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Development of Novel Microscale System as Immobilized Enzyme Bioreactor

Francis Jones,[1] Zonghuan Lu,[2] and Bill B. Elmore*,[2]

[1]Chemical and Environmental Engineering, The University of Tennessee at Chattanooga, Chattanooga, TN 37403-2598; and [2]Department of Chemical Engineering, Louisiana Tech University, Ruston, LA 71272, E-mail: belmore@coes.latech.edu

Abstract

This study involves a novel method for immobilized enzyme catalysis. The focus of the work was to design and construct a microscale bioreactor using microfabrication techniques traditionally employed within the semiconductor industry. Enzymes have been immobilized on the microreactor walls by incorporating them directly into the wall material. Fabricated microchannels have cross-sectional dimensions on the order of hundreds of micrometers, constructed using polydimethylsiloxane cast on silicon/SU-8 molds. The resulting ratio of high surface area to volume creates an efficient, continuous-flow reaction system. Transverse features also containing enzymes were molded directly into the channels.

Index entries: Immobilized enzymes; microscale bioreactor; polydimethylsiloxane microreactor.

Introduction

Science and technology are undergoing sweeping changes through the fast-paced revolution in miniaturization technologies. Rapid development of microelectromechanical systems (MEMSs) and related technologies are changing both our understanding and our modes of interacting with the world around us. In the last few years, the MEMS field has incorporated biologic aspects into design criteria to establish the emerging area of BioMEMS. A broad range of miniaturized devices is being developed including biologic and chemical sensors, microscale pumps, valves, chemical separators, and reactors *(1)*. Miniature chemical process devices offer

*Author to whom all correspondence and reprint requests should be addressed.

several advantages. With fluidic conduits or channels having very small cross dimensions (usually on the order of tens to hundreds of micrometers), the relatively large ratios of surface area to volume facilitate rapid heat and mass transfer. In addition, for small-scale reactors, a more efficient use of catalyst with improved yield and selectivity is possible *(2,3)*. Microscale devices also provide an excellent setting for membrane reactors in which a rapid removal of reaction-inhibiting products can significantly boost product yields *(4,5)*.

Traditionally, microdevices are fabricated from silicon using the techniques developed in the microelectronics industry. Silicon features as small as 1 μm can be fashioned very precisely employing these techniques. These devices are robust at high operating temperatures and in other challenging environments. However, working with silicon wafers is expensive and difficult. Alternative materials are being sought for specialized applications *(6)*.

Biologic reactions using enzymes as catalysts typically do not require high temperatures or elevated pressures. Therefore, plastics may potentially be used as reactor fabrication material. The great advantages of enzyme technology are the selectivity of reaction products and simpler reactive systems with lower energy requirements in comparison to traditional industrial operations *(7)*.

Biologic chemicals require enzymes to act as catalysts for liquid-solid heterogeneous reactions. Traditional bioreactor design requires these enzymes to be fixed onto a substrate. Enzyme immobilization onto an insoluble matrix was first demonstrated by Grubhofer and Schleith in 1954 *(8)*. Well-documented potential exists for the deployment of immobilized enzyme systems in the pharmaceutical, biomedical, and chemical industries as well as in analytical chemistry *(9)*. For example, urease immobilized on tubes and in membranes has been used in artificial kidney devices *(10)*. Enzymatic methods are also used in the modification of porcine insulin and in the production of aspartame *(9)*. Recently, researchers have used catalase entrapped in gels to treat wastewater containing hydrogen peroxide as an example of the growing effort to develop biodegradation processes for industrial pollutants *(11)*.

More recently, enzymes have been incorporated directly into carbon-based plastics such as polymethylmethacrylate to create biocatalytic resins *(12)*. The enzymes entrapped within these resins often maintain an appreciable fraction of their activity in comparison to a free-enzyme solution. The porous nature of such plastics can make interior enzymes available for reaction along with those at the surface. Equivalent studies are not available for enzymes in silicon plastics.

The main objective of the present study was to design and construct a novel immobilized enzyme bioreactor with microscale channels and packing elements fabricated by molding polydimethylsiloxane (PDMS). Our first enzyme selection, urease, was incorporated directly into the PDMS matrix, so that all surfaces in the reactor channels are catalytically active.

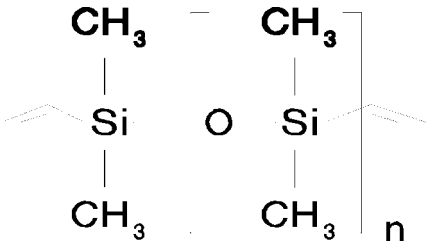

Fig. 1. Base unit of PDMS used in preparing immobilized enzyme microreactors.

PDMS is a particularly good material for micromolding because it is strong, flexible, and capable of maintaining precise, small features *(6)*. Although incorporation of the enzymes into the plastic matrix does change the material properties of PDMS, a robust bioplastic is obtained.

Materials and Methods

Enzymes and Equipment

Our study involves work with a variety of enzymes. Here we report results with urease (EC 3.5.1.5 Type IX; Sigma-Aldrich; from Jack Beans, ~60,000 U/g of solid). Preliminary results with amyloglucosidase (EC 3.2.1.3; Sigma-Aldrich, from Rhizopus mold, ~23,000 U/g of solid) are briefly mentioned.

Batch studies were conducted in 250-mL shake flasks in an environmentally controlled shaker/incubator. Continuous studies were performed in specially prepared microreactors molded from PDMS (Sylgard™ 84 silicone elastomer; Dow Corning) poured onto silicon wafer molds. The microreactor molds were prepared using 4-in. silicon wafers of Type P, crystal orientation <1-0-0>, 1 to 2 Ω resistivity, and 457–575 μm thickness from Silicon Quest, Santa Clara, CA.

SU-8, also used in the microreactor mold process, is a highly branched polymeric epoxy resin used as a negative photoresist. The SU-8 used in this work was obtained from Microchem.

Scanning electron microscopic images were taken by an AMRAY 1800 series scanning electron microscope having a resolution of 0.2 μm. All objects in this work were first sputtered with a nickel layer of a few nanometers thick in order to obtain an image.

Preparation of Biomaterial

Figure 1 illustrates the PDMS base formula. PDMS is supplied in two parts: a monomeric base and a "catalytic activator" or curing agent. Ten parts base are mixed with one part curing agent and allowed to cure at room temperature until hardened. The "bioplastic" was prepared with a mixture of enzyme and PDMS. Figure 2 illustrates the bioplastic matrix. After the completion of the enzyme/PDMS preparation, air bubbles were removed by placing the material in a vacuum chamber at about 100 mmHg for 20–30 min. Curing times of 3–5 d at room temperature were required.

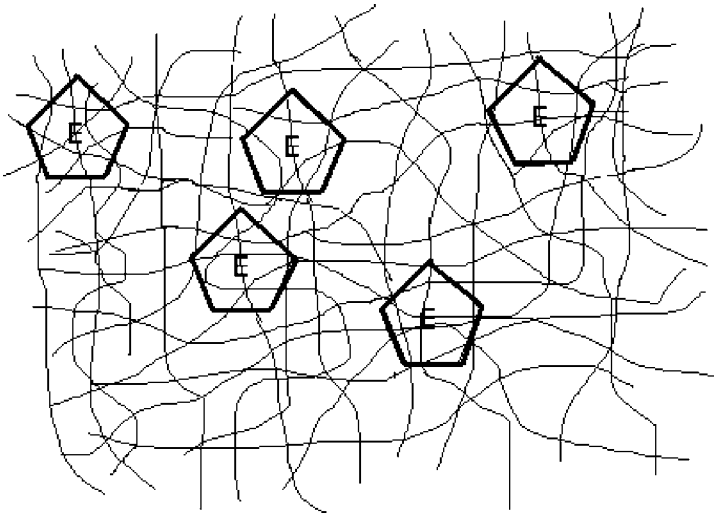

Fig. 2. Enzymes are immobilized in the PDMS crosslinked matrix by entrapment.

The bioplastic was cured at room temperature to maintain the viability of the enzyme preparation.

When urease was added to PDMS in fractions of approx 0.5–2.5 wt%, a clear, flexible material resulted, suitable for molding into a microreactor structure. Higher fractions of enzyme resulted in a yellow-brown bioplastic with little tensile or shear strength—likely a result of enzyme molecule interference with polymer crosslinking during the curing process. However, higher concentrations of enzymes in such polymers may be useful for "nonstructural" preparations to be used in batch reactors *(12)*.

Batch Studies

To assess the enzymatic activity of the polymer-enzyme complex, such nonstructural PDMS preparations with various enzyme fractions were prepared and cured in glass Petri dishes. On curing, these preparations were removed and cut into cubes nominally 3 mm on a side. Equal weights of these cubes (~10 g per reactor) were placed in 250-mL shake flasks. A 150-mL preparation of 0.1 mol/L of THAM (Tris[hydroxymethyl]aminomethane) buffer solution containing 0.1 mol/L of urea was placed in each of three flasks. The pH of the buffer/urea medium was adjusted to 7.5 by adding of HCl. Shake flasks were placed on a shaker incubator at 25°C and 200 rpm. Sample volumes of 2 mL were removed periodically for ammonia analysis.

Microreactor Fabrication

The biologic microreactors were fabricated using molds created by standard photolithographic techniques. The reactor design was first sketched as a "mask pattern" using specialized design software. When printed onto a transparency, this mask pattern was transferred via ultra-

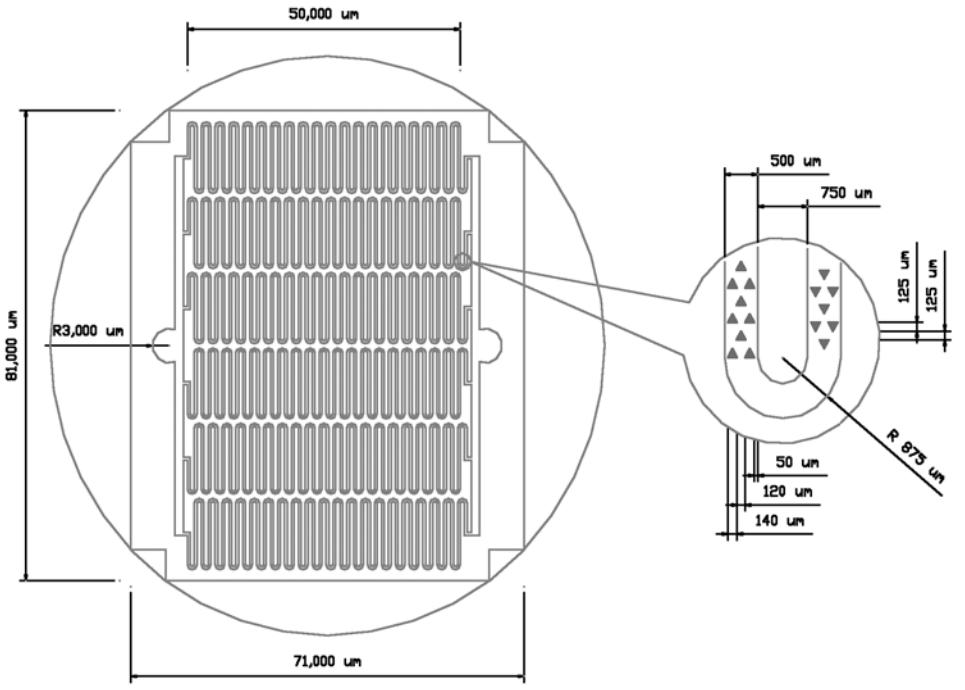

Fig. 3. L-edit microreactor design for photolithographic mask.

violet (UV) light exposure onto a layer of SU-8 photoresist spun-coated on a silicon wafer. By chemically etching the SU-8, a negative pattern of the microreactor channels was prepared having precise geometry and dimensions in relation to the mask design. These steps are presented in detail next.

Mask patterning

The mask pattern for the photolithographic process was drawn using integrated circuit design software (L-Edit version 5.17). Figure 3 presents details of the mask design. The mask was then transferred to a silicon wafer spun-coated with SU-8 photoresist. The mask was drawn with an internal unit equivalent of 1 μm and printed to a file in an encapsulated postscript format with a print ratio of 356:1. This ensured that the mask had the same dimensions as the actual design. The mask design was then transferred to a transparency sheet via a high-resolution printer.

Procedure for Micromold Fabrication

Preparation of Wafer

A <1-0-0> silicon wafer was first rinsed with deionized water, acetone, then isopropyl alcohol and blown dry with antistatic nitrogen. After drying, the wafer was placed on a hot plate at 250°C for 30 min for dehydration (hard baking) followed by cooling to room temperature.

Coating and Curing of SU-8 Photoresist

The prepared wafer was mounted on a spinning coater. About 5 mL of SU-8 was applied to the wafer and distributed by two spinning cycles of 60 s at 500 rpm each. The result was a coating thickness of about 200 μm. The wafer was baked for 45 min at 65°C in an oven to cure the SU-8 photoresist layer.

Mask Pattern Transfer

The SU-8-coated silicon wafer was mounted on a mask aligner, covered with the microreactor mask, and exposed to broadband (wavelength of 460 nm) UV light at an exposure rate of 24 mW/cm^2 for 60 s. The wafer was then baked for 15 min at 75°C.

Developing process for SU-8 Photoresist

After the pattern transfer, the wafer was placed in liquid SU-8 developer for 10 min. Then the SU-8 mold was rinsed in deionized water to remove the remaining SU-8 developer and blown dry with antistatic nitrogen.

Figure 4 summarizes the steps for microreactor mold preparation. Figures 5 and 6 show details of the micromold in scanning electron microscopic images. Parts of two neighboring channels are shown in Fig. 5. Triangular features are designed within the channels to promote mixing analogous to a structured "packing" used in macroscale system fluidic systems. Each triangular packing feature is an equilateral triangle 125 μm on a side and 125 μm in height as shown in Fig. 7. The reactor contains six channels, each 500 μm wide connected by manifolds at entry and exit. The triangular transverse "packing" features are separated by 125 μm both laterally and along the channel length. The overall footprint of the device is about 6.5 × 8 cm.

Bioreactor Molding and Setup Procedure

To prepare the microreactor, the PDMS/enzyme preparation was poured onto the mold in a layer about 5–8 mm thick. This was allowed to cure for 3–5 d until hardened. The reactor was then detached from the mold and placed into use. Figure 8 shows a bioreactor molded with PDMS containing 1.7 wt% urease. Figures 9 and 10 provide details of the channels. The direction of flow is from right to left in Fig. 10. The reactor channels were closed by compression between two acrylic plates—the top plate fitted with inlet and outlet ports for introducing fluid to the microreactor system.

Reaction System

For the urease enzyme system, a reactant solution of 0.1 mol/L of urea was fed to the microreactor by a peristaltic pump. Urease converts urea to ammonia and CO_2 by the following reaction:

$$NH_2CONH_2 + H_2O \rightarrow 2\ NH_3 + CO_2$$

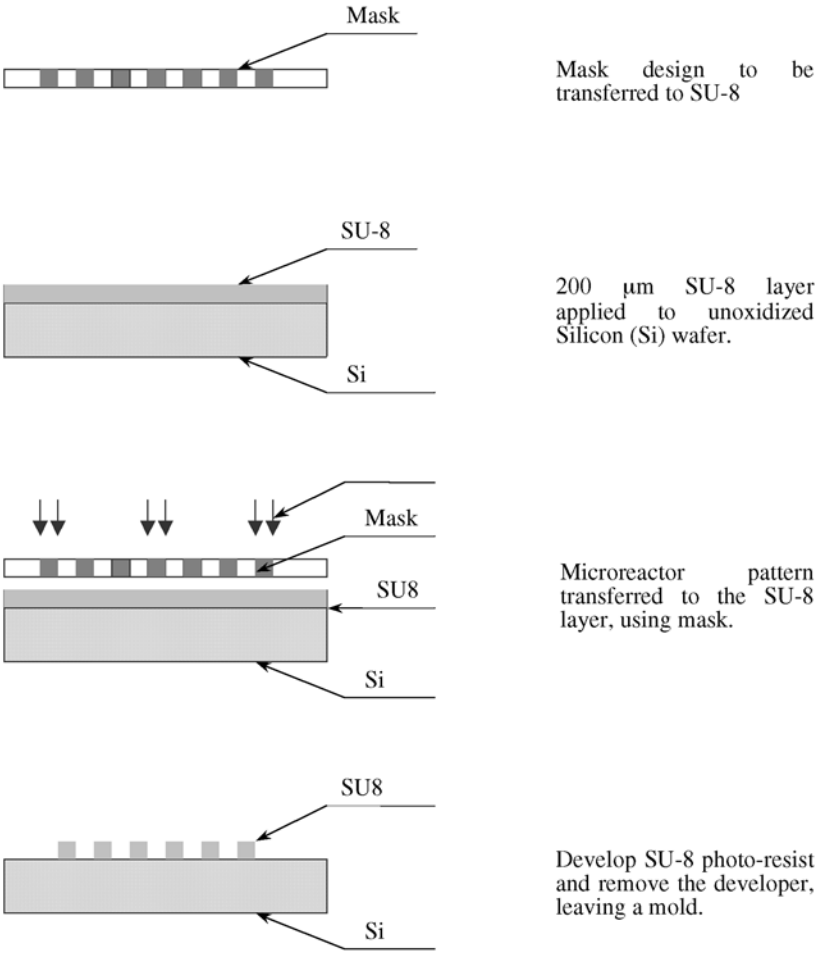

Fig. 4. Procedure for microreactor mold fabrication.

Reactor effluent was analyzed by an Orion 95-12 ammonia probe and a Fisher ph/mV meter. Variations in urea conversion were recorded as a function of flow rate for future modeling studies.

Results and Discussion

Batch Studies

Silicon rubbers have been found to absorb a maximum of 0.07 wt% water, which is thought to be owing to the presence of impurities *(13)*. Batch studies revealed that the presence of enzyme in the PDMS resulted in significant water adsorption. PDMS containing 1.7 wt% urease absorbed >4 wt% water after 6 h of exposure. It is expected that the added water absorption allowed enzymes within the interior of the polymer matrix to become available for reaction (along with surface enzymes). Reactant

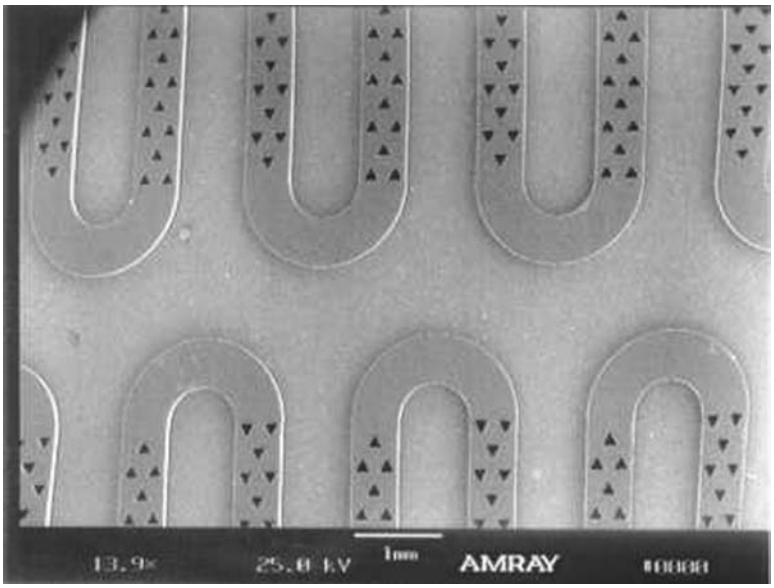

Fig. 5. Details of two channels of microreactor mold.

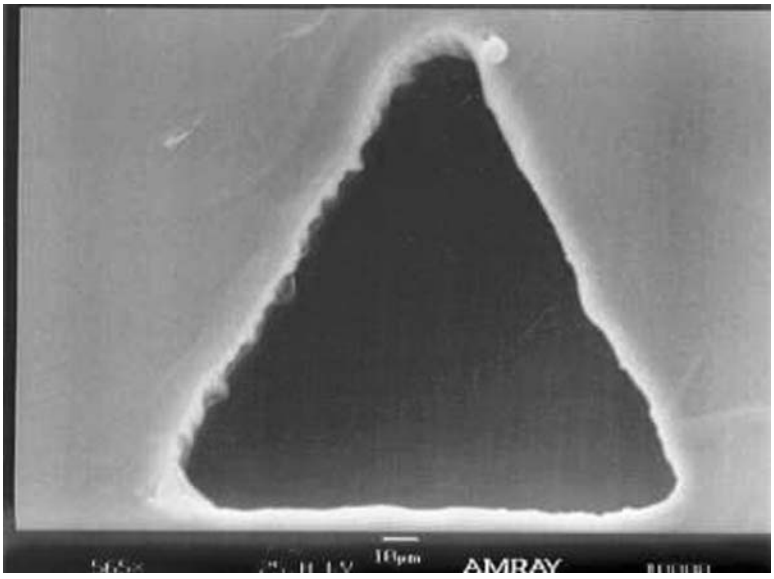

Fig. 6. Single triangular feature of microreactor mold.

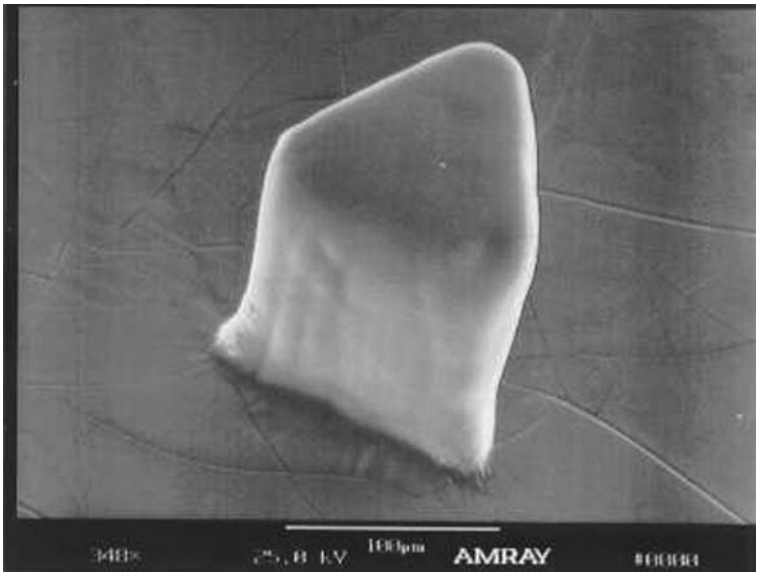

Fig. 7. Single triangular "mixing" feature of PDMS microreactor.

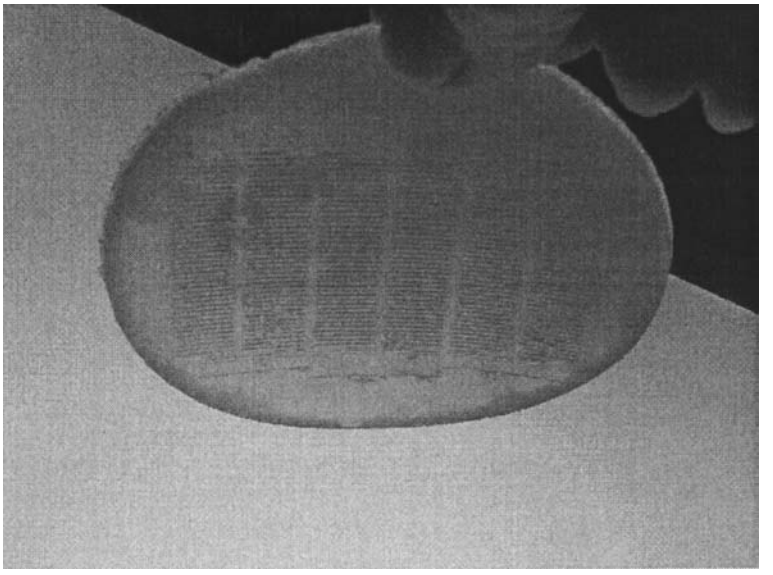

Fig. 8. Photograph of bioreactor molded with PDMS containing 1.7 wt% urease.

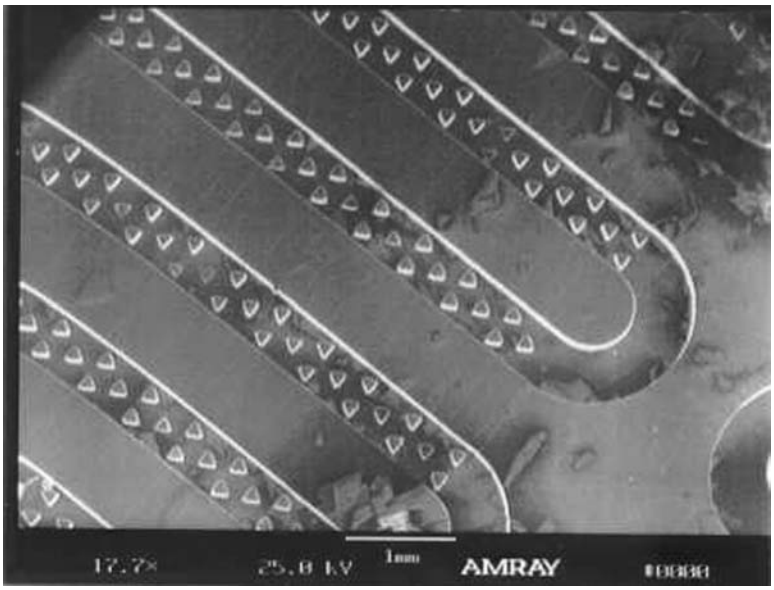

Fig. 9. Scanning electron microscopic image of a channel detail of bioreactor.

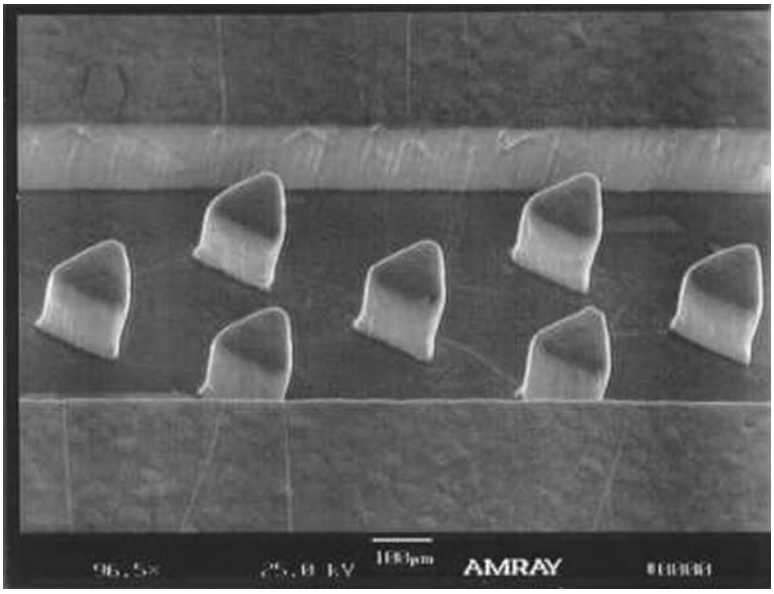

Fig. 10. Close-up of PDMS microreactor channel with transverse mixing features. Process fluid flow is from right to left.

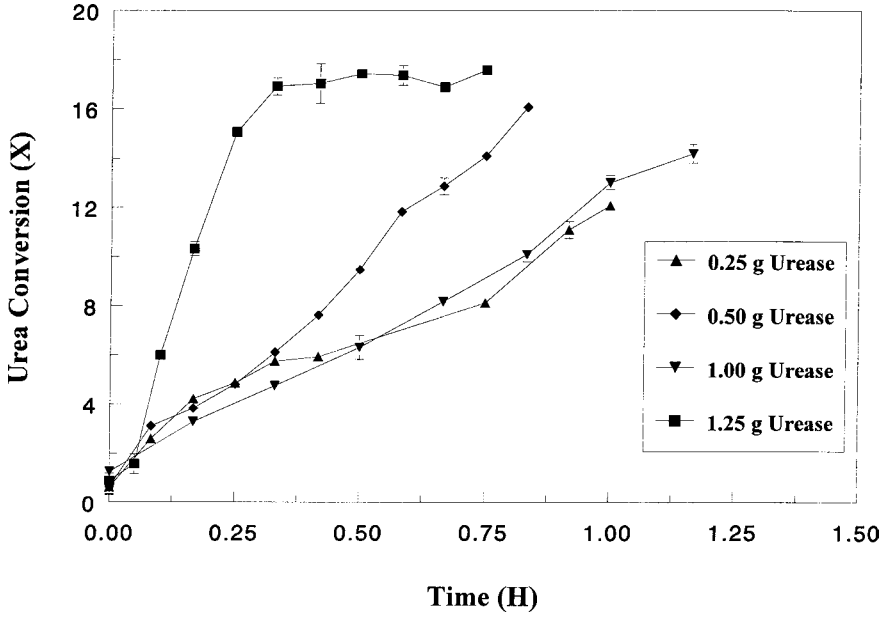

Fig. 11. Batch reactor results with urease in PDMS.

molecules could migrate to interior enzymes along water pathways much like gas-reactant molecules in traditional packed reactors migrate into the pores of the reactive porous packing material. However, additional analysis of our biomaterial is necessary to validate this possibility.

Figure 11 presents the results of experiments using bioplastic cubes in batch reactors. PDMS preparations were made containing 0.25, 0.5, 1.0, and 1.25 wt% urease. All PDMS-enzyme preparations were washed with 3 to 25 mL aliquots of deionized water before adding urea solution to remove any unattached enzyme present. All data points in Fig. 11 are averages of samples from three identical batch reactors. Error bars show one standard deviation from the mean. In general, conversions increase with increased enzyme content. With 1.25 wt% enzyme, conversion leveled off at about 17% after 20 min. This is likely a result of ammonia inhibition of the urease enzyme. A strong odor of ammonia product was present for batch reactors with higher enzyme concentrations. After about 1 h, the initial solution was removed, the polymer-enzyme resins were rinsed with deionized water (as done initially), and the experiments were repeated with fresh urea medium. Lower conversions were observed for the second run (data not shown).

A series of batch studies has been partially completed using amyloglucosidase with PDMS to convert starch. Similar success has been seen (data not shown) with this bioplastic material in converting the starch to glucose, thus demonstrating the "proof of concept" for other enzymes, as well.

Continuous Studies

To test the application of our bioplastic material as a structural material, a continuous-flow microreactor was fed the same urea solution (0.1 mol/L) as in the batch experiments. The PDMS microreactor material contained 1.4 wt% urease. Figure 12 presents the data for this system. The reactor was operated for approx 1 h prior to recording the data shown in Fig. 12 to eliminate possible effects of unattached enzymes potentially present within the microchannels (analogous to washing the 3-mm cubes in the batch experiments). Indeed, on start-up, a urea conversion of >50% was observed for a short period. After 1 h of operation, urea conversions ranged from 1.75% for a volumetric flow rate of 2.13 cm^3/min to 17.8% for a volumetric flow rate of 0.064 cm^3/min. These conversion values were reproducible over time as the flow rate was varied repeatedly from high to low.

The reactor volume was calculated to be 0.18 cm^3, giving a residence time, for a flow rate of 0.064 cm^3/min, of approx 2.81 min. The PDMS-enzyme resin in the batch systems had a nominal surface area of 200 cm^2 compared with a calculated surface area for the continuous reactor of 21 cm^2. The continuous microreactor performance compares quite favorably with batch reactor results (detailed comparisons are to be presented in the future).

Our microreactor design shown in Fig. 7 had several advantages over previous PDMS reactor designs attempting to attach enzymes to the PDMS microchannel walls by a covalent bonding procedure. In our previous work *(14)*, we achieved relatively low urea conversions. Our initial microreactor prototype consisted of channel diameters of 150 μm with no internal transverse mixing elements. Conversions using this latest microreactor configuration resulted in urea conversions an order of magnitude higher than our previous work *(14)* for equivalent residence times. Increased conversions using the present reactor are likely owing to improvements in enzyme coverage of microchannel surfaces, the availability of interior enzymes, and the presence of packing features that add to the reaction surface area and create superior fluid flow characteristics. The shallow depth of the channels (125 μm) provides for very short reactant diffusion lengths. This is one of the great advantages of microscale reactors. Small cross-channel dimensions also induce laminar flow. A reactor channel with no transverse features and a flow rate of 0.5 cm^3/min (typical for this work) has an approximate Reynolds number of 16. This means that reactant molecules can migrate to the walls owing to diffusion alone. The addition of transverse mixing features is designed to increase reaction surface area and to carry reactant directly to walls as in turbulent flow. Future studies will provide comparisons between open channels and those with transverse features.

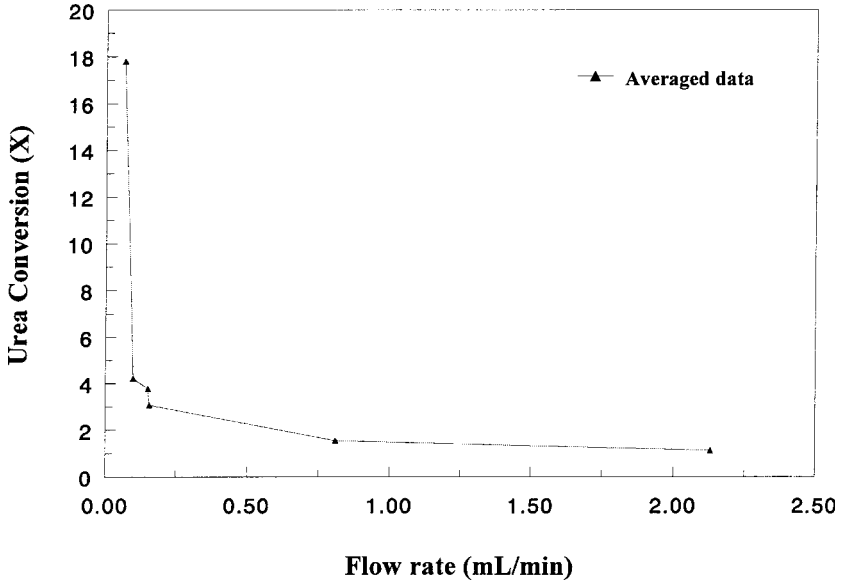

Fig. 12. Continuous-flow microreactor data.

Conclusion

A biologically active silicon plastic consisting of PDMS and enzymes has been developed. The enzymes incorporated into the PDMS polymer have been shown to retain significant enzyme activity for both urease and amyloglucosidase. A microscale bioreactor has successfully been designed and fabricated using this bioplastic as the structural material. The microreactor system produced significant rates of reaction in comparison to batch reactor systems for urease conversion of urea to ammonia. The potential advantages of such microscale systems for novel bioreactors warrant continued investigation.

Acknowledgments

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