Amperometric Biosensor for Glucose Based on a Nanometer-Sized Microband Gold Electrode Coimmobilized with Glucose Oxidase and Poly(*o*-phenylenediamide)

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Abstract

A nanometer-sized (88 mm) microband gold electrode for glucose detection is described. These noble metal ultramicrosensors are prepared by electrochemical copolymerization of *o*-phenylenediamide (PDA) and glucose oxidase at the nanometer-sized electrodes. Electropolymerization conditions of PDA at the microband surface are optimized. The current can rapidly (3s) reach a steady value upon the potential step from 0 to +0.6 V without stirring the solution. The permselectivity characteristics of polymer can eliminate the interference of ascorbic acid. The amperometric response to glucose shows a linear relation in the range of 0.5 mM to about 10 mM with the correlation coefficient of 0.9993. With further increasing of glucose concentration an obvious Michaelis-Menten's response can be observed. The maximum steady current density is ca. 4.4 ± 0.2 mA/cm^2 and the apparent Michaelis-Menten constant is 21.1 ± 0.2 mM.

Keywords: Glucose sensors, Nanometer-sized biosensors, *o*-Phenylenediamide, Glucose oxidase, Electrochemical polymerization, Microband gold electrodes

1. Introduction

In recent decades, electrochemical microsensors based on voltammetric microelectrodes have received considerable attention due to the needs and challenges of miniaturization of detectors and detection devices as well as the advantages of microelectrodes compared with conventionally sized electrodes [1–7]. These miniaturized biosensors are particularly attractive in ex vivo and in vivo clinical applications. Glucose is one of the most important biomolecules. The levels of glucose in blood or cerebrospinal fluid are related to many diseases such as diabetes, pulmonary tuberculosis and meningitis etc., thus, its detection is a routine project in clinic chemistry. Many methods have been reported for the determination of glucose [8] which has long been a popular target for the development of biosensors [9]. In these glucose sensors, amperometric sensors based on the electrodes modified with glucose oxidase or with glucose oxidase and a mediator have been well developed [10–15]. With the effort in miniaturization, many glucose sensors were based on the ultramicro- or microelectrodes such as carbon fiber microelectrodes [2, 16, 17], screen-printed carbon microdisk [18] and microband [19] electrodes. All these microbiosensors except those described in literature [2, 17] were prepared by using electropolymerization or electrodeposition, a flexible and readily controlled method for the immobilization of enzymes at electrode surfaces. With this method the thickness and spatial extent of the immobilized enzyme layer can be controlled by changing the electrolysis time, the potential range or the component of the solution. Kuwana used a polypyrrole film to immobilize glucose oxidase on a carbon fiber surface and obtained a microbiosensor for glucose with the Michaelis-Menten constant, K'_M, of 20 mM [16]. Wang prepared glucose microsensors by the coimmobilization of glucose oxidase and *o*-pheylenediamine at +0.65 V, Pt or Rh at −0.8 V on the screen-printed carbon microdisk array electrodes [18]. In the meantime, Bartlett also made a glucose sensor based on the carbon bands with 10 μm width by immobilizing the enzyme from a buffer solution containing 25 mM *o*-pheylenediamine at +0.4 V [19]. These glucose sensors were of microometer-sized diameter.

In this work we coimmobilized glucose oxidase on a nanometer-sized gold band electrode by electropolymerization of *o*-pheylenediamine at +0.65 V and obtained a nanoscopic (88 nm width) glucose sensor. This sensor responses to glucose with a higher current density than those reported previously and reaches a steady value within 3 s without stirring the solution, which would develop the application range of the glucose sensor.

In fact, the electropolymerization of *o*-pheylenediamine was first reported in 1958 [20]. The reaction mechanism of electropolymerization has been well studied [20–23]. Due to permselectivity characteristics of the membrane, it can produce a sensor virtually free from the interference of ascorbic acid and its fouling problem [24]. Prior to those reported in [18] and [19], a glucose fast-response amperometric sensor based on glucose oxidase immobilized in an electropolymerized poly(*o*-phenylenediamine) film on platinum electrode surface had been reported [25]. The adherent, highly reproducible thin membrane possessed a thickness of around 10 nm. With a rotating disk electrode a response time less than 1 s was obtained [25]. Our work utilized this thin film to immobilize glucose oxidase on a 88 nm wide gold band electrode.

2. Experimental

2.1. Apparatus

Electrochemical measurements were performed with a PAR M273/270 electrochemical analyzer (EG&G, PAR Co.) with a platinum wire counter-electrode, a saturated calomel reference electrode (SCE), against which all potentials were quoted in our results, and a modified microband gold working electrode (ca. 88 nm × 0.5 cm) at 25 ± 0.1 °C.

The method of fabrication of the microband gold electrode was similar to that reported in literature [26]. After the edge of a deposited gold film on glass plate was abraded with successively finer grades of sandpaper, the working electrode was polished with 0.3 μm and then 0.05 μm alumina slurry on microcloth pads (Buehler), followed by rinsing with distilled water and

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removal of traces of alumina from the surface by brief sonicating in acetone and twice distilled water, respectively. Prior to use, the electrode was pretreated by continuous cycling from 0 V to −0.2 V at 50 mV/s in phosphate buffer solution (pH 7.0) until a constant background was observed, yielding a gold band of real area of approximately 3.8×10^{-6} cm^2 which was determined by using single sweep voltammetry in 1.0 M KCl solution containing 1.0 mM $K_3[Fe(CN)_6]$.

2.2. Chemicals

Glucose oxidase (GOD, EC1.1.3.4, Type II, 23 800 U/g, *Aspergillus niger*) and *β*-D-glucose (97 %) were obtained from the Sigma Chemical Company and used as received. *o*-Phenylenediamine was purchased from the Chemical Reagent Factory of Shanghai (China) and was purified by triple crystallization from water. L-Ascorbic acid was from Sigma (reagent grade). All other chemicals were of analytical grade. All solutions were prepared with double distilled water. 0.1 M phosphate buffer solutions (PBS) with various pHs were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. The solutions of *β*-D-glucose were prepared in pH 7.0 phosphate buffer and were allowed to equilibrate 24 h prior to use.

2.3. Procedures

The poly(*o*-phenylenediamine) (poly-PDA) was electrochemically polymerized at +0.65 V in pH 5.8 0.1 M acetate buffer solution containing 5.0 mM PDA. Poly-PDA/GOD was prepared by using a constant applied potential of +0.65 V in a solution containing 500 U/ml GOD and 5 mM PDA for 5 min. After the film was rinsed with pH 7.0 PBS the electrode was kept in pH 7.0 PBS at less than 4 °C. All the experiments for preparing the poly-PDA and poly-PDA/GOD modified microband gold electrodes were carried out under nitrogen atmosphere after deaerating with pure N_2 for 10 min. The electrochemical measurements were performed inside a Faraday cage. The chronoamperometric experiments were carried out with a potential step from 0 to +0.6 V. In our experiments the solutions and the working electrodes were kept motionless.

3. Results and Discussion

3.1. Electrochemical Polymerization of *o*-PDA at a Nanometer-Sized Microband Gold Electrode

The voltammograms of 5.0 mM *o*-PAD in pH 7.0 PBS displayed a wide irreversible oxidation peak at about +0.6 V (shown in Fig. 1), which was different from those in literature [20, 22, 25]. In those reports, two irreversible oxidation peaks at about +0.35 V and +0.55 to +0.6 V had been observed in the first cycle, and then the first peak at +0.35 V disappeared. It was possible that the difference resulted from the size of electrodes. At a microelectrode the mass transport rate is very fast, at a low scan rate a sigmoidal wave can be obtained, so that the two peaks in the first cycle can not be distinguished. The decrease in current at the potential more than +0.6 V resulted from the formation of insulating film. With successive cyclic sweeps the peak current quickly decreased. After 3 to 4 cycles the peak disappeared and the wave trended to a background shape, indirectly, but clearly indicating that a compact and insulating film was formed and the film was substantially free from holes [25]. According to Yacynych's explanation, a conducting film was initially formed, and on the top of this a nonconducting film formed which eventually insulated the electrode [22]. Usually, it is difficult to form a film at a microelectrode due to its fast mass transport. However, with a wider scan range of potential an insulating polymer film can be obtained at a gold band electrode. Our experimental results showed that with a potential sweep range from 0 to +0.8 V the rate of current decrease was very slow in PDA solution, the voltammograms did not reach a steady shape even after the electrode was swept for over 20 cycles, and the film was very poor, from the evidence of the cyclic voltammograms of bare and film modified electrodes in 1.0 M Cl solution containing 1.0 mM $K_3[Fe(CN)_6]$. With a scan range of 0 to +0.9 V, some different appearances were observed, indicating that a compact and insulating film was formed. The film thickness was very small, and has been evaluated as ca. 10 nm from the intercept of Koutecky-Levich plot for the reduction of H^+ at a rotating poly-PDA modified disk electrode in a 0.2 M pH 3.0 trifluoroacetate buffer [25].

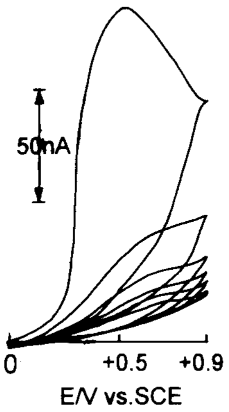

Fig. 1. Cyclic voltammogram of 5.0 mM *o*-phenylenediamide in pH 7.0 PBS at a nanometer-sized microband gold electrode at 50 mV/s.

3.2. Glucose Oxidase and Poly-PDA Coimmobilization at a Nanometer-Sized Microband Gold Electrode

Both the cyclic voltammogram (not shown) and the chronoamperometric curve (Fig. 2) of PDA solution containing glucose oxidase were different from those without glucose oxidase. With increasing GOD concentration in PDA solution the oxidation peak current of the voltammogram increased and the rate of the decrease in peak current reduced. Thus, it needed a greater number of sweep cycles to reach a steady voltammogram. In chronoamperometric experiments, the oxidation current in PDA solution containing

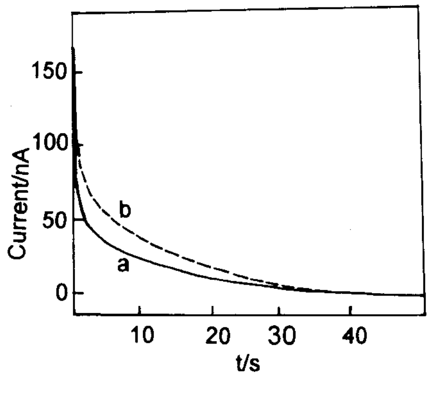

Fig. 2. Chronoamperometric curves of a) 5.0 mM PDA and b) 5.0 mM PDA + 500 U/mL GOD in pH 5.8 acetate buffer with a potential step from 0 to +0.6 V.

GOD at the potential of +0.65 V was larger than that without GOD. After an electrolysis time of 30 s the two curves tended to overlap (Fig. 2), the effect of GOD on the formation of poly-PDA film gradually decreased, and the curve reached a steady shape. The current was near to zero by an electrolysis time of 50 s, indicating that a layer of GOD/poly-PAD film on the surface was formed. Our experiments chose an electrolysis time of 5 min for the formation of GOD/poly-PDA film. The charge in current was different from those in [22] and [28], resulting from both the adsorption of GOD on the electrode surface prior to the electropolymerization [27] and the exposition of active dots of electrode surface due to the incorporation of GOD. The former decreased the oxidation rate of PDA and made the formation of an insulating polymer film slower. The latter was just the opposite. At the beginning the first factor dominated, resulting in a lower rate of insulating film formation. However, once the GOD was incorporated into the film the second factor became dominant. Finally, the insulting polymer films were formed, and the currents at both poly-PDA and GOD/poly-PDA approached the same value.

The effects of pH on the formations of both poly-PDA and GOD/poly-PDA were different. Poly-PDA film could be formed on the microband electrodes at nearly all pH values. The characteristics of all these films for the electrochemical response of 1.0 M KCl solution containing 1.0 mM $K_3[Fe(CN)_6]$ were similar. The responses of GOD/poly-PDA films formed at the solutions of various pHs to glucose in PBS (pH 7.0) changed greatly. When the films were formed at pH values lower than 4.8 or higher than 6.9, the response to glucose deteriorated. This pH effect is related to the isoelectric point of glucose oxidase [29]. A pH value of 5.8 was chosen for the electropolymerization of GOD/poly-PDA film in subsequent experiments.

3.3. Electrochemistry of Ascorbic Acid at the GOD/Poly-PDA Electrode

Figure 3 shows the cyclic voltammograms of 4.0 mM ascorbic acid in PBS (pH 7.0) at bare and GOD/poly-PDA nanometer-sized microband gold electrodes. At a bare electrode, the oxidation current begins to increase at the potential of about +0.1 V and reaches a maximum value at about +0.5 V. A quasi-steady-state voltammogram could be observed, indicating a fast electron transfer process. In contrast, at a GOD/poly-PDA film electrode the response is greatly diminished due to the permselectivity characteristics of poly-PDA polymer film.

3.4. Electrochemical Response of Glucose at the GOD/Poly-PDA Electrode

Figure 4 shows the linear sweep voltammograms of bare and GOD/poly-PDA film electrodes in PBS (pH 7.0) containing 5.0 mM glucose. A sigmoidal response can be seen at the GOD/poly-PDA electrode. The oxidation current begins to rise at the potential of +0.35 V and reaches a limiting value at +0.5 V, then stays at this value until +0.65 V. At a potential more positive than +0.65 V the current further increases due to the background. With increasing glucose concentration up to 25 mM the limiting value of oxidation current increases. At the bare electrode the oxidation current begins to rise at the potential of +0.22 V, but the change in current with positively scanning is very little, compared with that at a GOD/poly-PDA modified electrode. These results indicate that the GOD in polymer film catalyzes the oxidation of glucose at the microband electrode in the presence of oxygen. The electrode process can be described as follows:

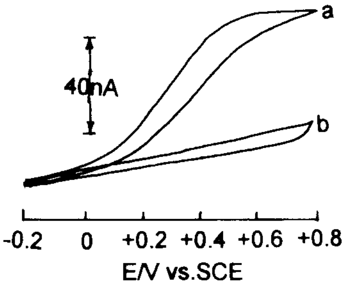

Fig. 3. Cyclic voltammograms of 4.0 mM ascorbic acid in pH 7.0 PBS at a) bare and b) poly-PDA modified microband gold electrodes at 50 mV/s.

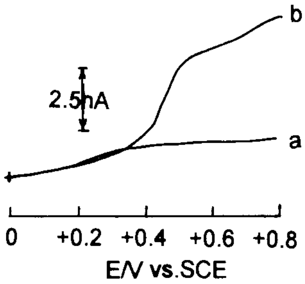

Fig. 4. Single sweep voltammograms of 5.0 mM glucose in pH 7.0 PBS at a) bare and b) GOD/poly-PDA modified electrodes at 5 mV/s.

$$GOD_{ox} + \beta\text{-D-glucose} \rightarrow GOD_{red} + \text{D-gluconic acid} \quad (1)$$

$$GOD_{red} + O_2 \rightarrow GOD_{ox} + H_2O_2 \quad (2)$$

and

$$H_2O_2 - 2e \rightarrow (1/2)O_2 + H_2O \quad (3)$$

With the increase of glucose concentration more H_2O_2 is produced, thus the oxidation current of H_2O_2 is related to the concentration of glucose in solution. According to the results in Figure 4, we chose a working potential of +0.6 V for the amperometric determination of glucose concentration. Furthermore, due to the fast mass transport rate of the microelectrode, a quasi-steady-state response can be obtained without stirring the solution, simplifying greatly the measurement procedures.

The chronoamperometric curves of the GOD/poly-PDA modified electrode in a static cell with pH 7.0 PBS containing various concentrations of glucose are shown in Figure 5. At various concentrations of glucose all of the response curves reached steady values quickly, indicating a considerably fast response time (for example, the current reached 90 % and 95 % of its steady-state value within 2 s and 3 s, respectively, for the glucose concentration as high as 20 mM). The steady value of current was related to the concentration of glucose. There was a linear relation between steady-state current and glucose concentration over the range of 0.5 to 10 mM, with a correlation coefficient of 0.9993. The microsensor exhibited good reproducibility for the determination of glucose with the concentrations within linear range. The relative standard deviation of results was 1.6 % for 6 successive determinations at 2.0 mM glucose concentration.

With the further increase of glucose concentration the oxidation current of chronoamperometric curve trended towards a constant value. From the plot of steady-state current vs. glucose concentration a Michaelis-Menten type response can be observed (Fig. 5 insert), showing a characteristic of Micheals-Menten kinetic mechanism.

At a thin polymeric film electrode, the reaction between substrates in solution and enzymes immobilized in film follows

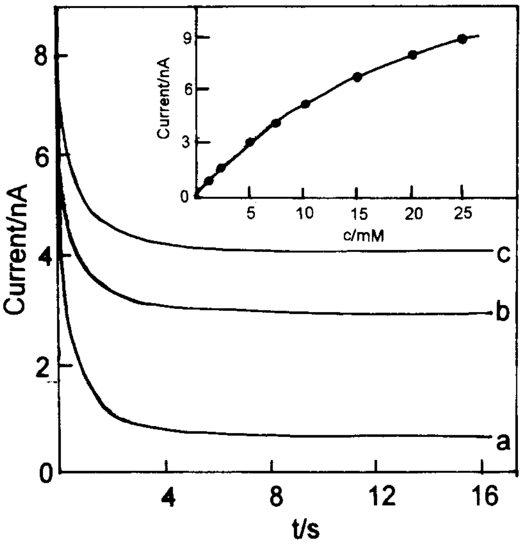

Fig. 5. Chronoamperometric curves of GOD/poly-PDA modified electrode in pH 7.0 PBS containing a) 1 mM, b) 2 mM and c) 5 mM glucose with a potential step from 0 to +0.6 V. Also shown is the plot of steady-state current vs. glucose concentration.

the Michaelis-Menten equation [30]:

$$i_{ss} = i_m[\text{substrate}]/(K'_M + [\text{substrate}]) \qquad (4)$$

where K'_M is the apparent Michaelis-Menten constant, i_{ss} is the steady-state current, i_m is the maximum current under saturating substrate conditions and

$$i_m = nFA\Gamma k_{+2} \qquad (5)$$

k_{+2} is the decomposition rate of intermediate complex in enzyme reaction. Γ is the surface concentration of enzyme. Both i_m and K'_M for the immobilized enzyme can be evaluated by using amperometric methods with Lineweaver-Burke transformation of the Michaelis-Menten equation [31, 32]:

$$1/i_{ss} = 1/i_m + K'_M/(i_m[\text{substrate}]) \qquad (6)$$

Figure 6 shows the plot ($R = 0.9997$ for $n = 8$) according to Equation 6. From the slope and intercept of the plot an average K'_M of 21.1 ± 0.2 mM and a maximum steady-state current of 16.7 ± 0.7 nA ($4.4 \pm 0.2\,\text{mA/cm}^2$) can be evaluated. The value K'_M is slightly more than that reported by Kuwana at a carbon fiber microelectrode [16]. Comparing the K'_M value with those reported in [16] and [25], it can be seen that with decreasing electrode size the K'_M value increases, though they were of the same order of magnitude as that for soluble glucose oxidase [32]. Furthermore, the steady-state current density at the nanometer-sized electrode is also very large. It is about 24 times larger than that reported in [25].

With the addition of ascorbic acid to the solution containing 5.0 mM glucose in pH 7.0 PBS, the response current kept nearly a constant value when the concentration of ascorbic acid was less than 0.6 mM, on further increasing the ascorbic acid concentration the current slightly decreased, ascribed to oxygen depletion [25]. Considering the physiological levels of ascorbic acid and glucose, the sensor can well exclude the interference of ascorbic acid for the determination of glucose in biological samples.

3.5. Stability of the GOD/Poly-PDA Electrode

The stability of the sensor was tested by the change in response current to 5 mM glucose. After the sensor was kept at 4 °C in pH 7.0 PBS for two weeks, the current did not change. With a passage of three weeks there only was a decrease in the current of about 14 %. Thus, the sensor possessed good stability and might be used in clinic analysis.

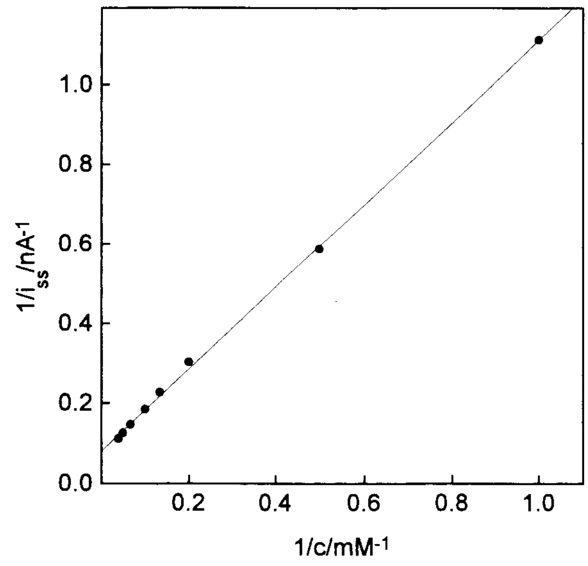

Fig. 6. Lineweaver-Burke analysis of the relationship between the steady-state current and glucose concentration. The experimental conditions are the same as in Figure 5.

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5. References

[1] R.M. Wightman, *Science* **1988,** *240,* 415.

[2] J. Wang, M. Lin, *Electroanalysis* **1989,** *1,* 151.

[3] O. Niwa, Y. Xu, H.B. Halsall, W.R. Heineman, *Anal. Chem.* **1993,** *65,* 1559.

[4] W.G. Kuhr, V.L. Barrett, M.R. Gagnon, P. Hopp, P. Pantano, *Anal. Chem.* **1993,** *65,* 617, and W.B. Nowall, W.G. Kuhr, *Electroanalysis* **1997,** *9,* 102.

[5] H.Y. Chen, H.X. Ju, Y.G. Xun, *Anal. Chem.* **1994,** *66,* 4538.

[6] T. Abe, Y.Y. Lau, A.G. Ewing, *J. Am. Chem. Soc.* **1991,** *113,* 7421.

[7] L. Huang, R.T. Kennedy, *Trends. in Anal.Chem.* **1995,** *14,* 158.

[8] G. Cooper, *Crit. Rev. Clin. Lab. Sci.* **1973,** *4,* 101.

[9] A.P.F. Turner, I. Karube, G.S. Wilson, in *Biosensors: Fundamentals and Applications,* Oxford University Press, Oxford, **1987**.

[10] P.N. Bartlett, J.M. Cooper, *J. Electroanal. Chem.* **1993,** *362,* 1.

[11] B.A. Gregg, A. Heller, *J. Phys. Chem.* **1991,** *95,* 5976 and *Anal. Chem.* **1990,** *62,* 258.

[12] T.J. Ohara, R. Rajagopalan, A. Heller, *Anal. Chem.* **1994,** *66,* 2451.

[13] Y. Kajiya, H. Matsumoto, H. Yoneyama, *J. Electroanal. Chem.* **1991,** *319,* 185.

[14] M. Marchesiello, E.M. Genies, *Electrochim. Acta* **1992,** *37,* 1987.

[15] E. Rohde, E. Dempsey, M.R. Smyth, J.G. Vos, *Anal. Chim. Acta* **1993,** *278,* 5.

[16] J.W. Furbee, Jr., T. Kuwana, R.S.Kelly, *Anal.Chem.* **1994,** *66,* 1575.

[17] E. Csöregl, L. Gorton, G. Marko-Vargo, *Anal. Chim. Acta* **1993,** *273,* 59.

[18] J. Wang, Q. Chen, *Anal. Chem.* **1994,** *66,* 1007.

[19] P.N. Bartlett, P.R. Birkin, *Anal. Chem.* **1994,** *66,* 1552.

[20] P.J. Elving, A.F. Krivis, *Anal. Chem.* **1958,** *30,* 1645 and 1648.

[21] P.J. Elving, A.F. Krivis, H.B. Mark, F.C. Anson, *Anal. Chem.* **1963,** *35,* 722.

[22] A.M. Yacynych, H.B. Mark, *J. Electrochem. Soc.* **1976,** *123,* 1346.

[23] W.R. Heineman, H.J. Wieck, A.M. Yacynych, *Anal. Chem.* **1980,** *52*, 345.

[24] S.V. Sasso, P.J. Plerce, R. Walla, A.M. Yacynych. *Anal. Chem.* **1990,** *62*, 1111.

[25] C. Malitesta, F. Palmisano, L. Torsi, P.G. Zambonin, *Anal. Chem.* **1990,** *62*, 2735.

[26] H.A.O. Hill, N.A. Klein, I.S.M. Psalti, N.J. Walton, *Anal. Chem.* **1989,** *61*, 2200.

[27] C. Bourdillon, J. Bourgeois, D. Thomas, *Biotechnol. Bioeng.* **1979,** *21*, 1877.

[28] Y. Ikariyama, S. Yamauchi, T. Yukiashi, H. Ushioda, *J. Electrochem. Soc.* **1989,** *136*, 702.

[29] N.C. Foulds, C.R. Lowe, *J. Chem. Soc.,Faraday Trans. 1*, **1986,** *82*, 1259.

[30] J.F. Castner, L.B. Wingard, *Biochemistry* **1984,** *23*, 2203.

[31] F.R. Shu, G.S. Wilson, *Anal. Chem*. **1976**, *48*, 1679.

[32] D.L. Scott, E.F. Bowden, *Anal. Chem*. **1994**, *66*, 1217.