Supplementary Material (ESI) for Chemical Communications

Supporting Information

Development of a microfluidic immobilised enzyme reactor and its application to biocatalyst characterisation and chemical transformation under flow conditions

Malene S. Thomsen[1], Peter Pölt[2] and Bernd Nidetzky[1]*

[1]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.
Fax: +43 316 873 8434; Tel: +43 316 873 8400; E-mail: bernd.nidetzky@tugraz.at

[2] Institute for Electron Microscopy, Graz University of Technology, Steyrergasse 17, A-8010 Graz, Austria

Materials

Silopren LSR 4070 (GE Bayer Silicones, Leverkusen, Germany), (3-Aminopropyl)triethoxysilane (Sigma-Aldrich, Vienna, Austria), Glutardialdehyde (Sigma-Aldrich, Vienna, Austria), PAMAM Dendrimer, Generation 5 solution (Sigma-Aldrich, Vienna, Austria), Trisoperl® - Size 100-200 μm, Pore size 50 nm, Area 79.81 m^2/g (Vitrabio, Steinach, Germany), 2-nitrophenyl-β-D-galactopyranoside (oNPGal; Sigma-Aldrich, Vienna, Austria).

Fabrication of microstructured element

The silicon master for polymer replication was prepared by mechanical micro-machining, using a micro-channel pattern designed with AutoCAD and industrial equipment for high-precision micro-milling.

A variotherm process of micro-injection moulding with industrial equipment was used for the fabrication of the micro-structured polymer plate, which is shown in Figure 1 of the main manuscript. Special features of the micro-structure are the inlet zone which facilitates homogeneous distribution of liquid into the microchannels, the passive mixing

elements that have been incorporated into each channel to enhance mass transfer, and sealing lips at the top of each channel wall. A two-component liquid silicone rubber, Silopren LSR 4070, which is a polydimethylsiloxane (PDMS) containing vinyl groups with added pyrogenic silicic acid, was applied. The PDMS was cured at 240 °C and pressure close to 40 MPa, and the following tempering was performed for 1 hour at 140 °C in saturated water vapour. After cooling, the ready PDMS element could be removed from the mould. Design of the micro-structured element was performed in collaboration with *dbs* - daily business support GmbH (Graz, Austria), and the plates were manufactured by *dbs*.

The housing for the microstructured element

The housing holds the PDMS microfluidic element in a sandwich-like construction. The upper and lower housing plates are made of poly(methyl methacrylate) (PMMA), and can be used in the temperature range -40 °C - 140 °C. A maximum pressure of 4 MPa is recommended during continuous use. On the one PMMA element, substrate supply can be applied. The other element consists partly of PMMA and a metal plate. The backside of the PDMS plate is placed on the metal plate and on the other side of the metal plate, through a meander structure in the PMMA, liquid from and external heating circulator is pumped through. In that way, the temperature of the process can be easily controlled. Three metal clamps made of chrome steel are holding the plates tightly together. The microreactor, disassembled and assembled, is shown in Figure S1.

The housing of microreactor was designed and fabricated by *dbs*.

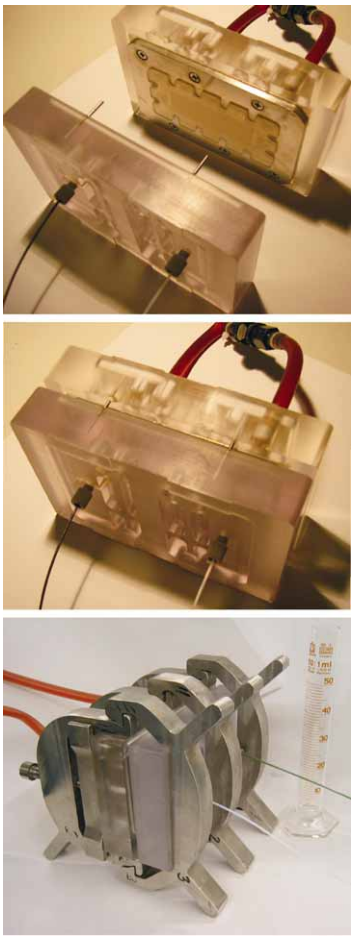

Figure S1: Housing for the PDMS microstructured plate.

Experimental setup of the microreactor

Figure S2 shows the basic system setup for experiments using the microreactor. The substrate was pumped through the microreactor (3) using a KNAUER Smartline Pump 1000 (2), which could deliver flow rates from 9.999 mL/min down to 0.001 mL/min. If the substrate was 2-nitrophenyl-β-D-galactopyranoside (oNPGal), a LINEAR UVIS 204 detector (4) was inserted subsequent to the microreactor in order to measure the product of enzymatic hydrolysis, 2-nitrophenol (oNP) at 405 nm. The volume of tubing connecting the microreactor and detector was around 350 µL. By way of comparison, the total volume of the microreactor was 167 µL. If lactose was used as substrate, samples were collected at the outlet of the tubing (5) and analysed offline using an enzymatic assay for glucose (Dipromed Glucose UV kit). A JULABO F25-MV Refrigerated/Heating Circulator (6,7) was used for temperature control.

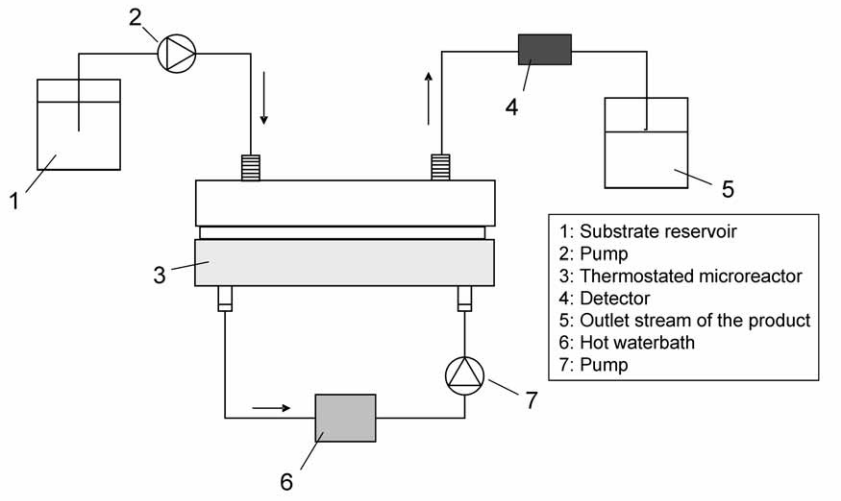

Figure S2: Experimental setup for the immobilised enzyme microreactor.

Enzyme production and immobilisation

Recombinant enzyme was produced (Splechtna et al., 2002) and partially purified by heat treatment using reported methods (Petzelbauer et al., 1999).

Immobilisation of CelB into the channels of the microstructured PDMS plate

The first step of the immobilisation procedure was washing of the microreactor plate for a few minutes with a dilute (30%) ”piranha solution" (conc. H_2SO_4: 30% H_2O_2; 7:3). Then, the plate was washed carefully with water, ethanol, and water again.

The immobilisation steps were performed having the microstructured PDMS plate placed in the housing, and pumping the different solutions through the microchannels. Usually the activation steps were carried out at low flow rate (e.g. 0.15 mL/min) and with intervals of 15 - 30 minutes with no flow to ensure time enough for reaction between the solution and the channel walls. The washing steps were usually performed continuously at a flow rate of 1 mL/min.

After first having washed the mirochannels with micro-filtered (0.2 μm filter pore size) water, a 15 mL solution of (3-aminopropyl)triethoxysilane (APTES) (10% in micro-filtered water; pH 3.5 – 4.0) was pumped through the channels at 80 °C for 3 hours. Then, the channels were washed 15 – 30 minutes by pumping filtered water through the channels. Then, a 10 mL glutardialdehyde solution (2.5% in Phosphate Buffered Saline (PBS); 140 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 8.4) was pumped through the channels for 2 hours at room temperature. Then, the microchannels were washed 15 – 30 minutes using PBS. Optionally, immobilisation of dendrimers was performed after the glutaraldehyde activation. It was done by applying 15 mL dendrimer solution ($6 \cdot 10^{-4}$ g/mL; in ethanol) and pumping it through the microreactor for around 10 – 15 hours. After the immobilisation of dendrimers, a washing step with PBS buffer was performed, and then a new step of glutaraldehyde activation and washing of the microchannels with PBS was performed. For immobilisation of CelB, 7 mL of the enzyme (0.095 mg/mL; PBS; pH 7.5) was pumped through the microchannels for 10 – 15 hours, recirculating the solution. The microchannels were washed in the end with sodium citrate buffer (20 mM; pH 5.5.) for 15 – 30 minutes. The amount of protein and enzyme activity bound to the plate was calculated from the difference of free protein and free enzyme activity in the CelB solution before and after loading on the glutardialdehyde-activated microreactor. Protein was determined with the Bio-Rad dye binding assay, adjusted to the used protein concentration range, and enzyme activity was measured by assaying for lactase activity as described below. Typically, around 20 % (8 to 30 %) of initial protein and activity were bound. The binding efficiency was calculated as the ratio between measured and calculated enzyme activities bound to the microstructured plate. The assay for immobilised enzyme activity is described below.

Immobilisation of CelB to porous glass beads

The method for immobilising CelB to porous glass beads corresponds to the method of immobilising CelB to the microstructured PDMS plate. The porous glass beads, Trisoperl®, were incubated overnight in concentrated "piranha solution" (70% conc. H_2SO_4, 30% H_2O_2), and were afterwards washed thoroughly with micro-filtered water. The carriers were then rinsed in 99% ethanol, and afterwards washed again with water. The carriers were then dried, and kept in a closed container until further use. The silanisation was performed by incubating the glass carriers in an excess of a freshly prepared solution of 10% APTES in water, with the pH adjusted to 3.5 – 4.0 with HCl, at 80 °C for 3 hours. Afterwards the carriers were washed three times with PBS (pH 7.0) with approximately ten times the volume of the original APTES solution added. The activation with glutardialdehyde was performed by incubating the silanised carriers with an excess of 2.5% glutardialdehyde in PBS buffer (pH 8.4) for 2 - 15 hours. Afterwards the carriers are washed thoroughly with PBS buffer. The glutardialdehyde-activated carriers were incubated with a suitable amount of CelB in PBS buffer (pH 7.0) for at least 2 hours.

Activity assays

For free enzyme

The measurement of lactase activity of CelB free in solution was carried out in a two-step assay. First, 1 mL of the reaction mixture containing 600 mM lactose in 20 mM sodium citrate buffer, pH 5.5, was pre-incubated for 10 min at 80 °C using an Eppendorf Thermomixer with agitation at 750 rpm. The reaction was initiated by adding 20 µL of enzyme solution. After exactly 5 min, the hydrolysis was stopped by immediately putting the tube on ice. Determination of the produced glucose was performed using the Dipromed Glucose UV kit. The activity could then be calculated from the definition that one unit of activity refers to 1 µmol glucose released per minute under the reaction conditions described above.

For enzyme immobilised to porous glass carriers

The same assay as above was adapted for the measurement of lactase activity of CelB immobilised to porous glass carriers. Here, 1 mL of 600 mM lactose in 20 mM sodium citrate buffer, pH 5.5, pre-incubated at 80 °C, was added to porous glass carriers with immobilised CelB, and incubated exactly 5 min after which the hydrolysis was stopped

by immediately putting the tube on ice. The amount of glucose produced was assayed using the Dipromed Glucose UV kit. The binding efficiency for CelB immobilised to the porous carriers was found to be 35% (±3) (Figure S3).

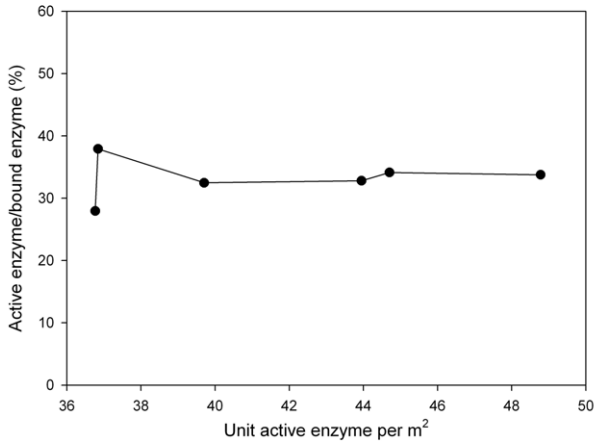

Figure S3: Immobilisation of CelB to porous Vitrabio carriers using glutardialdehyde yielded an average of 35% binding efficiency.

For enzyme immobilised in the microreactor

The measurement of lactase activity of CelB immobilised in the microreactor was performed by pumping 600 mM lactose in 20 mM sodium citrate buffer, pH 5.5, through the microreactor, which was temperature-controlled at 80 °C. The product was collected at the outlet of the microreactor, and determination of the produced glucose was performed using the Dipromed Glucose UV kit. As long as there was a linear relationship between residence time and converted lactose, the activity could be calculated. The residence time is the ratio of reactor volume and flow rate.

oNPGal assay

When using oNPGal as substrate, the activity of free CelB was assayed by adding 50 µL enzyme solution to 950 µL oNPGal (13 mM, sodium phosphate buffer, pH 5.5). This mixture was incubated at 80 °C for exactly 10 minutes. The reaction was stopped by immediately putting the tube on ice. The release of oNP was measured by determining the increase in absorbance at 405 nm (25 °C), and compared to a standard curve of oNP. One unit of oNPGal activity refers to 1 µmol of oNP released per min under the reaction conditions described above.

When oNPGal was used as substrate in the microreactor, a LINEAR UVIS 204 detector was inserted subsequent to the microreactor in order to measure the oNP at 405 nm. As long as there was a linear relationship between residence time and converted oNPGal, the activity could be calculated.

Scanning Electron Microscopy (SEM)

The SEM images were recorded with either a Zeiss DSM 982 Gemini or a Zeiss ULTRA 55 (both from Carl Zeiss SMT AG, Oberkochen, Germany) scanning electron microscope by use of the SE-detector (Everhart Thornley detector). To avoid charging of the electrically non-conductive specimens, they were coated with a thin Au-layer (thickness ~ 10 nm). This layer additionally enhances the image contrast. To avoid specimen damage and stay surface sensitive, in general an electron energy of 3 keV or 5 keV was chosen. At 3 keV the maximum information depth is around 0.2 μm, at 5 keV around 0.5 μm.

References

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