0273-2289/01/9601-3/0249/$12.25

Coimmobilization of Urease and Glutamate Dehydrogenase in Electrochemically Prepared Polypyrrole–Polyvinyl Sulfonate Films

Anamika Gambhir,*[,1] Manju Gerard,[1] A. K. Mulchandani,[2] and B. D. Malhotra[1]

[1]Biomolecular Electronics and Conducting Polymer Research Group, National Physical Laboratory, New Delhi 110012, India, E-mail anamikagulati@yahoo.com; and
[2]Chemical and Environmental Engineering Department, University of California, 92521-0425, CA

Abstract

Immobilization of urease and glutamate dehydrogenase enzymes in electrochemically prepared polypyrrole–polyvinyl sulfonate films (PPY-PVS) was carried out using physical adsorption and electrochemical entrapment techniques. Detailed studies on optimum pH, Fourier transform infrared spectroscopy, cyclic voltammetry, and scanning electron microscopy of the enzymes in the immobilized state were conducted. The value of the apparent Michaelis-Menten constant was experimentally determined to be 2.5 and 2.7 for physically adsorbed and electrochemically entrapped urease in PPY-PVS films, respectively.

Index Entries: Coimmobilization; urease; glutamate dehydrogenase; urea; biosensor.

Introduction

The technique of immobilization of enzymes has experienced phenomenal growth in the recent past, because it overcomes various limitations in the analytical uses of enzymes in aqueous solutions. Apart from being unstable, free enzymes lose their catalytic ability rapidly in aqueous solutions, from which they can neither be recovered nor can their activity be regenerated *(1)*. The free enzyme is thus immobilized by trapping it in an inert matrix such that the immobilized enzyme retains its catalytic properties

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

for a much longer duration, is stable over a wider temperature range as compared with the free enzyme, and can be used continuously for several analyses over a period of time. Several enzymes (e.g., invertase, cholinesterase, glucose oxidase, lactate oxidase, cholesterol oxidase, urease) have been immobilized on different organic and inorganic matrices *(2–4)* for various applications. The immobilization of enzymes in an inert and usually insoluble polymer matrix for application to a biosensor is currently drawing much attention.

Organic conducting polymers have emerged as a new class of electroactive materials as the immobilization matrix, because they facilitate rapid relay of biochemical signal to the detecting device of an electrochemical biosensor. Polypyrrole (PPY) has been widely used for biosensing application because of its facile polymerization, high conductivity, chemical stability, and, above all, its free-floating film can be readily prepared *(5–7)*.

Various conducting polyheterocyclic films have been investigated and PPY is of particular interest because the relatively low oxidation potential of the monomer enables film to be grown from aqueous solutions that are compatible with most of the biological elements *(8)*. In addition, electrochemical entrapment of enzyme in PPY matrix becomes feasible and has been attracting great interest as a favored immobilization technique.

Presently available colorimetric and kinetic methods for the determination of urea are based on hydrolysis of urea by urease. Compared to the colorimetric methods that are based on oxidative coupling of NH_3 with phenol or Nessler's reagent, kinetic methods involve coupling this reaction to another enzymatic reaction wherein NH_3 is accepted by α-ketoglutarate, and in the process, oxidation of NADH takes place, which can be monitored spectrophotometrically. Most biosensors described in the literature for the determination of urea are potentiometric and are based on NH_4^+- or HCO_3^--sensitive electrodes. Some urea sensors based on highly integrated biocatalytic systems such as microorganisms and tissue slices have also been prepared *(9–11)*. However, none of these studies has as yet led to urea analyzers suitable for routine applications. Gullbalt and Montalvo *(12)* have described conductometric sensors that measure change in conductivity as a result of urea hydrolysis.

In the present study, urease and glutamate dehydrogenase (GLDH) were coimmobilized in electrochemically prepared PPY–polyvinyl sulfonate (PPY-PVS) films for application to a urea biosensor. Hydrolysis of urea is catalyzed by urease as described in reaction 1. The ammonium ion released in the first reaction is coupled with α-ketoglutarate in the presence of GLDH and nicotinamide adenine dinucleotide (NADH) acts as a cofactor. Coimmobilization of urease and GLDH in conducting polymer matrix is significant since it generates an amperometric signal by oxidation of NADH to NAD^+:

$$(NH_2)_2CO + 3H_2O \xrightarrow{\text{UREASE}} 2NH_4^+ + OH^- + HCO_3^- \quad (1)$$

$$NH_4^+ + \alpha\text{-Ketoglutarate} + NADH \xrightarrow{\text{GLDH}} \text{L-glutamate} + NAD^+ \quad (2)$$

The characteristics of the PPY-PVS-urease/GLDH electrodes were studied using cyclic voltammetric, ultraviolet-visible, Fourier transform infrared (FTIR) techniques, respectively.

Materials and Methods

PPY-PVS films were electrochemically deposited on indium-tin-oxide (ITO) glass plates serving as working electrode, platinum as the counter-electrode, and saturated colomel electrode (SCE) as the reference electrode from a solution comprising predistilled pyrrole (0.1 *M*) and PVS (0.1 *M*) at a constant current of 2 mA *(13)*. The electrical conductivity of these films measured by four-points-probe technique was found to be about 10^{-3}S/cm.

The cyclic voltammetric studies of PPY-PVS electrodes were carried out using an eletrochemical interface (Sclumberger SI 1286) in the range of –0.8 to 1.4 V vs SCE at a scan rate of 50 mV/s. FTIR spectroscopy studies were carried out using a Nicolet 510 P spectrophotometer from 400 to 4800 cm^{-1} using KBr powder (spectroscopic grade).

Immobilization of Urease and GLDH

Physical Adsorption

Urease and GLDH stocks were prepared separately (2 IU/μL) in Tris buffer (0.1 *M*) at pH 7.0. For physical adsorption, 50 μL of each of the stock solutions was applied on the surface of electrochemically prepared PPY-PVS film (1×1 cm^2) and kept refrigerated under vacuum for drying. To prepare a film containing both urease and GLDH, 25 μL of both stocks was mixed together and applied on the film.

Electrochemical Entrapment

Aliquots from the stock solutions were added in the solution of predistilled pyrrole (0.1 *M*) and PVS (0.1 *M*) immediately after the deposition of film starts so that the final concentration in the total solution became nearly 2 IU/μL. The entire reaction of electrodeposition was carried out at constant current (2 mA). PPY-PVS films immobilized with the two enzymes (urease and GLDH) separately and in combination were prepared by the same procedure.

The activity measurements of urease catalyzing the hydrolysis of urea to NH_3 (reaction 3), which in turn forms an orange complex with Nessler's ($K_2Hg^{II}I_4$) reagent, was measured colorimetrically at 405 nm with a 160A Shimadzu spectrophotometer. Substrate urea and enzyme-immobilized PPY-PVS film were incubated for about 20 min in Tris buffer (0.1 *M*), pH 7.0, after which the film was removed and 0.1 mL of Nessler's reagent added. The final volume of the solution was adjusted to 20 mL and absorbance read at 405 nm. Quantitation of urea by kinetic methods is carried out by measuring oxidation of NADH in the presence of GLDH and urease at 350 nm (reactions 3 and 4).

$$\text{Urea} + H_2O \text{ --------> } 2NH_4^+ + 2HCO_3^- \quad (3)$$

$$\alpha\text{-Ketoglutarate} + NH_4^+ + NADH \text{ --------> L-glutamate} + NAD^+ + H_2O \quad (4)$$

The reaction was carried out in phosphate buffer (10 m*M*) at pH 7.8 with α-ketoglutarate (10 m*M*) as substrate for reaction 4 and adenosine 5'-diphosphate (0.8 m*M*) as cofactor.

Results and Discussion

Figure 1A shows a typical cyclic voltammogram obtained for the PPY-PVS film in 0.1 *M* Na PVS solution. In the first cycle, oxidation peaks are observed at –30 and 1140 mV. The –30 mV peak corresponding to the reduction in the polymer and its reversibility is only valid when the potential is kept below 0 mV. The 1140 mV peak is attributed to the degradation of the electroactive polymer. The second anodic sweep only shows a broad anodic maximum between 200 and 800 mV.

Figure 1B shows the cyclic voltammogram of PPY-PVS films with urease enzyme adsorbed in 0.1 *M* Na PVS solution at a scan rate of 50 mV/s. An increase in the current is observed and broad peaks are seen at 500 and –700 mV, respectively. Figure 1C shows the potentiodynamic behavior of the PPY-PVS electrode with two enzymes (urease + GLDH) physically adsorbed on it. Two anodic peaks are seen at –240 and 800 mV, and their corresponding cathodic peaks are observed, indicating the electroactive nature of the PPY-PVS-urease/GLDH films.

Figure 2, curve a exhibits an FTIR spectrum of PPY-PVS composite in KBr disc. The characteristic peaks for PPY at 1647 (C=N), 1385 (C-N), and (O=N) vibrations at 1188 cm^{-1} are seen. The 1040 cm^{-1} peak has been assigned to the symmetric stretching of the SO_3^- group. Figure 2, curve b shows the FTIR spectrum of urease immobilized in PPY-PVS films. Peaks for the amide II linkages can be clearly seen at 1550 cm^{-1}. Figure 2, curve c shows the FTIR spectrum of urease and GLDH immobilized on PPY-PVS films. Suppressed C-N vibrations seen at 1700 cm^{-1} can be assigned to the coupling of PPY to urease and GLDH. The peak at 3416 cm^{-1} corresponds to the N-H stretching.

The effect of pH on the activity of free and immobilized enzymes (urease and GLDH) is presented in Fig. 3. A shift in pH from 7.5 (solution) to 8.0 (entrapped and adsorbed in films) can be seen in PPY-PVS films. Interestingly, the rise and fall in activity was more gradual on both sides of the pH optima in the immobilized state. This may perhaps be owing to the presence of anionic charges on the PPY-PVS (when the amount of PVS is in excess) resulting in concentration of protons (thereby lowering the pH) around the enzyme. Thus, the immobilized enzyme is likely to have the optimum pH higher in solution as compared to that in the free state. Goldstein *(14)* observed a similar shift of 1 pH unit toward alkaline values of the pH optimum of chymotrypsin acting on acetyl-L-tyrosine ethyl ester when immobilized on the polyanion ethylene-maleic anhydride copolymer. Such displacements of the pH/activity profiles are known to depend

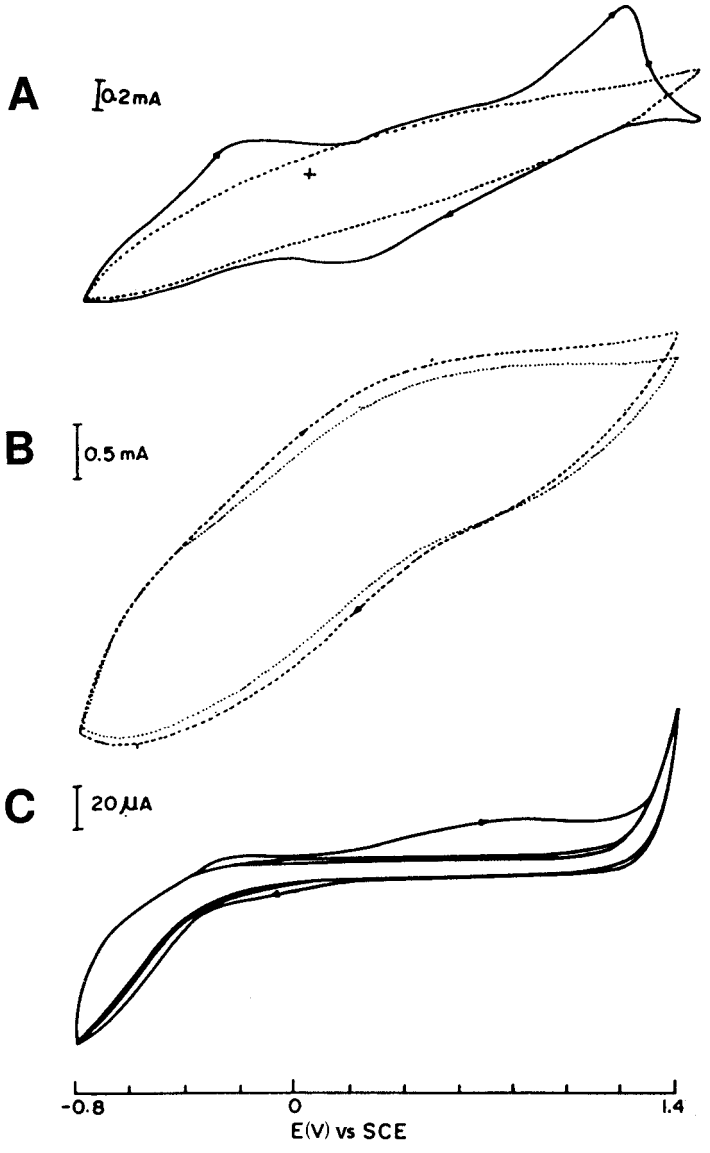

Fig. 1. **(A)** Cyclic votammograms of PPY-PVS films in 0.1 *M* sodium PVS solution: scan rate of 50 mV/s; **(B)** PPY-PVS films with physically adsorbed urease enzyme in 0.1 *M* sodium PVS solution: scan rate of 50 mV/s; **(C)** PPY-PVS films with physically adsorbed urease and GLDH in 0.1 *M* sodium PVS solution: scan rate of 50 mV/s.

on partitioning of protons affected by the presence of ionized groups on the polymer matrix *(15)*.

The leaching was found to be 5–10% in the case of entrapped enzymes over a period of 1 h in solution. However, in the case of adsorbed enzymes, this was found to be 15–20%. The half-life of this electrode was found to be about 30 and 35 d for adsorbed and entrapped enzymes, respectively.

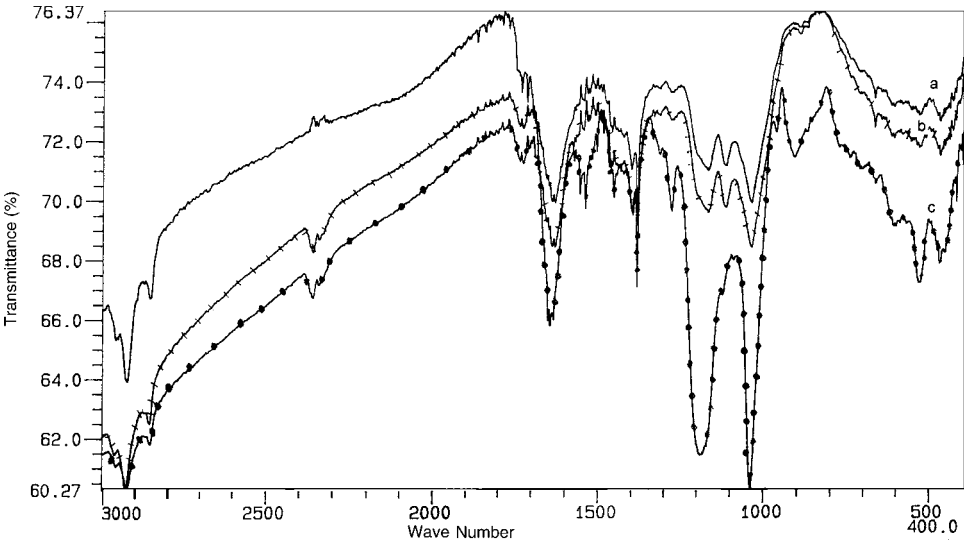

Fig. 2. FTIR spectrum of PPY-PVS composites (a) in KBr disc, (b) physically adsorbed urease, and (c) physically adsorbed urease and GLDH.

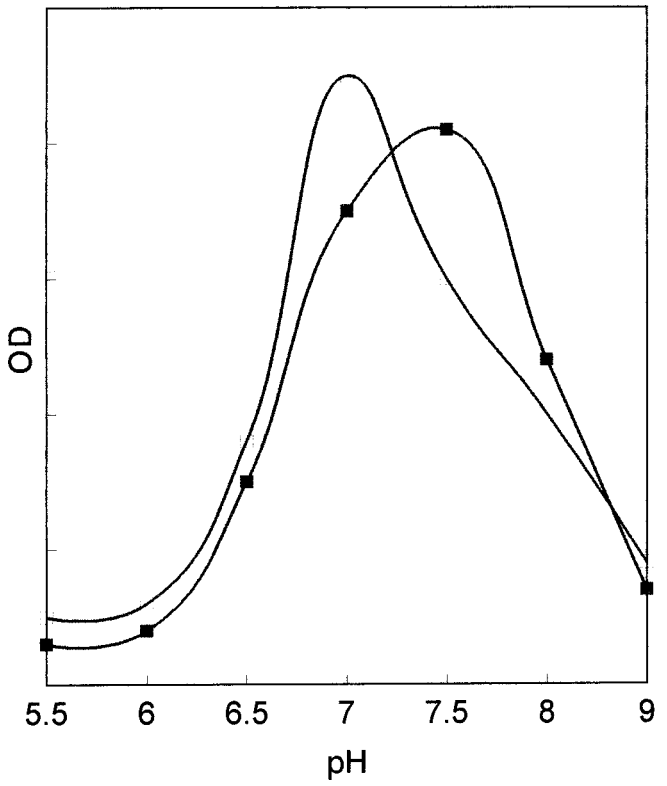

Fig. 3. Effect of pH on the activity of enzymes in (□) free and (■) immobilized state. OD, optical density.

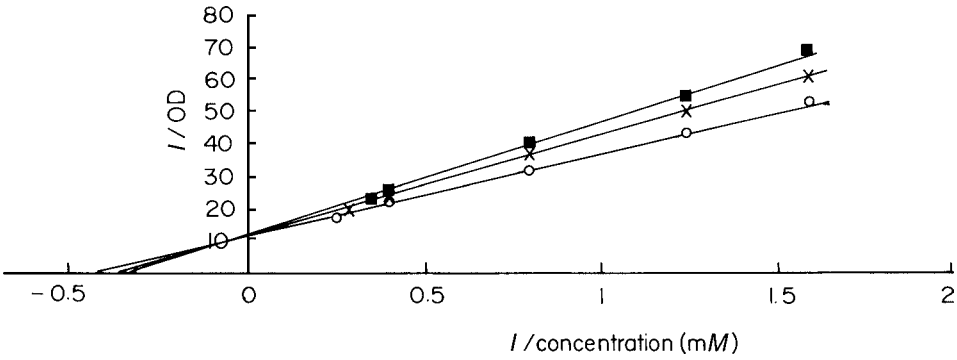

Fig. 4. Lineweaver-Burk plots for free (■), adsorbed urease (○), and entrapped urease (×). OD, optical density.

Lineweaver-Burk plots for the immobilized urease suggest that it still obeys Michaelis-Menten kinetics (Fig. 4). The apparent K_m (K_m^{app}) for adsorbed urease as obtained from the Lineweaver-Burk plot between 1/absorbance and 1/concentration m*M* was found to be 2.56 m*M* and for the entrapped state 2.77 m*M*. The K_m for free urease in dilute solution in a parallel experiment was found to be 2.9 m*M*. Several factors influence K_m^{app} including the substrate concentration range used and charge density around the substrate because the effect of substrate diffusion will be greater at low ionic strengths. Both these factors influence the apparent ease with which enzyme and substrate can associate and will thus affect K_m^{app}. In addition, ionic interactions between the polymer (PPY-PVS) and substrates are known to have a marked effect on the measured kinetic parameters of the enzyme. Owing to the presence of anionic charges on PPY-PVS, it tends to pull the substrate (urea) so that its concentration around the enzyme at the polymer surface is greater than the bulk phase concentration. Since K_m is inversely proportional to the enzyme activity and here K_m^{app} is lower than K_m, a higher affinity of the enzyme for the substrate is anticipated owing to diffusional and ionic effects. These studies helped us to understand the effect of the polymer matrix on the kinetics of the immobilized enzyme.

Figure 5A and B show the nodular surface consisting of the adsorbed enzyme and encapsulated structures consisting of entrapped enzymes, respectively. The presence of urease on the surface of the PPY-PVS film in the adsorbed state corroborate observed higher activity in this state. By comparison, the entrapped urease shows lower activity as a result of the partition effect of the polymer film where it rests intermolecularly.

Conclusion

It has been shown that urease and GLDH on PPY-PVS was successfully immobilized in electrochemically prepared PPY-PVS films. While physical adsorption is favored owing to its ease and speed of adsorption,

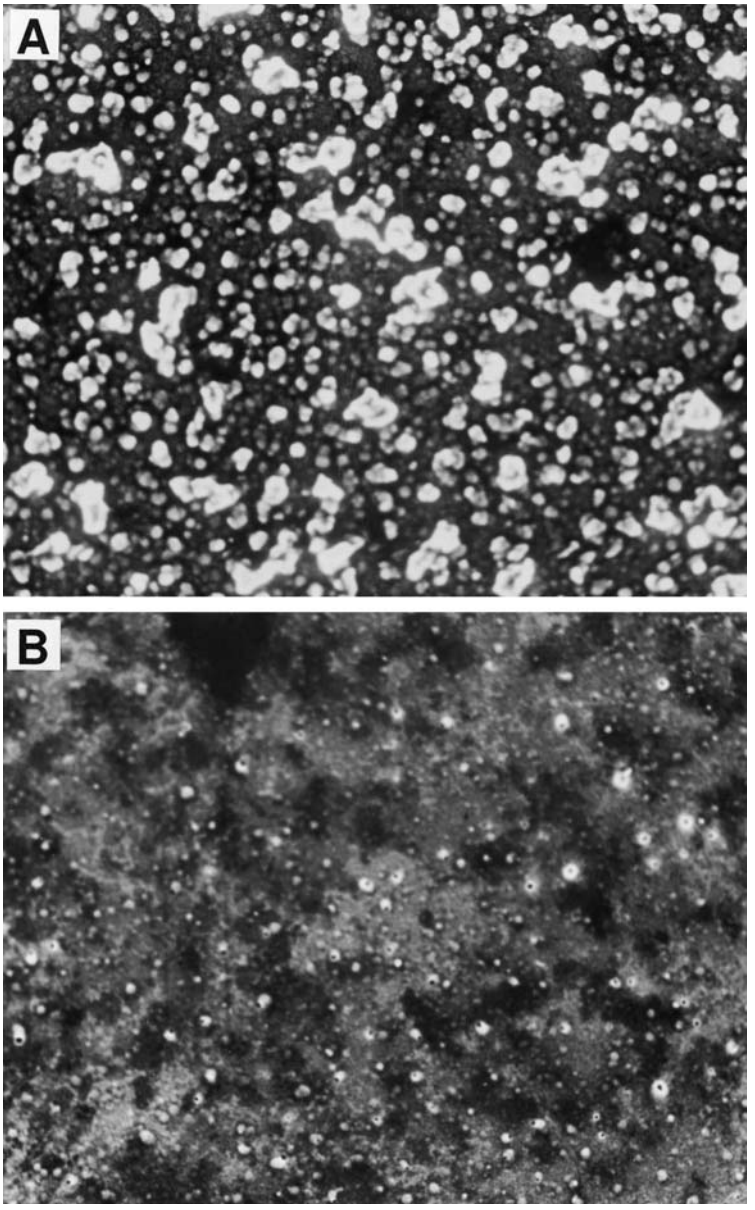

Fig. 5. Scanning electron micrographs of PPY-PVS films with immobilized urease and GLDH **(A)** adsorbed and **(B)** entrapped.

electrochemical entrapment gives the benefit of a one-step process in which spatial distribution of enzyme can be tailored simply by manipulating the preparation parameters. Level of interference is reduced since a conducting polymer possesses the size exclusion property, which is effective in eliminating the interference of electrooxidizable compounds such as ascorbate and urate.

Acknowledgments

We are grateful to Prof. A. K. Raychaudhury, Director, National Physical Laboratory, for all his help. A. G. is thankful to CSIR, India, for the award of a research associateship. This work was financially supported under the Indo-US project (DST/INT/US-404/93).

References

1. Weetall, H. H. (1974), *Anal. Chem.* **46,** 602A.
2. Alva, S. and Phadke, R. S. (1994), *Ind. J. Chem.* **33A,** 561–564.
3. Gerard, M., Ramanathan, K., Chaubey, A., and Malhotra, B. D. (1999), *Electroanalysis* **11(6),** 450–460.
4. Ramanathan, K., Ram, M. K., Malhotra, B. D., and Murthy, A. S. N. (1995), *Mater. Sci. Engg. C* **3,** 159–163.
5. Bartlett, P. N. and Whitaker, R. G. (1987), *J. Electroanal. Chem.* **224,** 37–48.
6. Shin, M.-C. and Kim, H.-S. (1996), *Biosens. Bioelectron.* **11(1/2),** 161–169.
7. Verghese, M. M., Ramanathan, K., Kamalasanan, M. N., Ashraf, S. M., and Malhotra, B. D. (1998), *J. Appl. Poly. Sci.* **70,** 1447–1453.
8. Asavapiriyont, S., Chandler, G. K., Gunawardana, G. A., and Plecher, D. (1984), *J. Electroanal. Chem.* **177,** 229–244.
9. Scheller, F. and Schubert, F., ed. (1992), in *Biosensors*, Elsevier, London, pp. 85–252.
10. Pandey, P. C. and Mishra, A. P. (1988), *Analyst* **113,** 329–331.
11. Karyakin, A. A., Vuki, M., Lukochova, L. V., Karyakina, E. E., Orlov, A. V., Karpachova, G. P., and Wang, J. (1999), *Anal. Chem.* **71,** 2534–2540.
12. Guilbalt, G. G. and Montalvo, J. (1969), *J. Anal. Chem. Soc.* **91,** 2164, 2165.
13. Otero, T. F. and Olazabal, V. (1996), *Electrochimica Acta* **41(2),** 213–220.
14. Goldstein, L. (1972), *Biochemistry* **11,** 4072–4078.
15. Trevan, M. D., ed. (1980) in *Immobilized Enzymes*, John Wiley & Sons, NY, pp. 11–53.