Lab on a Chip

Cite this: *Lab Chip*, 2012, **12**, 2743–2748

www.rsc.org/loc

PAPER

Functionalization of polycarbonate with proteins; open-tubular enzymatic microreactors†

D. Ogończyk,* P. Jankowski and P. Garstecki

Received 24th February 2012, Accepted 16th April 2012
DOI: 10.1039/c2lc40204a

This paper examines a set of techniques for the immobilization of enzymes on the surface of microchannels fabricated in polycarbonate (PC). Our experiments identify the method that uses combined physico-chemical immobilization on a layer of polyethyleneimine (PEI) as a reproducible vista for the robust immobilization of proteins. As an example, we demonstrate the fabrication, throughput and stability of an open-tubular reactor draped with alkaline phosphatase (ALP, EC 3.1.3.1) as a model enzyme. As PC is suitable for industrial applications the method could potentially be used to immobilize proteins in numbered-up implementations.

Introduction

Microreactor technology opened up new possibilities in the construction of flow-through reactors that yield superior control over chemical syntheses and biochemical reactions. Among other applications, microreactors can readily be used for the intensification of heterogeneous processes.[1–4] The rapid heat and mass transfer in microreactors enable significantly higher throughput of (bio)chemical processes than the classical batch reactors currently used in industry.[3,5] In a pronounced example, a synthesis reaction of oligosaccharide with the use of β-galactosidase in a continuous-flow microreactor was approximately five times faster than in the mixing reactor.[6] The intensification and greater reaction control has particular significance in the chemical, pharmaceutical and bio-based industries. Other advantages of the use of heterogeneous microreactors with immobilized catalysts include: i) reduced costs *via* higher yield and higher throughput of continuous processes in comparison to batch processing, ii) decreased environmental costs *via* smaller production of waste products and elimination of the problem of separation of the enzyme from the products, and iii) decreased risk and cost of failures. Often the immobilized biocatalysts exhibit improved stability and the ability to perform their function at more extreme reaction conditions in comparison to the dissolved enzymes.

Two strategies for the construction of reactors for heterogeneous biocatalysis are known: i) immobilization of enzymes on beads (*e.g.* on the surface of glass or magnetic beads) or ii) directly on the surface of the microchannels (open-tubular bioreactors). Here we focus on the open-tubular enzymatic microreactors as they combine satisfactory intensification of the enzymatic processes while offering good operational and storage stability and high throughput at much smaller pressure heads than those presented by bead-reactors. Importantly, in addition to their small-scale use in protein mapping that requires enzymatic cleavage of proteins into peptides, in medicinal chemistry (*e.g.* studies of inhibition or activation of enzymes) and in construction of biocells,[7] all of the advantages listed above could be of great use in industrial scaled-up (or rather numbered-up) applications. So far, enzymatic microreactors have been predominantly fabricated in materials such as glass, silicon, quartz or polymers (PDMS, epoxy-polymer SU8 and Teflon).[1,5] These materials demand fabrication schemes that are not suitable for numbering-up, and industrial implementations based on these materials are difficult or not cost-effective.[2,3,5]

Here we focus on the fabrication of heterogeneous bioreactors in polycarbonate (PC) – a material that offers an attractive set of characteristics including high toughness, impact strength, rigidity and heat resistance, low cost and commercial availability. Importantly, PC can be embossed or moulded in mass-production facilities, making this material particularly attractive for industrial applications. As PC is quite chemically inert, the modification of its surface requires the use of specific conditions and reagents that we review and test in this report.

The surface of PC may be activated by modification in three distinct regions: the methyl groups, the carbonate groups and the aromatic rings. The most commonly used approach is based on activation of the polymer with UV or plasma, most often resulting in activation of the methyl groups. For example Bora *et al.*[8] used a photolinker, 1-fluoro-2-nitro-4-azidobenzene (FNAB), to immobilize a protein ligand having an active nucleophilic group (*e.g.* amino group). Exposure of PC to ammonia plasma[9] creates ammonium groups while ozone/UV treatment results in a high concentration of carboxylic groups that can be used *e.g.* as bio-affinity adsorbents.[10] The use of PC allows for the construction of micropatterns of DNA on the surface of compact discs with the benefit of a convenient optical readout.[11–13] Suye *et al.*[14] used poly-(L-lysine), which reacts with

Institute of Physical Chemistry, Polish Academy of Sciences, Kasprzaka, 44/52,01-224, Warsaw, Poland. E-mail: ogonczyk@ichf.edu.pl
† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2lc40204a

the carbonate groups, to drape the surface with glutaraldehyde and glucose oxidase to obtain an amperometric glucose biosensor. Electrophilic reactions with aromatic rings of PC *via e.g.* the nitration of polycarbonate[15,16] can be used to yield thiol or carbonyl coatings.[15] Polycarbonate can also serve as a substrate for sophisticated multi-step modifications of the surface to attach ligands for molecular screening applications[17] and dyes for optical immunosensors.[18]

Unfortunately, although the UV and plasma treatments are effective in the activation of the surface of PC, they are difficult to apply inside microchannels (*i.e.* after bonding of the devices). In addition, the reactions with carbonate groups and aromatic rings are reported to present a rather low efficiency of the modification[14,15] or to use aggressive solvents (*e.g.* acetonitrile) that may destroy the microstructure of the channels.[17,18]

Here, we present a new and general method for the functionalization of the surface of PC with proteins and exemplify the use of this method in construction of an open-tubular enzymatic microreactor.

Our method uses the primary coating of the surface of PC with polyethyleneimine (PEI)[19] – a nontoxic, cationic polyelectrolyte. A layer of PEI presents a large density of free primary amino groups and thus the coating with PEI forms a convenient base for the further functionalization of PC. Here, we discuss three strategies for the immobilization of proteins on PC microchannels coated with PEI: *via* electrostatic adsorption, *via* covalent binding with a linker and *via* direct binding between PEI and an activated protein. In order to compare the stability of the immobilization and the activity of the biocatalyst over time we monitored the enzymatic turnover of the *p*-nitrophenyl phosphate (NPP) into *p*-nitrophenol that absorbs light at 405 nm. The results of the measurements of absorbance did not depend on the interval of time between the collection and measurement of samples. As a model enzyme we used the well known alkaline phosphatase (ALP, EC 3.1.3.1).

Importantly, as PEI is slightly hydrophilic (Fig.SI_1 in the ESI†) it is not necessary – in contrast to typical procedures of the immobilization of proteins on hydrophobic substrates – to use high ionic strength solutions. On the other hand, as we confirmed *via* an XPS analysis, PEI readily chelates cations. This may compromise the activity of some of the metalloproteins. In our experiments we thus first coat the microchannels fabricated in PC with a layer of PEI[19] and then we pass a solution of cations through the channel to saturate the chelating activity of PEI. In the case of the immobilization of alkaline phosphatase we rinsed the channels with a solution of $ZnCl_2$ (1 mM) and $MgCl_2$ (1 mM), while for the immobilization of urease we passivated the channels with a solution of $Ni(NO_3)_2$ (see the ESI† for details).

Results and discussion

Immobilization of an enzyme *via* electrostatic interactions

As PEI is a polyelectrolyte that has the ability to link proteins in aqueous media *via* the electrostatic attraction between the positively charged polymers and the negatively charged proteins, the easiest method for coating the surface with the enzymes is through physical immobilization (Fig. 1A). In the experiments we simply passed a solution of alkaline phosphatase (ALP) at a concentration of 15 mg mL^{-1} through the channels that were coated with PEI and passivated with cations. We found this coating to be unstable – the enzymatic turnover decreased sharply within a few hours of device operation (Fig. 2). Thus the physical adsorption of enzymes on the PEI-coated surfaces is not suitable for the long-term use of bioreactors.

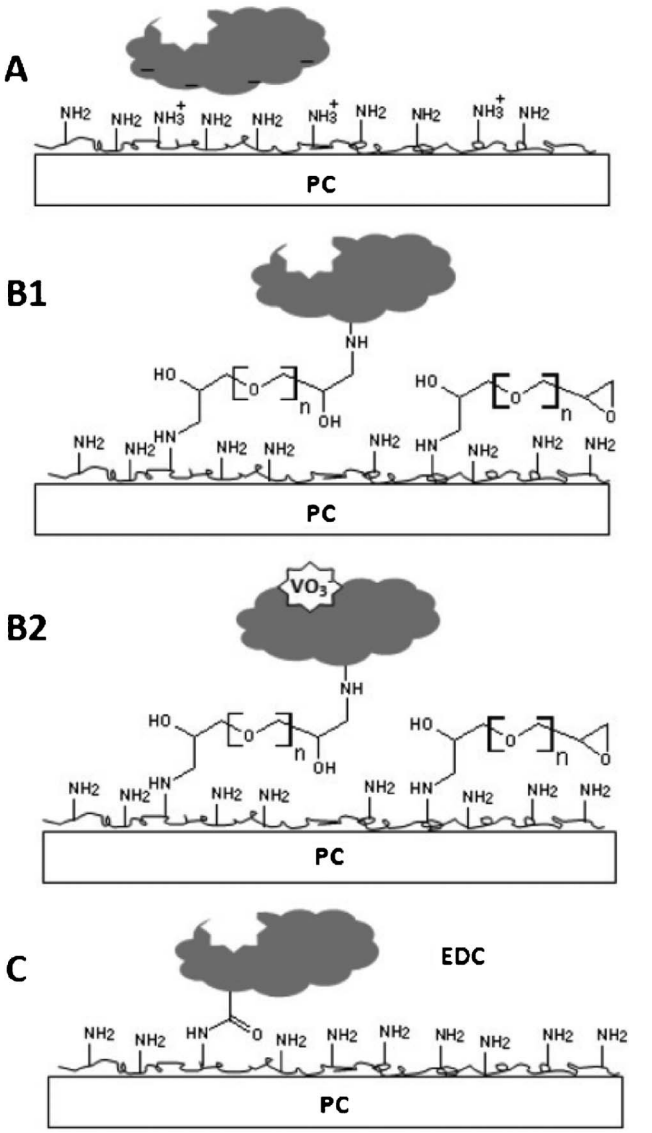

Fig. 1 (A) Physical immobilization of the enzyme *via* ionic interactions; (B1) binding with the use of poly(ethylene glycol) diglycidyl ether and (B2) a competitive inhibitor; (C) immobilization in the presence of an activator of the enzyme.

Immobilization of an enzyme with the use of linkers

A more stable immobilization of the enzyme can be obtained *via* covalent bonding of the protein to the PC/PEI surfaces. We tested a set of linkers that can bind to the amine groups of PEI and to amine groups presented by the protein. From the list of glutaraldehyde, poly(ethylene glycol) diglycidyl ether (PEGDE), trimethylolpropane triglycidyl ether and hexamethylene diisocyanate, we found PEGDE to yield the most stable coatings. In

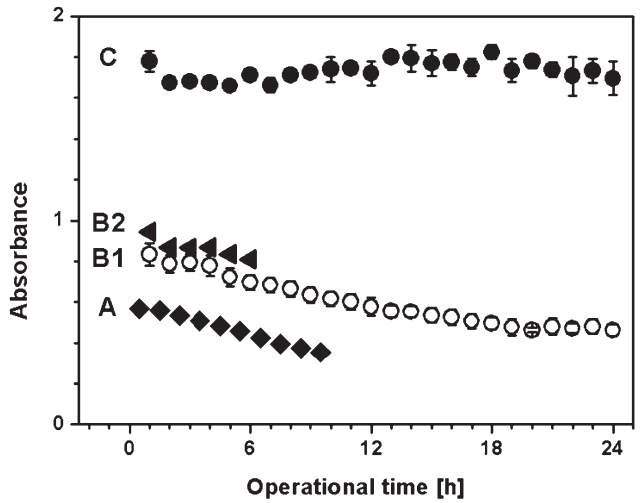

Fig. 2 Results of the test of conversion rate in continuous use of four types of enzymatic bioreactors: (A) (diamonds) modified with an aqueous solution of ALP (15 mg mL^{-1}); (B1) (open circles) modified with the use of PEGDE; (B2) (triangles) modified with the use of PEGDE in the presence of the competitive inhibitor; (C) (solid circles) prepared with the use of an activator of the enzyme. The ALP activity assays were performed in 0.2 M diethanolamine buffer (pH 9.5) using 1 mM NPP at room temperature and a fixed 10 mL h^{-1} rate of flow. Each data point represents an average of measurements taken in three independently prepared copies of the enzymatic bioreactors. Details of the immobilization procedures (A, B1, B2 and C) are given in the supplementary information file.†

all the experiments we first ran a solution of the linker through the PC-PEI channels. Next we used chemo-ligation to immobilize the protein *via* formation of bonds with the nucleophilic residues of the enzymes (Fig. 1(B1); please see the ESI† for details).

The procedure did not yield stably immobilized enzymes on the surface of the microchannels. Fig. 2 shows that the modification with the use of PEGDE (open circles) yields better results than the procedure that adsorbed ALP directly onto a layer of PEI. Still, in both cases the turnover (and hence the combination of the amount and activity of the immobilized protein) significantly decreased over time (38% and 26% over 10 h for the PEI-ALP and PEI-PEGDE-ALP, respectively).

Following the report of Mateo *et al.*,[20] we hypothesize that the observed decrease in turnover in the PEI–PEGDE–ALP system may result from the slow formation processes of the additional covalent linkages between the immobilized enzyme and the epoxy support, which deform the protein and decrease its activity (see also ESI, Fig.SI_2†). In order to eliminate the undesirable activity reduction of ALP caused by the subsequent formation of covalent bonds between the enzyme and the epoxy supports, we immobilized the enzyme in the presence of 100 μM of its competitive inhibitor, NH_4VO_3.[21] The inhibitor is meant to protect the conformation of the active site during coating and during the secondary step of the formation of the additional bonds with the epoxy groups (Fig. 1(B2)). We found, however, that the use of the inhibitor of ALP and the different reaction conditions of the immobilization did not solve the problem with the small operational stability (ESI, Fig.SI_3†).

Immobilization of an enzyme *via* activation with *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride

In the last set of experiments that we describe here—the ones that we found most efficient in the generation of stable coatings—we used *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimidehydrochloride (EDC) to activate the carboxylate groups on the protein and to bind them with the amine groups on PEI (Fig. 1C). EDC is a commonly used, water-soluble carbodiimide zero-length cross-linker that can easily couple carboxylate groups[22] of biocatalysts with primary amines of PEI. In order not to alter the conformational flexibility of the biocatalyst, the coating process required optimization. We screened for the optimal concentrations and proportions of ALP, EDC and *N*-hydroxysuccinimide (NHS: an agent that improves the efficiency of EDC).[23] We did not optimize the interval of reaction: since immobilization with the use of EDC requires a relatively long time,[24] we fixed the reaction time to overnight.

Following a set of experiments with varied parameters (Fig. 3, Fig. SI_4 and Fig. SI_5†) we found the optimum combination. The optimal procedure comprises: filling the passivated PC–PEI channels with an aqueous solution of ALP, EDC and NHS (1 mg of ALP in 1 mL of water was mixed with 0.5 mg of EDC for 0.5 h and then 0.5 mg of NHS was added) and passing it through the channel at room temperature overnight at a rate of 3 μL h^{-1}. After modification, we washed the bioreactor for one hour by passing through it a working buffer (0.2 M diethanolamine buffer (DEA) pH 9.5; V = 20 mL h^{-1}). As polyethylene may adsorb the enzyme, we always used fresh tubing in this last step.

The data shown in Fig. 2 (solid circles) shows that the optimized coating that uses EDC provides stable immobilization of ALP and that ALP apparently does not change its activity over prolonged continuous use of the microreactor. Fig. 3 shows a comparison of the activity tests for different methods of

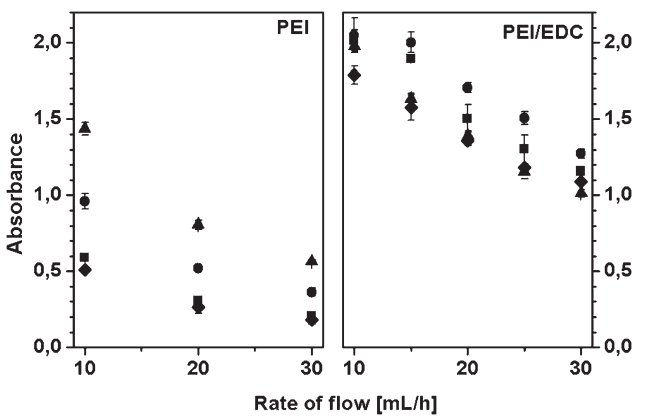

Fig. 3 Tests of the conversion rates in the ALP bioreactors as a function of the flow rate of the solution of NPP for two types of bioreactor: with ALP applied directly on the layer of PEI (left) and PEI/EDC (right). The PEI/EDC chips were modified with a solution of ALP, EDC and NHS at a mass ratio of 2 : 1 : 1. The charts show the influence of the concentration of ALP used for modification: 0.1 mg mL^{-1}, solid triangles, 1 mg mL^{-1}, solid circles, 10 mg mL^{-1}, solid squares and 15 mg mL^{-1}, solid diamonds. The enzyme activity assays were performed in 0.2 M diethanolamine buffer (pH 9.5) using 1 mM NPP at room temperature. Each data point represents an average of three series of measurements.

immobilization of the enzyme; a microreactor with ALP immobilized through only ionic interactions and a microreactor in which the immobilization of ALP was additionally chemically assisted with EDC. The bioreactor prepared in the absence of EDC yields a lower degree of bioconversion (Fig. 3 and Fig. SI_6†) and, as we have shown previously, exhibits a short life-time (Fig. 2). The use of EDC increases the rate of conversion (Fig. 3) in comparison to the reactors prepared with the use physical immobilization. We hypothesize that the main reason behind the increased conversion rate is the larger amount of protein immobilized on the walls of the reactor with the use of EDC.

Stability of the immobilized enzyme

We have also tested the stability of the coating and the activity of the enzyme immobilized with the use EDC against prolonged storage. We stored the dry microreactors in a refrigerator at +4 °C and after a given interval of time we ran a solution of NPP through the reactor at a standardized flow rate to measure the enzymatic conversion of the substrate. Fig. 4 compares the conversion rates measured after 6, 17 and 25 days and shows that there is no change in the activity of the enzyme and the conversion rate in the reactor.

Throughput of the reactors

We have also tested the potential for increasing the throughput of the open-tubular enzymatic bioreactor by decreasing the cross-section of the channel. A decrease in the width and height of the channels increases the surface-to-volume ratio (S/V) and increases the rate of diffusional transport between the bulk and the surface. Fig. 5 compares the absorbance from *p*-nitrophenol as a function of the flow rate through channels of different widths (w) and lengths: 0.2 × 0.2 × 1310 mm, 0.3 × 0.3 × 582 mm and 0.4 × 0.4 × 328 mm (S/V = 20, 13.3 and 10 mm^{-1}, respectively). As the volume of the microchannels was the same,

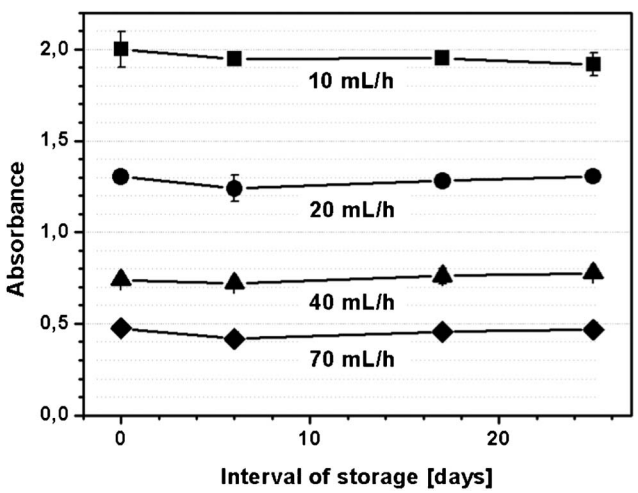

Fig. 4 Quantitative characterization of the stability against the storage time. The dry reactors were stored for several days at +4 °C. The ALP activity assays were performed in 0.2 M diethanolamine buffer (pH 9.5) using 1 mM NPP at room temperature at the beginning of the experiment (0 days) and after 6, 17 and 25 days of storage. Each data point represents an average of the measurements in two bioreactors.

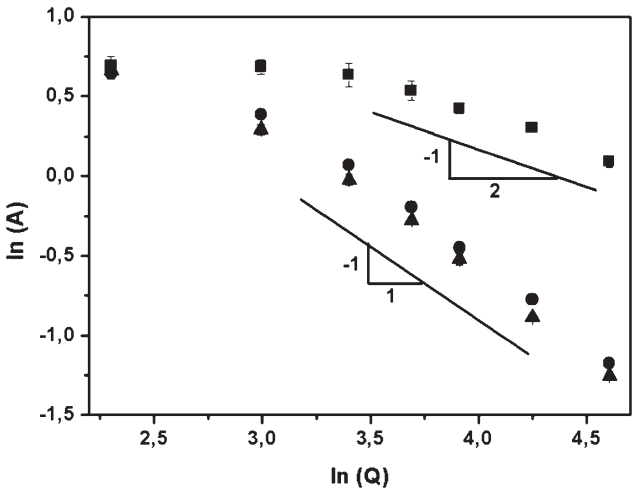

Fig. 5 Log–log plot of the absorbance from the product of the enzymatic reaction as a function of the flow rate of the solution of the substrate (NPP). Squares: 0.2 × 0.2 mm (length 1310 mm), circles: 0.3 × 0.3 mm (length 582 mm) and triangles: 0.4 × 0.4 (length 328 mm). The enzyme activity assays were performed in 0.2 M diethanolamine buffer (pH 9.5) using 1 mM NPP at room temperature. Each data point represents an average of the measurements in two bioreactors.

the average time or residence of the substrate in the channels was the same for the same rate of flow of the solution. This allows us to relate directly the concentration of the product with the rate of the reaction.

We flowed the substrate solution through each of the bioreactors at a range of flow rates $Q \in (10, 100)$ [mL h^{-1}]. The Reynolds number for these flows, calculated as $\mathrm{Re} = Q\rho/w\mu$ for $\mu \sim 1$ mPa s and $\rho \sim 10^3$ kg m^{-3} ranged between *ca.* 7 and *ca.* 140, indicating laminar flow. The measured conversion rate shows that i) the turnover rate is much higher in the channel with the smallest cross-section than in the two larger ones, and ii) that the concentration of the product decreases with the square root of the flow rate in the case of the smallest channel, while in the case of the two remaining ones (and all other cases reported here) it decreases with the first power of the flow rate. This last observation suggests that while in the larger channels conversion is limited by the reaction time at the surface of the channel (time to react $\propto t \propto Q^{-1}$), in the channel with the smallest lumen we have reached the regime of a diffusion-limited reaction (distance to diffuse $\propto \sqrt{t} \propto Q^{-1/2}$).

Immobilization of urease

The procedure that we describe can also be used to immobilize other proteins. For example, we used the same preparation procedure for a bioreactor with urease as the active enzyme (see the ESI for details†). The reactor catalyzes the hydrolysis of urea and causes the alkalinization of the environment according to the equation: $H_2N\text{-}CO\text{–}NH_2 + 3\ H_2O \rightarrow 2\ NH_4^+ + HCO_3^- + OH^-$. The enzyme activity assays were performed in 5 mM phosphate buffer using 10 mM urea with the use of a potentiometric glass ion-selective electrode (Fig. 6). The results show that the proposed method can be used for the immobilization of urease, and probably also a wide range of other proteins.

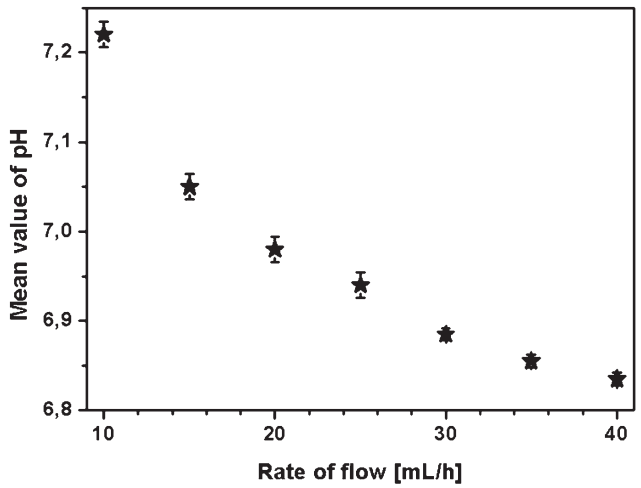

Fig. 6 The activity tests of urease reactors as a function of the flow rate of the urea. The assays were performed in 5 mM phosphate buffer composed of equal amounts of KH_2PO_4 and K_2HPO_4 in 0.1 M NaCl using 10 mM urea. Each data point represents an average of the measurements with a glass ion-selective electrode for two bioreactors.

Conclusions

We have presented the first test of a methodology for the immobilization of proteins on the surface of microchannels fabricated in polycarbonate. We tested three different strategies for coating the layers of PEI with proteins. The method that uses EDC as an activator of the protein for covalent binding between the enzyme and PEI allows the construction of stable bio-active coatings of microchannels fabricated in PC. Moreover, we demonstrate the utility and versatility of the developed bioreactor preparation procedure for other proteins. As PC is an attractive material that can be used in the mass-production of microfluidic devices, we believe that the method we describe here will be useful not only in laboratory or small-scale applications, but could also be interesting for industrial implementations of microreactors.

Enzymatic microreactor technology has a big potential not only due to the smaller amount of enzymes used but also to the increase in the surface of the phase boundary, thus enabling intensification of the processes of bioconversion.

For example, Froehlich and Bertau[25] showed the significant superiority of microreactor technology expressed in terms of the efficiency of the reaction. The functionalization of carbanosiloxanes and formosiloxanes was found to provide 40 and 60% of conversion after 1 h, respectively, while in traditional reactors 27 and 43% were recorded after 96 h. In another example, ceramic or glass microreactors presenting immobilized lipase[1] showed a yield 1.5 times better than that of batch reactors.

Clearly the use of microdevices enables a significant reduction of costs, and should make the microreactor technology attractive from an industrial point of view. In industrial implementations, the scale-up of throughput should be obtained *via* parallelization of the microreactors ('numbering-up'). Polycarbonate could be the material of choice for such implementations not only for its malleability and durability, but also for its efficiency in coating with proteins and the resulting large conversion rates in the reactors. For example, a tubular reactor with ALP immobilized using EDC on the inner wall of 1/16-inch poly(vinyl chloride) (PVC) tubing[26] offered smaller throughput than the reactors presented here, while it used *ca.* 20-fold larger amount of the enzyme in the immobilization procedure and the enzyme presented *ca.* 4 time higher activity than the one used here. In addition, the PC reactors present attractive storage stability: no change was observed in the activity of the enzyme after 25 days, compared to 17 and 50% decreases of the turnover rate after 3 and 9 days, respectively, in the PVC device. We note here that implementations of the procedures described in our report will certainly require further optimization for the surface coverage of the enzyme and for the yields of the reactions.

In summary, the combination of the attractive features of polycarbonate as a material suitable for mass production of microfluidic chips and the efficiency of the immobilization *via* the combined physico-chemical method using EDC with the observed high rates of conversion make the method potentially suitable not only for laboratory or analytical applications but also for industrial implementations.

Materials and methods

We fabricated the microfluidic channels in polycarbonate (PC) by micromilling. To prepare the microfluidic devices (μFLDs) we used milled PC plates with a thickness of 5 mm and plane PC plates with a thickness of 0.75 mm (Makrolon, Bayer, Germany). The bonding procedure includes: i) cleaning the slabs with isopropanol and an ultrasonic cleaner (1 h, 40 °C), ii) exposing the PC to oxygen plasma (2 min) and iii) bonding the pieces of PC in a thermal press (30 min, 130 °C). In the modification process we used digitally controlled syringe pumps (PHD 2000, Harvard Apparatus, US) and a thermostat (ED3, Julabo, Germany). A spectrophotometer (Nicolet Evolution 201 PC Control, Thermo, US) allowed for visualization of the results of the enzymatic reactions. In the bioreactor preparation process, alkaline phosphatase (ALP, EC 3.1.3.1, isolated from calf intestine, 10–30 U mg^{-1}, lyophilized powder; Sigma-Aldrich, Germany) and *p*-nitrophenyl phosphate (NPP; Sigma-Aldrich, Germany) were applied as a model enzyme and substrate, respectively. For the cross-linking of molecules of the protein, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; Sigma-Aldrich, Germany) and *N*-hydroxysuccinimide (NHS; Sigma-Aldrich, Germany) were used. Other reagents and solvents were obtained from Chempur (Poland). All chemicals were pure and analytical grade and were used without further purification. For the preparation of the aqueous solutions we used Milli-Q water.

Acknowledgements

This project was supported financially by the Ministry of Science and Higher Education (grant Iuventus Plus, IP2010 028970) and operated within the Foundation for Polish Science Team Programme co-financed by the EU European Regional Development.

References

1 M. Miyazaki and H. Maeda, *Trends Biotechnol.*, 2006, **24**, 463.
2 P. S. Dittrich, K. Tachikawa and A. Manz, *Anal. Chem.*, 2006, **78**, 3887.

3 E. S. Borovinskaya and V. P. Reshetilovskii, *Russ. J. Appl. Chem.*, 2011, **84**, 1094.
4 S. Matosevic, N. Szita and F. Baganz, *J. Chem. Technol. Biotechnol.*, 2011, **86**, 325.
5 M. P. C. Marques and P. Fernandes, *Molecules*, 2011, **16**, 8368.
6 K. Kanno, H. Maeda, S. Izumo, M. Ikuno, K. Takeshita, A. Tashiro and M. Fujii, *Lab Chip*, 2002, **2**, 15.
7 J. Krenková and F. Foret, *Electrophoresis*, 2004, **25**, 3550.
8 U. Bora, P. Sharma, S. Kumar, K. Kannan and P. Nahar, *Talanta*, 2006, **70**, 624.
9 S. J. Chang, S. M. Kuo, J. W. Lan and Y. J. Wang, *Artif. Cells, Blood Substitutes, Biotechnol.*, 1999, **27**, 229.
10 M. A. Witek, S. D. Llopis, A. Wheatley, R. L. McCarley and S. A. Soper, *Nucleic Acids Res.*, 2006, **34**, e74.
11 Z. Wang and R.-X. Li, *Nanoscale Res. Lett.*, 2006, **2**, 69.
12 Y. Li, Z. Wang, L. M. L. Ou and H.-Z. Yu, *Anal. Chem.*, 2007, **79**, 426.
13 Y. Li, L. M. L. Ou and H.-Z. Yu, *Anal. Chem.*, 2008, **80**, 8216.
14 S. Suye, Y. Kumon and A. Ishigaki, *Biotechnol. Appl. Biochem.*, 1998, **27**, 245.
15 M.-J. Banuls, *Bioconjugate Chem.*, 2008, **19**, 665.
16 J. Tamarit-LÓpez, S. Morais, M.-J. Bañuls, R. Puchades and A. Maquieira, *Anal. Chem.*, 2010, **82**, 1954.
17 J. J. L. Clair and M. D. Burkart, *Org. Biomol. Chem.*, 2003, **1**, 3244.
18 P. Najmabadi, K.-S. Ko, J. J. La Clair and M. D. Burkart, *J. Assoc. Lab. Autom.*, 2008, **13**, 284.
19 P. Jankowski, D. Ogończyk, W. Lisowski and P. Garstecki, *Lab Chip*, 2012, DOI: 10.1039/C2LC21297H.
20 C. Mateo, V. Grazu, J. M. Palomo, F. Lopez-Gallego, R. Fernandez-Lafuente and J. M. Guisan, *Nat. Protoc.*, 2007, **2**, 1022.
21 K. M. Holtz, B. Stec and E. R. Kantrowitz, *J. Biol. Chem.*, 1999, **274**, 8351.
22 N. Nakajima and Y. Ikada, *Bioconjugate Chem.*, 1995, **6**, 123.
23 L. S. Wong, F. Khan and J. Micklefield, *Chem. Rev.*, 2009, **109**, 4025.
24 G. K. Robinson, *Biochem. Educ.*, 1997, **25**, 232.
25 M. Bertau and P. Fröhlich, *Chem. Ing. Tech.*, 2010, **82**, 51.
26 B. Rozum, K. Gajownik, Ł. Tymecki and R. Koncki, *Anal. Biochem.*, 2010, **400**, 151.