 1950–1956; (*j*) E. Garcia, M. S. Hasenbank, B. Finlayson and P. Yager, *Lab Chip*, 2007, **7**, 249–255.

10 A pulse of tracer colour (100 μL 0.1 mM Procion Red H-8B) applied to the microstructured device operated continuously at flow rates of 0.25–1.00 mL min^{-1} gave a residence time distribution that was superimposable to that of just the Teflon tube connecting pump and reactor.