Original Papers

Amperometric Biosensors Based on a Biocatalyst Electrode with Entrapped Mediator

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A mathematical model of a biocatalyst electrode with entrapped mediator is presented. The electrode is a benzoquinone (BQ)-mixed carbon paste electrode (CPE), on which glucose oxidase (GOD) is immobilized by coating with a polymer film or semipermeable membrane (s.m., a film-coated GOD-BQ-CPE). BQ is dissolved in the immobilized-enzyme layer(e.l.) to mediate the electron transfer between the electrode and the enzyme. The model is focused on the diffusion accompanied with enzyme reaction of substrate (D-glucose) and mediators (BQ and its reduced form) in the e.l. and the diffusion in the s.m. The amperometric response of the electrode is analyzed as a function of transport, kinetic, and geometrical parameters and compared with experimental results. A glucose sensor based on a film-coated GOD-BQ-CPE has a wide concentration range of response and is not affected by oxygen in test solution. Biosensors for D-gluconate and ethanol based on film-coated gluconate dehydrogenase-BQ- and alcohol dehydrogenase-NAD-CPE's, respectively, are presented.

Keywords Biocatalyst electrode, biosensor, glucose sensor, gluconate sensor, ethanol sensor

The biocatalyst electrodes, that is, the oxidoreductase-immobilized electrodes, in which the electrode behaves as a substitute of an electron acceptor or donor of the enzyme reaction could be used in such novel applications as biosensors, bioreactors and biofuel cells (a review[1]). In these biocatalyst electrodes the presence of an electron transfer mediator between the electrode and the enzyme or enzyme reaction is useful to accelerater the electrocatalysis or bioelectrocatalysis at the electrodes. Thus it has been shown that, for instance, *p*-benzoquinone and its derivatives[2] and ferrocene and its derivatives[3] are useful to mediate the electron transfer between a graphite electrode and glucose oxidase (GOD) immobilized on the electrode surface. The carbon paste electrodes, first introduced by Adams[4] in the field of solid electrode electrochemistry, have a wide range of cathodic and anodic utility.[5] Ikeda *et al.*[6] mixed benzoquinone (BQ, a mediator) with carbon powder and paraffin liquid to make a BQ-mixed carbon paste electrode (BQ-CPE) and immobilized GOD on the surface of the electrode by coating with a polymer film. It was shown that BQ was dissolved and retained in the immobilized enzyme layer on the electrode surface and worked satisfactorily to mediate the electron transfer between the electrode and the enzyme. In this paper theory of the biocatalyst electrode with electron transfer mediator will be discussed with reference to its use as biosensors. Some experimental results are also presented. Use of the biocatalyst electrode in organic synthesis shall be discussed elsewhere.[7]

Theory

The model considered here is of a homogeneous immobilized-enzyme layer (e.l.) of thickness l which is placed immediately adjacent to the electrode surface and is confined there by means of a semi-permeable membrane (s.m.) of thickness l_m. We assume that the enzyme reaction is of ping-pong mechanism such as (for instance, for oxidation)

$$S + E_{ox} \underset{k_{1-}}{\overset{k_1}{\rightleftharpoons}} SE_{ox} \xrightarrow{k_2} P + E_{red} \quad \cdots\cdots\cdots\cdots \quad (1)$$

$$M_{ox} + E_{red} \underset{k_{3-}}{\overset{k_3}{\rightleftharpoons}} ME_{red} \xrightarrow{k_4} M_{red} + E_{ox} \quad \cdots \quad (2)$$

where S and P are the substrate (D-glucose in the case of GOD) and product, E_{ox} and E_{red} the oxidized and reduced forms of enzyme, and M_{ox} and M_{red} the oxidized and reduced forms of mediator, respectively. k_1, $\cdots$ are the rate constants of the indicated steps, respectively. The reduced mediator is oxidized at the electrode surface by releasing n electrons to the oxidized mediator,

$$M_{red} - ne \underset{\overleftarrow{k}}{\overset{\overrightarrow{k}}{\rightleftharpoons}} M_{ox} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (3)$$

where $\overrightarrow{k}$ and $\overleftarrow{k}$ are the rate constants of the charge transfer reaction at the electrode and are given, for

instance, by Butler–Volmer equations, respectively, as a function of the electrode potential. In this one-dimentional problem (see Fig. 1), the steady-state equations for the system are given by[8]

$$D_S\frac{d^2c_S}{dx^2} - v_E = 0 \quad \cdots\cdots (4a)$$

$$D_P\frac{d^2c_P}{dx^2} + v_E = 0 \quad \cdots\cdots (4b)$$

$$D_O\frac{d^2c_O}{dx^2} - v_E = 0 \quad \cdots\cdots (4c)$$

$$D_R\frac{d^2c_R}{dx^2} + v_E = 0 \quad \cdots\cdots (4d)$$

where D_j and c_j are the diffusion coefficients and the concentrations of j species (j=S, P, O for M_{ox}, and R for M_{red}) respectively, in the e.l. and v_E is the steady-state rate of the enzyme reaction per unit volume and given by

$$v_E = \frac{k_{cat}E}{1+(K_1/c_S)+(K_2/c_O)} \quad \cdots\cdots (5)$$

where the (maximum) enzyme reaction rate constant k_{cat} and the Michaelis constants K_1 and K_2 for the substrate and mediator, respectively, are related to the rate constants $k_1\cdots$ in eqs. (1) and (2) (see, for instance, eqs. (2) (3) and (4) in ref. 2). E is the concentration of the enzyme in the e.l. These equations have been solved by previous authors[8-10] under slightly different conditions than the conditions we shall use here. The solutions, hence the amperometric response of the biocatalyst electrode should depend on the Thiele modulus or its simplified form σ defined by

$$\sigma^2 = k_{cat}El^2/K_1D_S. \quad \cdots\cdots (6)$$

The σ parameter represents the ratio of the rate constant of enzyme reaction, $k_{cat}El/K_1$, to the rate of diffusion, D_s/l, in the e.l. (see eq. (7) below).

We first consider the case when the enzyme reaction is first order with respect to the substrate, that is, K_1/c_S is much larger than $1+(K_2/c_O)$ in eq. (5), then we have

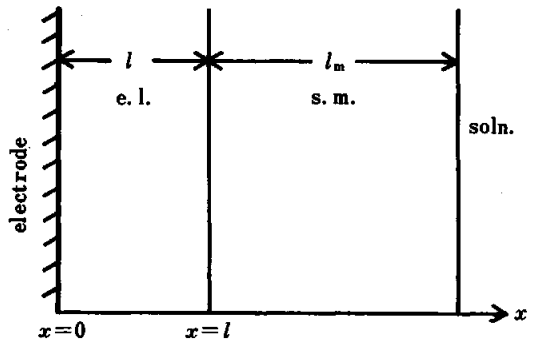

Fig. 1 Scheme of the electrode.

$$v_E = k_{cat}Ec_S/K_1. \quad \cdots\cdots (7)$$

Under the boundary conditions that $dc_S/dx=0$ at the electrode surface ($x=0$) and $c_S={}^lc_S$ at the boundary between the e.l. and the s.m. ($x=l$), the solution of eq. (4a) is readily obtained as

$$c_S = {}^lc_S\cosh(\sigma x/l)/\cosh(\sigma). \quad \cdots\cdots (8)$$

Equation (8) is illustrated in Fig. 2. As seen in Fig. 2, the concentration polarization of S becomes negligible when σ approach zero. In the steady state the rate of conversion of S to P in the e.l. is equal to the flux of S at $x=l$, f_S, which is derived from eq. (8) as

$$f_S = (k_{cat}El/K_1)[\tanh(\sigma)/\sigma]{}^lc_S \quad \cdots\cdots (9)$$

for the linearlization approximation by eq. (7). For the general case where the enzyme reaction is not first order with respect to the substrate and/or the mediator the eq. (4a) etc. cannot be directly solved in terms of an easily interpretable solution but be solved by employing numerical technique with a computer. This was actually performed by Ikeda *et al.*[11] to obtain the numerical solutions for various dimentionless parameters under the appropriate boundary conditions. Here we shall not discuss the results, which will be published elsewhere, but use an approximate equation using the apparent or effective Michaelis constants K_1' and K_2' instead of K_1 and K_2 in eq. (5);

$$f_S = \frac{k_{cat}'El}{1+(K_1'/{}^lc_S)+(K_2'/{}^oc_O)} \quad \cdots\cdots (10)$$

where ${}^oc_O=c_O$ at $x=0$. The apparent Michaelis constants are introduced to take into account the correction for the concentration polarization of S and M_{ox} in the e.l. (see Fig. 2), hence it is expected $K_1'>K_1$ and $K_2'>K_2$.

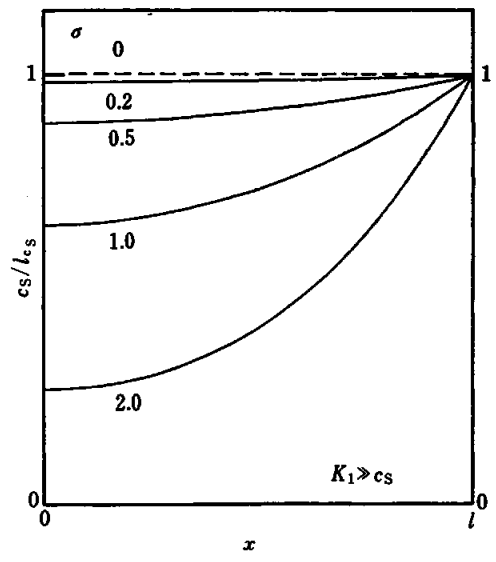

Fig. 2 Concentration profiles of substrate in the enzyme layer (e.l.) for varoius parameters σ.

In eq. (10) k_{cat} is also replaced by the apparent rate constant k_{cat}'. Comparison of eq. (9) with eq. (10) indicates that $k_{cat}'/K_1'=(k_{cat}/K_1)[\tanh(\sigma)/\sigma]$ for the linearization approximation. Also note that k_{cat}'/K_1' approaches k_{cat}/K_1 as σ approaches zero. The substrate is transported to the e.l. through the s.m. from the bulk of solution. Accordingly we have

$$f_S = P_{S,m}[{}^*c_S-({}^lc_S/\beta_S)] \quad \cdots\cdots (11)$$

where $P_{S,m}$ is the permeability of the s.m. to S, ${}^*c_S$ the concentration of the substrate in solution immediately adjacent to the s.m. and β_S the distribution coefficient of the substrate between the e.l. and the solution. Substitution of eq. (11) for ${}^lc_S$ in eq. (10) leads to the flux f_S as a function of ${}^*c_S$ (see Discussion).

The steady-state current is related to the flux of the reduced mediator at the electrode surface, f_R;

$$I/nFA = f_R = D_R(dc_R/dx)_{x=0} \quad \cdots\cdots (12)$$

where F is the Faraday constant and A the electrode surface area. In general, however, all of the reduced mediator produced in the e.l. are not oxidized at the electrode but a part of them is transported to and diffused out through the s.m. into the solution, except in the ideal case where the s.m. is impermeable to the mediator. Accordingly we define the collection factor f_c which represents the ratio of the amount of reduced mediator collected by electrode to the total amount of reduced mediator produced by the enzymatic reaction in the e.l. Therefore we have at the steady-state

$$I/nFA = f_R = f_c f_S \quad \cdots\cdots (13)$$

where f_R is the flux of reduced mediator at $x=0$ and is obtained by solving eq. (4d) under the appropriate boundary conditions; at the boundary of the e.l. and the s.m.

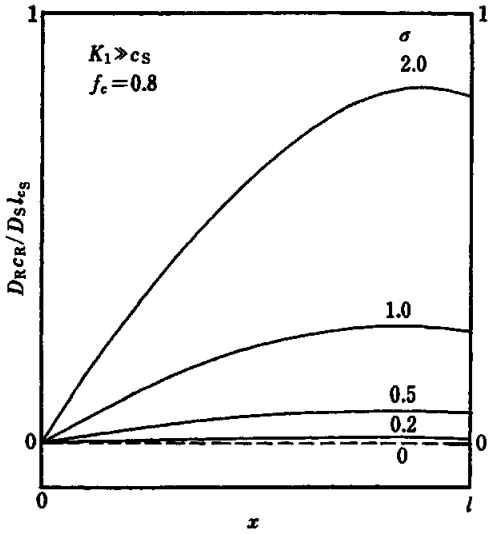

Fig. 3 Concentration profiles of reduced mediator in the e.l. for various parameters σ at a collection factor $f_c=0.8$.

$$x = l: \quad D_R(dc_R/dx)_{x=l} = P_{R,m}{}^lc_R/\beta_R \quad \cdots\cdots (14)$$

and at the electrode surface for the limiting current

$$x = 0: \quad {}^oc_R = 0 \quad \cdots\cdots (15)$$

where $P_{R,m}$ is the permeability of the s.m. to the reduced mediator, ${}^lc_R$ the concentration of the reduced mediator in the e.l. at $x=l$, β_R the distribution coefficient of the reduced mediator between the e.l. and the solution. The solution for the case of eq. (7) is given by

$$f_R = (k_{cat}El/K_1)[\tanh(\sigma)/\sigma]{}^lc_S f_c \quad \cdots\cdots (16)$$

with

$$f_c = 1 - \frac{(P_{R,m}/P_R)}{1+(P_{R,m}/P_R)}F(\sigma) \quad \cdots\cdots (17)$$

where $F(\sigma)=1-[\cosh(\sigma)-1]/\sigma\sinh(\sigma)$ and $0.5<F(\sigma)<1$ for $0<\sigma<\infty$, and P_R is the permeability, defined by $P_R=D_R\beta_R/l$, of the e.l. to the reduced mediator. Figure 3 shows the concentration profiles of the reduced mediator in the e.l. The f_c value of 0.8 in Fig. 3 was used to make prominent the effect of the collection factor (see Discussion).

Theory of the current–potential curves, where the boundary condition eq. (15) is replaced by, for instance, $f_R=\vec{k}^o c_R-\overleftarrow{k^o}c_O$ (see eq. (3)), will be discussed elsewhere.[7]

Response time. The transient response of amperometric enzyme electrodes has been discussed by Bergel and Comtat[10] and Mell and Maloy[12] using the digital simulation technique. Here we shall take a slightly different approach to this problem. Most of the solution for diffusion in a plane sheet of length l are given in the form of infinite series which can be written as $M_t/M_\infty=1-\Sigma f(x)\exp(-\nu t)$, M_t and M_∞ being the solutions at time t and $t\to\infty$, respectively. Here $f(x)$ and ν, both being not a function of t and ν not of x, are different for each term in the series.[13,14] Accordingly, the transient course of the process, as M_t approaches M_∞, may be discussed in terms of an exponential function of ν_1, usually the first term of the series. Thus we can discuss the "response time τ" of the present biocatalyst electrode by $\tau=1/\nu$, ν being that of the solution of the diffusion or diffusion–reaction process concerned. Under the assumption that the enzymatic reaction rate is approximately given by ν_E defined by eq. (7) even in non-steady state diffusion equations, in stead of eqs. (4a–d), we get the response time for the diffusion–reaction of substrate (eq. (8) as $\tau=(4l^2/\pi^2D_S)$-$[1/(1+4\sigma^2/\pi^2)]$[14], and finally the response time for the flux of the reduced mediator as given by

$$\tau_{e.l.} = (4l^2/\pi^2D_R) + (4l^2/\pi^2D_S)[1/(1+4\sigma^2/\pi^2)]. \quad \cdots\cdots (18)$$

Also we have the response time for diffusion of substrate in the s.m. as given by

$$\tau_{s.m.} = (4l_m^2/\pi^2 D_{S,m}). \quad \cdots\cdots (19)$$

Accordingly, the response time of the biocatalyst electrode with mediator may be evaluated by

$$\tau = \tau_{e.l.} + \tau_{s.m.} \quad \cdots\cdots (20)$$

If the enzyme reaction is first order with respect to the mediator in stead of eq. (7), a slightly different expression of the response time for the flux of the reduced mediator in place of eq. (18) is derived. These are only an approximate approach to the problem of the "response time" of the amperometric response of the biocatalyst electrode with mediator, but are instructive to show which factors are important to determine the response time.

Experimental

Figure 4 shows the construction of a film-coated GOD-immobilized BQ-mixed carbon paste electrode (a film-coated GOD-BQ-CPE).[6,15] The method of preparing the electrode was described previously.[6] In brief, a weighed amount of mediator (here BQ) was mixed with paraffin liquid and carbon powder to make a BQ-mixed carbon paste electrode, on which enzyme (here GOD) was immobilized by coating or covering with a semipermeable membrane (here a polymer film such as a nitrocellulose film or dialysis membrane). The whole electrode was covered by a nylon net to give the electrode physical strength. The geometrical surface area of the electrode was 0.09 cm^2. The electrolysis current was measured at a constant applied potential using a three electrode system at 25°C. The test solution was stirred with a magnetic rotor at 600 r.p.m, when the concentration polarization in the solution side adjacent to the s.m. surface seemed negligibly small.[15] Other details on experimental methods were described elsewhere.[15]

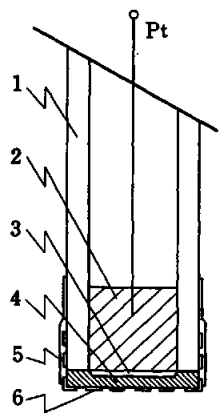

Fig. 4 A film-coated GOD-immobilized BQ-mixed carbon paste electrode. 1, glass tube; 2, mediator(BQ)-mixed carbon paste electrode; 3, immobilized enzyme(GOD)-layer; 4, semipermeable membrane (coating film); 5, heat shrinkable tube: 6, nylon net.

Results and Discussion

Previous experiments with film-coated GOD-BQ-CPE's have shown that in these electrodes BQ was dissolved in the e.l. on the electrode surface to reach a steady-state concentration of BQ there, that the loss of BQ due to leakage through the film was small enough compared with the supply of BQ from the CPE as a BQ reservoir, and that the concentration of BQ in the e.l. increased with increasing concentration of BQ in the carbon paste, m_{BQ}. The catalytic oxidation current of D-glucose observed with this electrode increased with increasing m_{BQ} to reach a saturation value at m_{BQ} larger than 20% (see Fig. 5 in ref. 15), indicating that $K_2'/{}^oc_O \ll 1$ in eq. (10) at $m_{BQ} > 20\%$. The collection factor in eq. (13) is supposed to be nearly equal to unity for the usual electrodes since $l/l_m \ll 1$ (see below). Thus the steady-state limiting current is expressed by

$$I/nFA = f_S = \frac{k_{cat}' El}{1+(K_1'/{}^lc_S)} \quad \cdots\cdots (21)$$

and using eq. (11) it is given as a fuction of ${}^*c_S$, the bulk concentration of the substrate:

$$\frac{I}{nFA}\left[1+\frac{k_{cat}' El\beta_S}{K_1' P_{S,m}}+\frac{\beta_S}{K_1'}\left({}^*c_S-\frac{(I/nFA)}{P_{S,m}}\right)\right] = \frac{k_{cat}' El\beta_S}{K_1'}{}^*c_S. \quad \cdots\cdots (22)$$

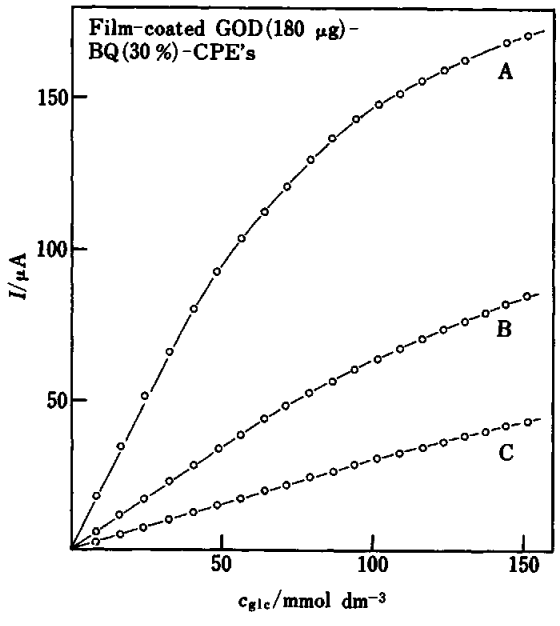

Fig. 5 Dependence of the current(I) on the concentration of D-glucose (c_{glc}) obtained with film-coated GOD(180 μg)-BQ(30%)-CPE's; A, nitrocellulose film of 50 μm thickness; B and C, dialysis membrane (Visking Co.) of 50 and 100 μm thickness, respectively. The current was measured at 0.5 V *vs.* SCE.

This equation predicts that I increases first linearly with increasing $^*c_S$ but deviates downward from the linearity at large $^*c_S$. The linearity extends to the concentration larger than K_1 owing to (a) the diffusion-reaction in the e.l. (that is, $K_1' > K_1$) and (b) the diffusion in the s.m. (eq. (11)). This prediction was experimentally verified by Ikeda *et al.*[15] Some of the results are reproduced in Fig. 5. These results indicate that the film-coated GOD–BQ–CPE can be designed to make D-glucose sensors for use at from low to high concentration range by the selection of $P_{S,m}$, $k_{cat}'El$, and K_1' (and $K_2'/{}^oc_O$ if necessary). When $P_{S,m}$ is sufficiently small, eq. (22) is reduced to $I/nFA = P_{S,m}c_S$, that is, the current becomes permeability-controlled. Also, this D-glucose sensor can be designed to show the current response independent of the oxygen tension in the test solution, as seen in Fig. 6. When the concentration of mediator in the e.l. is sufficiently high ($K_2'/{}^oc_O \ll 1$ in eq. (10), so that eq. (21)), the current response becomes independent of the concentration of any oxidants (oxygen etc.) that can function as the mediator. Setting the electrode at sufficiently positive potential to oxidize all possible reduced mediators, if any, promotes to make this advantage perfect. The sufficiently positive potential setting of the electrode also hepls to eliminate the interference caused by an occurrence that the reduced mediator (or mediators) might be oxidized by coexisting oxidant(s) in the e.l., provided that the resulting reduced form of the oxidant(s) is oxidizable at the electrode.

Interference by coexisting oxidizable substances, such as L-ascorbic acid, uric acid and others due to their oxidation at the electrode can be eliminated by placing an electrochemical filter (a pre-grid electrode[16]) or other filters (a selective permeability membrane etc.) in front of the film-coated electrode.

Response time observed with the film-coated GOD–BQ–CPE's examined in this study was 20 to 100 s. This result indicates that the response times of these electrodes are determined mainly by diffusion through the s.m. (the coating film, eq. (20)); the thickness of the s.m. was 25 to 100 μm whereas that of the e.l. was supposedly in the order of 1 μm or less.

Choice of the s.m. (the coating film) is important in designing the electrodes for use as sensors. It not only concerns with the immobilization of enzyme but also protects the enzyme from undesirable contaminations from the test solution. When the permeability of the s.m. to substrate is small and the current is permeability-controlled, then the current response of the electrode becomes apparently independent of the rate of the enzyme reaction (the reaction mode, the life time of immobilized enzyme) in the e.l., resulting in an increased life time and stability (for instance, not effected by pH of test solution in spite of the inherent pH dependence, if any, of enzyme reaction etc.), though in the cost of decreased sensitivity (small $P_{S,m}$) and prolonged response time (larger $\tau_{s.m.}$ in eq. (19)). Permeability of the s.m. to mediator should be as small as possible or preferably null to retain the mediator effectively in the e.l. and to make the collection factor (nearly) equal to unity. This is also concerned with the choice of mediator. Polymer mediators or redox polymers could be used but probably in the cost of prolonged response time since their diffusion coefficients (or apparent charge transfer diffusion coefficients of redox polymers) are usually not large.[17]

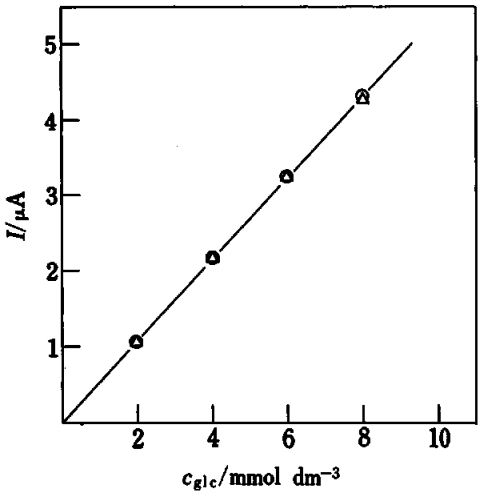

Fig. 6 Calibration curves of D-glucose obtained with a gold minigrid-attached film(dialysis membrane, 50 μm)-coated GOD(30 μg)–BQ(30%)–CPE in air-saturated (Δ) and deaerated (O) solutions. The current was measured at 0.5 V *vs.* SCE.

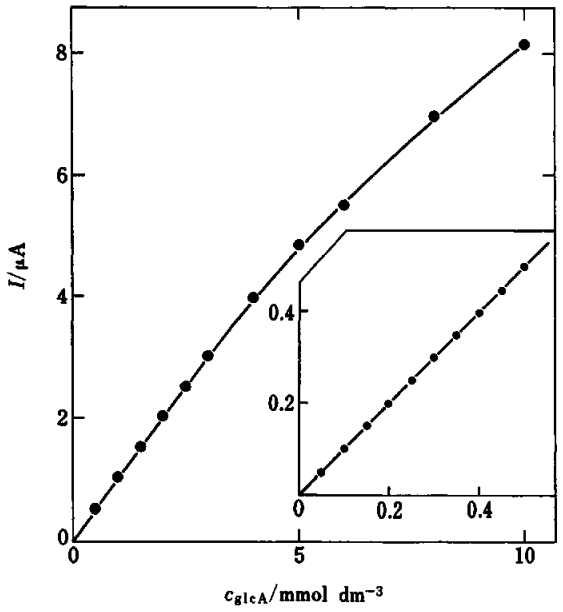

Fig. 7 Dependence of the current(I) on the concentration of D-gluconate (c_{glcA}) obtained with a film-coated GADH(56 μg)–BQ(1.3%)–CPE.

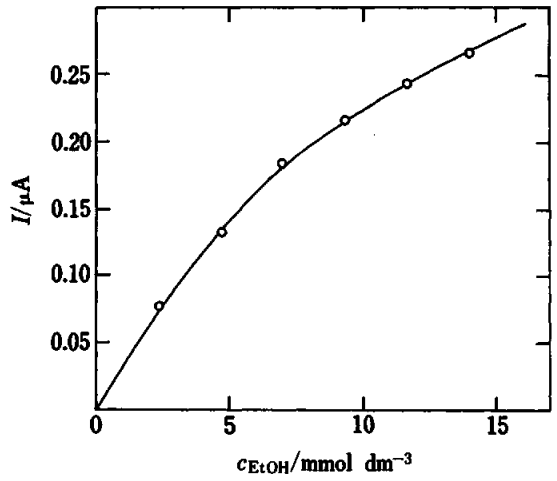

Fig. 8 Dependence of the current(I) on the concentration of ethyl alcohol (c_{EtOH}) obtained with a film-coated ADH(100 μg)-NAD(6%)-CPE.

Further problem in the choice of mediator, especially from electrochemical or synthetic point of view, has been discussed elsewhere.[7]

The biocatalyst electrodes can be constructed in principle on any kinds of dehydrogenase as well as oxidase, provided that the way to conjugate the electron transfer between the electrode and the enzyme or enzyme reaction is available. Here we shall show two examples of the biocatalyst electrodes based on mediator- and cofactor-mixed carbon paste electrodes. A film (dialysis membrane of 20 μm thickness)-coated D-gluconate dehydrogenase(GADH, 56 μg)-immobilized BQ(1.3%)-mixed carbon paste electrode (a film-coated GADH-BQ-CPE) was constructed as described in Experiment. GADH was prepared from *Pseudomonas fluorescens* FM-1.[18] Figure 7 shows the dependence of the current, measured at 0.4 V *vs.* SCE with this electrode, on the gluconate concentration in 0.1 mol dm^{-3} acetate buffer of pH 4.5. In the GADH electrode ubiquinone also functioned satisfactorily as the mediator.[18] Figure 8 shows the calibration curve of ethylalcohol obtained with a film(nitrocellulose film of 50 μm thickness)-coated alcohol dehydrogenase(ADH, 100 μg)-immobilized NAD^+(6%)-mixed carbon paste electrode (a film-coated ADH-NAD-CPE). ADH was purchased from Sigma Co., (lot No. 68F-8017). The current was measured at 0.55 V *vs.* SCE on 0.24 mol dm^{-3} pyrophosphate buffer of pH 8.5.

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