Available online at www.sciencedirect.com
ScienceDirect
Catalysis Today 120 (2007) 30–34
CATALYSIS TODAY
www.elsevier.com/locate/cattod

Activity and lifetime of organophosphorous hydrolase (OPH) immobilized using layer-by-layer nano self-assembly on silicon microchannels

Scott R. Forrest, Bill B. Elmore, James D. Palmer *

Louisiana Tech University, Chemical Engineering Program, 600 W. Arizona, P.O. Box 10348 TS, Ruston, LA 71272, United States

Available online 7 September 2006

Abstract

Organophosphorous hydrolase (OPH) enzyme has been immobilized and layered onto the walls of silicon microchannels manufactured at Louisiana Tech University. Enzyme immobilization was performed using layer-by-layer (LbL) nano self-assembly. The polycation used was poly(ethylenimine) (PEI). The polyanion used was poly(styrenesulfonate) (PSS). Enzyme microreactors were constructed with one and two layers of enzyme to compare activity performance.

The substrate utilized for this study was methyl-parathion (MPT) which is hydrolyzed by the OPH enzyme to *para*-nitrophenol (PNP). Reactor performance was characterized by feeding 100 μM MPT through the microreactors at a variety of flow-rates and measuring the concentration of PNP in the effluent using a UV/vis spectrometer. The reactor conversion versus residence time was used to determine the observed first order rate kinetics. The first order rate constant for the two bilayers of enzyme was double that observed for the single layer architecture, however the activity of both architectures dropped substantially over a period of 2 days. Microreactors with two channel dimensions were tested, one with 98 parallel channels 60 μm wide and another with 1000 parallel channels 5 μm wide. The microreactor with the smaller channel width demonstrated superior performance that was proportional to the increase in available surface area.

Keywords: Organophosphorous hydrolase; LbL; MPT

1. Introduction

Enzymes immobilized in silicon microchannels have potential for applications in sensors due to the small size and low liquid holdup in the system which increases the response time of the sensor. In addition, fundamental studies of the enzyme/fluid interaction can be facilitated by the well-characterized laminar flow inside the microchannel. Many researchers have studied the incorporation of enzymes into microchannels. Researchers at Pacific Northwest National Laboratory have covalently linked the *organophosphorous hydrolase* (OPH) enzyme in silica using amino-derivitized self-assembled monolayers [1]. Other researchers have demonstrated that the OPH enzyme can be entrapped using casting in a Nafion solution [2]. A similar technique was demonstrated for the *urease* enzyme in polydimethylsiloxane (PDMS) [3].

Layer-by-layer nano self-assembly (LbL) utilizes the interaction of oppositely charged molecules to create ultra-thin, self-limiting films of a material. Multiple layers can be produced by sequential exposure of the surface to complimentary charged polymers, proteins, nanoparticles, etc. [4–10]. LbL self-assembly is a method that has been demonstrated in the literature as an effective means for the immobilization of enzymes. The technique is simple, inexpensive, and applicable to conformal surfaces. The immobilization of *glucose oxidase*, *horseradish peroxidase*, *urease*, and OPH using the LbL technique have been demonstrated previously in the literature [11–17].

The authors have previously reported on the immobilization of *urease* in a microchannel reactor using LbL nano self-assembly [18]. This study extends that work by utilizing the OPH enzyme. The difference is important for two reasons. First, the isoelectric points of the two enzymes are different. *Urease* has an isoelectric point of 5 versus 8.3 for OPH. Second, the product of the *urease* reaction produces ammonia which can create product inhibition, an issue avoided with the OPH enzyme and hydrolysis of methyl-parathion.

* Corresponding author. Tel.: +1 318 257 2885; fax: +1 318 257 2562.
E-mail addresses: sforrest@latech.edu (S.R. Forrest), jpalmer@latech.edu (J.D. Palmer).

0920-5861/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.cattod.2006.07.037

2. Experimental

The silicon microchannels were produced at Louisiana Tech using standard photolithography and inductively coupled plasma (ICP) etching. ICP etching has the advantage of producing high aspect ratio vertical sidewalls as compared to wet etching techniques. Fig. 1 is an SEM photo showing the inlet and the microchannel sidewalls of the manifold area of a microreactor used in this study. Microreactors with two different dimensions were fabricated and tested for this study. The first microreactor had 98 parallel channels with dimensions of 2.7 cm, 60 μm and 65 μm in length, width and depth, respectively. The second microreactor had 1000 parallel channels with dimensions of 2.7 cm, 5 μm and 50 μm in length, width and depth, respectively. The manifold of the entrance and exit region was 0.25 cm in length for both designs. Each microfluidic device was mounted in a pyrex holder that sealed the top of the channels and allowed an interface with the 1/8 in. tubing which connected the inlet to a syringe pump and the outlet to the sample collection vessel.

The methyl-parathion (MPT, neat, 99.5% purity), methanol, Trizma–hydrochloride (Tris–HCl), Tris[hydroxyl methylaminomethane] (Tris–base), and polyelectrolytes were all obtained from Sigma–Aldrich and used without further purification. Type 1 Reagent Grade Water (RGW) obtained using a Barnstead Series 1090 E-Pure reverse osmosis purifier was utilized for all experiments. The polycation utilized was polyethylenimine (PEI) with molecular weight of 25k. The polyanion utilized was polystyrenesulfonate (PSS) with molecular weight of 70k. *Organophosphorous hydrolase* (OPH) was obtained from Dr. Vipin Rastogi of the US Army Edgewood Chemical-Biological Center.

The low solubility of methyl-parathion in an aqueous environment required the addition of methanol to the feed solution. The feed solution was 20% (w/v) methanol in water with 100 μM MPT, 116 mM Tris–HCl, and 384 mM of Tris–base. The concentrations of Tris–HCl and Tris–base were chosen to produce a pH of 8.6.

The literature suggests that the optimum pH for OPH activity is approximately 8.6. The isoelectric point of OPH is 8.3. Therefore, all layering and the feed solution was buffered to a pH of 8.6 and the complimentary polyelectrolyte for OPH was chosen to be PEI with an isoelectric point of 11. Previous experiments have demonstrated that a small number of precursor layers on a silicon surface will enhance the performance of the immobilized enzyme, and were therefore employed in this study with bilayers of PEI and PSS. The PSS, PEI, and OPH solutions for layering were 2 g/l with Tris–HCl and Tris–base sufficient to produce a pH of 8.6. The layering solutions were pumped through the microchannels at a rate of 0.001 ml/min. The polyelectrolyte solutions were fed for a period of 10 min through the microchannels while the OPH solution was fed for 20 min. The layering time for the enzyme was increased to compensate for the much smaller difference in the OPH's isoelectric point versus the deposition pH.

Fig. 2 depicts the experimental apparatus in operation. The feed solutions were metered using a 74900 Series syringe pump from Cole-Parmer. The effluent was collected and transferred to a quartz micro-cuvette for analysis on an Ocean Optics SD-2000 UV–vis Spectrometer with an AIS Model Mini-DTA UV–vis light source. The absorbance of the sample was measured at 400 nm and compared with a standard curve to determine the concentration of *para*-nitrophenol (PNP). The reactor performance was quantified by adjusting the feed

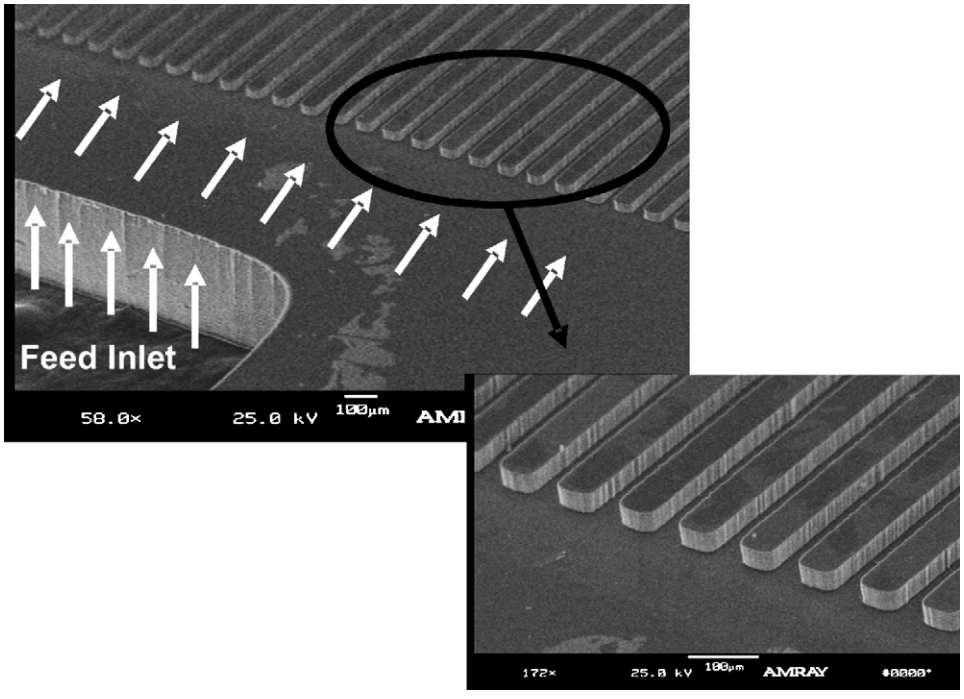

Fig. 1. SEM photo of inlet and manifold of silicon microreactor.

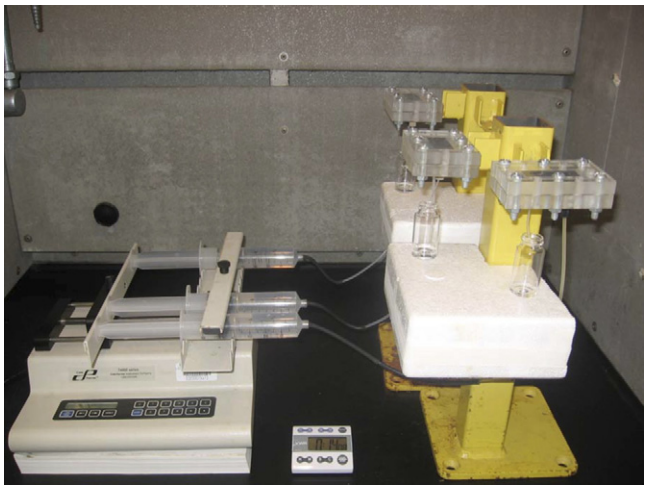

Fig. 2. Experimental setup with multiple reactors.

flow-rate, waiting an equivalent of three residence times through the system, and measuring conversion of substrate in the effluent using the spectrometer. Enough kinetic data was obtained each day to perform a first-order rate analysis using the integral method.

3. Results

A previous study in which *urease* was immobilized in a microreactor using LbL nano self-assembly by the authors indicated that the polycation PEI results in a much higher enzyme activity than immobilization of enzyme using PDDA [18]. Initial screening experiments with the limited amount of OPH available provided a similar trend. Therefore, PEI was used as the polycation for all of the architectures presented in this paper. In addition, a precursor layer of PEI and PSS was deposited onto the silicon surface before the subsequent PEI/enzyme layers for all the experiments in this study because of the benefits noted in the prior work. A single layer of immobilized OPH enzyme was compared with an architecture containing two layers of immobilized enzyme. Both architectures were layered in the microreactor consisting of 98 parallel channels, 60 μm wide, 65 μm deep, and 2.7 cm long. Fig. 3 depicts the difference in

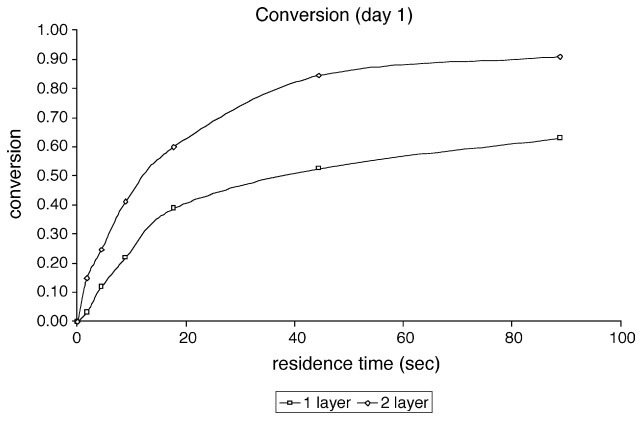

Fig. 3. Comparison of conversion rates for single and double layer reactors.

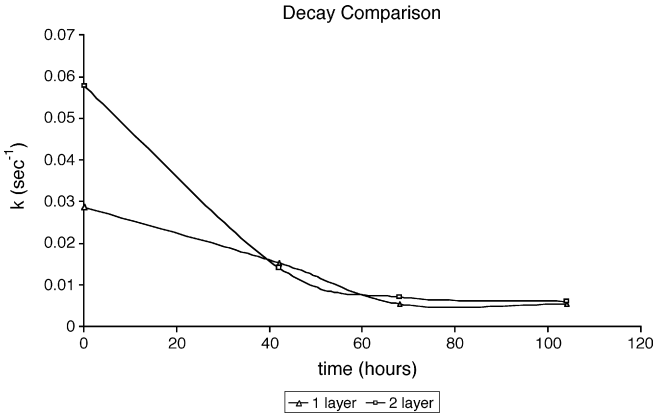

Fig. 4. Comparison of decay rate of single and double layer reactors.

activity of the single and two layer architectures on the first day of testing, and Fig. 4 depicts the difference in first order rate constant over time for the two systems. The performance increase in the first day appeared to scale linearly with the amount of layers present. On the first day, a 44 second residence time in the microreactor resulted in a 85% conversion for the two layer architecture and a 53% conversion for the single layer architecture. The day one first order rate constant for the dual layer architecture was 0.058 s^{-1} versus 0.029 s^{-1} for the single layer architecture. Interestingly, the day one performance of the single layer of OPH immobilized using this technique was similar to that obtained using an identical architecture with *urease*. An attempt was conducted to quantify the OPH immobilized in the microreactor by feeding a 1 M solution of NaCl and measuring the effluent for protein using the Lowry method. Unfortunately, the protein in solution appeared to be below the measurable level of the instrument.

The enzyme performance was measured immediately after the immobilization was conducted, and at three subsequent intervals (42, 68 and 104 h from the initial layering time). The feed solution was continuously pumped through the channels for the entire study to determine the stability of the layers. A decrease in performance was observed at the 42 h interval for both the single and two layer architectures. Indeed, the performance of the two layer architecture was dramatic and dropped to the level of the single layer architecture by the 42 h interval. The two layer architecture remained equivalent to the single layer for the subsequent intervals examined. One possible reason for the dramatic decrease in activity could be physical detachment and loss of the enzyme from the surface of the microchannel. However, this hypothesis appears unlikely as a number of the effluent samples were monitored over time and no samples exhibited any measurable increase in substrate conversion. This phenomenon should have been most clearly evident with the high feed flows which generated the highest internal shear forces and lowest initial conversion of substrate in the effluent, but as stated above no additional conversion was identified as the samples were monitored over time. The authors believe one contributor to the high decrease in enzyme activity over time for the OPH versus *urease* enzyme was the use of methanol in the feed solution for OPH. Methanol was required

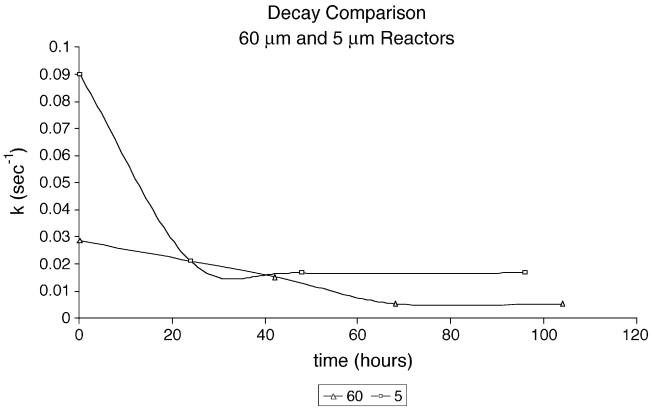

Fig. 5. Comparison of decay rate of 60 and 5 μm reactors.

to dissolve the desired concentration of MPT but could have denatured the OPH enzyme through continuous exposure in the experiment.

An experiment was conducted to determine if smaller channel widths would substantially increase the performance of the reactor due to the reduced diffusion distances between the catalytic surface and the bulk concentration. A microreactor with 1000 parallel channels, 5 μm wide, 50 μm deep, and 2.7 cm long was tested and compared to the results above using a microreactor with 60 μm wide channels. Fig. 5 depicts the first order rate constant obtained for the two reactor geometries versus hours of operation. The initial activity of the reactor with 5 μm channels exhibited a three-fold higher rate constant than the reactor with 60 μm wide channels. The activity of both reactors decayed substantially over the course of the 100 h in which the systems were monitored. Regardless, the reactor with 5 μm channels did retain a three- to four-fold higher activity than the reactor with 60 μm channels at the end of the experiment as the performance of the latter system declined at 68+ hours. The available surface area of each reactor was calculated and compared to determine if the activity performance increase was greater proportion of available enzyme. Using the area from both the channels and the manifolds, the available area of the reactor with 5 μm channels was 31 cm^2 while the available area of the reactor with 60 μm channels was 8.6 cm^2. Therefore, the ratio of surface area of the reactor with 5 μm channels was 3.7 times higher than that of the reactor with 60 μm channels which was similar to the level of increase in catalytic activity that was measured.

4. Conclusions

The OPH enzyme was successfully immobilized in microreactors using LbL nano self-assembly. An experiment with two layers of enzyme produced the expected result of doubling the first order rate constant versus a reactor with only a single layer for the initial day of operation. The activity decreased substantially over a period of 42 h and remained at relatively low levels over the rest of the 100 h of the tests. The rate of decay was not expected but may have been exacerbated from the use of methanol in the feed solution. The decay rate of free enzyme in an aqueous and 20% methanol solution should be measured to determine the extent of which methanol denatures OPH enzyme. A protein method with a higher sensitivity than the Lowry assay is being explored to quantify the protein immobilized in each layer. The reactor with 5 μm channels did exhibit a higher activity than the reactor with 60 μm channels, but the reactor performance was not higher than the expected proportion in exposed area. Therefore, the smaller channels did not exhibit process intensification for this particular system. The rate of reaction at the surface was likely not sufficient to deplete the concentration of substrate close to the surface. Indeed, the calculated mass transfer coefficient for the 5 μm channel is an order of magnitude higher than that of the 60 μm channel, but the surface reaction rate must be sufficiently high to ensure that the mass transfer is either the controlling resistance or a large contributor before this phenomenon will be evident.

Acknowledgements

The authors would like to thank Dr. Vipin Rastogi of the US Army Edgewood Chemical-Biological Center for providing the OPH enzyme that made this study possible. The authors would also like to thank Dr. Yuri Lvov at Louisiana Tech's Institute for Micromanufacturing for his valuable insight into the layer-by-layer self-assembly process.

References

[1] PNNL enzyme microreactor research. http://www.pnl.gov/microcats/aboutus/research/enzymaticr.html.
[2] A. Mulchandani, P. Mulchandani, W. Chen, J. Wang, L. Chen, Amperometric thick-film strip electrodes for monitoring organophosphate nerve agents based on immobilized organophosphorous hydrolase, Anal. Chem. 71 (1999) 2246–2249.
[3] B. Elmore, Z. Lu, F. Jones, The development of a novel micro-scale system as an immobilized enzyme bioreactor, Appl. Biochem. Biotechnol. (2002) 98–100.
[4] R. Iyer, Multilayers of colloidal particles, J. Colloid Interf. Sci. 21 (1966) 569.
[5] G. Decher, F. Essler, J.D. Hong, K. Lowack, J. Schmitt, Y. Lvov, Layer-by-layer adsorbed films of polyelectrolytes, proteins or DNS, Polym. Prep. Am. Chem. Soc. Polym. Chem. Div. 34 (1) (1993) 745.
[6] D. Hong, K. Lowack, J. Schmitt, G. Decher, Layer-by-layer deposited multilayer assemblies of polyelectrolytes and proteins: from ultrathin films to protein arrays, Prog. Colloid Polym. Sci. 93 (1993) 98–102.
[7] G. Sukhorukov, E. Donath, H. Lichtenfeld, E. Knippel, M. Knippel, A. Buddle, M. Möhwald, Layer-by-layer self-assembly of polyelectrolytes on colloidal particles, Colloids Surf. A: Physicochem. Eng. Aspects 137 (1998) 253.
[8] Y. Lvov, G. Decher, H. Möhwald, Assembly, structural characterization and thermal behavior of layer-by-layer deposited ultrathin films of poly(finylsulfate) and poly(allylamine), Langmuir 9 (1993) 481–486.
[9] D. Feldheim, K. Grabar, M. Natan, T. Mallouk, Electron transfer in self-assembled inorganic polyelectrolyte/metal nanoparticle heterostructures, J. Am. Chem. Soc. 118 (1996) 7640.
[10] D. Yoo, A. Wu, J. Lee, M. Rubner, New electro-active self-assembled multilayer thin films based on alternately absorbed layers of polyelectrolytes and functional dye molecules, Synth. Met. 85 (1997) 1425–1426.

[11] J. Hodak, R. Etchenique, E. Calvo, Layer-by-layer self-assembly of glucose oxidase with a poly(allylamine)ferrocene redox mediator, Langmuir 13 (1997) 2708–2716.
[12] S. Rao, K. Anderson, L. Bachas, Controlled layer-by-layer immobilization of horseradish peroxidase, Biotechnol. Bioeng. 65 (4) (1999).
[13] X. Yan, X. Shi, K. Hill, H. Ji, Microcantilevers modified by horseradish peroxidase intercalated nano-assembly for hydrogen peroxide detection, Anal. Sci. 22 (2006) 205–208.
[14] F. Caruso, C. Schuler, Enzyme multilayers on colloid particles: assembly, stability, and enzymatic activity, Langmuir 16 (2000) 9595–9603.
[15] Y. Lvov, A. Antipov, A. Mamedov, H. Möhwald, G. Sukhorukov, Urease encapsulation in nanoorganized microshells, American Chemical Society, Nano Lett. 1 (2001) 125–128.
[16] Y. Lvov, F. Caruso, Biocolloids with ordered urease multilayer shells as enzymatic reactors, Anal. Chem. 73 (2001) 4212–4217.
[17] Y. Lee, I. Stanish, V. Rastogi, T. Cheng, A. Singh, Sustained enzyme activity of organophosphorus hydrolase in polymer encased multilayer assemblies, Langmuir 19 (2003) 1330–1336.
[18] S. Forrest, B. Elmore, J. Palmer, Appl. Biochem. Biotechnol. 121 (2005) 85–92.