

Enzyme and Chemical Encapsulation in Polymeric Microcapsules*

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SYNOPSIS

Polypyrrole microcapsules (prepared via the template method) were used for immobilization of both enzymatic and chemical catalytic systems. Enzymes immobilized include glucose oxidase, catalase, trypsin, subtilisin, and alcohol dehydrogenase. The chemical catalytic system investigated consisted of immobilized Pd nanoparticles for the catalysis of hydrogen peroxide decomposition. Microcapsules loaded with glucose oxidase (GOD) were found to have higher enzymatic activity than that of GOD-loaded thin films, a competing encapsulation method. Trypsin was used to explore the possible leakage of small proteins from the capsules; no leakage was observed. Subtilisin was used to show that these microcapsules can be used in nonaqueous solvents. The effect of the capsule wall thickness on the rate of the enzymatic reaction was also explored. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

Enzyme immobilization has emerged as an important method for the development of biosensors and bioreactors.¹⁻²⁰ Current methods of enzyme immobilization include adsorption or covalent attachment to a support,²⁻⁴ microencapsulation,^{5,6} and entrapment within a membrane/film^{7,8,11-20} or gel.⁹ An ideal immobilization method should employ mild chemical conditions, allow for large quantities of enzyme to be immobilized, provide a large surface area for enzyme-substrate contact within a small total volume, minimize barriers to the mass transport of substrate and product, and provide a chemically and mechanically robust system.

We recently developed a new approach for the immobilization of enzymes. This approach entails immobilization of the desired enzyme in template-synthesized²¹⁻²⁶ polymeric tubules. While the template approach allows for easy preparation of such tubular structures,^{21-24,27-29} the tubules typically obtained are open on both ends and, therefore, not useful for enzyme immobilization. In this article, we describe in detail³⁰ synthetic strategies to yield

capped versions of such microtubules (i.e., microcapsules). These microcapsules are prepared from the polymer polypyrrole and are arranged in a high-density array in which the individual capsules protrude from a surface like the bristles of a brush. We have developed procedures for filling these polypyrrole microcapsules with high concentrations of enzymes. The enzyme-loaded microcapsule arrays function as bioreactors in both aqueous solution and organic solvents.

EXPERIMENTAL

Materials

The monomer, pyrrole (Aldrich 99%), was distilled prior to use. Purified water, obtained by passing house-distilled water through a Milli-Q (Millipore) water-purification system, was used to make all solutions. FeCl₃ was used as received. Poretics microporous polycarbonate membrane filters (Poretics Corp.) were used as the template membranes.²¹⁻²⁴ The membranes used for these studies had a pore diameter of 400 nm, a pore density of 1×10^8 pores/cm², and were ca. 10 μm in thickness. Unless otherwise noted, the enzymes and substrate molecules were used as received, and enzyme solutions were prepared using a pH 7.0 phosphate buffer (0.05 M).

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Formation of the Polymeric Microcapsules

We have shown that when polypyrrole is synthesized from the monomer in the presence of the polycarbonate template membrane the polymer preferentially nucleates and grows on the pore walls.^{21,31} This leads to the formation of polypyrrole tubules within the pores of the membrane. However, these tubules are not capped and are, therefore, not useful for enzyme immobilization. The primary challenge in developing an immobilization method is developing methods for capping these tubules.³⁰ The capped tubules used in most of the studies described here were prepared using a method that combines both electrochemical and chemical synthesis of polypyrrole within the template membrane. We also developed a purely chemical method for preparing the desired polypyrrole microcapsules. Both of these methods are described here.

The Electrochemical/Chemical Method

Unless otherwise noted, this is the procedure that we have used for most of the work reported here. A schematic of this strategy for preparing polypyrrole microcapsules is shown in Figure 1. Polypyrrole was synthesized using a two-step procedure as follows: First, polypyrrole (PPy) "caps" are electrochemically³² deposited at one face of the membrane. To accomplish this, one surface of the microporous polycarbonate template membrane is first sputtered with ca. 50 nm of gold [Fig. 1(A)]. The gold coats the surface of the membrane but does not block the pores. This thin gold layer serves as the electrode for the electrochemical polymerization of the PPy caps.

PPy was galvanostatically deposited at this Au surface film/electrode from a solution containing 0.2M pyrrole and 1M NaCl in a 50 : 50 methanol : water mixture. The methanol was necessary to ensure proper wetting of the pores. A thick surface layer of PPy is deposited on the gold film electrode, and the polymer subsequently grows inward into the pore. The PPy that grows into the pore will become the cap for the microcapsule. The thickness of this cap can be varied by varying the charge used during the electropolymerization. A charge of 0.21 C per cm² of membrane surface area was found to result in a cap of ca. 1 μm thickness within the pore [Fig. 1(B)].

After electropolymerization of the PPy caps, PPy microtubules are synthesized chemically²¹⁻²⁴ within the pores of the membrane [Fig. 1(C)]. This is done by immersing the membrane in a solution containing 0.5M FeCl₃ as the oxidant and 0.2M pyrrole as the monomer.³¹ A polymerization time of 5 min was

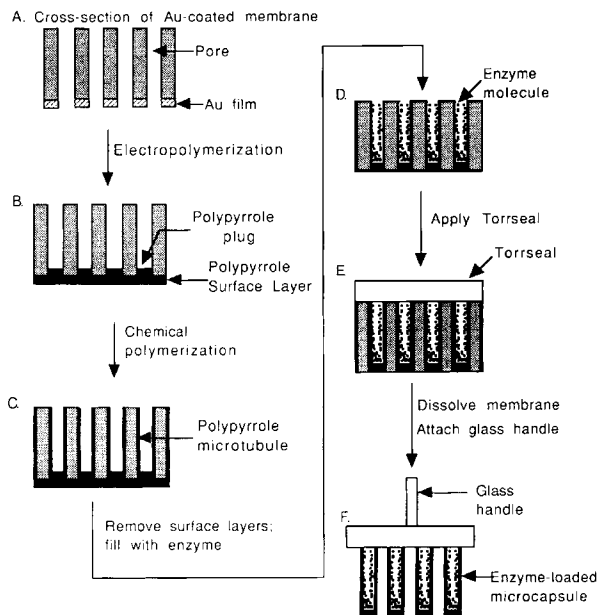


Figure 1 Schematic diagram of methods used to synthesize and enzyme-load the microcapsule ensemble using the electrochemical/chemical route.

used. The PPy grows within the pores as well as on both surfaces of the membrane. The surface layers are removed by polishing the surfaces with 1 μm alumina powder using a Kim wipe wetted with methanol. The membrane is then sonicated and thoroughly rinsed in the pH 7.0 buffer solution repeatedly to remove traces of the monomer, oxidant, and alumina powder. This two-step synthesis results in the formation of the desired capped tubules or microcapsules within the pores of the membrane [Fig. 1(C)].

These PPy microcapsules are filled with the enzyme by vacuum filtration of an enzyme solution through the capsule-containing membrane [Fig. 1(D)]. The microcapsules are first wetted with water by ultrasonating the membrane in a buffer solution. The membrane is then placed in a vacuum filtration apparatus with the cap-side down. A buffered solution of the enzyme is filtered through the open (upper) side of the membrane [Fig. 1(D)]. The water from the enzyme solution pervaporates through the PPy cap.³³ The enzyme, being a large molecule, cannot permeate the PPy cap and is thus retained within the microcapsule.

Vacuum filtration is continued overnight until all the water has pervaporated through the PPy microcapsules. We found that, during this process, enzyme crystallizes on the upper surface of the membrane. This excess enzyme is wiped off using a wet Kim wipe. Care must be taken while wiping excess en-

zyme from the surface because an excessively wet Kim wipe can wick the enzyme solution from the interior of the microcapsule. Alternatively, a Kim wipe dipped in hexane can be used to remove the excess enzyme from the surface. Hexane is useful because the aqueous enzyme solution is not wicked out of the capsules. Torrseal epoxy is then applied to the face of the membrane that is not capped [Fig. 1(E)]. The epoxy is too viscous to go into the microcapsules. A glass rod is inserted into the epoxy so that the ensemble of the microcapsule can be conveniently handled. Once the epoxy cures, the polycarbonate host membrane is dissolved using copious quantities of dichloromethane. This results in an ensemble of PPy microcapsules with the enzyme contained within them [Fig. 1(F)].

Finally, pieces of the template membrane of a 0.5 cm² surface area were used to prepare the microcapsule ensembles described here. This means that these ensembles contained 5×10^7 enzyme-loaded microcapsules. Each microcapsule has an outside diameter of 400 nm, an inside diameter of 375 nm, a length of 10 μ m (the thickness of the membrane), and a cap thickness of 1 μ m.

Chemical Method for Microcapsule Formation

A schematic of this method is shown in Figure 2. The polycarbonate membrane was immersed into 10 mL of a 0.2M pyrrole solution and 10 mL of 0.5M FeCl₃ was added.³¹

Polymerization was allowed to proceed for 1 min. During this period, a thin skin of PPy is deposited onto the walls of the pore [Fig. 2(A)]. The membrane was removed from this solution and rinsed in water. After drying, a piece of Scotch-brand tape was applied to one face of the membrane. This tape prevents further polymerization of PPy on the face of the membrane covered by the tape.

The membrane/tape composite was then reimmersed into the polymerizing solution for an additional 30 min. During this time interval, the desired PPy microtubules are deposited within the pores of the membrane. PPy was then removed from the surface of the membrane not covered by the tape using a wet Kim wipe [Fig. 2(B)]. Polymerization was then repeated twice more by immersion of the membrane/tape composite into a polymerization solution that was 1M in FeCl₃ and 0.2M in pyrrole. Polymerization was allowed to proceed for 5 min in the above two cases. During these polymerization periods, the PPy walls of the tubules thicken and ultimately caps of PPy are formed at the face of the membrane not covered with the tape [Fig. 2(C)].

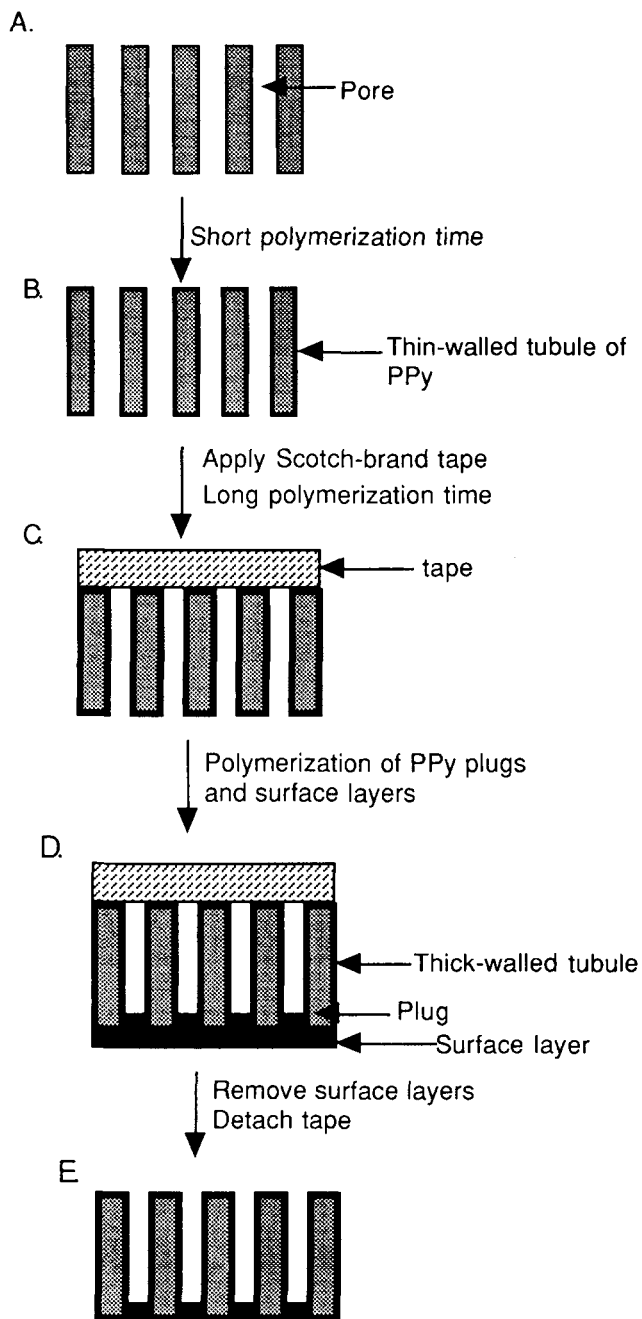


Figure 2 Schematic diagram of methods used to prepare microcapsule ensemble using the purely chemical route.

The surface PPy layer on the cap-side is removed by polishing with alumina powder. The membrane was then cleaned by ultrasonication in water. Finally, the Scotch-brand tape was detached from the membrane surface by repeated rinsing of the membrane in diethyl ether [Fig. 2(D)]. The ether dissolves the adhesive from the tape. At this stage, the PPy capsules can be loaded with the enzyme and

would result in structures similar to the case of Figure 1(D).

Transmission Electron Microscopy (TEM)

TEM images of the microcapsules prepared by the electrochemical/chemical method were obtained as follows: The microcapsule-containing polycarbonate membrane was placed on a copper TEM grid. The membrane was held in place by a vacuum and was then washed with copious quantities of dichloromethane. The dichloromethane dissolved the polycarbonate, leaving the PPy microcapsules on the TEM grid.

We also attempted to image the encapsulated enzyme within the PPy microcapsule using TEM. The enzyme cannot be imaged directly because of insufficient contrast between the PPy and enzyme. However, a solution containing horseradish peroxidase (HRP) that is covalently attached to 20 nm colloidal gold particles is available from the Sigma Chemical Co. This solution was used to load the microcapsules with Au-tagged HRP. Gold, being electron dense, can be easily observed within the PPy capsules using the TEM. The microcapsules used for these studies were prepared by the purely chemical method.

Monitoring Enzyme Activity

We have encapsulated five enzymes and one chemical catalytic system within microcapsules prepared via the electrochemical/chemical method described above. UV-visible spectroscopy was used to assay the activity of the encapsulated enzymes. The experimental setup used is shown in Figure 3. The enzyme-loaded microcapsule array is inserted (using the glass rod) into a cuvette (3 mL) that contains the substrate for the enzyme and the enzyme assay chemistry (see below). The cuvette is present in the sample chamber of a Hitachi U-3501 UV-vis spectrometer. A magnetic stir bar is used to stir the solution in the cuvette. Prior to the monitoring of the enzyme activity, the enzyme-loaded microcapsule array was immersed in the buffer solution at 4°C. The solution was stirred for a period of 2 days. This allows any enzyme from defective tubes to leach out so that only enzymatic activity from encapsulated enzyme is assayed.

Catalase

Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen. The enzyme-mediated decay in hydrogen peroxide concentration can be

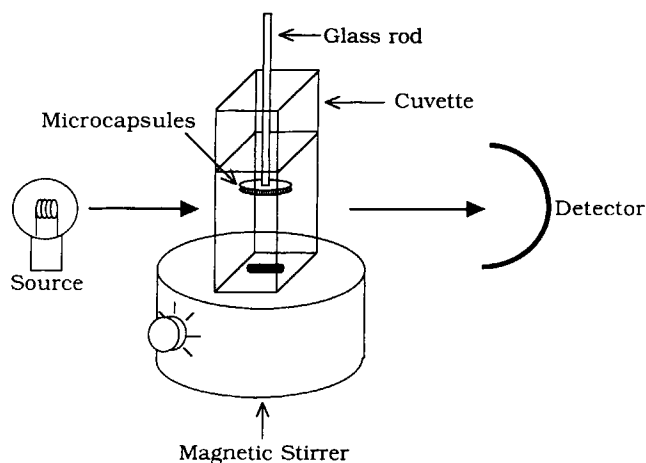


Figure 3 Experimental setup used for UV-visible spectroscopy.

conveniently monitored at 240 nm.³⁴ The catalase-loaded microcapsule array was introduced into a cuvette containing 3 mL of 35 mM H₂O₂ in the pH 7.0 buffer. The decay in absorbance of hydrogen peroxide was monitored at 240 nm. A control experiment was done, wherein an ensemble of empty PPy capsules (i.e., with no catalase) was introduced into the cuvette solution. The response at 240 nm was again monitored as a function of time.

A simple test was performed to see if catalase leached out of the PPy microcapsules during the enzymatic assay. The catalase-loaded microcapsules were introduced into the cuvette solution as described above, and the decay in absorbance at 240 nm was monitored. The catalase-loaded microcapsules were then removed from the cuvette solution and the monitoring of absorbance was continued. If no catalase leached from the capsules, no further decay in H₂O₂ concentration should be observed during the time interval that the capsules were removed from the solution. In contrast, if catalase leached out, the H₂O₂ concentration should continue to decay even though the capsules are not present in the assay solution. This process of insertion and removal of the catalase-loaded microcapsules was repeated twice.

Glucose Oxidase (GOD)

The activity of encapsulated GOD was evaluated using the standard peroxidase-*o*-dianisidine assay method.³⁵ GOD catalyzes the oxidation of glucose, in the presence of air, to yield gluconolactone and hydrogen peroxide. In the presence of the enzyme peroxidase, the H₂O₂ produced oxidizes the reduced form of dianisidine to the red oxidized form. The

absorbance of the oxidized dianisidine is monitored at 500 nm.

In a typical experiment, the enzyme-loaded microcapsules were introduced into a cuvette solution containing glucose (92 mM), peroxidase (180 units), and *o*-dianisidine dihydrochloride (0.17 mM). The solution was stirred, and the absorbance of oxidized dianisidine was monitored as a function of time. This assay procedure was done at two levels of enzyme loading. The results of these experiments have been published elsewhere.³⁰ Unless otherwise noted, PPy capsules with wall thicknesses of ca. 25 nm (corresponding to a polymerization time of 5 min) were used for all the studies. However, to explore the effect of wall thickness on the kinetics of glucose oxidation, a set of experiments was done on microcapsules with walls that were ca. 90 nm thick (30 min polymerization time).

Finally, while the majority of the experiments described here were done using microcapsules prepared via the electrochemical/chemical method, encapsulation in the chemically prepared microcapsules was also studied. This was accomplished by loading the chemically prepared capsules with 0.1 mg (18 units) of GOD and evaluating the enzymatic activity using the dianisidine/peroxidase method.

Trypsin

Trypsin is a relatively small enzyme (23,500 Daltons); it is useful for investigating whether small proteins can be leached from the capsules through the capsule walls. Trypsin catalyzes ester hydrolysis, and its catalytic activity was evaluated using *N*- α -benzoyl-L-arginine ethyl ester as the substrate molecule. The microcapsules were loaded with 1 mg of trypsin in 0.5 mL of pH 7 buffer. The trypsin-loaded microcapsules were introduced into an aqueous solution of the substrate and the absorbance due to the product of the enzymatic reaction (*N*- α -benzoyl-L-arginine) was monitored at 253 nm. After the assay, these microcapsules were stored in pH 3 buffer at 4°C for a period of 45 days. After 45 days, the enzymatic activity of the microcapsules was reevaluated. This was done to determine if the enzyme was retained within the microcapsule for long periods of time.

Alcohol Dehydrogenase (ADH)

ADH catalyzes alcohol oxidation using NAD⁺ as a soluble cofactor. Because NAD⁺ is a large molecule (660 Daltons), the ADH/NAD⁺ system can be used to investigate whether molecules of this size can

permeate through the PPy capsule wall. This was accomplished by loading the ADH (without NAD⁺) into the capsules and then immersing the capsules into a solution containing ethanol (6.5%) and NAD⁺ (1.4 mM). For the enzymatic reaction to occur, ethanol and NAD⁺ must diffuse through the capsule wall and find the ADH within the capsule.

The enzymatic reaction was followed spectroscopically by monitoring the NADH absorbance of the external at 340 nm.³⁴ Note, again, that after the reaction with the enzyme the NADH must diffuse through the capsule wall into the external solution. Finally, analogous experiments were conducted with an NAD⁺ that was covalently bound to a 40K Dalton dextran chain (Sigma Chemical Co). As before, the NAD⁺/dextran was added only to the external solution. We anticipated that, in this case, no enzymatic reaction would be observed because the dextran chain would prevent the NAD⁺ from permeating the capsule wall.

Subtilisin

Subtilisin catalyzes transesterification reactions. Zaks and Klibinov³⁶ carried out reactions catalyzed by subtilisin in nonaqueous media. They showed that the enzyme retains its activity even when it is not soluble in the solvent medium. Because PPy is insoluble in nearly all solvents, our microcapsules might be useful for such nonaqueous enzymatic processes. To explore this point, we investigated a transesterification reaction catalyzed by subtilisin in acetone.

The subtilisin was loaded into the microcapsules from an aqueous buffer solution. After evaporation of residual water, the capsules were immersed into an acetone solution of *N*-acetyl-L-phenylalanine ethyl ester (5 mM) and propanol (1 M). This transesterification reaction was monitored using a gas chromatography-mass spectrometry (GC-MS) method. An HP GC (5890 A) (with a DB-1 column) and an HP MS (5970) were used. GC conditions used were as follows: detector temperature: 290°C; injector temperature: 275°C; oven temperature: 200°C; and temperature ramp: 10°C/min for 5 min. The progress of the reaction was monitored as the ratio of the area under the product (*N*-acetyl-L-phenylalanine propyl ester) peak to the area under the reactant (*N*-acetyl-L-phenylalanine ethyl ester) peak.

Because subtilisin is insoluble in acetone,³⁶ this enzyme system can be used in an important control experiment. In the case of the subtilisin-nonaqueous system, there can be no leaching of the enzyme through the PPy walls as the enzyme is insoluble in

the solvent medium. But incomplete removal of the enzyme from the surface of PPy could lead to some enzyme being stuck to the torr seal surface. To prove that the enzymatic reaction is not due to extraneous sources of the enzyme, control experiments were carried out. The controls that were chosen for this study were microcapsules that had both ends of the capsules blocked and fibers (solid tubules) of PPy. The reason for choosing these controls is that, in contrast to the microcapsules used for all the preceding work, enzymes cannot be loaded into these microstructures after they are fabricated. The PPy fibers were made by using long times of electropolymerization. By controlling the time of electropolymerization, the PPy fibers grow through the entire length of the pore. PPy capsules were made chemically as described earlier. In both cases, after removing the surface layers, a solution of subtilisin was poured onto the upper surface of the PPy-containing membrane and a vacuum was applied to the lower surface. The enzyme would not, however, go through the capsule or fiber and was therefore only retained on the surface of the membrane. The surface was then wiped clean (as before) and the composite was attached to a glass rod using torr seal epoxy.

Palladium Nanoparticles

In addition to biocatalysts, we have shown that these microcapsules can be used to encapsulate a chemical catalytic system. Palladium nanoparticles were prepared by mixing equal volumes of a solution of palladium(II) chloride (0.01M) and a solution of SnCl₂ (0.025M) in 0.1M HCl.³⁷ The resulting Pd nanoparticles were then filtered through the PPy microcapsules [as per Fig. 1(D)]. The encapsulated Pd nanoparticles were used to catalyze the decomposition of hydrogen peroxide, using the methods described for the enzyme catalase.³⁴

RESULTS AND DISCUSSION

Electron Microscopy

Figure 4(A) shows a scanning electron micrograph (SEM) of typically PPy microcapsules prepared via the electrochemical/chemical methods. This SEM shows that the microcapsules are capped and that the capsules protrude from the torr seal surface like the bristles of a brush. The exposed PPy surface area in such a microcapsule array is 12 cm² of the capsule surface area per cm² of the substrate epoxy

area. This figure could be increased by using membranes with higher porosity (the porosity of this membrane is only 12%) or by using thicker membranes (and thus making longer microcapsules).

The wall thickness in these microcapsules can be controlled by varying the polymerization time. Unless otherwise noted, a polymerization time of 5 min was used for these studies. This yields a wall thickness of ca. 25 nm [see Fig. 4(B)]. These thin walls ensure that diffusion of the substrate and product molecules through the capsule is facile. In spite of these thin walls, these capsules have extraordinary mechanical strength.³⁸ Shorter polymerization times yield microcapsules with poor mechanical strength, and broken or flattened capsules are observed in the TEM after dissolution of the membrane. Longer polymerization times cause diffusional mass transport through the capsules to be slower (see below).

While the electrochemical/chemical route for preparing microcapsules allows for the greatest amount of control over the capsule growth process, this method requires two separate synthetic steps, and the electrochemical step is more difficult than is the chemical step. We have, therefore, investigated purely chemical routes for preparing such microstructures. We found that if the membrane is immersed in the polymerization solution for long times (2 h), the tubules formed an ultimate cap on both ends to yield bottle-like microstructures [see Fig. 4(C)]. While these are interesting microstructures, the caps on both ends make it difficult to load these structures with the enzyme, unless, of course, the enzyme was dissolved in the polymerization solution. This is undesirable because the pH of this solution is quite low (pH ca. 1).

This led us to idea of masking one surface of the membrane with Scotch tape. By using this approach, caps cannot form on the masked surface and open microcapsules are obtained. These microcapsules can then be filled with the enzyme via the simple filtration method described in Figure 1. Figure 4(D) shows a TEM of microcapsules prepared via this chemical approach. These capsules had been loaded with a low concentration solution of horseradish peroxidase that had been tagged with a 20 nm Au nanoparticle. The Au nanoparticles are clearly evident in this TEM.

Catalase

When the catalase-loaded microcapsules were immersed into the cuvette solution containing hydrogen peroxide [Fig. 5(A), time *a*], a rapid decay in H₂O₂ absorbance was observed due to the catalytic

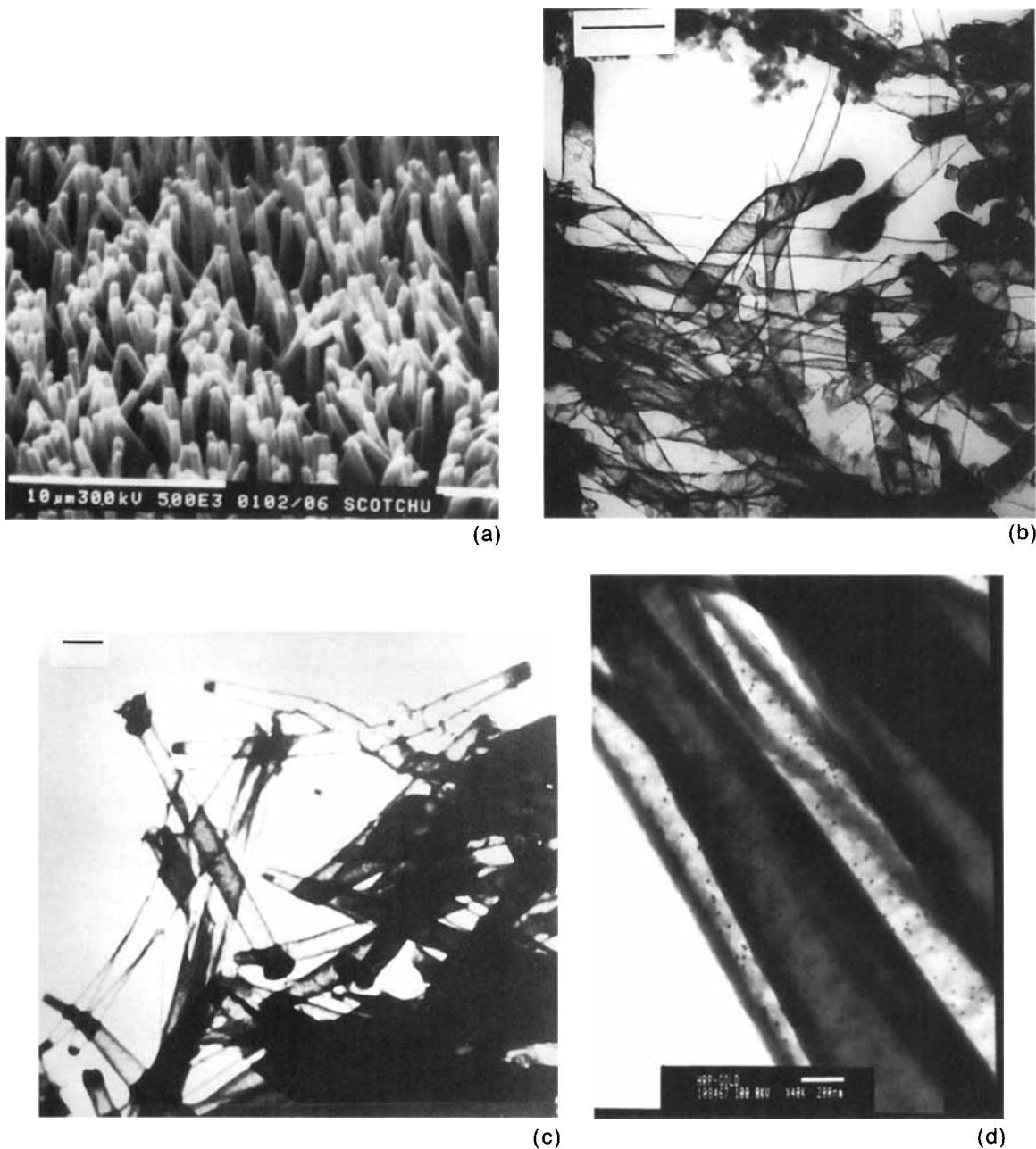


Figure 4 (a) Scanning electron micrograph of a typical microcapsule ensemble showing PPy microcapsules extending from the torr seal surface. (b) Transmission electron micrograph of microcapsules that had not been attached to the epoxy surface. Scale bar in upper-left corner is 1.0 μm . (c) Transmission electron micrograph of free PPy microcapsules prepared using the chemical method. Scale bar in the upper-left corner is 1.0 μm . (d) Transmission electron micrograph of microcapsules that contain horseradish peroxidase that has been labeled with 20 nm-diameter gold particles.

decomposition of the H_2O_2 . At time b, the capsule array was removed from the cuvette solution. The decay in H_2O_2 concentration slows to that corresponding to the natural (i.e., noncatalyzed) rate. This process was repeated twice more, and similar

responses were obtained [Fig. 5(A)]. This indicates that the catalase does not leach out of the microcapsules. If catalase leached from the microcapsules, then the higher (catalytic) rate of decay would be observed after the capsules were removed from the

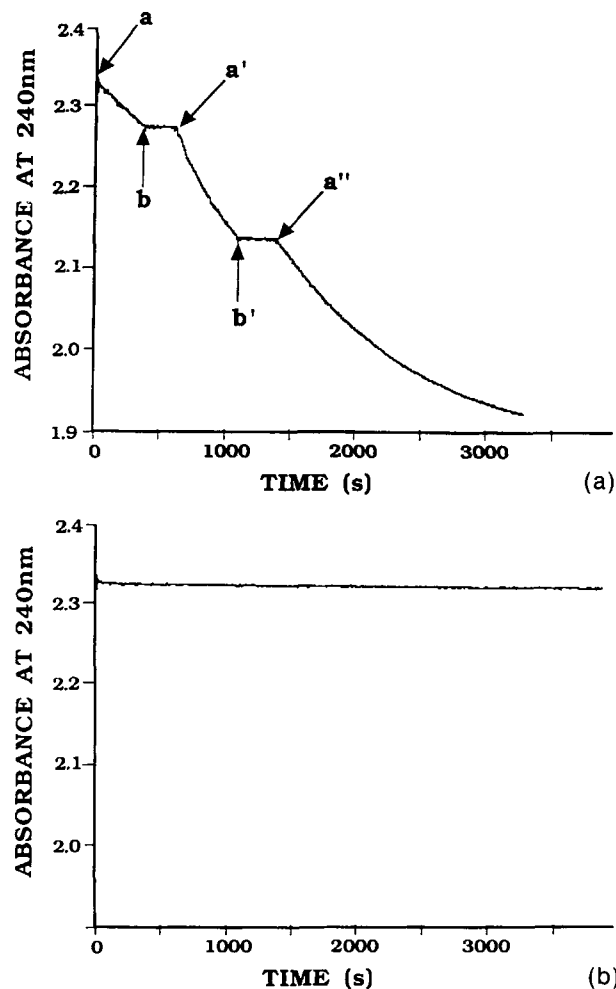


Figure 5 (a) Absorbance due to H_2O_2 upon insertion (points a , a' , a'') and removal (points b and b') of a catalase-loaded microcapsule ensemble into a solution that was 35 mM in H_2O_2 , pH 7.0 phosphate buffer. (b) Absorbance at 240 nm after immersion of empty PPy microcapsules into a 30 mM solution in H_2O_2 .

cuvette. Finally, as a control, empty PPy capsules were introduced into the cuvette solution. As expected, little change in absorbance (corresponding to the natural decay of H_2O_2) is observed [Fig. 5(B)].

Glucose Oxidase

The standard *o*-dianisidine peroxidase assay procedure was used to determine the catalytic activities of GOD within the microcapsules. In our previous article,³⁰ we showed that the amount of enzyme within the microcapsule can be controlled easily. We also showed that large quantities of the enzyme can be loaded within these microcapsules. A comparison of the activities of our GOD-loaded microcapsules

was made with thin films containing GOD. Higher enzymatic activities were achieved with our immobilization method and thus show promise for the development of new types of enzymatic biosensors.

The effect of the PPy wall thickness on the kinetics of glucose oxidation was also investigated. In all the experiments described so far, capsules with a wall thickness of 25 nm were employed. These thin walls minimize diffusional mass-transport barriers. To explore this point more fully, capsules with 90 nm-thick walls were also investigated. Figure 6 shows plots of rate of the enzymatic reaction vs. concentration of glucose for capsules with 25 nm and 90 nm-thick walls. Note the dramatically slower rate for the capsules with the thicker walls. Assays of the quantity of enzyme loaded indicated that both the 90 and 25 nm-thick capsules contained the same amount of GOD. Hence, the lower rate for the thicker-walled capsules is due to slower mass transfer. Finally, in both cases, the enzymatic rate ultimately levels at a high substrate concentration, indicating that typical Michaelis-Menten behavior is observed for these capsules.

Microcapsules prepared by the purely chemical route were also used to encapsulate GOD. That the GOD in these capsules is enzymatically active is shown by the data in Figure 7. We currently prefer the electrochemical/chemical method for preparing such microcapsules. This is because it is easier to control the wall thickness in the chemical step after the caps have been formed electrochemically. However, it seems possible that with careful attention to polymerization time and conditions procedures could be developed that yield optimal microstructures from the simpler purely chemical method.

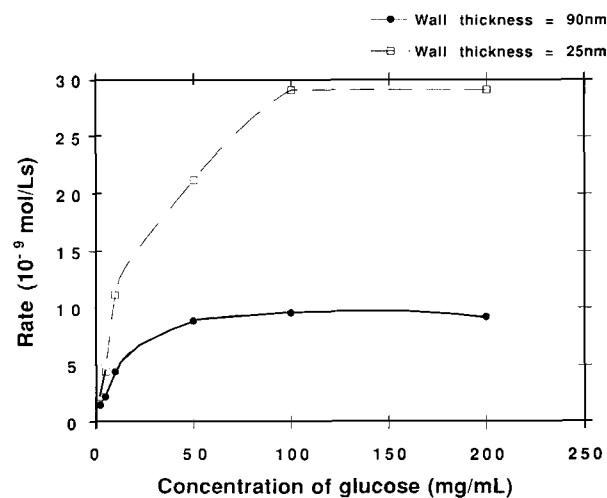


Figure 6 Plot of rate vs. concentration of glucose for two different microcapsule wall thicknesses.

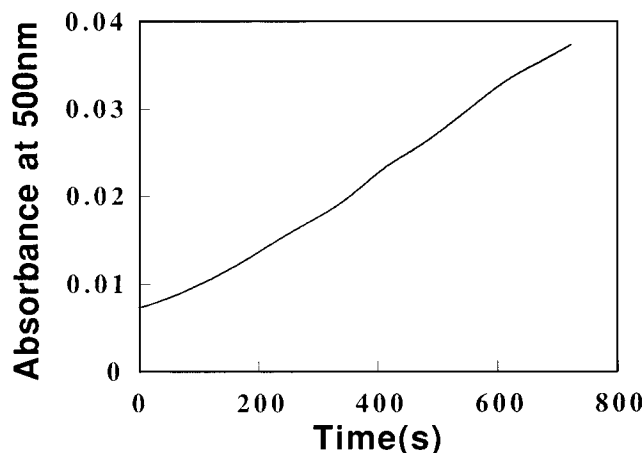


Figure 7 Absorbance for the oxidized form of *o*-dianisidine after immersion of chemically prepared glucose oxidase microcapsules into an assay solution.

Trypsin

The encapsulated trypsin was used to catalyze the hydrolysis of *N*- α -benzoyl-L-arginine ethyl ester. An increase in absorbance was observed at 253 nm, corresponding to the formation of the product *N*- α -benzoyl-L-arginine [Fig. 8, curve (a)]. When the catalytic activity was reevaluated after 45 days, no loss of enzymatic activity was observed within experimental error [Fig. 8, curve (b)]. These results indicate that trypsin is not leached out of the capsules. Again, as trypsin is one of the smallest of enzymes, this means that nearly any enzyme can be permanently immobilized within these microcapsules.

Alcohol Dehydrogenase

ADH was loaded into the microcapsules and its catalytic activity was measured by monitoring the NADH produced (at 340 nm) in the external solution phase. An increase in absorbance at 340 nm was observed corresponding to the formation of NADH [Fig. 9, curve (a)]. The only way that NADH could have been detected in the external solution is by alcohol and NAD^+ diffusing through the walls of the capsule and reacting with the encapsulated ADH. These experiments suggest that even large molecules such as NAD^+ and NADH can permeate through the walls of the capsules. Because substrate molecules for most enzymatic processes are smaller than this, these experiments show, again, that these capsules can be used with nearly any enzymatic system. It is possible for large molecules to permeate the capsule wall because polypyrrole is nanoporous³⁹ and

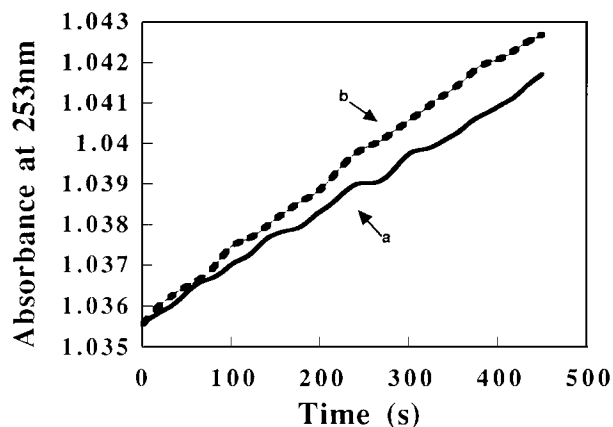


Figure 8 Absorbance for *N*- α -benzoyl-L-arginine when trypsin-loaded capsules were immersed into the assay solution. Solid line is before and dashed line is after 45 day storage at 4°C.

because the walls are thin. In another experiment, dextran-linked NAD^+ was used in the external solution. No enzymatic activity was observed [Fig. 9, curve (b)]. This is because the large dextran chain (mol wt = 40,000) cannot diffuse through the walls of the PPy microcapsule.

Subtilisin

The transesterification reaction catalyzed by the encapsulated subtilisin was monitored using GC-MS. Figure 10 shows typical GC-MS data and compares the chromatogram at time = 0 and at 67 h into the course of the enzymatic reaction. The appearance of the product ester is clearly seen. The

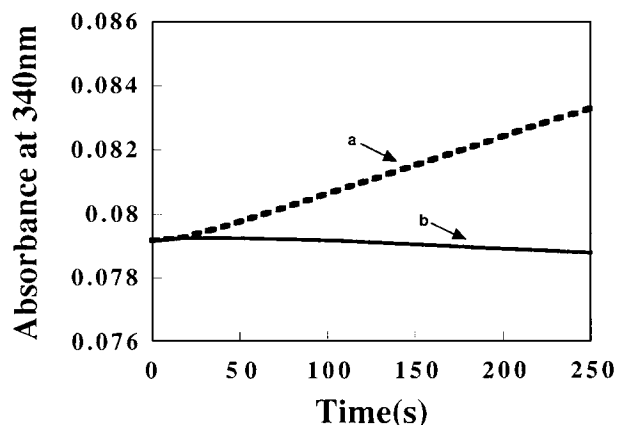


Figure 9 Assay of alcohol dehydrogenase activity. Dashed line is the absorbance due to NADH upon insertion of alcohol dehydrogenase-loaded microcapsules. Solid line is the analogous experiment done with dextran-linked NAD.

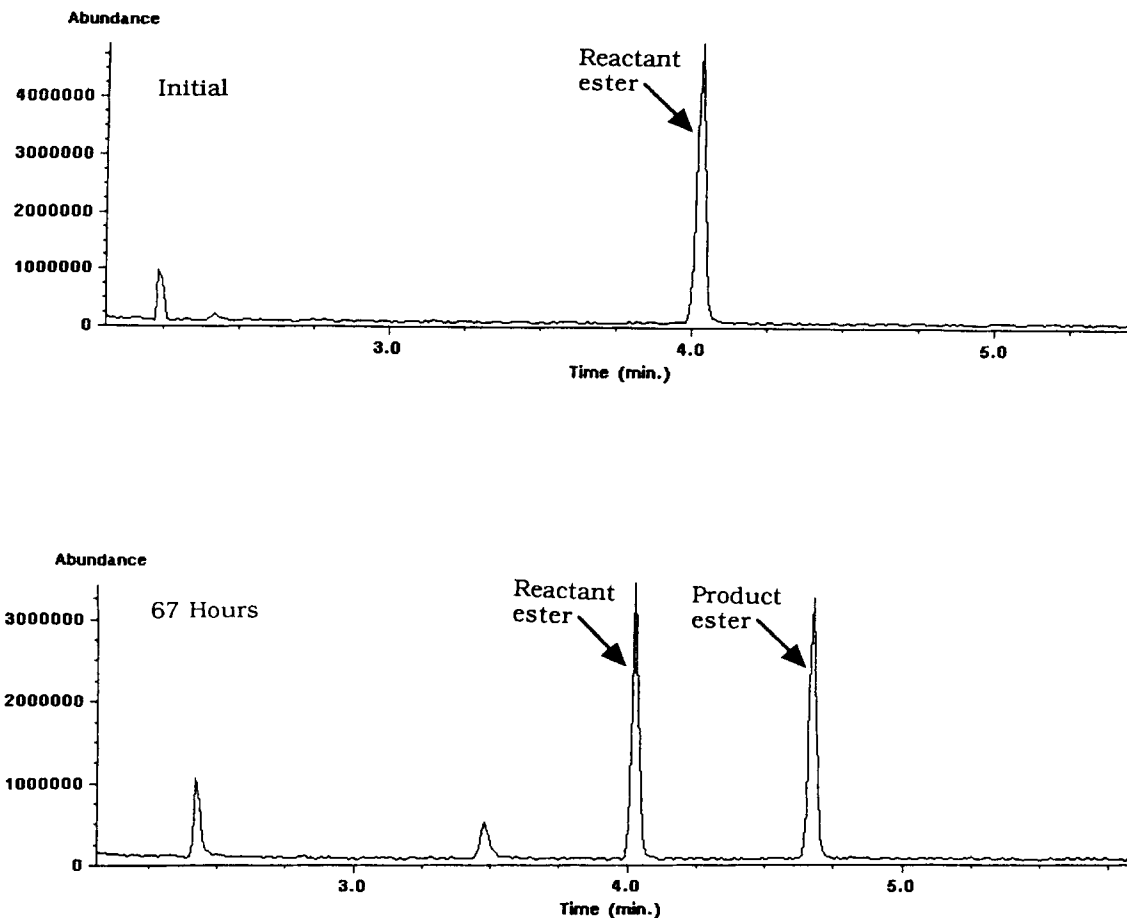


Figure 10 Gas chromatograms from the subtilisin-catalyzed transesterification reaction mixture as a function of time.

progress of the reaction was monitored by ratioing the area of the product ester peak to the area of the reactant peak. A plot of the progress of the reaction vs. time is shown in Figure 11 for both subtilisin-loaded and empty microcapsules. No reaction is observed for the empty capsules. Finally, this reaction occurs at a much slower rate than do any of the other enzymatic processes investigated. This is because the transesterification reaction is slow in the absence of water.³⁶ The upward curvature in the progress vs. time plot for the subtilisin-loaded capsules is undoubtedly due to the incorporation of water into the acetone from water vapor in the air.

The activities of subtilisin in three microstructures: microcapsules (with both ends of the capsule blocked), microcapsules (with one end blocked as described previously), and fibers are shown in Figure 12. The fibers and capsules (both ends blocked) practically showed no response in the GC-MS. As expected, the capsules (with one end blocked) showed an enzymatic response. This implied that

there was negligible contribution from the enzyme stuck to the torseal surface.

Palladium Nanoparticles

That the Pd nanoparticles incorporated into the microcapsules are catalytically active is proven by the data in Figure 13. A rapid decay in H_2O_2 concentration is observed upon immersion of the Pd-loaded microcapsules. The decay goes back to the slow background rate when the microcapsules are removed from the solution.

CONCLUSION

Polymeric microcapsules were prepared and evaluated as encapsulating agents for bio and chemical catalysts. The polymer used to prepare these capsules—polypyrrole—has several important attributes: First, it is known to be nanoporous, and this

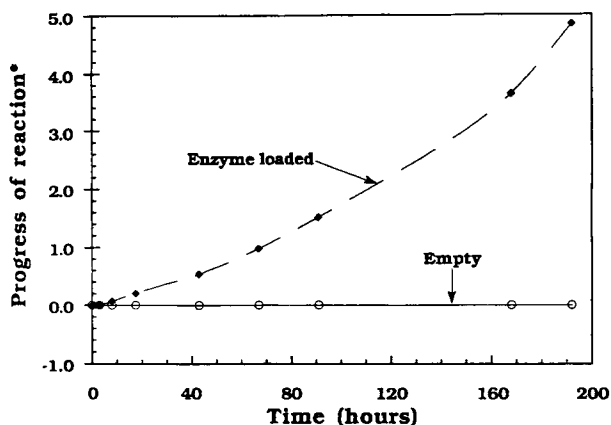


Figure 11 Progress of transesterification reaction after immersion of enzyme-loaded and empty microcapsules into the reaction mixture. Progress of the reaction was monitored by ratioing the area under the product ester peak to the area under the reactant ester peak.

allows for facile diffusion of substrate and product molecules through the capsule walls. Our work has shown that the molecular weight cutoff for the nanopores in polypyrrole is between 660 (NAD which does permeate the capsule wall) and 23,000 Daltons (trypsin which does not permeate the capsule wall). This molecular weight cutoff makes this polymer ideal for enzyme immobilization. The second important feature of this polymer is its good mechanical properties. We have shown that tubule PPy microstructures with extremely thin walls have remarkable mechanical strength. This ability to make encapsulants with extremely thin walls again minimize mass-transport barriers in the encapsulation systems.

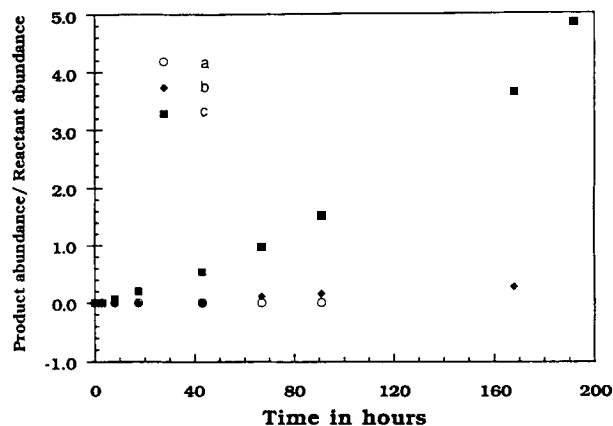


Figure 12 Relative activities of subtilisin in three different PPy microstructures: (a) fibers; (b) microcapsules (with both ends blocked); (c) microcapsules (with one end blocked).

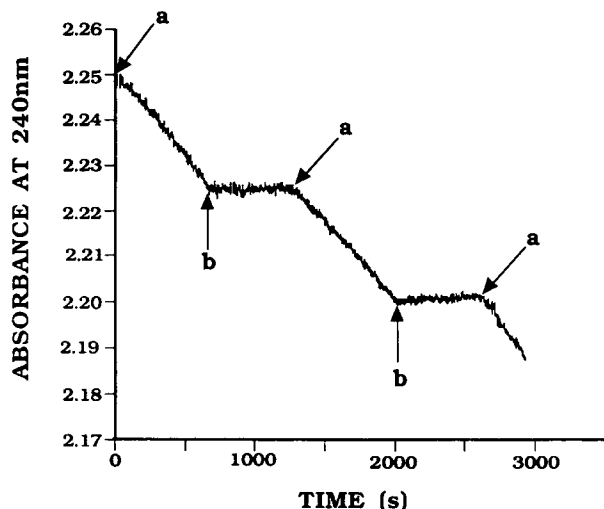


Figure 13 Absorbance at 240 nm upon (a) insertion and (b) removal of palladium-loaded microcapsules.

The encapsulation method that we developed offers a number of important advantages: First, it is very general. Any enzyme or combination of enzymes can be loaded into these microstructures. Second, the enzyme-loading process is chemically benign. The enzyme never sees harsh chemical environments. Finally, this encapsulation method provides for a large surface area for enzyme/substrate contact. In the current system, there are 12 cm² of encapsulant surface area per cm² of substrate area. This number could, in fact, be significantly higher. For example, an Anopore microporous alumina membrane has a porosity of ca. 55%, a pore diameter of 200 nm, and a thicknesses of 55 μ m. If analogous microcapsules could be prepared in these membranes, an encapsulation system that offers 192 cm² of surface area per cm² of substrate area would be obtained.

We are currently exploring various possible applications for these microcapsule ensembles. We are particularly interested in applications in biosensors.

REFERENCES

1. J. Woodward, Ed., *Immobilized Cells and Enzymes—A Practical Approach*, IRL Press: Washington, DC, 1985.
2. J. Woodward, in *Immobilized Cells and Enzymes—A Practical Approach*, J. Woodward, Ed., IRL Press, Washington, DC, 1985, Chap. 1.
3. S. E. Wolowacz, B. F. Y. Yon-Hin, and C. R. Lowe, *Anal. Chem.*, **64**, 1541 (1992).
4. B. F. Y. Yon-Hin, M. Smolander, T. Crompton, and C. R. Lowe, *Anal. Chem.*, **65**, 2067 (1993).

5. K. F. Gu and T. M. S. Chang, in *Bioreactor Immobilized Enzymes and Cells*, M. Moo-Young, Ed.; Elsevier, New York, 1988.
6. H. E. Klei, D. W. Sundstrom, and D. Shim, in *Immobilized Cells and Enzymes*, J. Woodward, Ed., IRL Press, Washington, DC, 1985, Chap. 4, p. 49.
7. P. N. Bartlett, Z. Ali, and V. Eastwick-Field, *J. Chem. Soc. Faraday Trans.*, **88**, 2677 (1992).
8. G. Fortier and D. Belanger, *Biotech. Bioeng.*, **37**, 854 (1991).
9. L. M. Ellerby, C. R. Nishida, F. Nishida, S. A. Yamanaka, B. Dunn, J. S. Valentine, and J. I. Zink, *Science*, **255**, 1113 (1992).
10. R. Maida and A. Heller, *Anal. Chem.*, **64**, 2889 (1992).
11. N. C. Foulds and C. R. Lowe, *J. Chem. Soc. Faraday Trans.*, **82**, 1259 (1986).
12. M. Marchesiello and E. M. Genies, *Electrochim. Acta*, **37**, 1987 (1992).
13. M. Marchesiello and E. M. Genies, *Electrochim. Acta*, **35**, 358 (1993).
14. N. F. Almeida, E. J. Beckman, and M. M. Ataii, *Biotech Bioeng.*, **42**, 1037 (1993).
15. P. N. Bartlett and R. G. Whitaker, *J. Electroanal. Chem.*, **224**, 37 (1987).
16. Y. Kajiya, H. Sugai, C. Iwakura, and H. Yoneyama, *Anal. Chem.*, **63**, 49 (1991).
17. M. Umana and J. Waller, *Anal. Chem.*, **58**, 2979 (1986).
18. Z. Sun and H. Tachikawa, *Anal. Chem.*, **64**, 1112 (1992).
19. N. C. Foulds and C. R. Lowe, *Anal. Chem.*, **60**, 2473 (1988).
20. T. Tatsuma and T. Watanabe, *J. Electroanal. Chem.*, **356**, 245 (1993).
21. Z. Cai and C. R. Martin, *J. Am. Chem. Soc.*, **111**, 4138 (1989).
22. C. R. Martin, L. S. Van Dyke, Z. Cai, and W. Liang, *J. Am. Chem. Soc.*, **112**, 8976 (1990).
23. C. R. Martin, *Adv. Mater.*, **3**, 457 (1991).
24. C. R. Martin, R. Parthasarathy, and V. Menon, *Syn. Met.*, **55-57**, 1165 (1993).
25. C. R. Martin, *Acc. Chem. Res.*, **28**, 61 (1995).
26. C. R. Martin, *Science*, **266**, 1961 (1994).
27. R. Pool, *Science*, **247**, 1410 (1990).
28. J. H. Georger, A. Singh, R. P. Price, J. M. Schnur, P. Yager, and P. E. Schoen, *J. Am. Chem. Soc.*, **109**, 6169 (1987).
29. C. G. J. Koopal, M. C. Feiters, R. J. M. Nolte, B. de Ruiter, R. B. M. Schasfoort, R. Czajka, and H. Van Kempen, *Syn. Met.*, **51**, 397 (1992).
30. R. Parthasarathy and C. R. Martin, *Nature*, **369**, 298 (1994).
31. V. P. Menon and C. R. Martin, to appear.
32. R. M. Penner, L. S. Van Dyke, C. R. Martin *J. Phys. Chem.*, **92**, 5274 (1988).
33. R. W. Baker, E. L. Cussler, W. Eykamp, W. J. Koros, R. L. Riley, and H. Strathmann, Eds., *Membrane Separation Systems, Recent Developments and Future Directions*, Noyes Data Corporation, Park Ridge, NJ, 1991, Vol. 2, p. 151.
34. *Worthington Enzyme Manual: Enzymes, Enzyme Reagents, Related Biochemicals*, Worthington Biochemical Corporation, Freehold, NJ, 1972, p.41.
35. S. K. Dalvie and R. E. Baltus, *Biotech. Bioeng.*, **40**, 1174 (1992).
36. A. Zaks and A. M. Klibanov, *J. Biol. Chem.*, **263**, 3194 (1988).
37. A. M. T. van der Putten, J.-W. G. de Bakker, and L. C. J. Fokkink, *J. Electrochem. Soc.*, **139**, 3475 (1992).
38. C. R. Martin, R. Parthasarathy, and V. Menon, *Electrochim. Acta*, **39**, 1309 (1994).
39. P. Burgmayer and R. W. Murray, in *Handbook of Conducting Polymers*, Marcel Dekker, New York, 1986, Vol. 1, Chap. 15.

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