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Benzoquinone-mediated enzyme biosensor for amperometric determination of glucose

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Abstract. An amperometric enzyme sensor for the analysis of glucose is described. The sensor uses benzoquinone as a mediator for electron transfer between immobilized glucose oxidase and a pyrolytic graphite electrode. A linear current response, proportional to glucose concentration in the range 1–50 mM, is observed. The influence of oxygen, *p*H and operating potential on the sensor performance is examined.

Keywords. Benzoquinone; amperometric biosensors; glucose oxidase; enzyme sensor; cyclic voltammetry.

1. Introduction

Amperometric biosensors have the advantage of combining the specificity of an enzyme with the sensitivity of an electrode surface for biomolecular recognition. Sensors which use an electron transfer reaction of a mediator at an electrode surface coupled to a redox enzyme have been found to be attractive for the development of biosensors. The topic has been reviewed (Turner *et al* 1987; Cardosi and Turner 1991). Carbon surface containing chemical functionalities or suitably modified by external reagents are being widely used as sensors for biological substances (Wring and Hart 1992). Cass *et al* (1984) have pioneered the development of a glucose sensor by employing ferrocene derivatives as electron transfer mediators. The commercially available glucose sensor (Medi Sense, Abingdon, UK and Cambridge, USA) incorporates glucose oxidase (GOD) and a ferrocene mediator into a carbon electrode matrix covered by an absorbent membrane (Matthews *et al* 1987; Cass 1990). Although quite popular, the sensor received a fair amount of criticism (Cardosi and Turner 1991).

While ferrocene derivatives have established themselves as efficient mediators, the viability of other electron transfer mediators such as tetrathiafulvalene (Turner *et al* 1987), tetracyanoquinodimethane (Hendry and Turner 1988; Pandey *et al* 1993), ruthenium complexes (Crumbliss *et al* 1986) and *tris* (4,4′ substituted 2,2′-bipyridine) complexes of iron(II), ruthenium(II) and osmium(II) (Zakeeruddin *et al* 1992) have been considered for glucose/GOD system.

It was our interest to provide an alternative route for the development of a glucose sensor. Quinones offer themselves as potential electron transfer mediators. They act as electron acceptors for flavoproteins in biological systems for example, ubiquinones in the respiratory chain. Quinones have some similarities to ferrocenes for use as

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mediators. The solubility of quinones and ferrocenes are alike in aqueous buffers (Weast *et al* 1964–1965). The structure of a quinone can be changed at will to suit a desired potential region. They can be attached to electrode surfaces (Narasimhan and Wingard 1986). Quinones possess well behaved electrochemistry particularly on graphite based electrodes, whose surfaces are ideally suited for sensor fabrication. The rate of reaction between the reduced enzyme and benzoquinone (BQ) is comparable to that with the ferrocene derivatives (Murthy and Anita 1993). Bourdillon *et al* (1986) have simulated enzyme electrocatalysis of glucose-GOD-BQ system and concluded that coupling would be more efficient when the enzyme is immobilized onto the electrode surface (reticulated vitreous carbon). BQ could be adsorbed easily on a carbon electrode surface for electron transfer from GOD (Hu and Turner 1991).

Since the electrochemistry of BQ is highly reversible at pyrolytic graphite (PG) electrodes rather than on either Pt or carbon paste electrodes (Murthy and Anita 1993), we considered it interesting to immobilize GOD on a PG electrode by a simple and effective method (di Gleria and Hill 1992). This involves generation of $-NH_2$ groups on a PG electrode by treatment with laurylamine and coupling GOD by using carbodiimide reagent. BQ was adsorbed by the dip-coating method. Our overall objective was to explore the feasibility of employing quinone derivatives as alternative mediators for a variety of enzymes and substrates. Glucose/GOD system is chosen as a model to test the viability of *p*-benzoquinone as a mediator for fabricating a glucose sensor at a future date.

2. Material and methods

GOD [(EC 1.1.3.4) from *Aspergillus niger*; molecular weight 186,000; activity 81 IU/mg], 1 cyclohexyl 3-(2-morpholino ethyl) carbodiimide metho-*p*-toluene sulphonate and β-D(+) glucose (Sigma) were used as received. Benzoquinone (Aldrich) was recrystallized from *n*-hexane. Laurylamine (SRL, India) was distilled before use. All solutions were prepared in double distilled water. The supporting electrolyte was 0·2 M phosphate (K_2HPO_4 and KH_2PO_4) buffer, *p*H 7·1. Glucose solutions were stored overnight to allow equilibration between α- and β-anomers. To prevent any competitive oxidation by dissolved O_2, all solutions were thoroughly deoxygenated by purging with N_2 for at least 30 min prior to use.

Cyclic voltammetry experiments using BAS voltammograph (CV27) and an *X-Y-t* chart recorder were performed in a conventional three-electrode electrochemical cell having a working volume of 5 ml. A basal-plane (area 0·183 cm^2) PG electrode, supplied by Le Carbone Lorraine, France and polished with 600 grit SiC paper and 1 μm diamond paste served as the working electrode. The counter electrode was the Pt wire. All potentials were referred to the Ag/AgCl reference electrode.

2.1 *Construction of GOD electrode*

The preparation of the electrode involves two steps – one is the adsorption of the mediator (here *p*-benzoquinone) onto the electrode and the other the immobilization of the enzyme. The basal-plane pyrolytic graphite (BPG) electrode was dipped in a solution of 200 mM BQ in propanol for about 2 hours at room temperature. After drying, the electrode was washed thoroughly with distilled water and phosphate buffer to remove any traces of the organic solvent which might tend to denature the enzyme. The electrode retained BQ activity for several hours as determined by cyclic voltammetry.

The electrode was then immersed in a saturated methanolic solution of laurylamine for 10 min and sonicated for 5 min. To a 100 μl, 0·1 mM aqueous solution of GOD, 100 μl, 10% aqueous solution of cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulphonate was added at 5°C and the previously modified electrode was again immersed in it for half an hour. The electrode was washed several times with buffer and stored in the same buffer at 4°C when not in use. The electrode response was stabilized by continuous operation of the electrode under potentiostatic control at 0·20 V in 5 mM glucose over a 2 hour period. Thereafter the electrode was found to give stable responses.

3. Results and discussion

The cyclic voltammogram (CV) of a BQ/GOD modified electrode in phosphate buffer, *p*H 7·1 is shown in figure 1a. The electrochemistry of BQ is reversible unlike on Pt or carbon paste electrodes. The E_{pa}, E_{pc} and $E^{\circ\prime}$ values are 0·18, 0·12 and 0·15 V respectively. A comparison of CV's (not shown) of unmodified and modified BPG electrodes in phosphate buffer shows a decrease in capacitance of the modified electrode thereby indicating that GOD is immobilized onto the electrode. It can be seen from figure 1b that upon addition of glucose (20 mM) a large catalytic current flows at oxidising potentials. This is indicative of enzyme-catalysed oxidation of glucose electrochemically coupled to BQ

$$\text{Glucose} + \text{GOD (FAD)} \longrightarrow \text{gluconolactone} + \text{GOD (FADH}_2\text{)}, \qquad \text{(i)}$$

$$\text{GOD (FADH}_2\text{)} + \text{BQ(ox)} \xrightarrow{k} \text{GOD (FAD)} + \text{H}_2\text{Q(red)}, \qquad \text{(ii)}$$

$$\text{H}_2\text{Q(red)} \rightleftharpoons \text{BQ(ox)} + 2e^- + 2\text{H}^+. \qquad \text{(iii)}$$

This behaviour is particularly apparent at slower scan rates and indicates the regeneration of BQ from hydroquinone (H_2Q) (reduced form) by the enzyme GOD in

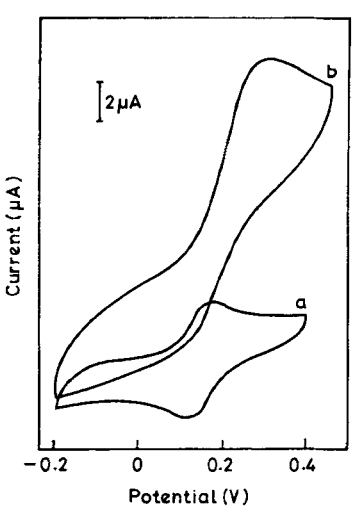

Figure 1. Cyclic voltammograms of (a) BQ/GOD modified electrode, (b) as in (a) with 20 mM glucose solution. Scan speed: 5 mV/s in 0·2 M phosphate buffer (*p*H 7·1)

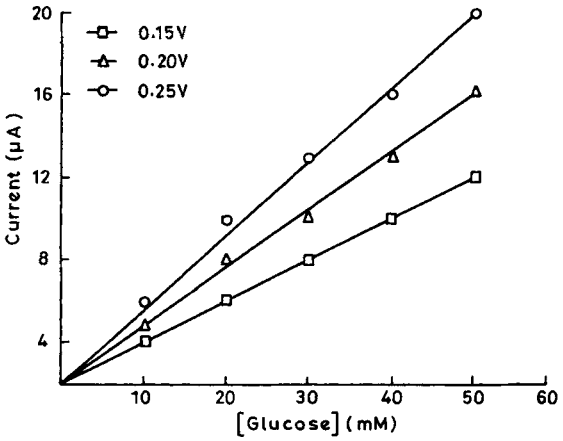

Figure 2. Effect of operating potential on the response to glucose. (□) 0·15 V, (Δ) 0·20 V and (○) 0·25 V vs. Ag/AgCl. Scan speed: 5 mV/s in 0·2 M phosphate buffer (*p*H 7·1).

its reduced state. The latter is maintained in the reduced state by the presence of substrate.

3.1 *Effect of operating potential*

The variation of catalytic currents with concentration of glucose at different operating potentials using the enzyme electrode is shown in figure 2 from where it can be inferred that the sensitivity is affected by the applied potential, while the linear range shown is identical (0–50 mM). This can be attributed to an increased driving force for the electrochemical reaction which results in more rapid reoxidation of the FAD centres of GOD.

3.2 *Effect of O_2 saturation*

Comparison of calibration curves for the glucose sensor in N_2 and O_2 saturated phosphate buffer solutions is shown in figure 3. The data in the plot are based on the average of measurements carried out on two modified electrodes. The difference between the values from both the electrodes was marginal. A 6% decrease in the current is observed for the O_2 saturated buffer solution. Under the conditions of normal blood glucose concentration (4–8 mM), the decrease in current upon O_2 saturation is $\approx 4\%$ with ferrocenecarboxylic acid (FCA) as mediator (Cass *et al* 1984).

The measured amperometric current (at 0·25 V) for different concentrations of glucose at the modified electrode varies in the range 1–50 mM. A straight line was obtained by linear regression analysis and the correlation coefficient (r) was found to be 0·998 [$y = 1{\cdot}4x + 0{\cdot}3$]. The apparent Michaelis–Menten constant, K'_m, is found to be 47 mM. The value is in fair agreement with that calculated from the Eadie Hofstee form (figure 4) of the Michaelis–Menten equation,

$$j_s = j_{max} - K'_m(j_s/C), \tag{1}$$

where j_s is the steady state current density, j_{max} is the maximum current density under saturating substrate conditions and C is the concentration of glucose in solution. The value of K'_m using (1) is found to be 46 mM.

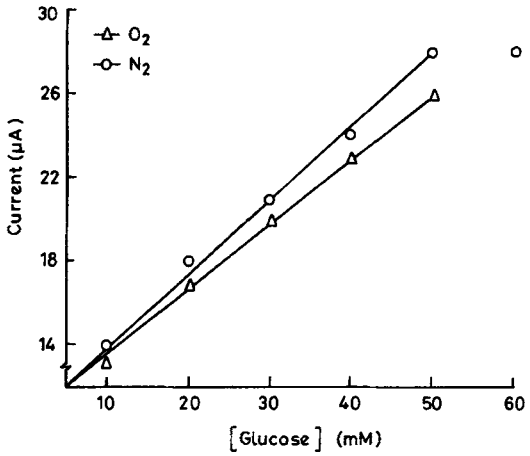

Figure 3. Calibration curves for the GOD modified electrode poised at 0·25 V vs Ag/AgCl in (○) N_2 saturated and (Δ) O_2 saturated phosphate buffer (*p*H 7·1). Scan speed: 5 mV/s.

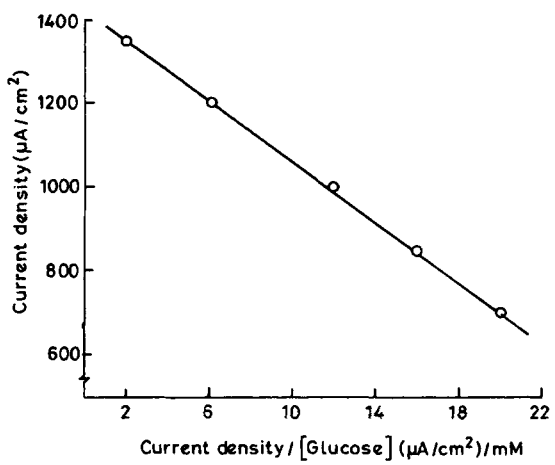

Figure 4. Eadie–Hofstee plot for the BQ/GOD modified electrode.

3.3 *Effect of pH*

A detailed *p*H profile study was carried out in the *p*H range 5·7–8·0 and the results obtained are shown in figure 5. It is seen that peak current increases at first, attains a limiting value at *p*H ~ 7·0 and then decreases. All measurements were therefore made at *p*H 7·1

3.4 *Stability*

The modified electrode gave stable current response over a period of 48 h. After that the current was reduced to half the initial value.

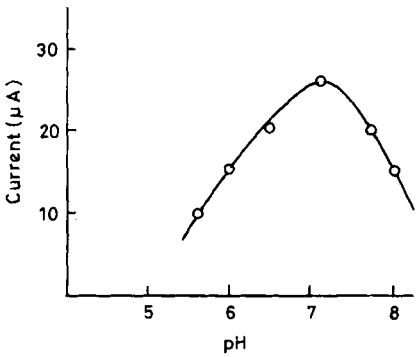

Figure 5. *p*H profile of the BQ/GOD modified electrode with 20 mM glucose. Scan speed: 10 m V/s in 0·2 M phosphate buffer (*p*H 7·1).

Table 1. Rate constants for the reaction of reduced glucose oxidase with oxidized mediators.

Mediator	$k(M^{-1}s^{-1})$	Reference
Benzoquinone	$1{\cdot}9 \times 10^6$	Present study
Ferrocene derivatives	$0{\cdot}3–5{\cdot}3 \times 10^5$	Cass *et al* (1984)
$[Ru(CN)_6]^{4-}$	1×10^4	Crumblis *et al* (1986)
$Ru(NH_3)_5Py^{2+}$	1×10^5	Crumblis *et al* (1986)
Tris(4,4′-substituted 2,2′-bipyridine) complexes of Fe(II), Ru(II) and Os(II)	$1 \times 10^5 - 1{\cdot}5 \times 10^7$	Zakeeruddin *et al* (1992)

3.5 *Kinetic aspects*

An approximate estimate of the surface coverage of the electrode was made by using the method of Sharp *et al* (1979) the peak current is related to the surface coverage by the relation,

$$i_p = (n^2F^2A\Gamma v)/(4RT), \tag{2}$$

where n represents the number of electrons involved in the reaction, $A(cm^2)$ is the area of the electrode, Γ (mol cm^2) is the surface coverage and other symbols have their usual significance. Using relation (2) the surface coverage was calculated to be $5{\cdot}8 \times 10^{-9}$ mol cm^{-2}.

We attempted to determine the approximate rate of the reaction, k ((2)) by using the method followed by Andrieux and Saveant (1978) who derived an expression relating the peak current with the concentration,

$$i_p = 0{\cdot}496\, nFA(DnFv/RT)^{1/2}.C, \tag{3}$$

where n is the no. of electrons involved in the reaction, A (cm^2) is the area of the electrode, C is the bulk concentration, D is the diffusion coefficient and other parameters have their usual significance. The constant 0·496 is given as a function of log $[k\Gamma/(DnFv/RT)^{1/2}]$ in figure 1 of Andrieux and Saveant (1978). From our data of the modified electrode with a coverage $5{\cdot}8 \times 10^{-9}$ mol cm^{-2}, this constant is found to be

0·389. Using this value, the rate constant, k for the reaction (2) was calculated to be $1{\cdot}9 \times 10^6\ M^{-1}s^{-1}$. This value is compared with the k values for other mediators reported in the literature (table 1).

4. Conclusion

It has been shown that BQ/H_2Q couple acts as an effective mediator between reduced GOD and a PG electrode. Its performance rests on a number of features: the rapid rate of electron transfer between reduced GOD and BQ; the well behaved electrochemistry of BQ and the low solubility of BQ.

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References

Andrieux C P and Saveant J M 1978 *J. Electroanal. Chem.* **93** 163
Bourdillon C, Laval J M and Thomas D 1986 *J. Electrochem. Soc.* **133** 706
Cardosi M F and Turner A P F (eds) 1991 In *Advances in biosensors* (London: Jai) vol 1, pp 125–169
Cass A E G (ed.) 1990 In *Biosensors – A practical approach* (Oxford: IRL Press)
Cass A E G, Davis G, Francis G D, Hill H A O, Aston W J, Higgins I J, Plotkin E V, Scott L D L and Turner A P F 1984 *Anal. Chem.* **56** 667
Crumbliss A L, Hill H A O and Page D J 1986 *J. Electroanal. Chem.* **206** 327
di Gleria K and Hill H A O 1992 *Inorg. Chim. Acta* **198** 863
Hendry S P and Turner A P F 1988 *Horm. Metab. Res. Suppl. Ser.* **20** 37
Hu J and Turner A P F 1991 *Anal. Lett.* **24** 15
Matthews D R, Holman R R, Brown E, Steemson J, Watson A, Hughes S and Scott D 1987 *Lancet* (April 4) 778
Murthy A S N and Anita 1993 *electroanalysis* **5** 265
Narasimhan K and Wingard L B 1986 *Anal. Chem.* **58** 2984
Pandey P C, Glazier S and Weetall H H 1993 *Anal. Biochem.* **214** 233
Sharp M, Petersson M and Edstorm K 1979 *J. Electroanal. Chem.* **95** 123
Turner A P F, Hendry S P and Cardosi M F 1987a *The World Biotech. Report* **1** 125
Turner A P F, Karube I and Wilson G S (eds) 1987b In *Biosensors – Fundamentals and Applications* (Oxford University Press)
Weast R C, Selby S M and Hodgman C D (eds) 1964–1965 In *Handbook of chemistry and physics* (Cleveland, OH: The Chemical Rubber Co.) pp C-201
Wring S A and Hart J P 1992 *Analyst* **117** 1215
Zakeeruddin S M, Fraser D M, Nazeeruddin M K and Gratzel M 1992 *J. Electroanal. Chem.* **337** 253