Electrochemical Poly(1,3-phenylenediamine) Synthesis as Enzyme Immobilization Media

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Received 23 April 1997; accepted 17 September 1997

ABSTRACT: Electrochemical polymerization of the 1,3-phenylenediamine in the presence of glucose oxidase with KCl aqueous electrolyte at a potential of 0.800 V versus Ag–AgCl produces adherent poly(1,3-phenylenediamine) containing enzyme (glucose oxidase) film on a platinum electrode. Polymeric sensor prepared in this one-step procedure can be used to determine hydrogen peroxide formed as the result of the enzymatic reaction between glucose and glucose oxidase in the presence of O_2. The amperometric responses of the resultant enzyme electrode to glucose were rapid, reaching steady-state values within 4–5 s, and there was a linear relationship between glucose concentration and obtained current up to 6 m*M*. Polymeric sensor was stable for more 3 months. The glucose selectivity of enzyme electrode was determined in the presence of some interfering substances, such as lactose, sucrose, urea, uric acid, paracetamol, and ascorbic acid. Also, the effects of buffer concentration, storage conditions, and temperature on the steady-state amperometric responses were studied. Moreover, the Arrhenius activation energy for the enzymatic reaction was calculated. © 1998 John Wiley & Sons, Inc. J Appl Polym Sci 68: 145–152, 1998

Key words: poly(1,3-phenylenediamine); glucose; glucose oxidase; enzyme electrode; amperometric biosensor

INTRODUCTION

Electrochemically synthesizable polymers on the electrode surface have various technological application areas, such as in batteries,[1,2] energy storage,[3] electrochromic displays,[4,5] and corrosion protection.[6] In recent years, amperometric biosensors have gained a great deal of attention due to their specificity, simplicity, and sensitivity for determination of clinically significant substrate glucose.

In the biosensor construction, conducting or nonconducting polymeric films, such as polypyrrole,[7,8] poly(*N*-methylpyrrole),[9,10] polyphenylene,[11–14] poly(vinyl alcohol),[15] poly(vinyl alcohol)–butyl acrylate,[16,17] and polythiophene,[18] have been used to prevent electroactive interferences and fouling of the electrode surface by protein and other substances to immobilize enzyme and to entrap mediators.

Sasso et al.[12] used poly(1,2-diaminobenzene) films to prevent interferences and fouling of the working electrode surface in the already immobilized glucose oxidase biosensor. Then, poly(1,2-diaminobenzene) itself was used to immobilize glucose oxidase by Malitesta et al.[14] Also, Reynolds and Yacynych[19] employed poly(1,3-diaminobenzene) to prevent interferences in the biosensor construction using platinized, disk-type carbon ultramicroelectrodes (8 μm diameter) with glucose oxidase immobilized by glutaraldehyde crosslinking or covalent attachment to the electrode surface using carbodiimide.

In the present article, we report synthesis, electrochemical characterization, and sensor applica-

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Journal of Applied Polymer Science, Vol. 68, 145–152 (1998)
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tion (as enzyme immobilization matrix) of the poly(1,3-phenylenediamine) itself obtained by the electrochemical polymerization of the related monomer in the presence of enzyme (in a one-step procedure).

EXPERIMENTAL

Reagents

1,3-Phenylenediamine, glucose oxidase (EC 1.1.3.4), type X-S (181,600 U/g) from *Asperigillus Niger,* and D-(+) glucose were purchased from Sigma Company (St. Louis, Missouri). Glucose and KCl were used without further purification. The glucose stock solution (0.40*M*) was prepared in doubly distilled water and left at room temperature for 24 h before use to ensure the presence of β-D-glucose form (controlled by polarimeter).

Other reagents, such as lactose, sucrose, urea, ascorbic acid, and uric acid, were analytical grade and supplied either by Sigma Chemical Company or E. Merck (Darmstadt, Germany).

Apparatus

Electrochemical techniques, such as polymerization, cyclic voltammetry (CV), and amperometric measurements, were carried out by a BAS (Bioanalytical Systems, Inc.) 100BW electrochemical analyzer in a three-electrode cell with a platinum working electrode, Ag–AgCl (BAS, MF-2063) reference electrode, and a Pt wire coil auxiliary electrode. The pH measurements were performed with a Jenway 3010 pH meter.

Electrochemical Polymerization

The poly(1,3-phenylenediamine) films containing enzyme (glucose oxidase) were obtained by electrochemical polymerization of relevant monomer at a constant potential (0.800 V versus Ag–AgCl) in the presence of glucose oxidase in aqueous KCl solution under an atmosphere of nitrogen at a room temperature.

Monomer and enzyme concentrations were 0.10 mol/L and 100 U/mL, respectively. The thickness of the polymeric films was controlled by the amount of charge (1.20 mC) passed during the electropolymerization.

The polymeric films thus prepared were thoroughly rinsed with doubly distilled water to eliminate the weakly bound enzyme to the polymer and stored in the phosphate-buffered salts (PBS) containing 125 m*M* NaCl, 2.7 m*M* KCl, and 5 m*M* phosphate buffer) solution pH 6.5 for further electrochemical studies.

Amperometric Measurements

Amperometric responses of the poly(1,3-phenylenediamine)–GOx electrode to glucose injections were measured by application of a potential of 0.70 V (versus Ag–AgCl) that was predetermined by linear sweep voltammetry as being in the diffusion-controlled plateau region for hydrogen peroxide oxidation on the polymer electrode at a scan rate of 200 mV s^{-1}.[20]

All the solutions used in the amperometric studies were aerated by bubbling air for 15 min prior to use. The cell system containing 10.0 mL of PBS was kept at a room temperature under gentle stirring, a constant potential predetermined was applied to the cell, and the background current was allowed to decay before aliquots of the stock glucose solution then current due to hydrogen peroxide formed as a result of the enzymatic reaction between glucose and glucose oxidase was measured as a function of time. In order to determine the linearity of the enzymatic electrode response, successive additions of the required amount of substrate were injected, and the current–time graph was continuously recorded.

RESULTS AND DISCUSSION

Electropolymerization of 1,3-Phenylenediamine

The required potential for electrochemical polymerization of the monomer was determined by cyclic voltammetry. Figure 1 shows the current–potential curve of the bare Pt electrode in the absence and presence of 1,3-phenylenediamine in aqueous KCl solution. On the voltammogram, irreversible oxidation currents appeared at 560 and 810 mV peak potentials.

These irreversible peaks decreased to almost the background level on the following cycles show the blocking the poly(1,3-phenylenediamine) film formation of the access of monomer to the electrode surface on the subsequent cycles.

The polymeric films, prepared as described in the experimental section, were stable both in air and in phosphate buffer. The visual inspection of polymer revealed a thin, homogeneously covered film of a brownish red color.

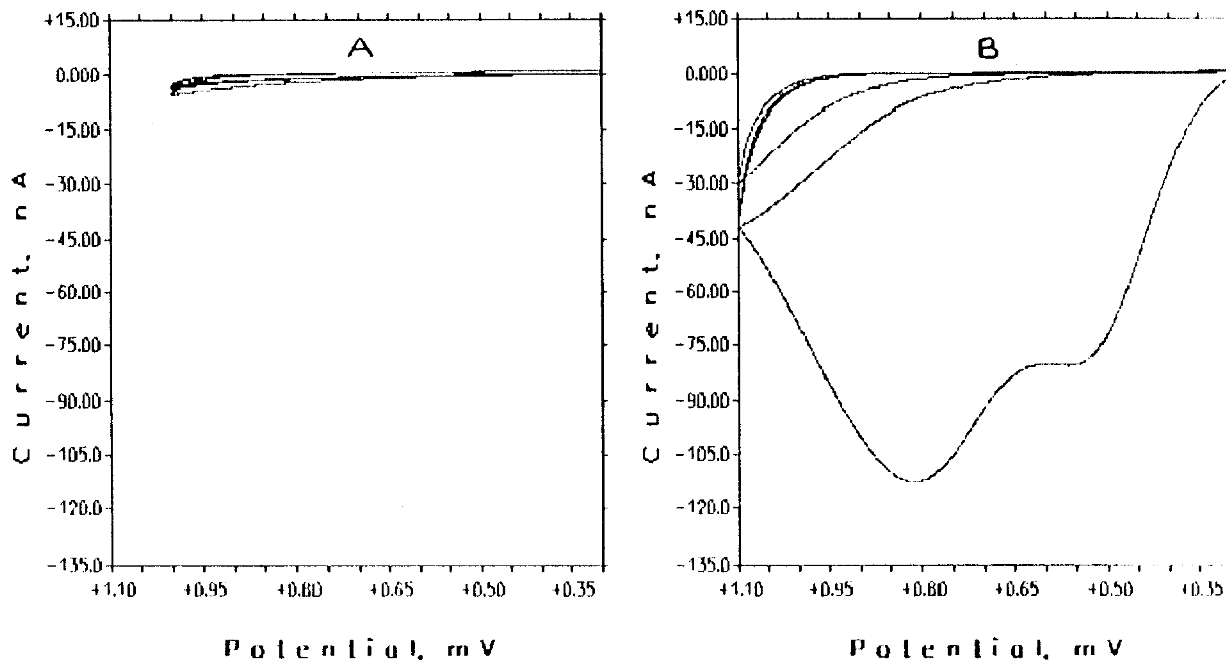


Figure 1 Cyclic voltammograms of the bare pt electrode in 0.1*M* KCl (A) and 0.1*M* KCl + 0.10*M* 1,3-phenylenediamine (B). Scan rate: 50 mV s^{-1}.

Cyclic voltammograms of the poly(1,3-phenylenediamine) and poly(1,3-phenylenediamine)–glucose oxidase electrodes are indicated in Figure 2. Upon comparison of the voltammograms, differences confirm that the polymer has been affected by enzyme (glucose oxidase) incorporation.

Glucose oxidase from *Asperigillus niger* has an isoelectric point of 4.2 and is, therefore, negatively

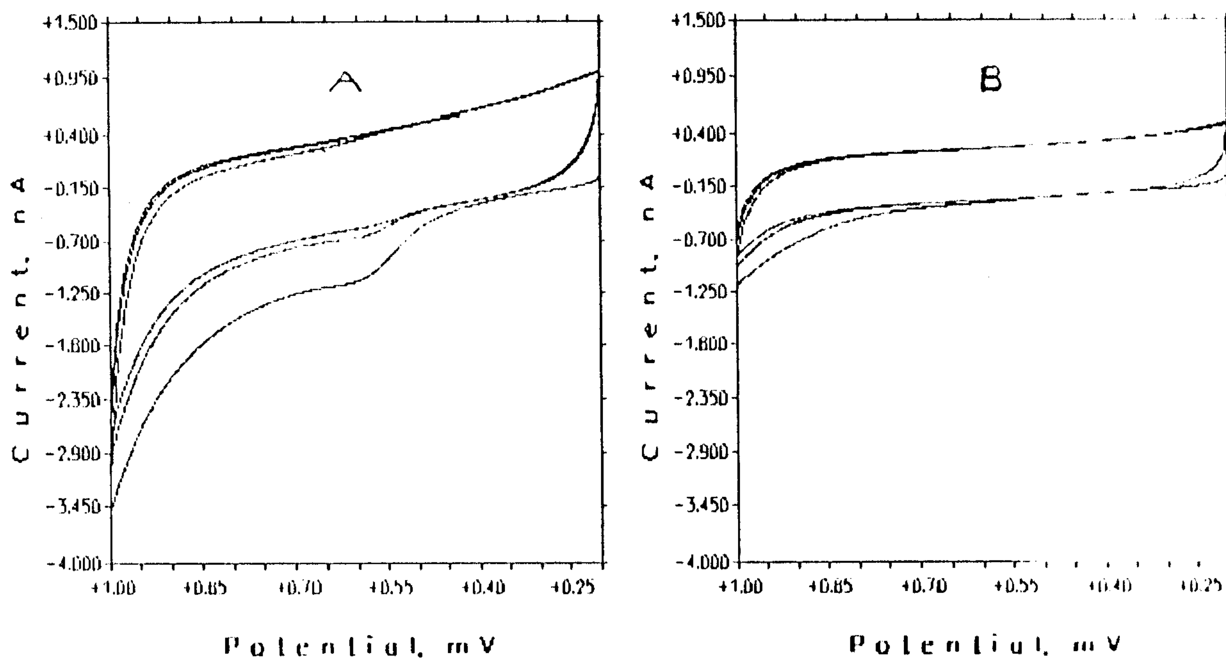


Figure 2 Cyclic voltammograms of the poly(1,3-phenylenediamine) (A) and poly(1,3-phenylenediamine)–glucose oxidase (B) electrodes in 0.1*M* KCl.

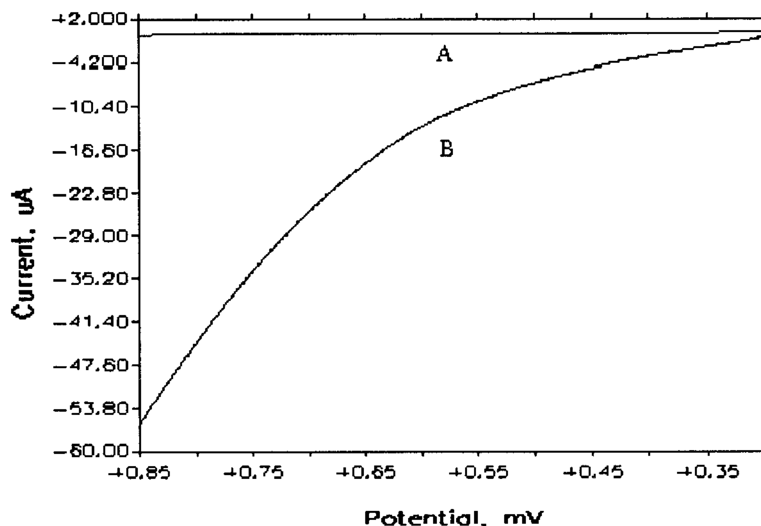
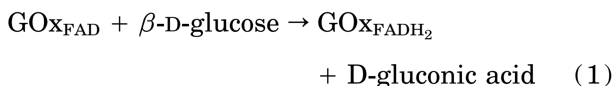


Figure 3 The linear sweep voltammograms of the bare pt electrode in the absence (A) and presence of 50 m*M* hydrogen peroxide (B) in PBS.

charged at the pH 6 used for the electrodeposition and is presumably incorporated as the counter ion into the polymer matrices.[21] Therefore, polymerization of the 1,3-phenylenediamine monomer was conducted at a pH of 6.5.

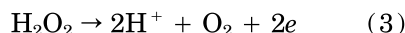
Use of the Enzyme Electrode as a Glucose Sensor

The overall reaction of enzyme (glucose oxidase) in the presence of O_2 as an electron acceptor involves the catalyzed oxidation of β-D-glucose to gluconic acid and electroactive hydrogen peroxide. The enzymatic reaction can be described as follows.



$$\mathrm{GOx_{FAD}} + \beta\text{-D-glucose} \rightarrow \mathrm{GOx_{FADH_2}} + \text{D-gluconic acid} \quad (1)$$

$$\mathrm{GOx_{FADH_2}} + O_2 \rightarrow \mathrm{GOx_{FAD}} + H_2O_2 \quad (2)$$



$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e \quad (3)$$

The formed hydrogen peroxide as a result of enzymatic reaction then diffuses towards the platinum surface, and the amperometric response is determined by measuring the anodic current.[22] The linear sweep voltammograms of the polymer electrode in the absence and the presence of 50 m*M* hydrogen peroxide in PBS are shown in Figure 3. As could be easily seen from the figure, we decided that electroactive hydrogen peroxide could be determined directly at a potential of 0.700 V versus Ag–AgCl on the poly(1,3-phenylenediamine) electrode.

In order to determine the range of operation for poly(1,3-phenylenediamine)–glucose oxidase electrode, the steady-state amperometric responses to the addition of stock glucose solution were measured as a function of time, as indicated Figure 4. Also, Figure 5 shows the typical response curve for enzyme electrode. As shown in Figure 6, the electrode gave a linear, steady-state amperometric response up to 6 m*M* glucose and became nonlinear above 6 m*M*. It is important to obtain this linear relation between glucose concentration and amperometric current as human blood glucose concentration lies within the narrow limits of 3.5 to 5 m*M*.[22]

Effect of Temperature

The effect of temperature on the steady-state amperometric response of the enzyme electrode was investigated between 293–353 K. The amperometric current increased with increasing temperature (approximately 0.18 nA/K), reaching a maximum response at approximately 323 K, and then decreased, as demonstrated in Figure 7. The enzyme electrode used in temperature measurement then gave a minimum response to glucose injection when compared with a previous response, due probably to thermal inactivation of the enzyme immobilized in polymeric matrix.

The Arrhenius form of the temperature depen-

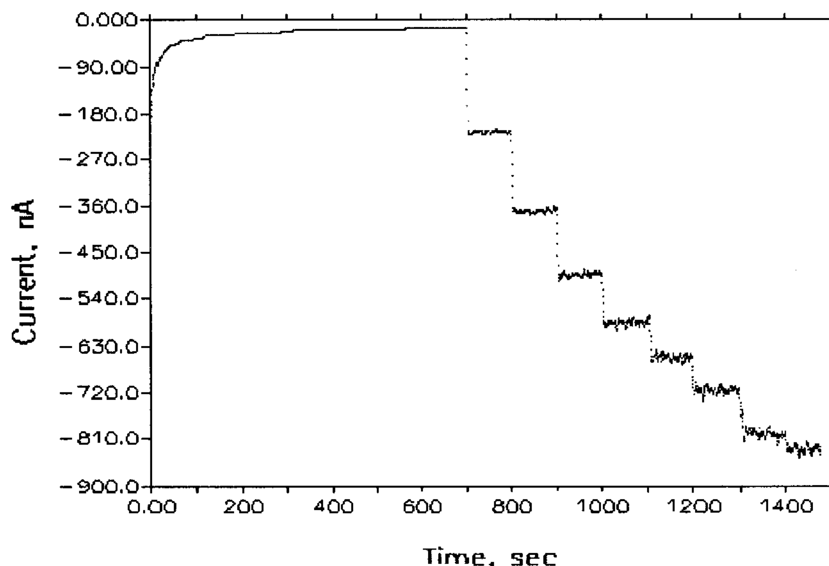


Figure 4 The steady-state amperometric responses to the addition of stock glucose solution of the enzyme electrode.

dence of the amperometric current is shown in the following equation:

$$I = I^0 \exp(-E_a/RT)$$

where I^0 represents a collection of constants, R is the gas constant, T is the temperature in Kelvin degrees, and E_a is the activation energy. The activation energy for enzymatic reaction was calculated to be 36.3 kJ/mol from the slope of the line of best-fit of a graph in which I is plotted on the logarithmic scale versus the reciprocal of the temperature.[23]

Effect of Buffer Concentration

The effect of buffer concentration at a constant salt concentration (NaCl and KCl) on the amperometric response of the biosensor was examined from 0–15 mM for buffer concentrations. As depicted in Figure 8, the optimal buffer concentration was found to be 5 mM.

Specificity to Glucose of the Biosensor

Figure 9 reveals that the amperometric response to glucose injections of the poly(1,3-phenylenediamine) electrode that contains no enzyme. As ex-

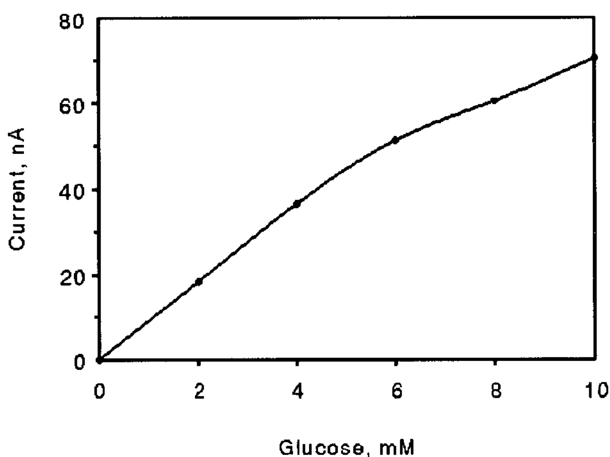


Figure 5 Calibration curve for glucose of the enzyme electrode.

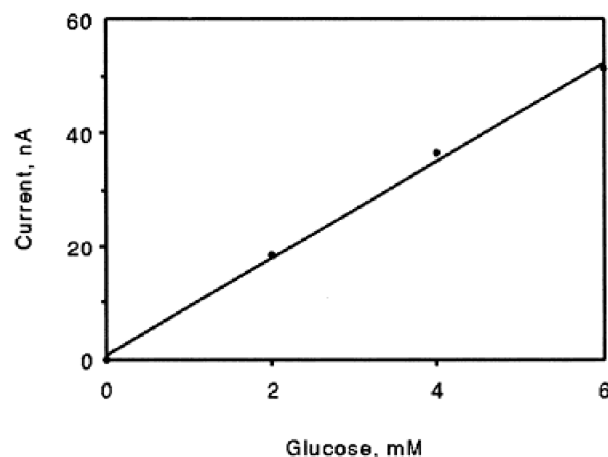


Figure 6 The linear portion of the calibration curve for glucose of the enzyme electrode.

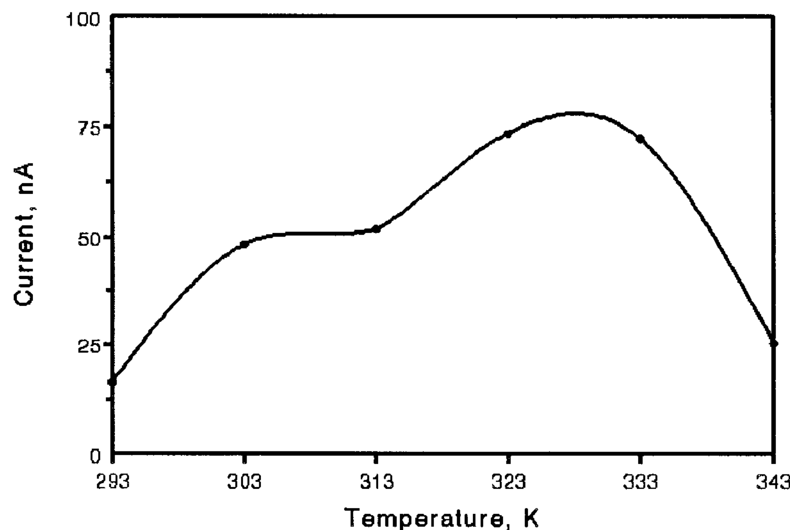


Figure 7 Effect of temperature on the response of the enzyme electrode.

pected, the polymer electrode did not give any anodic response to the successive glucose injections. This evidence confirms that amperometric responses obtained by using enzyme electrode are the result of the enzymatic reaction between glucose and glucose oxidase in the poly(1,3-phenylenediamine) matrix.

On the other hand, the required potential to oxidize the hydrogen peroxide generated as a result of enzymatic reaction is sufficiently anodic (700 mV versus Ag–AgCl). This anodic potential is enough for the oxidation of possible interferants, such as ascorbic acid, paracetamol, and uric acid. Therefore, these anodic currents from the interferants mentioned could contribute to the amperometric response observed due to glucose.

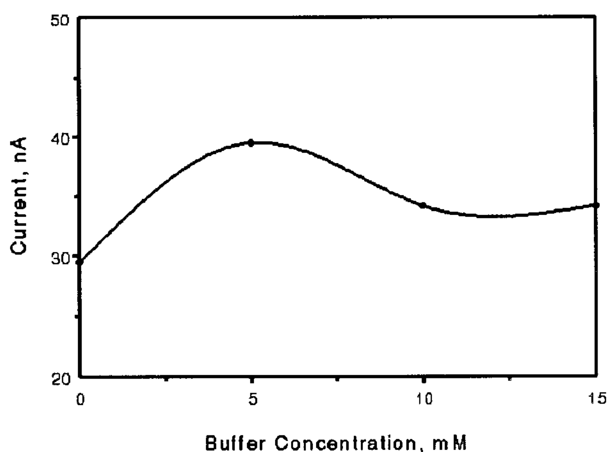


Figure 8 The effect of buffer concentration on the amperometric response of the enzyme electrode.

The glucose specificity of the enzyme electrode in PBS solution containing interfering substances, such as lactose, sucrose, urea, ascorbic acid, paracetamol, and uric acid, was tested by recording the current generated for successive 2 m*M* glucose injections. As could be seen in Figure 10, the sensor gave response to the successive glucose injections in the presence of all the interferants.

The Stability of the Poly(1,3-phenylenediamine)–GOx Electrode

The stability of the enzyme electrodes was monitored periodically by measuring their steady-state amperometric responses obtained by 2 m*M* glucose injections. In order to determine the ideal storage medium, electrodes were stored both in air (−10°C) and in PBS solution (+4°C) when not in use. A plot of steady-state amperometric response versus age is shown in Figure 11. As can be easily seen in figure, sensors were very stable. However, it is also seen that the glucose sensitivity of the electrode stored in PBS solution at 4°C is higher than that of the other electrode. The increase in the amperometric response to glucose injections in the first stages of the stability measurements may be attributed to the opening of the polymeric channels. Then, as expected, amperometric response decreases due to denaturation or leaching out of enzyme.

When compared to the other sensors, such as poly(*o*-phenylenediamine),[14] polyindole,[20] polypyrrole,[24] poly(*p*-aminophenol),[25] and poly(*p*-phe-

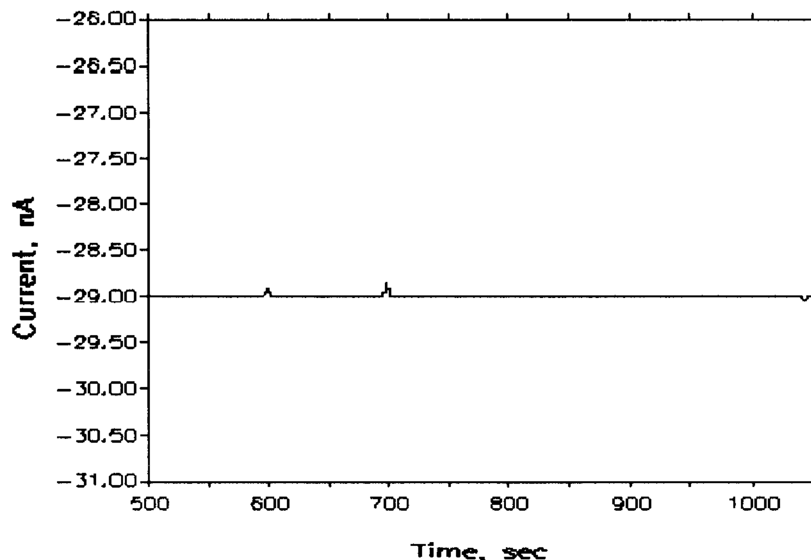


Figure 9 The amperometric response to glucose injection of the poly(1,3-phenylenediamine) electrode, starting from the 600th s, 2.0 mM glucose was injected at every 100th s. Spikes belong to disturbance of the system.

nylenediamine),[26] reported in the literature, it is clear that the sensor shows much higher stability.

As a result, we have demonstrated that the sensor based on poly(1,3-phenylenediamine) could be prepared easily by the electropolymerization of the relevant monomer. The some advantages of the resultant polymeric sensor are as follows.

1. The sensor has been prepared in a one-step procedure that took approximately 25 min (for example, a short immobilization period).
2. The steady-state amperometric response to glucose of the sensor is fast (for example, rapid glucose determination).
3. When compared to other polymeric sensors, the stability of this sensor seems quite satisfactory.
4. The linear relation up to 6 mM glucose allows

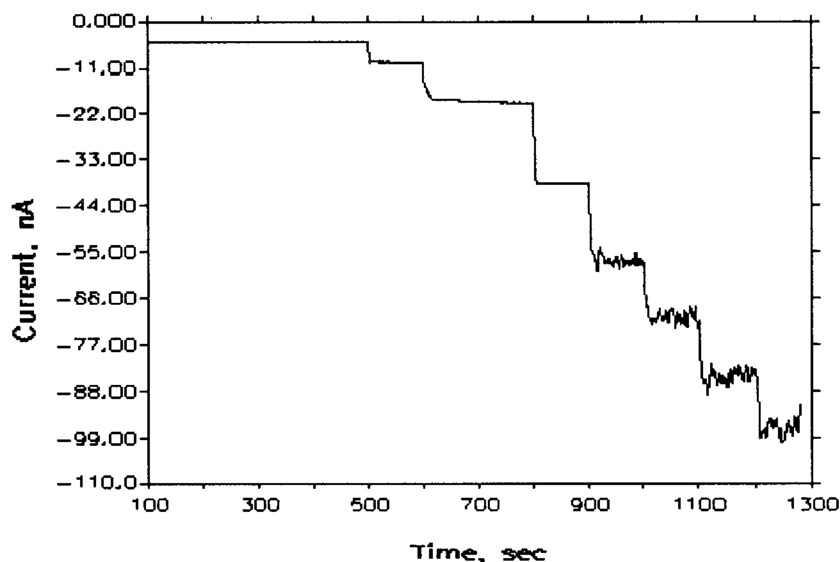


Figure 10 The specificity of the enzyme electrode. Injections are as follows: 200th s, sucrose; 300th s, urea; 400th s, lactose; 500th s, ascorbic acid; 600th s, paracetamol; 700th s, uric acid. Starting at the 800th s, 2.0 mM glucose injections were made at every 100th s.

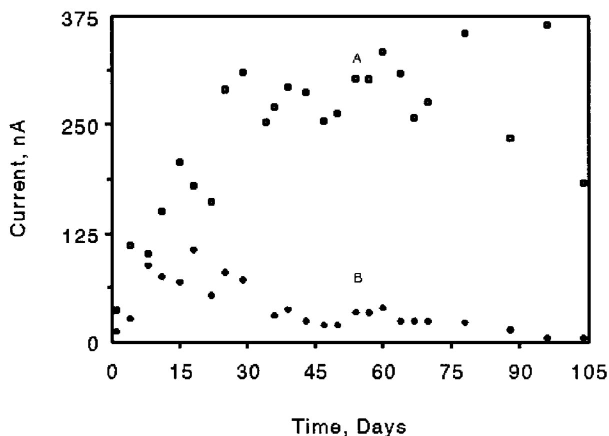


Figure 11 Stability of the amperometric response of the enzyme electrodes in PBS (A) and in air (B).

the sensor to be used in biomedical analysis for diabetic patients.

5. The fact that sensor response was unaffected in the presence of the interfering substances reveals that it could be used in the blood matrix.

This work has been supported by the Scientific and Technical Research Council of Turkey (TÜBİTAK) through grant KTÇAG-DPT-6.

REFERENCES

1. N. Mermillod, J. Tanguy, and F. Petiot, *J. Electrochem. Soc.,* **133,** 947 (1986).
2. T. Osaka, K. Naoi, H. Sakai, and S. Ogano, *J. Electrochem. Soc.,* **134,** 285 (1987).
3. T. Matsunaga, H. Difulen, T. Nakajima, and T. Kawage, *Polym. Adv. Technol.,* **1,** 33 (1990).
4. S. Kuwabata, H. Yoneyama, and H. Tamura, *Bull. Chem. Soc. Jpn.,* **57,** 2247 (1984).
5. R. Bjorklund, S. Andersson, S. Allenmark, and I. Lundstrom, *Mol. Cryst. Liq. Cryst.,* **122,** 263 (1985).
6. S. Sathiyanarayanan, S. K. Dhawan, D. C. Trivedi, and K. Balakrishnan, *Corros. Sci.,* **33,** 1831 (1992).
7. N. C. Foulds and C. R. Lowe, *J. Chem. Soc.,* **82,** 1259 (1986).
8. G. Fortier, E. Brassard, and D. Belanger, *Biosens. Bioelectron.,* **5,** 473 (1990).
9. P. N. Bartlett and R. G. Whitaker, *J. Electroanal. Chem.,* **224,** 37 (1987).
10. P. De Taxis du Poet, S. Miyamoto, T. Murakami, J. Kimura, and I. Karube, *Anal. Chim. Acta,* **263,** 235 (1990).
11. R. Hintsche, G. Neumann, I. Dransfeld, G. Kampfrath, B. Hoffmann, and F. Scheller, *Anal. Lett.,* **22,** 2175 (1989).
12. S. V. Sasso, R. J. Pierce, R. Walla, and A. M. Yacynych, *Anal. Chem.,* **62,** 1111 (1990).
13. D. Centonze, A. Guerrieri, C. Malitesta, F. Palmisano, and P. G. Zambonin, *Ann. Chim.,* **82,** 2219 (1992).
14. C. Malitesta, F. Palmisano, L. Torsi, and P. G. Zambonin, *Anal. Chem.,* **62,** 2735 (1990).
15. J. Liu, M. Chen, and M. Lin, *J. Appl. Polym. Sci.,* **40,** 2161 (1990).
16. G. H. Hsiue, Z. S. Chou, K. P. Hsiung, and N. Yu, *Polym. Mater. Sci. Eng.,* **57,** 825 (1987).
17. G. H. Hsiue, Z. S. Chou, N. Yu, and K. P. Hsiung, *J. Appl. Polym. Sci.,* **34,** 319 (1987).
18. T. Yamatoto, K. Sanechica, and A. Yamatoto, *J. Polym. Sci., Polym. Lett. Ed.,* **18,** 9 (1980).
19. E. R. Reynolds and A. M. Yacynych, *Electroanalysis,* **5,** 405 (1993).
20. P. C. Pandey, *J. Chem. Soc.,* **84,** 2259 (1988).
21. A. F. Diaz, J. I. Castillo, J. A. Logan, and W.-Y. Lee, *J. Electroanal. Chem.,* **129,** 115 (1981).
22. G. Fortier, E. Brassard, and D. Belanger, *Biotechnol. Tech.,* **2,** 177 (1988).
23. G. Fortier, E. Brassard, and D. Belanger, *Biosens. Bioelectron,* **5,** 473 (1990).
24. E. Ekinci, M. Özden, A. A. Karagözler, H. M. Türkdemir, and A. E. Karagözler, *Doğa, Tr. J. Chem.,* **19,** 170 (1995).
25. E. Ekinci, A. A. Karagözler, and A. E. Karagözler, *Electroanalysis,* **7,** 1 (1995).
26. E. Ekinci, A. A. Karagözler, and A. E. Karagözler, *Synth. Met.,* **79,** 57 (1996).