Role of mediators in electron transport from glucose oxidase redox centre to electrode surface in a covalently coupled enzyme paste electrode†

D SAVITRI and CHANCHAL K MITRA*

Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India

**Corresponding author (Fax, 91-40-3010 120; Email, ckmsl@uohyd.ernet.in).*

We have studied the glucose oxidase immobilized carbon paste electrodes in the presence and absence of small mediator molecules. We have used p-benzoquinone and riboflavin as mediators in our studies. The effect of mediator molecules on the electron transfer between the enzyme redox centre and the electrode surface was explained from the cyclic voltammograms and rotating disk electrode data. In the absence of oxygen, we have noted that the mediators play a central role in the electron transfer. We have also proposed a possible mechanism for the electron transfer from enzyme active site to the electrode surface via mediators, based on our observations.

1. Introduction

Research and development of biosensors has been a topic of central interest in bioelectrochemistry, owing to their commercial potential and scientific significance. In biosensors, the biocatalyst is usually immobilized onto a supporting matrix close to the electrode. The performance of biosensors depends not only on the technique used for immobilization and the nature of biocatalyst but also on the other adsorbed species such as mediators and additives. In case of paste electrodes, it also depends on the composition of the paste, method of construction, storage, etc. As to the nature of the enzyme and their interaction with the electrode surface, a wide range of investigations have been undertaken especially on glucose oxidase (Weibel and Bright 1971; Dixon and Webb 1979; Razumas *et al* 1984). However, how enzyme molecules interact with electrode surfaces and how the various reaction conditions influence the characteristics of enzyme is not yet fully understood and more studies on these aspects are needed.

Various methodologies have been used to provide efficient electrical contact between redox proteins and electrodes. Diffusional electron mediators such as ferrocenes (Cass *et al* 1984, 1985; Bourdillion *et al* 1993), ferrocyanides (Mor and Guamaccia 1977; Montagne and Marty 1995) and quinone derivatives (Foulds and Lowe 1986; Janada and Weber 1991) have been employed as charge transporters that connect the active redox centre of the enzyme to the electrode surface. Willner *et al* (1992, 1994) have studied the mediated electron transfer between enzymes and gold electrodes focusing on structure of mediator molecules. We have studied carbon paste based modified electrodes to examine/explore the role of mediator in establishing the electrical contact between redox centre of enzyme and electrode. We have

Keywords. Covalent enzyme electrodes; biosensors; mediated electron transfer

†Dedicated to the memory of Professor J Das

Abbreviations used: GOD, glucose oxidase; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; CV, cyclic voltammetry; RDE, rotating disk electrode; SCE, saturated calomel electrode; FAD, flavin adenine dinucleotide; $FADH_2$, reduced flavin adenine dinucleotide; DCC, dicyclohexyl carbodiimide; POD, peroxidase (horse-radish)

also proposed a possible mechanism for electron transfer from the active centre of an enzyme covalently immobilized onto the electrode surface.

In the work presented here, glucose oxidase (GOD) molecules were immobilized through covalent coupling onto glassy carbon powder as supporting matrix, which also acts as the electrode. We have used p-benzoquinone and riboflavin, adsorbed onto the covalent matrix, as electron mediators. The performance of the GOD electrodes was tested with various concentrations of glucose solutions in both deoxygenated and oxygenated samples. A blank paste electrode prepared with activated carbon (no mediators) served as control to characterize the blank electrode performance.

2. Methodology

2.1 *Instrumentation*

For the rotating disk electrode studies, we have used a Tacussel (Villeurbanne, France) rotating disk electrode and motor speed control unit (CTV101T) with an analogue voltage input for speed control that was connected to a data acquisition board (daqboard 216A). The digital to analogue converter (DAC) channel of the daqboard was used for rotational speed control of the rotating disk electrode. The electrochemical cell and other instrumentation details for this as well as for cyclic voltammetry and flow-injection studies are as described earlier by Savitri and Mitra (1998).

All necessary softwares for data collection were written by us in Turbo Pascal (Version 7). All graphs were plotted using a commercial program (Sigmaplot, Jandel Scientific, CA, USA).

2.2 *Reagents*

GOD enzyme (Cat No: G-2133) was procured from Sigma Chemical Company (USA). 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDC) and parabenzoquinone were from Merck (Darmstadt, Germany). All other reagents used were AR grade from Qualigens (Mumbai).

2.3 *Carbon paste electrode*

Glassy carbon powder (Sigradur-G from HTW, Germany), that are spherical in shape with an average particle size of ~ 1 μm was used as supporting matrix for enzyme immobilization in our studies. We have studied three sets of electrodes in our studies: (i) blank paste electrode (without enzyme) as control; (ii) covalently immobilized GOD electrode without mediators and (iii) covalently immobilized GOD electrode with quinone and riboflavin as mediators. The matrix activation and other immobilization procedures were performed following the protocol standardized by us earlier (Sree Divya *et al* 1998; Savitri and Mitra 1998). We have immobilized the GOD covalently onto the matrix using an ethylenediamine spacer arm and following EDC condensation procedure.

2.4 *Assay of GOD*

The assay for estimating the activity of the enzyme loaded onto the matrix by covalent immobilization was performed following the colorimetric method as described in the Sigma catalogue:

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{GOD}} \delta\text{-gluconolactone} + H_2O_2$$

$$H_2O_2 + \text{o-dianisidine(reduced)} \xrightarrow{\text{POD}} \text{o-dianisidine (oxidized)} + H_2O.$$

The colour developed was measured spectrophotometrically at 500 nm. The activity was calculated comparing the absorbance of the test mixture with the absorbance of a standard enzyme concentration taken under same reaction conditions.

2.5 *Solid electrode*

Glassy carbon rod of 3 mm diameter fitted in a teflon holder served as solid electrode. The electrode surface was polished with diamond paste and was treated with nitric acid to remove any greasy material.

2.6 *Experiments*

2.6a *Cyclic voltammetry*: (i) The optimum potential window for enzyme electrode performance has been chosen by taking cyclic voltammograms for the GOD electrode in the presence of substrate glucose. A potential scan was taken from – 500 mv to + 1000 mv (vs SCE) and the potential between – 300 mv to – 100 mv is found to be optimum for enzyme electrode performance. (ii) The effective surface area of the paste electrode and solid electrode were determined by cyclic voltammetry using ferrocyanide/ferricyanide couple. The surface area of the paste electrode is found to be 20% higher than the solid electrode and the reason can be attributed to the porosity of the paste electrode. The background currents (with the supporting electrolyte alone) for blank paste electrode are found to be ~ 30% higher than the blank solid electrode which is expected from the difference in their surface area. (iii) A cyclic voltammetry (CV) scan was also taken for enzyme electrode with and without mediators, in the presence/absence of substrate, in an oxygenated/deoxygenated sample, to observe the role of the mediator species and bound cofactors on the electron transfer. Deoxygenation was accomplished by slow

bubbling of nitrogen gas for 20–25 min through the supporting electrolyte to remove the dissolved oxygen prior to the experiment.

The whole of the experimental procedure was programmed and data was saved to a file and processed using Sigmaplot. The data averaging was done using Sigmaplot *runavg* transform with a 64 point window size to reduce the noise.

2.6b *Rotating disk electrode studies*: The experiments with rotating disk electrode (RDE) set-up were performed for the solid, blank paste and enzyme electrodes (with and without mediators) in the presence and absence of dissolved oxygen. The response of the electrodes was checked for various substances—hydrogen peroxide, quinone, riboflavin and glucose solutions. The bias was fixed at –300 mv (vs SCE reference) in all the experiments. The electrode was rotated at various speeds from 0 to 1300 rpm, in steps of 9, 36, 81, ... 1296 rpm. The change in current with the changing rotational velocity of the electrode was plotted on the recorder and the current data was also saved to a file. The data were plotted using Sigmaplot and analysed. Data averaging using a 32 point window size was also performed to reduce the noise further.

2.6c *Flow-injection set-up*: The enzyme paste was used to pack a micropipet tip electrode to check the linearity of response. The response of the electrode was checked at – 100 mv bias potential (vs AgCl/Ag reference) for different concentrations of glucose solutions: 0·1 mM, 0·5 mM and 1 mM. Since the effective surface area and the electrode surface area exposed to the solution is small, we expect a better linearity and response with this set-up. Hence this set of experiments was used to check performance of the enzyme electrode.

The electrodes were stored in a humid atmosphere at 4°C after experiment until further use. The equilibration time of the electrode, before the response with supporting electrolyte was a flat base line, ranged from 1–1·5 h (subject to packing errors). The experiments were carried out in 0·1 M KCl solution at 37°C.

3. Results and discussions

Though there has been considerable interest in the electrochemical behaviour of GOD, only few reports are available in the literature directly related to the interaction between GOD and electrodes. Aided with sensitive techniques such as differential pulse voltammetry, it has been shown that GOD undergoes direct electron exchange on mercury or graphite or platinum electrodes (Scheller *et al* 1979; Narasimhan and Wingard 1986). We have studied the mechanism of electron transfer in paste electrode systems. In our work the role of mediator was found to be central for an efficient electrochemical contact with electrode surface and a mechanism has been proposed. We have also observed that the FAD cofactor bound to GOD enzyme aids in electron transfer to the electrode surface that was found to be irreversible as observed by cyclic voltammetry experiments. Further the electrode surface reactions in the presence of oxygen (dissolved) were found to be electrochemically different (hydrogen peroxide gives a reduction current) from the reactions in the absence of oxygen (oxidation current).

3.1 *Mediated electron transfer from enzyme active site to the electrode*

Cyclic voltammogram of enzyme electrode (without mediators) in the presence of the substrate (glucose) shows a single clear irreversible peak (figure 1) at a potential ~ 100 mv (vs SCE) although a second small peak can be seen around ~ 225 mv. These peaks are due to the buried FAD which undergoes 2 electron redox

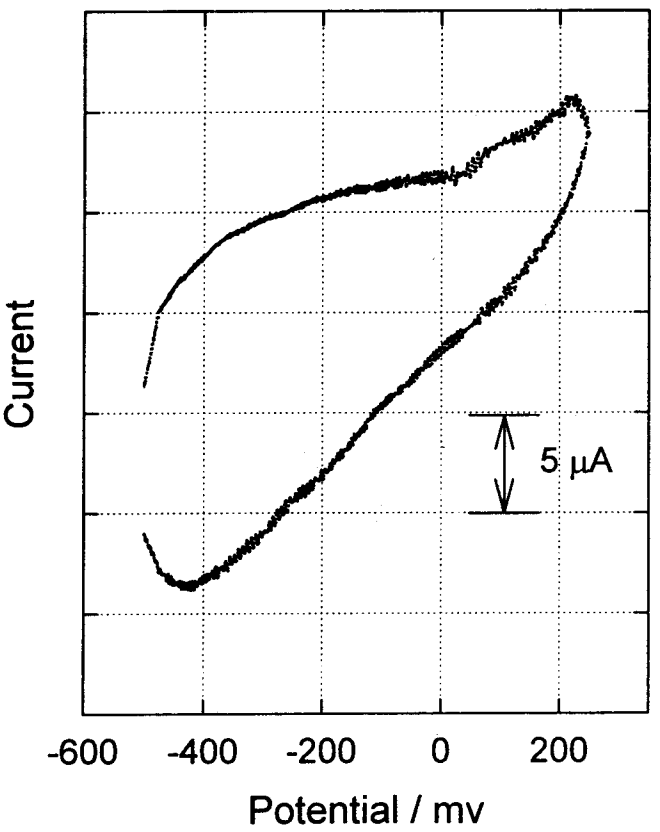

Figure 1. CV of enzyme electrode with no adsorbed mediator species. The potential scan was taken in the presence of glucose (1 mM). Two small irreversible peaks can be observed that correspond to the reduction of the enzyme bound FAD cofactor. Scan rate: 0·5 mv/s; electrode surface area: ~ 2·2 cm^2.

reactions. The peak potentials are considerably shifted from native FAD immobilized (Savitri and Mitra 1998), due to binding with the enzyme. The electron exchange also becomes irreversible in the holoenzyme as seen in figure 1. CV of enzyme electrode with the adsorbed mediator species-quinone and riboflavin shows three clear peaks one symmetrical reversible peak and two irreversible peaks as shown in figure 2. This symmetrical reversible peak at ~ – 170 mv (vs SCE) is due to the adsorbed quinone. The redox peaks due to riboflavin are not seen in the potential range used. The irreversible peaks are similar to the ones observed in case of electrode with no mediators. The CV of the enzyme electrode with adsorbed quinone in the presence of glucose shows a clear peak due to quinone at a potentials of ~ – 100 mv (vs SCE) as can be seen in figure 3. This clearly shows that in the presence of the second mediator (riboflavin), there is a shift in the potential of quinone (~ 70 mv), suggesting a strong interaction between these two mediators. The peak due to the bound FAD (enzyme) also appears considerably sharper due to rapid electron exchange in the presence of mediators (figure 2). We also note that electrochemical detection is optimum in the potential window of – 300 to – 100 mv, close to the quinone peak.

3.2 *Interaction of redox centre of GOD with mediators*

Covalent enzyme electrode with no mediator species or with one of the mediators shows no significant concentration dependence (for the substrate glucose). Figure 4 shows plot of $\omega^{1/2}$ vs current for enzyme electrodes with and without mediator species with glucose as substrate. For enzyme electrode alone or with one of the mediators present, the currents are practically same for all the three different glucose concentrations studied (figure 4a). In the case of the enzyme electrode with both the mediators present as adsorbed species, there is a clear and significant concentration dependence for the three different concen-

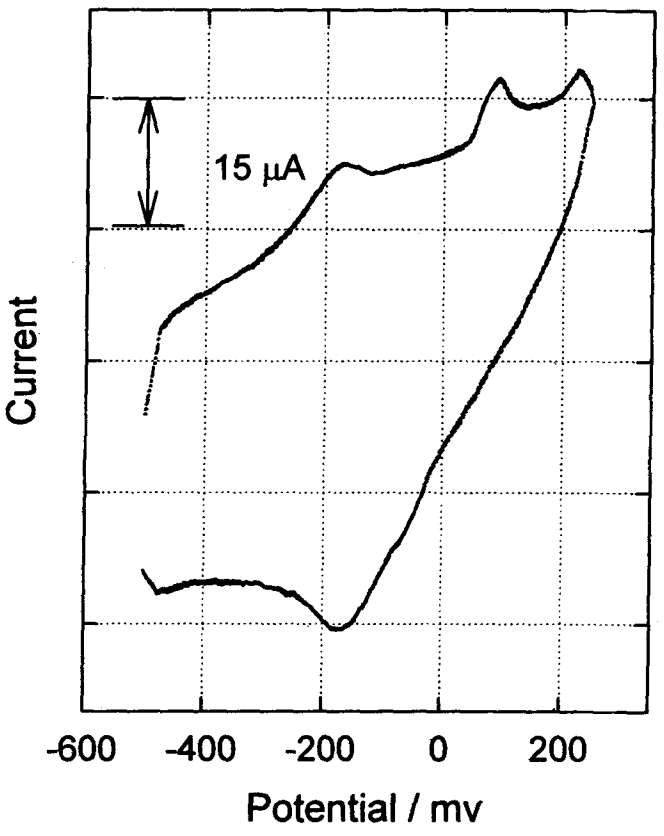

Figure 2. CV of enzyme electrode with two adsorbed mediators: riboflavin and quinone. Here three clear peaks are observed, one reversible peak corresponding to quinone reduction and two irreversible peaks corresponding to FAD reduction (as in figure 1). Riboflavin peaks are out of range of the potential range used. Scan rate: 2 mv/s.

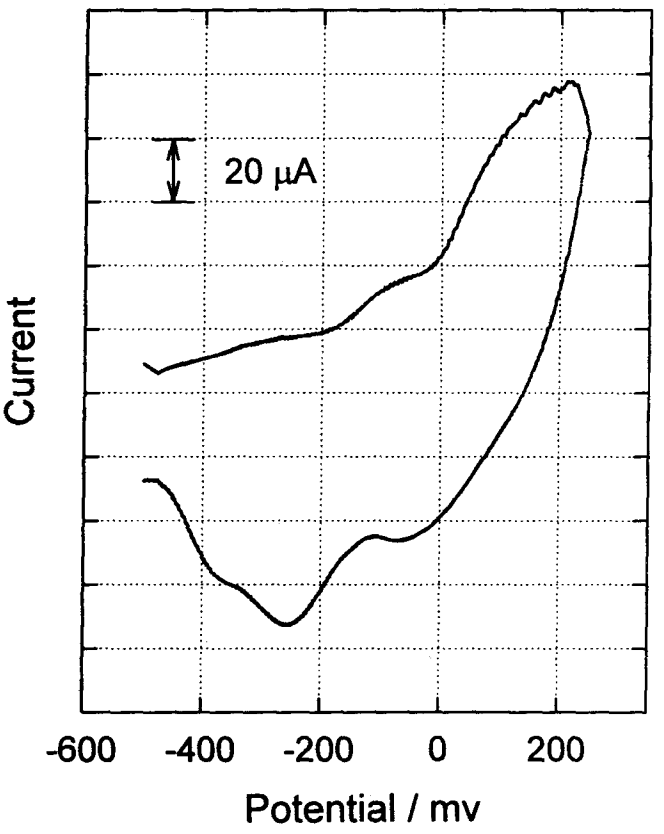

Figure 3. CV of enzyme electrode with adsorbed quinone in the presence of substrate (glucose) in a deoxygenated sample. The peaks due to quinone are clearly seen. Scan rate: 1 mv/s. Other conditions are similar to figure 2.

trations of the substrate (figure 4b). This is observed only in a deoxygenated sample. In the presence of oxygen, no significant response can be seen in the presence of glucose as substrate, as the oxygen acts as the electron acceptor (from the $FADH_2$ of the GOD enzyme directly) instead of the mediators (graph not shown).

From the above observations and from the CV of the enzyme electrode with the mediators (figures 2 and 3), it is clear that the second mediator riboflavin is playing the role of mediating the electron transfer to the electrode surface from the redox centre of enzyme via reduced quinone. Janada and Weber (1991) have reported quinone mediated electrochemical communication in glucose oxidase electrode immobilized in polypyrrole. A mechanism is proposed for the electron transfer on the basis of our experimental results.

3.3 *Mechanism of electron transfer*

The sequence can be described as follows:

$$\text{GOD (FAD)} + \text{glucose} \rightarrow \text{GOD } (FADH_2) + \delta\text{-gluconolactone}$$

$$\text{GOD } (FADH_2) + \text{riboflavin} \rightarrow \text{GOD (FAD)} + \text{reduced riboflavin}$$

$$\text{reduced riboflavin} + \text{quinone} \rightarrow \text{riboflavin} + \text{reduced quinone}$$

$$\text{redued quinone} \rightarrow \text{quinone} + 2H^+ + 2e.$$

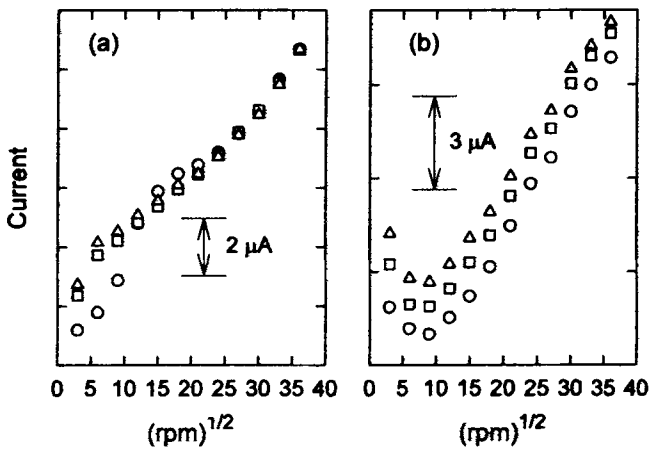

Figure 4. Plot of $\omega^{1/2}$ vs current for enzyme electrode with or without mediators in deoxygenated samples at – 300 mv bias. The electrode response was checked at three different concentrations of glucose solutions 0·1 mM, 0·5 mM, 1 mM. (**a**) The response of enzyme electrode (no mediators) and shows no effect of substrate concentration on the electrode response. (**b**) The response of enzyme electrode with mediators. The graph shows a small but clear concentration dependence. (o), 0·1 mM; (□), 0·5 mM; (Δ), 1 mM glucose solution.

The final current is produced by the oxidation of the reduced quinone and hence is oxidation current.

3.4 *Performance study*

The performance of enzyme electrode was characterized by checking the linearity and detection limits with the substrate glucose using a flow-injection set-up at – 100 mv bias potential. Figure 5 shows the plot of current values against time on X-axis for enzyme electrode with both the mediators for three different concentrations of glucose solution. This electrode showed a linearity with increasing substrate concentration till 1 mM glucose concentration. Essentially it is the response curve at various substrate concentrations.

3.5 *Enzyme assay*

The amount of enzyme loaded onto the matrix in case of covalent immobilization was estimated to be 0·3 units/mg of the carbon matrix, as determined by a colorimetric assay. This corresponds roughly to about 0·2% of enzyme loading, viz., 2 μg of enzyme per mg of carbon powder. Assuming all the glassy carbon powder particles are all equal in size (1 μm) and using an approximate value for the footprint size of the enzyme

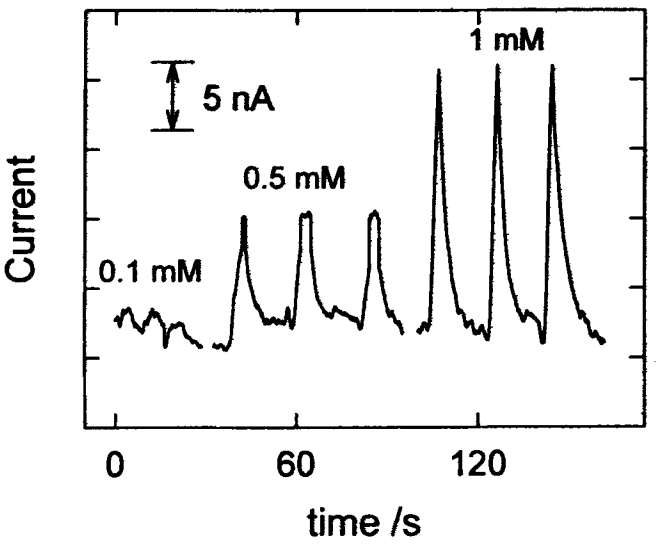

Figure 5. Plot of current values against time for enzyme electrode with both the mediators, in a flow-injection set-up, for three different concentrations of glucose. Each concentration of glucose was injected three times. The data files for all the concentrations were merged and running average is taken for the collected current values with a window size equal to the acquisition frequency. We see a clear linearity between current and increasing substrate concentration. The experiment was carried out at – 100 mv bias potential in supporting electrolyte solution (from Savitri and Mitra 1998).

(80×10^{-18} m^2 as calculated geometrically from the X-ray diffraction studies data of Hecht *et al* 1993), we estimate that approximately 40% of the surface is covered by the enzyme. This is small and studies have been undertaken to increase the enzyme loading further.

Acknowledgement

One of the authors (DS) wishes to thank the University Grants Commission, New Delhi for a senior research fellowship.

References

Bourdillon C, Demaille C, Moiroux J and Saveant J-M 1993 New insights into the enzymatic catalysis of the oxidation of glucose by native and recombinant glucose oxidase mediated by electrochemically generated one-electron redox co-substrates; *J. Am. Chem. Soc.* **115** 2–10

Cass A E G, Davis G, Francis G D, Hill H A O, Aston W J, Higgins J J, Plotkin E V, Scott L D L and Turner A P F 1984 Ferrocene mediated enzyme electrode for amperometric determination of glucose; *Anal. Chem.* **56** 667–671

Cass A E G, Davis G, Green M J and Hill H A O 1985 Ferricinium ion as an electron acceptor for oxidoreductases; *J. Electroanal. Chem. Interfacial. Electrochem.* **190** 117–127

Dixon M and Webb E C 1979 *Enzymes* 3rd edition (London: Longmann Press)

Foulds N C and Lowe C R 1986 Enzyme entrapment in electrically conducting polymers-immobilization of glucose oxidase in polypyrrole and its applications in amperometric biosensors; *J. Chem. Soc. Faraday Trans. 1* **82** 1259–1264

Hecht H J, Kalisz H M, Hendle J, Schmid R D and Schomburg D 1993 Crystal structure of Glucose oxidase from *Aspergillus niger* refined at 2.3 Å resolution; *J. Mol. Biol.* **229** 153–172

Janada P and Weber J 1991 Quinone mediated glucose oxidase electrode with the enzyme immobilised in polypyrrole; *J. Electroanal. Chem. Interfacial. Electrochem.* **300** 119–127

Montagne M and Marty J-L 1995 Bi-enzyme amperometric d-lactate sensor using macromolecular NAD^+; *Anal. Chim. Acta* **315** 297–302

Mor J R and Guamaccia R 1977 Assay of glucose using an electrochemical enzymatic sensor; *Anal. Biochem.* **79** 319–328

Narasimhan K and Wingard L B 1986 Enhanced direct electron transport with glucose oxidase immobilized on (aminophenyl)boronic acid modified glassy carbon electrode; *Anal. Chem.* **58** 2984–2987

Razomas V J, Jasaitis J J and Kulys J J 1984 Electrocatalysis on enzyme modified carbon materials; *Bioelectrochem. Bioenerg.* **12** 297–322

Sree Divya P, Savitri D and Mitra C K 1998 Covalent enzyme immobilization on to glassy carbon matrix-implications in biosensor design; *J. Biosci.* **23** 131–136

Savitri D and Mitra C K 1998 Electrochemistry of reconstituted glucose oxidase on carbon paste electrodes; *Bioelectrochem. Bioenerg.* **47/1** 67–73

Scheller F, Strnad G, Numann B, Kuhn M and Ostroniski W 1979 Polarographic reduction of prosthetic group in flavoprotein; *Bioelectrochem. Bioenerg.* **6** 117–122

Weibel M K and Bright H J 1971 The Glucose oxidase mechanism – interpretation of the pH dependence; *J. Biol. Chem.* **246** 2734–2744

Willner I, Katz E, Riklin A and Kasher R 1992 Mediated electron transfer in glutathione reductase organised in self-assembled monolayers on Au electrodes; *J. Am. Chem. Soc.* **114** 10965–10966

Willner I, Lapidot N, Riklin A, Kasher R, Zahany E and Katz E 1994 Electron transfer communication in glutathione reductase assemblies: electrocatalytic, photocatalytic and catalytic systems for the reduction of oxidised glutathione; *J. Am. Chem. Soc.* **116** 1428–1441

MS received 4 September 1998; accepted 17 December 1998

Corresponding editor: DIPANKAR CHATTERJI