The steady-state current at microcylinder electrodes modified by enzymes immobilized in conducting or non-conducting material

M. Somasundrum *, K. Aoki

Department of Applied Physics, Faculty of Engineering, Fukui University, 3-9-1 Bunkyo, Fukui-shi 910-8507, Japan

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Abstract

A diffusion-kinetic model is presented for an enzyme-modified microcylinder electrode, where the enzyme reaction generates an electro-active product. Simple, approximate expressions are derived for the steady-state current in cases where the enzyme is immobilized in a metallically conducting, or a non-conducting matrix. The model is also extended to the chemical sensor case, of a conducting polymer without enzyme. The model is used to analyze steady-state signals for glucose produced by Pt-coated carbon fibres, on which glucose oxidase has been entrapped in poly(1,2-diaminobenzene). © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The use of microelectrodes is becoming increasingly common in biosensors [1–3]. This is due to factors such as fast response times, high signal: noise ratios, and the ability to operate in low conductivity media, sub-micro volumes and in vivo [4]. Among the possible microelectrode geometries, microcylinders such as carbon fibres, are often used. This is because they are cheap, readily available, their form is suited to implantation [5] and because much is known about their surface characteristics [6]. Also, the rates of some electrode reactions at carbon fibres can be enhanced by chemical or electro-chemical pretreatment [7–10]. Enzymes have been immobilized at carbon fibres by methods such as dip-coating in enzyme/cross-linking solutions [7,11,12], the biotin–avidin–biotin binding method [13] and entrapment during the electrodeposition of a conducting polymer [14] or a metal [15]. Carbon fibre biosensors have been described for analytes such as pyruvate [16], glucose [10,15,17], hydrogen peroxide [11,13], lactate [12,18], 4-aminophenyl phosphate [19], acetylcholine [20], hypoxanthine [21] and glutamate [22]. These sensors have been used for batch measurement, as well as in flow-through systems [14], in cell culture media [21] and in vivo [12], and have been used in both amperometry [7] and more complex applied waveforms, such as DPV [12]. Although less common, micro-cylinder biosensors using other materials, such as platinum, have also been reported [23].

In biosensor research, mathematical models are often useful in the understanding and optimization of electrode modifications. Rate limiting factor(s) can be identified, and the extraction of kinetic and physical data enables results from different systems to be compared meaningfully.

Approximate analytical solutions have previously been derived for enzymes in solution [24,25] and entrapped in redox [26], conducting [27,28] and non-conducting polymers [29,30], or held in thin solution layers behind membranes [31]. In the immobilized enzyme models it has been assumed either that diffusion was planar, or that the films were so thin that concentration gradients did not exist [29]. The last case has been used to analyze microelectrode results [29]. However, in practice it is often the case that thicker films are used, and numerical simulations have suggested that even below 1 μm film thickness, concentration polariza-
tion of reactants can be significant [32]. Hence, when concentration polarization occurs, none of the previous planar solutions can be applied to a microcylinder. Fluxes at a microcylinder biosensor have been plotted by digital simulation [33], but so far there have been no analytical expressions of the current. However, analytical solutions would be useful, since they enable various limiting cases to be indentified, along with the effect of experimental parameters on the flux. Therefore the aim of this work is to describe a mathematical model suitable for micro-cylinder electrodes. The model is written for an enzyme reaction to generate an electro-active product (e.g. hydrogen peroxide from an oxidase enzyme), that either reacts at an immobilization matrix which is metallically conducting (applicable to an electrodeposited metal, or a polymer which contains sufficient conducting sites/particles, see Fig. 1); or that the product reacts at the underlying electrode. It is assumed that the sensor is used for batch measurement in a stirred solution, which would provide a simple means to characterize the sensor, prior to its use in a more complex system (e.g. in a flowing stream or in vivo). Extension of the model to chemical sensors using microcylinders, is also described.

2. Theory

2.1. Mass balances

The system presented here is a cylindrical rod electrode which is concentrically coated with a porous material that may be electrically conducting, immobilizing an enzyme E such as an oxidase. The coated electrode is present in a stirred solution which contains the enzyme substrate S and an excess of supporting electrolyte. Assuming Michaelis–Menten kinetics, the reaction within the film is

\[ S + E \xrightarrow{k_1} \frac{1}{k_{-1}} [E, S] \xrightarrow{k_2} P + E_2 \]  

When the film contains so much conducting material as to cause electric percolation [34], the electrochemical oxidation also occurs in the conducting material itself, as is shown in Fig. 1. The consumption rate of S is given by \( k_1 c_S c_E - k_{-1} c_{ES} \), where \( c_i \) denotes the concentration of species \( i \). The rate is equivalent to \((k_{cat}/K_M) c_S c_E\), where \( K_M \) is the Michaelis constant, defined as \( K_M = (k_{-1} + k_{cat})/k_1 \). The rate soon reaches a steady-state partly because the solution is stirred at a uniform speed and partly because mass transport in the film is fast enough to be completed. The consumption rate of S in the film is compensated by diffusion. The mass balances for S in the film can be written in cylindrical coordinates:

\[ \frac{D_S}{r} \frac{d}{dr} \left[ r \frac{d c_S}{dr} \right] - \frac{k_{cat} c_E c_S}{K_M} = 0 \]  

where \( D_S \) is the diffusion coefficient of S.

The product P in reaction (1) is usually hydrogen peroxide, which is oxidized electrochemically to oxygen at the electrode. When the conducting material in the film is electrically percolated to the electrode and hence can work as an electrode, hydrogen peroxide is consumed by electrochemical oxidation even at the conducting material. The rate of consumption will be \( \nu(r) = k c_H \), where \( k \) is the rate constant for the hydrogen peroxide reaction, and \( c_H \) is the peroxide concentration. Then the equation of continuum for hydrogen peroxide is generally expressed in the steady-state by

\[ \frac{D_H}{r} \frac{d}{dr} \left[ r \frac{d c_H}{dr} \right] + \frac{k_{cat} c_E c_S}{K_M} - \nu(r) = 0 \]  

Under the condition of limiting current, the boundary conditions at the electrode surface \( (r_0) \) and at the film surface \( (r_1) \) are given by

\[ r = r_0 : \frac{d c_S}{dr} = 0, \quad c_{S*} = 0 \]  
\[ r = r_1 : \quad c_S = c_S^* \quad c_H = 0 \]  

where \( c_S^* \) is different from the bulk concentration of the substrate by the partition coefficient of the film. The
oxidation of hydrogen peroxide at a local site in the film generates the current, which flows through the electrically percolated domain of the film to the electrode (see Fig. 1). The current is provided by the consumption rate at each site. Thus, the total current at an electrode of length \( L \), is expressed by

\[
I = I_0 + I_1 - 2 \pi k_{\text{cat}} n F \int_{r_0}^{r_1} r v \, dr
\]

According to the theory of percolation [34], the electric connection is either present or absent, showing a phase transition at a concentration threshold of the conducting material. For the former case, the diffusion term is neglected. Then Eq. (6) is re-written as

\[
I = 2 \pi L L_{\text{H}}(dc_{\text{H}}/dr)_{r=r_0}
\]

For the latter case, \( v(r) \) in Eq. (3) is neglected and \( v \) in Eq. (6) is replaced by the diffusional flux at \( r_0 \)

\[
I = 2 \pi L r_0 \cdot L_{\text{H}}(dc_{\text{H}}/dr)_{r=r_0}
\]

The analytical expression for the current can be obtained as follows: the expression for \( c_S \) is derived from Eq. (2) under the assumption of a uniform value of \( c_{E} \). Expressions for \( v \) and \( c_{1H} \) are obtained from Eq. (3) for known \( c_E \). Eqs. (7) or (8) give expressions for the current. The solution of Eq. (2) is in the form of modified Bessel functions of zeroth order, \( I_0(\chi r) \) and \( K_0(\chi r) \), where \( \chi^2 = k_{\text{cat}} c_E / D_S K_M \). Application of Eqs. (4) and (5) gives

\[
c_S = c_S^0 \left[ \frac{K_1(\chi r_0) I_0(\chi r) - I_1(\chi r_0) K_0(\chi r)}{K_1(\chi r_0) I_0(\chi r_1) + I_1(\chi r_0) K_0(\chi r_1)} \right]
\]

2.2. Electrode reaction at conducting material in the film

When the conducting material is percolated to the electrode, the current is expressed by Eq. (7). Inserting Eq. (9) into Eq. (7) and carrying out the integration, we obtain

\[
\frac{I}{nF c_S^0 D_S L} = g_A(r_1/r_0, \chi r_0)
\]

\[
= 2 \pi \chi r_1 \left[ \frac{K_1(\chi r_0) I_0(\chi r) - I_1(\chi r_0) K_0(\chi r)}{K_1(\chi r_0) I_0(\chi r_1) + I_1(\chi r_0) K_0(\chi r_1)} \right]
\]

This is the expression for the current as a function of the kinetic parameters \( \chi r_0 \) and \( \chi r_1 \).

When \( \chi r_0 \) is so large that \( I_n(x) \approx e^{-x} / \sqrt{(2\pi x)} \) and \( K_n(x) \approx e^{-x} / \sqrt{(\pi 2x)} \), Eq. (10) is reduced to

\[
I/nF \approx 2 \pi c_S^0 D_S \chi L r_1 \tanh(\chi(r_1 - r_0))
\]

This is essentially the same as the expression for the current at a planar electrode, derived by Bartlett and Whitaker [27]. Further simplification of Eq. (10) by use of \( \tanh(\chi(r_1 - r_0)) = 1 \) (for high enzyme activity and/or thick films) yields

\[
I/nF = (2\pi r_1) L c_S^0 D_S \chi. 
\]

This suggests \( \chi \) is the thickness of the reaction layer. Reaction is at the edge of the film and therefore the current is independent of film thickness. Alternatively, for low enzyme activity and/or thin films, applying the approximations of modified Bessel functions for small arguments \( I_n(x) = 1, I_1(x) = x/2, K_0(x) = -\ln(x), K_1(x) = 1/x \) to Eq. (10) gives

\[
I/nF \approx c_S^0 (k_{\text{cat}} c_E / K_M) L \pi (r_1^2 - r_0^2)
\]

This equation implies that the enzymic reaction occurs uniformly in the film volume, \( L \pi (r_1^2 - r_0^2) \).

As indicated in Eq. (10), the dimensionless current, \( g_A \), can be described in terms of a dimensionless parameter for film thickness \( r_1/r_0 \) and enzyme kinetics \( \chi (r_0) \). In Fig. 2 numerical values of \( g_A \) are plotted against \( r_1/r_0 \) for different values of \( \chi (r_0) \). It can be seen that \( g_A \) continues to increase with film thickness (although in practice the response would eventually be limited by mass transport of substrate in solution). This is because, since \( H_2O_2 \) is assumed to undergo rapid oxidation adjacent to where it is generated, without diffusing to the electrode, the response is always controlled by enzyme kinetics. In order to evaluate \( g_A \) readily, we obtained the simple approximate equation:

\[
g_A(\alpha, x) = 2 \alpha x \tan(x^2(\alpha - 1))
\]

where \( \beta \) is an empirical constant dependent on the value of \( \alpha \) (\( \beta r_1/r_0 \)), as listed in Table 1. The values of \( \beta \) for each corresponding range of \( \alpha \) are chosen such that \( g_A \) calculated from Eq. (13) does not vary by more than 5% from the Eq. (10) result. For values of \( \alpha \) obtained from geometric measurements, we can plot a curve. Since the
expression for $D$ does not have to be known because boundary conditions are known for $E$ (Eqs. (4) and (5)). In fact, to calculate the current we only require an expression for $\frac{D}{c_s}$ term on the right hand side of Eq. (10) is evaluated from experiments, we can evaluate $x(=\chi r_0)$ from the curve. If $D_s$ and $c_{s0}$ are estimated, we can determine a value for $k_{cat}/K_M$.

### 2.3. Reaction at electrode only

If hydrogen peroxide reacts at only the underlying electrode, then Eq. (3) holds without $v$:

$$\frac{D_H}{\gamma} \frac{dc_{H1}}{dr} (\frac{dc_{H1}}{dr} + \chi^2 D_s c_s = 0$$ (14)

Since the analytical expression for $c_s$ has already been obtained (Eq. (9)), we integrate Eq. (14) twice under the boundary conditions (Eqs. (4) and (5)). In fact, to calculate the current we only require an expression for $(dc_{H1}/dr)_{r=r_0}$. However, the second integration is necessary because boundary conditions are known for $c_{H1}$, but not for $dc_{H1}/dr$. Note that the first integration of the expression for $c_s$ (Eq. (9)) converts $I_0(\chi r)$ and $K_0(\chi r)$ to $I_1(\chi r)$ and $K_1(\chi r)$ respectively. The second integration does not have a closed solution, but can be computed numerically. Thus when the expression for $c_{H1}$ is inserted into Eq. (8) we have

$$\frac{I}{nF c_{s0} D_s L} = g_{s}(\chi r_0, x) \equiv \frac{2\pi}{\ln x}$$

$$K_{1}(\chi r_0) \int_{r_i}^{r_f} I_1(\chi r) \frac{dr}{r} - I_1(\chi r_0) \int_{r_0}^{r_f} K_1(\chi r) \frac{dr}{r}$$

$$K_1(\chi r_0) I_0(\chi r_f) + I_1(\chi r_0) K_0(\chi r_f)$$ (15)

Applying the asymptotic forms of the modified Bessel functions to Eq. (15) for large values of $\chi r_0$ we obtain

$$\frac{I}{nF} \approx 2\pi L c_{s0} D_s / \ln x$$ (16)

The rapid enzymic reaction makes the current become controlled by diffusion of substrate through the region of film near the solution interface. Since hydrogen peroxide is generated near the interface, and must diffuse to the electrode, increasing $x (= r_1/r_0)$ causes more peroxide to be lost to the solution, and so the response decreases.

In contrast, the current for small values of $\chi r_0$ is expressed by

$$\frac{I}{nF} \approx \frac{\pi(r_1 - r_0) L r_0 c_{s0}^2 k_{cat} c_E}{2 K_M}$$ (17)

This equation suggests the current is kinetically controlled.

Fig. 3 shows curves of $g_{s}$ as a function of $r_1/r_0$, computed from Eq. (15), where the integration was performed using commercial software (MATHCAD, version 7). The response has a maximum corresponding to the changeover from kinetic to diffusion control. The optimum thickness decreases as either enzyme activity or electrode radius increase. We obtained an approximate equation in the following form:

$$g_{s}(\alpha, x) = \{2\pi/\ln(x)\} \{1 - \text{sech}(\chi^2 (x - 1))\}$$ (18)

where again $\beta$ is an empirical constant. The value of $\beta$ corresponding to a given value of $x$, is shown in Table 2. The tabulated values are such that Eq. (18) produces results not more than 5% different from the Eq. (15) values.

### 2.4. Extension to chemical sensors

Conducting polymer films are sometimes used in electroanalysis without the entrapment of enzyme. In this case the analyte reacts directly at the polymer backbone [35]. The advantage of this modification above that of a bare electrode, is that reaction occurs through a volume, rather than on a surface. Carbon fibre electrodes modified with poly(3-methylthiophene) have been used in this manner to detect amino acids [36] and catecholamines [37]. In such a system, at steady-state the current will be equivalent to the flux of analyte entering the film, $D_{s}(dc_{s}/dr)_{=r_1}$. If we assume reaction

<table>
<thead>
<tr>
<th>$x$</th>
<th>$\beta$</th>
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<tbody>
<tr>
<td>$&lt; 1.3$</td>
<td>1</td>
</tr>
<tr>
<td>$1.3 &lt; x &lt; 2.4$</td>
<td>1.12</td>
</tr>
<tr>
<td>$2.4 &lt; x &lt; 2.9$</td>
<td>1.22</td>
</tr>
<tr>
<td>$2.9 &lt; x &lt; 16$</td>
<td>1.3</td>
</tr>
<tr>
<td>$16 &lt; x &lt; 25$</td>
<td>1.32</td>
</tr>
</tbody>
</table>

For $x > 0.1$, $\beta = 1.0$ for all $x$.

Fig. 3. $\text{H}_2\text{O}_2$ oxidation at electrode: dimensionless response as a function of dimensionless film thickness, for different values of the kinetic parameter $\chi r_0$. Curves plotted from Eq. (15).
at the polymer can be characterized by a first order rate constant $k$, then 

\[ \chi^2 = k/D_S. \]

Under these conditions Eqs. (10)–(13) can be used to describe the observed current.

### 3. Experimental

#### 3.1. Reagents

All reagents were provided by Wako Chemicals Ltd. unless otherwise stated. Glucose oxidase (GOD) was from *Aspergillus niger*, type 10 (246 U mg$^{-1}$). Molar concentrations of GOD were calculated by measuring the absorbance of stock solutions at 450 nm using a Beckman DU 7500 UV-vis spectrophotometer, and assuming an extinction coefficient of 1.4 $\times$ 10$^4$ M$^{-1}$ cm$^{-1}$ [38]. Stock solutions of $\phi$-glucose were prepared in the buffer solution, 0.1 M phosphate at pH 7.0, and were left to mutarotate for at least 24 h before use. Carbon fibres were a gift from Tsukuba Materials Information Laboratory.

#### 3.2. Apparatus

All experiments employed a three-electrode cell of 10 ml volume, with Pt coil counter electrode, and a Ag/AgCl reference electrode connected to the cell via a salt bridge. Potential manipulation and data collection were performed with a computer-controlled potentiostat (Autolab µStat).

#### 3.3. Procedures

Working electrodes were prepared by connecting a single carbon fibre to a copper wire, using conducting adhesive. This wire was then sealed inside a narrow glass tube with Araldite two-component epoxy-resin, leaving approximately 3–9 mm of fibre protruding.

Pt was deposited onto the fibre from a quiescent solution of 2 mM $K_2PtCl_6$ in 0.1 M perchloric acid. The potential was swept from 0.5 to $-0.6$ V ten times, at 10 mV s$^{-1}$. After deposition, the diameter and length of the fibre were measured using a microscope. Diameters of coated fibres were typically between 10 and 20 μm.

GOD was immobilized on the Pt-coated fibre through the polymerization of 1,2-diaminobenzene (1,2-DAB). The unsterilized polymerization solution contained 5 mM 1,2-DAB + GOD (24.6–174.7 U ml$^{-1}$) + phosphate buffer, pH 7.0. The potential was swept from 0.0 to 0.8 V 15 times at 50 mV s$^{-1}$. The modified fibre was then placed in stirred buffer solution for 1 h at open circuit, to ensure the removal of weakly adsorbed enzyme, before transfer to the electrochemical cell. Steady state currents were recorded to glucose at 0.65 V in stirred buffer solution. All experiments were performed at room temperature.

### 4. Results and discussion

As stated earlier, application of Eq. (13) requires sufficient conducting sites in the polymer, while Eq. (18) requires that the polymer does not conduct. The second case can be applied unambiguously for an appropriate coating. Therefore poly(DAB) was chosen to immobilize GOD, as it is known not to conduct. The polymerization of 1,2-DAB in the presence of GOD is shown in Fig. 4 (1st and 15th scans). Since the polymer is non-conducting, it insulates the electrode as it is formed. The flattening out of the current suggested that 15 scans were enough to coat the electrode.

Fig. 5 shows a background-subtracted linear sweep voltammogram of 4.4 mM $H_2O_2$ in phosphate buffer, at a Pt-modified carbon fibre (5 mV s$^{-1}$ sweep rate). It can be seen that above 0.6 V the oxidation of $H_2O_2$ satisfies the boundary condition in Eq. (4). Hence, steady state currents to glucose were recorded at 0.65 V. A relatively fast stirrer speed was used, and further increases in the stirrer speed from this level did not change the response.
The current responses are shown in Fig. 6, for fibres prepared using 0.73–4.38 μM GOD in the polymerization solution.

To estimate the thickness of the poly(DAB) films we considered the charge passed during polymerization. Each polymerization voltammogram for a single fibre was replotted as a current–time curve, and the area under the curve integrated. The total charge (combined redox and capacitive), was thus found to be 6.6 × 10⁻⁴ C. Hence, based on the density of 1,2-DAB (1.13 g cm⁻³) and area of the fibre, we estimated the maximum possible film thickness to be 0.8 μm. For the electrode radii used here, this gives 1.08 ≤ z ≤ 1.16. Clearly, the real value of z must be less than this, since some of the charge must be capacitive (and in fact Yacynych and Emr have suggested that 1,2-DAB films are less than 10 nm thick [39]). However, from Table 2, when z ≤ 1.25, β = 1 for any value of z in Eq. (18). Therefore β can be taken as 1 in this case. In Fig. 7 each gradient in Fig. 6 is converted to a dimensionless response by approximating 1/D₀K to 2.5 × 10¹¹ cm⁻² s (where K is the partition coefficient of glucose), from the GOD/poly(N-methylpyrrole) result of Bartlett and Whitaker [40]. Hence the data are fitted to Eq. (18) (solid line), where cES refers to the concentration of GOD in bulk polymerization solution. From the fit, the kinetic parameter kcat/DSKₐ was found to be 2.2 × 10¹⁵ cm mol⁻¹ (where cₑ = γES). Using enzyme mass instead of molar concentration, this value corresponds to 1.6 × 10⁷ cm mg⁻¹, which is significantly lower than the value found by Bartlett and Whitaker for GOD inside poly(N-methylpyrrole) films, grown on planar Pt electrodes [40] (5.6 × 10⁹ cm mg⁻¹). However, to compare the catalytic activity of the two systems, we would also need to know either the molar enzyme concentration or the enzyme activity, used in their case.

5. Conclusion

We have presented a kinetic-diffusion model of the steady state at an enzyme-modified microcylinder electrode. The model is applicable to an enzyme entrapped in a polymer which is either insulating or contains enough conducting sites to conduct electrically via percolation. The model suggests that these two cases may be distinguished by the way the response varies with film thickness. If the film thickness and electrode radius are known, then simple approximate expressions can be used to describe the steady state current. From these expressions, kinetic parameters may be estimated by altering experimental conditions. As an illustration, for GOD in poly(DAB) films on platinum-coated carbon fibres, the kinetic parameter kcat/DSKₐ was estimated by varying the enzyme concentration in the polymerization solution.
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References