

Immobilization of Glucose Oxidase onto Electrochemically Prepared Poly(aniline-co-fluoroaniline) Films

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ABSTRACT: Poly(aniline-co-fluoroaniline) [poly(An-FAn)] films were electrochemically deposited on indium tin oxide glass plates. The characterization of these conducting copolymer films was carried out with ultraviolet-visible and Fourier transform infrared techniques. The enzyme glucose oxidase (GOX) was immobilized onto the conducting poly(An-FAn) films by a physical adsorption method. Amperometric

response experiments carried out with poly(An-FAn)/GOX films revealed linearity from 0.5 to 22 mM glucose, and the Michaelis-Menten constant was found to be about 23.5 mM. The shelf life of these poly(An-FAn)/GOX electrodes was measured to be about 15 days, and the electrodes were stable up to 45°C. © 2004 Wiley Periodicals, Inc. *J Appl Polym Sci* 91: 3999–4006, 2004

INTRODUCTION

Analytical devices combining the specificity of enzymatic reactions and the high sensitivity of electrochemical transduction have attracted increasing interest in the last decade.^{1,2} Efforts have recently been directed toward the fabrication of a biosensor for the estimation of glucose with glucose oxidase (GOX).³ Puig-Lleixà et al.⁴ trapped GOX in an acrylated polyurethane photopolymeric membrane. The sensor showed a sensitivity of 1.68 A/mM in a linear range of 0.1–5 mM glucose. The response time was 30 s, and the lifetime was about 4 weeks. Conducting polymers have been used as potential systems for the immobilization of enzymes.^{5–7} In these systems, there is a direct transfer of electrons to and from the enzymes. The entrapment of enzymes in conducting polymer films provides a controlled method of localizing biologically active molecules in defined areas on the electrodes. These enzyme-immobilized polymer electrodes have been shown to have enhanced lifetimes and fast response times and to suppress the effect of

interfering reactions.^{8–10} Most of the work on the immobilization of GOX in conducting polymer films has been concentrated on conducting polymers such as polypyrrole (PPY) and polyaniline (PANI).^{11–16} The entrapment of enzyme (GOX) in a growing conducting polymeric film in a one-step process during electropolymerization has also been reported.^{17–21}

Foulds and Lowe²² entrapped GOX in a PPY matrix electrochemically deposited on a printed platinum electrode. Cooper and Hall²³ reported the electrochemical preparation of GOX-loaded PANI films. It has been found that PANI films exhibit an enhanced loading of GOX after self-exchange and, therefore, can be used for the fabrication of third-generation glucose biosensor.²⁴ Recently, Kotowski et al.²⁵ immobilized GOX with glutaraldehyde in a conductive lecithin PPY bilayer membrane. The peak current exhibited a non-linear dependence on the concentration of glucose between 1 and 20 mmol/L.

Copolymers, in general, exhibit different physical and mechanical properties than homopolymers. The properties of copolymers can also be modified through the variation of either the ratio of the various constituents or the manner in which they are chemically attached. Copolymerization is known to be a powerful method for effecting systematic changes in polymer properties.²⁶ Nazzal and Street²⁷ synthesized a conducting copolymer by grafting pyrrole with derivatized polystyrene. The copolymer exhibited improved electrical conductivity that was comparable to that of other polymers. It was recently reported that considerable improvements in the mechanical properties of PPY were obtained by copolymerization with phenylene oxide²⁸ and naphthalene oxide.²⁹ Pandey et al.²⁶ synthesized poly(aniline-co-orthoanisidine) by

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Contract grant sponsor: DST Project; contract grant number: SP/S2/M-52/96.

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electrocopolymerization, and the electrical conductivity of the copolymer was found to be 1.2×10^{-2} S/cm, two orders of magnitude less than that of electrochemically synthesized PANI. This electrical behavior was due to the incorporation of *o*-anisidine moieties into PANI chains. The chemical and electrochemical synthesis of a copolymer of aniline and fluoroaniline {poly(aniline-*co*-fluoroaniline) [poly(An-FAn)]} was reported in an earlier publication.³⁰ Substituted PANIs possess enhanced processability and high thermal stability,^{13,14,30} and so they can be used for biosensor applications.

In this study, efforts were made to immobilize GOX on electrochemically synthesized poly(An-FAn) films. These conducting copolymer films were characterized with ultraviolet-visible (UV-vis), Fourier transform infrared (FTIR), electrical conductivity, scanning electron microscopy (SEM), and electrochemical techniques. To the best of our knowledge, no previous efforts were made to immobilize enzymes on conducting copolymers for the fabrication of biosensors.

EXPERIMENTAL

Aniline (Sigma) and 2-fluoroaniline (Fluka) were distilled before use. D(+)-Glucose, hydrochloric acid (HCl), dipotassium hydrogen orthophosphate (K_2HPO_4), potassium dihydrogen orthophosphate ($KH_2PO_4 \cdot H_2O$), and methanol (CH_3OH), all from Merck, and GOX (EC 1.1.3.4), from Sigma, were used as received without further purification. Aqueous solutions were prepared in doubly distilled deionized water obtained with a Millipore Milli-RO 10 TS water purification system.

The electrochemical polymerization and cyclic voltammogram studies of poly(An-FAn) were carried out with a Schlumberger SI 1286 electrochemical interface. Electrical conductivity measurements of electrochemically synthesized copolymer films were conducted with a four-point-probe technique. UV-vis measurements were carried out on a Shimadzu 160 A UV-vis spectrophotometer, and FTIR spectra of the copolymer films were recorded with a Nicolet 510 P spectrometer. SEM micrographs were obtained with a Leo 440 instrument.

Preparation of the conducting poly(An-FAn) films

Aniline (0.1 mL) and 0.1 mL of fluoroaniline (predistilled) were dissolved in 15 mL of 1M HCl. Poly(An-FAn) films (1.0 cm²) were electrochemically polymerized onto indium tin oxide (ITO) glass plates (1.5 cm \times 1.0 cm) at a constant current of 2 mA, with ITO-coated glass plates used as the working electrodes and platinum foil (1.5 cm \times 1.0 cm) used as a counter electrode. These conducting polymer films were washed three times with deionized water and dried in air.

Preparation of the glucose solution

A glucose stock solution (100 mL, 50 mM) was prepared in a 0.1M phosphate buffer solution (pH 7.0). The stock solution was left for about 24 h for meta rotation before use. Glucose solutions of different concentrations (0.5–28 mM) were prepared by further dilution with a phosphate buffer.

Fabrication of the enzyme electrode

GOX (5 mg) was dissolved in 1 mL of a phosphate buffer (0.1M, pH 7.0) to prepare a 1 unit/ μ L solution of GOX. One unit of GOX is defined as the amount of enzyme that will oxidize 1 μ m of glucose to gluconic acid per minute. Five microliters of this solution was immobilized on conducting poly(An-FAn) films, which were coated onto ITO glass plates by a physical adsorption technique. These films were then washed with water for the removal of excess GOX from the surface and were dried in air for about 25°C.

RESULTS AND DISCUSSION

Electrical conductivity

The electrical conductivity of an electrochemically synthesized poly(An-FAn) film on an ITO glass plate was measured by a four-point-probe technique and was found to be about 2.72×10^{-3} S/cm. This value of electrical conductivity was two orders of magnitude higher than that of poly(2-fluoroaniline) and three orders of magnitude less than that of PANI. This could be attributed to the incorporation of less conducting fluoro moieties into the PANI chain during electrocopolymerization.⁴

FTIR studies

Figure 1 shows the FTIR spectra of electrochemically synthesized poly(An-FAn) films on ITO glass plates with physically immobilized GOX. The spectra show characteristic peaks at 1593, 1508, 1304, 1163, 1020, and 827 cm⁻¹. The 1593-cm⁻¹ peak arose from C=C due to quinoid rings, whereas the 1508-cm⁻¹ peak was attributed to a combination of C—N in quinoid and benzanoid sequences. The peaks observed at 1163 and 1304 cm⁻¹ were ascribed to the presence of halogen (fluoro) groups in the copolymer film.²⁵ The 827-cm⁻¹ peak was attributed to the C—H vibration band of the para-linked phenyl ring.

UV-vis studies

Figure 2(a) shows the UV-vis spectrum of electrochemically synthesized poly(An-FAn) cast onto ITO glass plates obtained with reference to the ITO glass plates. The spectra shows absorption bands at 343 and

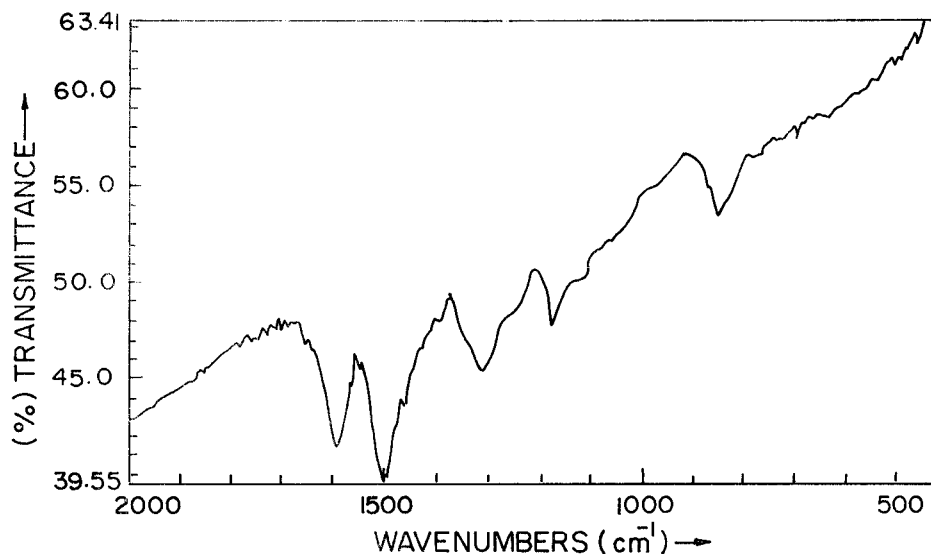


Figure 1 FTIR spectra of electrochemically synthesized poly(An-FAN) films.

422 nm. These peaks were assigned to $n-\pi^*$ and $\pi-\pi^*$ transitions corresponding to C=C and C—N chromophores, respectively.

The band gap of the electrochemically synthesized copolymer from the aforementioned absorption spectra was calculated from a plot of $(\alpha h\nu)^{-2}$ versus photon energy ($h\nu$) shown in Figure 2(b).³¹ The energy band gap was estimated to be 2.39 eV, which is lower than that of chemically synthesized poly(An-FAN).³⁰

SEM studies

A SEM micrograph [Fig. 3(a)] shows the fibrillar morphology of a poly(An-FAN) film. This fibrillar structure of the copolymer provided increased space for the adsorption of the enzyme (GOX). The globular structure shown in Figure 3(b) indicates the presence of GOX³² on the copolymer film.

Cyclic voltammetry of poly(An-FAN)

Figure 4 shows a cyclic voltammogram of poly(An-FAN) films obtained in 1M HCl at a scanning rate of 50 mV/s. Two anodic peaks, similar to those of PANI (0.28 V)³³ and polyfluoroaniline (0.47 V)⁴ versus Ag/AgCl, can be clearly seen. They were attributed to the radical cations of PANI and polyfluoroaniline obtained because of the copolymerization. The first oxidation peak obtained for poly(An-FAN) was shifted toward a higher potential than PANI and toward a lower potential than polyfluoroaniline. The third oxidation peak (0.7 V) was attributed to the second oxidation peak of polyfluoroaniline. In light of these results, we expected that the conjugation length of the copolymer would lie between

those of PANI and polyfluoroaniline, as observed by Genies and Lapkowski.³³

To ascertain the electroactive nature of the GOX/poly(An-FAN) films, we carried out cyclic voltammetry experiments in the presence of glucose (10 mM) in a phosphate buffer (0.1M, pH 7.0). The GOX/poly(An-FAN) immobilized films were used as working electrodes, platinum was used as the counter electrode, and Ag/AgCl was used as the reference electrode. The cyclic voltammogram (Fig. 5) reveals that the oxidation of glucose began after 0.2 V, with the maximum oxidation at 0.48 V. For this reason, 0.2 V was used as the bias voltage for the amperometric measurements as a function of the glucose concentration.

Enzyme activity measurements

The activity measurements for GOX immobilized in poly(An-FAN) films were carried out by a spectrophotometric method (Sigma). The reaction mixture consisted of glucose (2.4 mL; 10%, w/v) and *o*-dianisidine (0.5 mL) in a sodium acetate buffer (0.05M, pH 5.1) in the presence of horseradish peroxidase (100 μ L). The films were incubated for about 2 min at room temperature, and the color was monitored at 500 nm.

The apparent enzyme (GOX) activity immobilized on conducting poly(An-FAN) films was estimated with the following equation:

$$U \text{ (cm}^{-2}\text{)} = AV / \epsilon st \quad (1)$$

where A is the difference in the absorbance at 500 nm before and after the incubation of a poly(An-FAN)/GOX film in a glucose solution, V is the total volume of the assay (3.0 mL), ϵ is the millimolar extinction

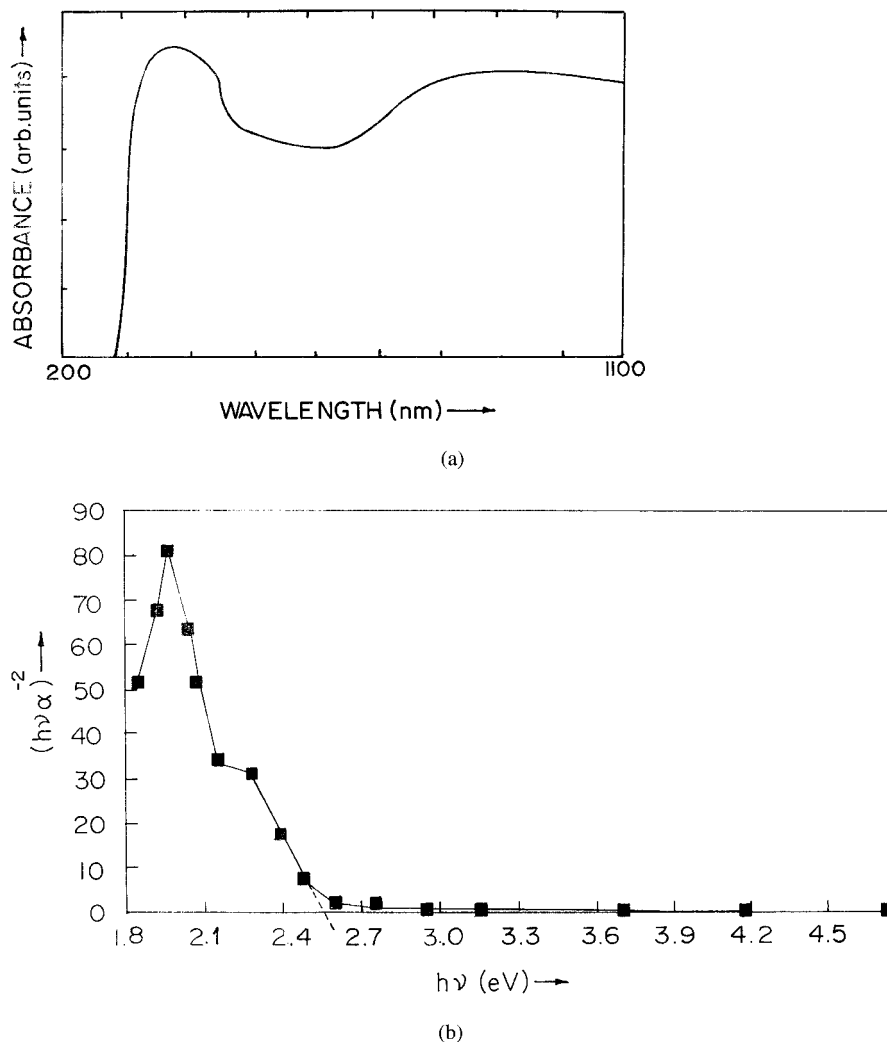


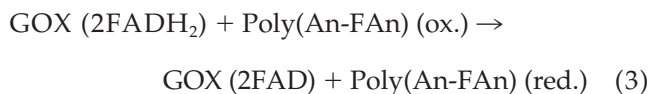
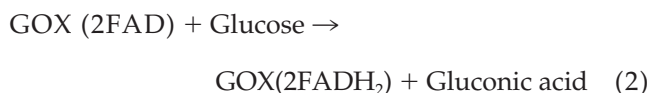
Figure 2 (a) UV-vis spectrum of chemically prepared poly(An-FAn) films and (b) plot of $h\nu$ versus $(\alpha h\nu)^{-2}$ for the band gap of electrochemically synthesized poly(An-FAn).

coefficient (7.5) of *o*-dianisidine, s is the surface area of the film, and t is the incubation time (2 min). U is the enzyme activity of GOX [unit/cm² of poly(An-FAn)/GOX film]. One unit of GOX is defined as the amount of enzyme that will oxidize 1 μ m of glucose to gluconic acid per minute. The activity of GOX in the poly(An-FAn)/GOX films was found to be about $2U$. These experiments revealed that the enzyme remained active in the immobilized state.

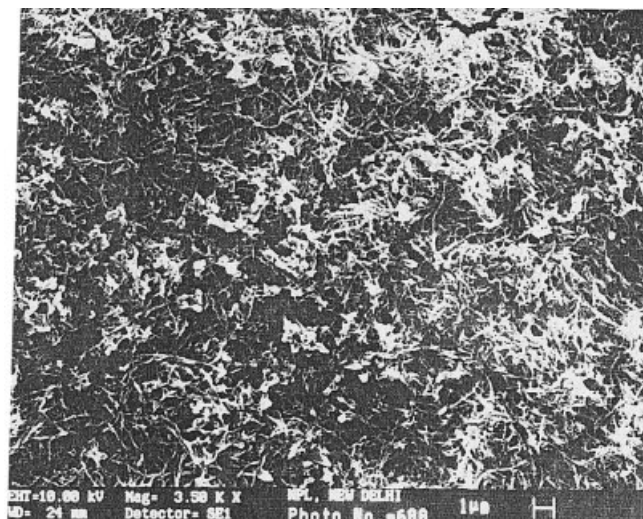
Amperometric response

Amperometric responses were determined in a two-electrode cell containing a poly(An-FAn)/GOX-coated ITO glass plate as a working electrode and Pt foil as a counter electrode in a 0.1M phosphate buffer and glucose solution (pH 7.0).

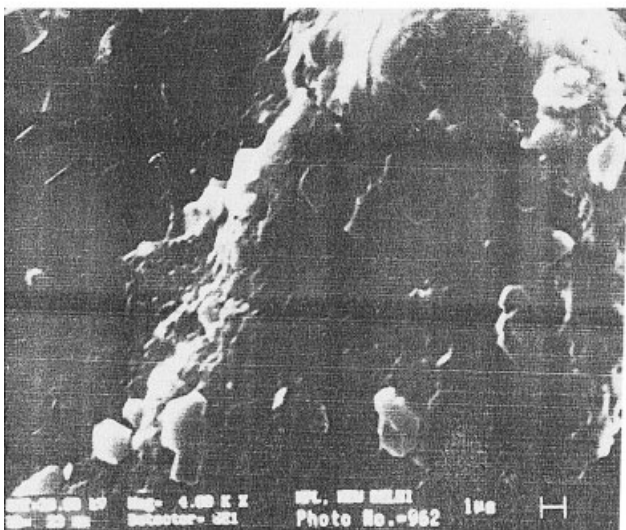
The reaction between GOX and its substrate glucose occurred as follows:⁹



This reaction suggests that the conducting copolymer substrate not only acted as a matrix for the immobili-



(a)



(b)

Figure 3 SEM micrographs of poly(An-FAn) films (a) with the enzyme and (b) without the enzyme.

zation but also acted as an electron-transferring medium during the electrochemical process.

Figure 6 shows the response curve of (GOX)/poly(An-FAn) films with various concentrations of glucose. These (GOX)/poly(An-FAn) electrodes exhibited linearity with 0.5–22 mM glucose and had a response time of about 60 s.

Substrate kinetics

The relationship between the substrate concentration and reaction rate may be described by the Michaelis-Menten equation :

$$v_0 = v_{\max} S / (K_m + S) \quad (5)$$

where v_0 is the initial rate of reaction, v_{\max} is the maximum rate of reaction, S is the solution concentration (glucose in this case), and K_m is the Michaelis-Menten constant. The K_m value characterizes the affinity between the substrate and the enzyme. A low K_m value reflects high affinity. At low substrate concentrations ($S \ll K_m$), the reaction rate is directly proportional to the substrate concentration (first-order reaction); at high concentrations ($S \gg K_m$), the reaction is zero-order and no longer dependent on the substrate concentration but is only dependent on the enzyme activity.

K_m can be calculated from a Lineweaver-Burke plot. A Lineweaver-Burke plot contains reciprocal values of v_0 and S on the Y and X axes, respectively. The Lineweaver-Burke equation is as follows:

$$1/v_0 = 1/v_{\max} (1 + K_m/S) \quad (6)$$

The relevant plot gives K_m and v_{\max} at the intercepts of the curve with the abscissa and the ordinates, respectively.

A Lineweaver-Burke plot (Fig. 7), obtained from Figure 6, gave a K_m value for poly(An-FAn)/GOX electrodes through extrapolation of 23.5 mM.

Shelf life

The shelf life of an enzyme electrode is defined as the storage time of the electrode up to which it can be used for the detection of a substrate. Poly(An-FAn)/GOX electrodes were tested for stability under the same operating conditions used for amperometric response measurements. The responses of the poly(An-FAn)/GOX electrodes were measured once a week. The responses were measured on fresh electrodes. The enzyme electrodes were stored at 4–10°C when not in use. Figure 8 shows the amperometric responses of poly(An-FAn)/GOX electrodes in a 5.1 mM glucose solution in a phosphate buffer (pH 7) as a function of the time (measured in days). The response current was found to be about 80% of its maximum value in 15 days. Therefore, these poly(An-FAn)/GOX electrodes can be used for glucose estimation within 15 days.

Effect of the temperature

Poly(An-FAn)/GOX electrodes were tested for thermal stability by an amperometric method. All measurements were taken in a measuring cell with a poly(An-FAn)/GOX electrode as the working electrode and Pt foil as the counter electrode. Each measurement was taken on a fresh electrode with a glucose concentration of 5.1 mM. Figure 9 shows the response current of a poly(An-FAn)/GOX electrode as a function of temperature. The response current of the poly(An-FAn)/GOX electrodes increased up to 45°C, after which the response current decreased abruptly at

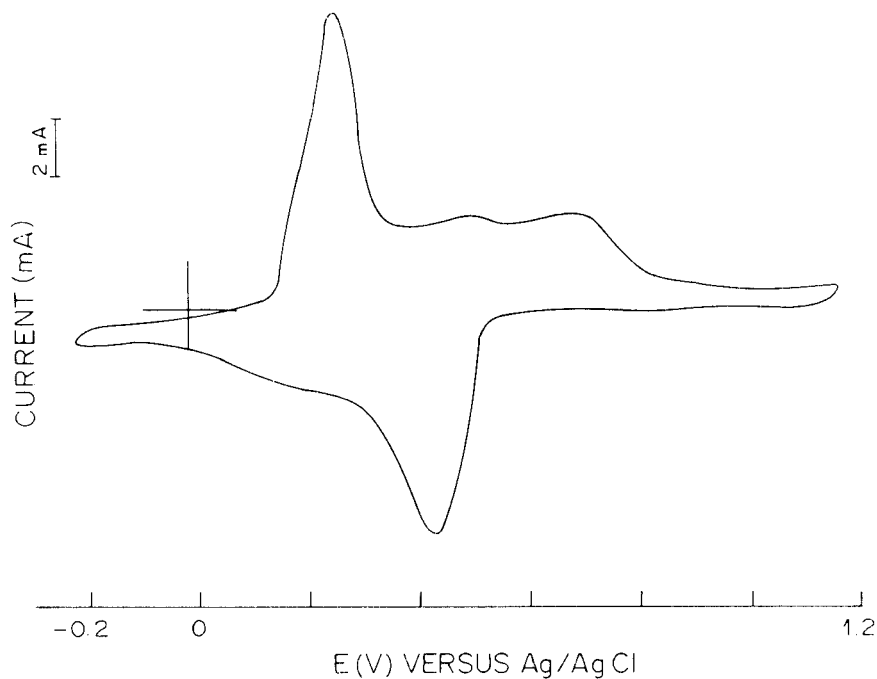


Figure 4 Cyclic voltammetry of a poly(An-FAn) film.

50°C. These poly(An-FAn)/GOX electrodes can be used for the estimation of glucose over a wide range of temperatures, from 25 to 45°C.

Effect of the interferents

Certain interferents such as ascorbic acid and uric acid affect the response of glucose biosensors. These are the

major interferents in blood. The effect of interferents was studied in the presence of their physiologically normal levels with 5.1 mM glucose. This was accomplished through the measurement of the response current for 5.1 mM glucose at pH 7.0 and 30°C in the presence of ascorbic acid (0.1 mM) or uric acid (0.5 mM). This is the normal level present in blood. The error in the response current was within 5%, and this

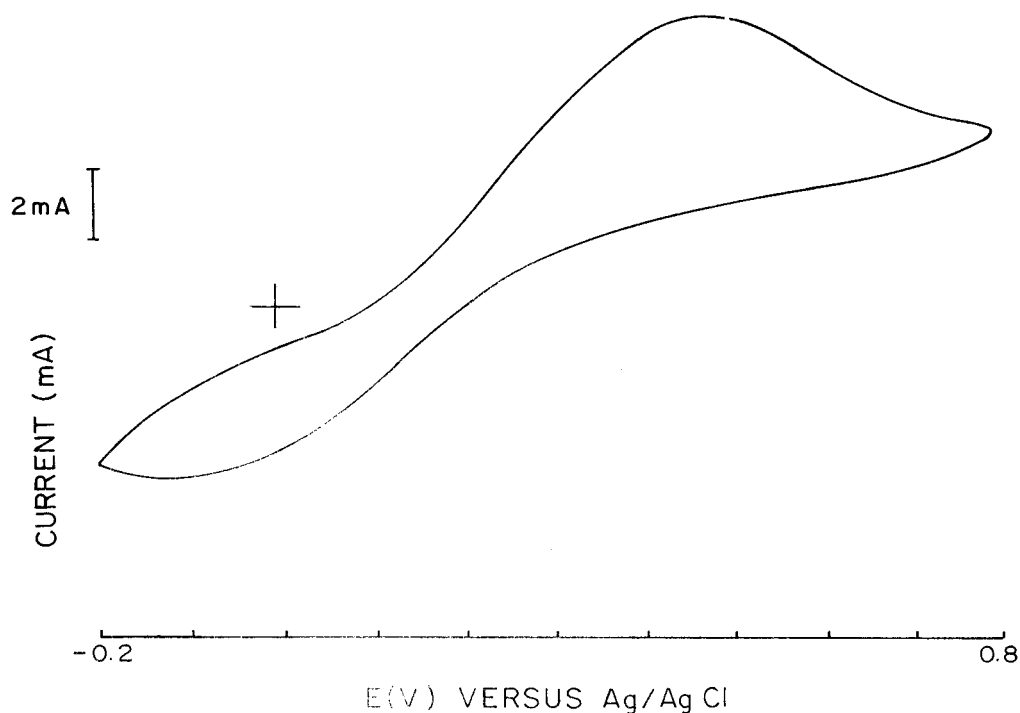


Figure 5 Cyclic voltammogram of a poly(An-FAn)/GOX film with GOX.

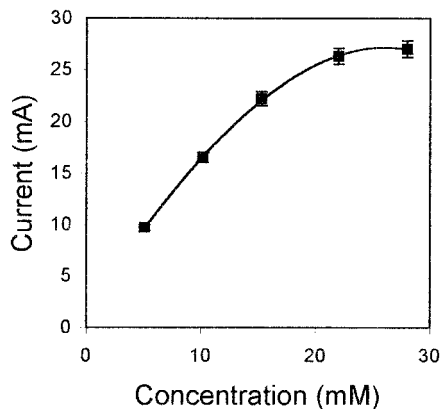


Figure 6 Amperometric response of enzyme poly(An-FAn)/GOX films obtained as a function of the glucose concentration.

indicated that there was no significant effect of the interferent on the glucose sensor based on a poly(An-FAn)/GOX film.

Table I compares the results obtained for a poly(An-FAn)/GOX electrode and other conducting-polymer-based glucose biosensors reported in the literature.

CONCLUSIONS

It has been demonstrated that electrochemically prepared poly(An-FAn) films can be used for the physical adsorption of GOX for the fabrication of glucose biosensors. These poly(An-FAn)/GOX electrodes

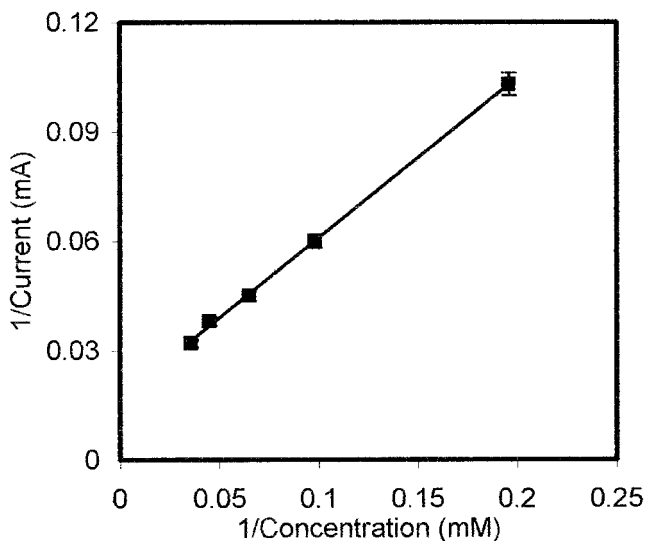


Figure 7 Lineweaver-Burke plots for GOX immobilized on poly(An-FAn) films.

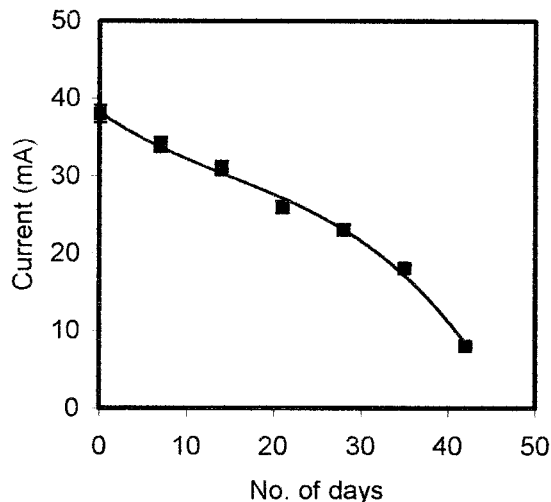


Figure 8 Response of poly(An-FAn)/GOX electrodes as a function of the storage time in the presence of glucose (5.1 mM) in a phosphate buffer (pH 7).

can be used to estimate glucose concentrations from 0.5 to 22 mM, and they have a response time of about 60 s. The poly(An-FAn)/GOX films are stable up to 45°C, and the shelf life of these electrodes is 15 days at 4°C.

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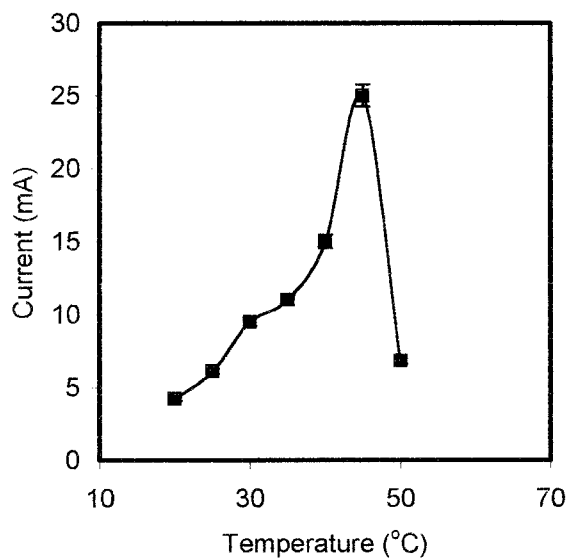


Figure 9 Effect of the temperature (25–50°C) on the activity of GOX immobilized on poly(An-FAn) films in a phosphate buffer (pH 7.0).

TABLE I
Responses of the Various Conducting Polymer Biosensors Using GOX

Matrix	Principle	Linearity (mM)	Stability (days)	K_m	Reference
Polyindole/GOX	Amperametric	1–20	35	27.3	9
PPY/GOX	Amperametric	1–10	21	30.7	6
PPY/GOX	Amperametric	1–6	—	—	16
PANI/GOX	Amperametric	1–4	5	—	11
PANI–ferricnide/GOX	Amperametric	1–8	—	—	24
PPY/GOX/PPD	Amperametric	1–6	14	—	35
PPY/GOX	Amperametric	1–6	15	22.7	35
Poly(An-FAn)/GOX	Amperametric	0.5–22	15	23.5	This work

References

- Mizutani, F.; Asai, M. In *Bioinstrumentation*; Wise, D. L., Ed.; Butterworths: Boston, 1990; p 317.
- Scheller, F. W.; Schubert, F.; Renneberg, R.; Muller, H. G.; Janchen, M.; Weise, H. *Biosensors* 1985, 1, 135.
- Wilson, R.; Turner, A. P. J. *Biosens Bioelectron* 1992, 7, 165.
- Puig-Lleixà, C.; Jimenez, C.; Bartroli, J. *Sens Actuators B* 2001, 72, 56.
- Sharma, A. L.; Gerard, M.; Singhal, R.; Annapoorni, S.; Malhotra, B. D. *Appl Biochem Appl Biotechnol* 2001, 96, 155.
- Pandey, P. C. *J Chem Soc Faraday Trans* 1988, 84, 2259.
- Collins, G. E.; Buckley, L. J. *Synth Met* 1996, 78, 93.
- Belanger, D.; Nadrean, J.; Fortier, G. J. *J Electrochem Soc* 1989, 274, 143.
- Foulds, N. C.; Lowe, C. R. *J Chem Soc Faraday Trans* 1986, 82, 1259.
- Umana, M.; Waller, J. *Anal Chem* 1986, 58, 2979.
- Ramanathan, K.; Annapoorni, S.; Malhotra, B. D. *Sens Actuators B* 1994, 21, 165.
- Kamin, R. A.; Wilson, G. S. *Anal Chem* 1980, 52, 1199.
- Jinqing, K.; Huaiguo, X.; Shaolin, M.; Hong, C. *Synth Met* 1997, 87, 205.
- Yamato, H.; Koshiba, T.; Ohwa, M.; Wernet, W.; Matsumura, M. *Synth Met* 1997, 87, 231.
- Ramanathan, K.; Sundaresan, N. S.; Malhotra, B. D. *Electroanalysis* 1995, 7, 579.
- Bartlett, P. N.; Whitaker, R. G. J. *Electroanal Chem* 1987, 224, 37.
- Iwakura, C.; Kajiyama, Y.; Yoneyama, H. J. *J Chem Soc Chem Commun* 1988, 1019.
- Sharma, A. L.; Annapoorni, S.; Malhotra, B. D. *Curr Appl Phys* 2003, 3, 239.
- Yabuki, S.; Shinohara, H.; Aizawa, M. J. *J Chem Soc Chem Commun* 1989, 945.
- Malitesta, C.; Palmosano, F.; Torsi, L.; Zambonin, P. G. *Anal Chem* 1990, 62, 2735.
- Kajiyama, Y.; Sugai, H.; Iwakura, C.; Yoneyama, H. *Anal Chem* 1991, 63, 49.
- Foulds, N. C.; Lowe, C. R. *Anal Chem* 1988, 60, 2473.
- Cooper, J. C.; Hall, E. A. H. *Biosensors* 1992, 7, 473.
- Vergese, M. M.; Ramanathan, K.; Ashraf, S. M.; Malhotra, B. D. *J Appl Polym Sci* 1988, 70, 1447.
- Kotowski, J.; Janes, T.; Tien, H. T. *Bioelectrochem Bioeng* 1988, 19, 277.
- Pandey, S. S.; Annapoorni, S.; Malhotra, B. D. *Macromolecules* 1993, 26, 3190.
- Nazzari, A. I.; Street, G. B. *J Chem Soc Chem Commun* 1993, 375.
- Kumar, N.; Malhotra, B. D.; Chandra, S. *J Polym Sci Polym Lett Ed* 1985, 23, 57.
- Malhotra, B. D.; Kumar, N.; Ghosh, S.; Singh, H. K.; Chandra, S. *Synth Met* 1989, 31, 155.
- Sharma, A. L.; Saxena, V.; Annapoorni, S.; Malhotra, B. D. *J Appl Polym Sci* 2001, 81, 1460.
- Mott, N. F.; Davis, E. A. *Electronic Process in Non-Crystalline Materials*, 2nd ed.; Clarendon: New York, 1979; p 288.
- Nakamura, S.; Hayashi, S.; Koga, K. *Biochim Biophys Acta* 1976, 45, 294.
- Genies, E. M.; Lapkowski, M. *J Electroanal Chem* 1987, 236, 189.
- Vidal, J.-C.; Garcia, E.; Mendez, S.; Yarnoz, P.; Castillo, J.-R. *Analyst* 1999, 123, 319.