Diffusion-controlled Mediated Electron Transfer-type Bioelectrocatalysis Using Microband Electrodes as Ultimate Amperometric Glucose Sensors

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We performed numerical simulations on an extremely fast, mediated, electron transfer-type bioelectrocatalytic reaction using a microband electrode. The simulations under fast-enzyme-kinetics conditions predicted that the decrement of the current density by increasing the microband thickness would effectively improve the upper limit of detection. These predictions were accurate for an ultrathin-ring with thickness of 100 nm and gold leaf with thickness of 10 μm electrodes, acting as novel amperometric glucose sensors with FAD-dependent glucose dehydrogenase. The gold leaf electrode provided pseudo-steady-state currents which were proportional to the glucose concentration up to a concentration of 20 times higher than the mediator concentration.

Keywords Amperometry, pseudo-steady-state current, microband electrode, bioelectrocatalysis, glucose sensor, FAD-dependent glucose dehydrogenase

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Introduction

Electrochemical biosensors consist of an electrode intimately combined with a molecular recognition element, such as an enzyme, antibody, aptamer, or microbe.1–3 In particular, enzymes have received significant attention in the construction of practical biosensors, because they are biological catalysts that convert analytes into products with high specificity. Enzymatic biosensors enable selective and highly sensitive detection and play a significant analytical role in the fields of medicine, agriculture, food safety, bioprocesses, and industrial monitoring.4–8

Amperometric biosensors, such as commercialized blood glucose sensors, are based on mediated bioelectrocatalysis.9–11 Extensive studies have been carried out to improve the detection range, sensitivity, stability, and reliability of amperometric biosensors.12–17 Amperometry, a detection method that measures current under a constant potential, is common in enzymatic biosensing, since it balances the detection time, accuracy, and usability.18 Practical applications of amperometric sensors are limited when their current response is time dependent, because the device requires time control. Therefore, user-friendly and convenient amperometric sensors should display a time-independent current response, i.e., steady-state current. Thus, the concentration profile of the redox-active species in the system should be independent of time.

Several techniques or materials have been adopted to achieve steady-state currents,19,20 such as rotating electrodes,21 permeation membranes on the electrode surface,22,23 microelectrodes,24–26 and enzymatic reactions.27,28 The steady-state conditions arising from microelectrodes appear to be the most stable for amperometric sensors, because the limiting current \( i_{\text{lim}} \) is simply determined by the analyte diffusion coefficient and the electrode size.26,29

The steady-state current arising from non-linear diffusion around a micro-disk electrode is expressed as follows:30

\[
\begin{align*}
    i_{\text{lim}} &= 4nFDc_0r, \\
    &\quad \text{where } n, F, D, c_0, \text{ and } r \text{ are the number of electrons, Faraday constant, diffusion coefficient, concentration in the bulk phase, and radius of the electrode, respectively. Although using microelectrodes allows system miniaturization, the microelectrode signals are quite small (typically less than a few nA) compared with the noise from external disturbance. It is thus necessary to increase the signal intensity for practical sensors.}
\end{align*}
\]

Ultrathin-ring electrodes are a type of ultimate thin microband electrodes composed of a sputtered gold ring buried in an insulator.31 The thickness of the ring must be quite small (~100 nm) compared with its diameter to sufficiently form a toroidal diffusion layer. The electrochemical behavior of ultrathin-ring electrodes is similar to that of band electrodes. Ultrathin-ring electrodes provide a large pseudo-steady-state current density, as described by

\[
\begin{align*}
    \frac{i(t)_{\text{lim}}}{nFDc_0d} &= \frac{1}{\sqrt{\pi t}} + 1 \quad (\tau < 2) \\
    &\quad \text{where } t, \tau, n, F, D, c_0, \text{ and } d \text{ are the time, time constant, number of electrons, Faraday constant, diffusion coefficient, concentration in the bulk phase, and diameter of the electrode, respectively.}
\end{align*}
\]
where \( \tau = \frac{D_l w^2}{I} \), \( I \), and \( w \) are the dimensionless time, microband length, and microband thickness, respectively. The current response of an ultrathin-ring electrode is sufficiently large for detection without a Faraday cage. In addition, decreasing the ring thickness decreases the response time, which is defined as the time that the variation of current is less than the threshold.

Under steady-state current flow, the behavior of an amperometric sensor is understood through the concept of a serial resistance circuit. In mediator-type amperometric biosensors without a permeation membrane, the resistances correspond to the inverse of the diffusion-limited current \( i_d \), the enzyme reaction current \( i_k \), and the electrode-reaction-limited current \( i_e \). The inverse of the steady-state current \( i \) is expressed by the sum of these resistances:

\[
\frac{1}{i} = \frac{1}{i_d} + \frac{1}{i_k} + \frac{1}{i_e}.
\]

The minimum-current term is regarded as the rate-determining step. Here, the value of \( i_d \) is unstable and difficult to control, since the enzyme activity is unstable. However, for an extremely fast enzymatic reaction under limiting-current conditions, the situation is regarded as \( i_k, i_e \gg i_d \). Thus, the current is controlled by the diffusion of the analyte.

The current produced by a mediated bioelectrocatalytic oxidation reaction of glucose on a micro-disk electrode with an extremely fast enzymatic reaction rate has been investigated. Numerical simulations for this system have supported the experimental data and revealed that a pseudo-steady-state catalytic current controlled by substrate diffusion can be obtained when the enzymatic reaction is sufficiently fast. The steady-state catalytic limiting current is proportional to the diffusion coefficient and the concentration of the substrate over the mediator concentration \( c_M \) because the substrate flow is almost completely converted to the mediator flow at the reaction plane near the electrode surface, which behaves as a virtual electrode surface that can oxidize the substrate. In addition, when the enzyme activity is sufficiently high \( i_k \gg i_d \), the steady-state current becomes independent of the enzyme activity. However, the sensitivity of glucose was low \( (13 \mu A M^{-1}) \) because of the using of a micro-disk electrode. Employing the ultrathin-ring electrode will augment the sensitivity.

In this work, we used numerical simulations to study the effects of the microband thickness on the catalytic current and constructed two types of band electrodes: ultrathin-ring and gold leaf ones. These electrodes were used to detect a mediated bioelectrocatalytic reaction. We utilized FAD-dependent glucose dehydrogenase (FAD-GDH), which exhibits thermostability, high substrate specificity, and a large Michaelis constant toward glucose. The numerical simulation and experimental results indicate the advantages of the gold leaf electrode in the measuring range. Additionally, immobilization of enzyme at the gold leaf electrode surface was attempted to improve the availability of the biosensor from a practical application viewpoint.
Experimental

Reagents and chemicals

FAD-GDH was obtained from BBI Solutions (GLD3, 483 U mg⁻¹, UK). Ferrocenedimethanol (Fc) and d-(+)-glucose were purchased from Wako Pure Chemicals (Japan). 1,2-Naphthoquinone (1,2-NQ) was purchased from Tokyo Chemical Industry (Japan). All reagents were of analytical reagent grade and used without further purification. A d-glucose stock solution into the buffer solution containing 0.2 mM while increasing t

Electrode fabrication

An ultrathin-ring electrode with a thickness of 100 nm was fabricated as described in a previous report² with slight modifications. Firstly, a UV-resin rod with the diameter of 2 mm was fabricated and bound with a copper wire. A 100 nm-thick gold film was then sputtered on the each semi-cylindrical surface of the rod using a desktop quick coater (SC-704, Sanyu Electron Co., Ltd.) (Fig. 1A). The thickness of the gold film was controlled by the current and time of the DC sputtering. After Au film deposition, the rod was potted in epoxy resin. The rod was then cut horizontally with a turning machine to reveal the ring (Fig. 1B). The tip of the ring electrode was made flat by polishing with waterproof abrasive paper (#2000) (Fig. 1C).

The microband electrode (10 μm thick, 6 mm wide) was fabricated with gold leaf with a thickness of 10 μm. After the connection of a lead wire, both sides of the gold leaf were covered with insulating tape (N-300, Nitto Denko Co., Ltd.) (Fig. 1a), and then cut with a razor blade (Fig. 1b) to expose a cross-section (Fig. 1c).

Enzyme immobilization at the electrode surface

A 20-μL drop of FAD-GDH stock solution (200 mg mL⁻¹) containing 1% glutaraldehyde was cast onto the gold leaf electrode surface. The drop was dried under room temperature for 30 min. The prepared enzyme film was washed with a buffer solution to remove any un-immobilized enzyme.

Electrochemical measurements

Cyclic voltammetry was performed with an electrochemical analyzer (CV-100W, BAS) equipped with a low-current module (CV-100W, BAS) as the reference electrode and counter electrode, respectively. Chronoamperometry was carried out at 0.6 V positive to 0.2 V negative with a scan rate of 100 mV s⁻¹. After Au film deposition, the rod was potted in epoxy resin. The rod was then cut horizontally with a turning machine to reveal the ring (Fig. 1B). The tip of the ring electrode was made flat by polishing with waterproof abrasive paper (#2000) (Fig. 1C).

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Results and Discussion

Numerical simulations

Previously, the steady-state current arising from a mediated enzymatic reaction on a microdisk electrode has been characterized using theoretical analysis and numerical simulations.²⁵ Because of the difference in the geometrical symmetry, the steady-state characteristics of a microband electrode is weaker than those of the microdisk electrode.²² Numerical simulations were performed to elucidate the pseudo-steady-state characteristics of the mediated enzymatic reaction on a microband electrode.

Figure 2a shows the effect of the microband electrode thickness on the numerically calculated amperograms for a catalytic oxidation current at microband electrodes of varying w; the triangles, squares, and circles represent w = 0.1, 1, and 10 μm, respectively. The potential was stepped to 0.6 V positive of the formal potential of mediator at cグルコース = 0.5 mM. The curves in Fig. 2a show amperograms for the direct oxidation of glucose at each microelectrode surface calculated from Eq. (2). The overlapping of the symbol and curve demonstrates that the current is determined by the diffusion of glucose. When
When $w = 100$ nm, the current quickly reaches a pseudo-steady-state. However, when $w = 10$ m, the current decays over time. The time dependence of the catalytic oxidation current at the microband electrode is strongly affected by changes in $w$.

Figure 2b shows the calibration curves. The triangles, squares, and circles in Fig. 2b represent the calculated current at $t = 8$ s for $w = 0.1$, 1, and 10 $\mu$m, respectively. The numerical simulations indicate that increasing the electrode thickness is an effective method to increase the upper limit of the linear relationship between $c_{\text{glucose}}$ and $i_{\text{lim}}$. Increasing the electrode thickness decreases the current density since the (pseudo) steady-state current at the microband electrode is insensitive to the thickness of the electrode.33 The decrease in the current density increases the distance of the reaction plane from the electrode surface.

On the other hand, the calculations also demonstrate that a very thick microband electrode does not display a steady-state current response. Consequently, there is a trade-off between expanding the linear range of the calibration curve and achieving steady-state characteristics in an ultra-fast mediated bioelectrocatalytic reaction at a microband electrode.

Although microband electrodes with a large $w$ display poor steady-state current characteristics and charging current characteristics, there are several advantages to using such electrodes in real measurements, including easy preparation, easy handling, and electrode toughness. The numerical simulations predicted that increasing the electrode thickness is an effective way to improve the detection upper limit.

**Preparation of microband electrodes**

We attempted to prepare ultrathin-ring electrodes according to a method reported in our previous work.32 However, fabricating thicker ring electrodes was difficult using this methodology because of poor reproducibility. Therefore, microband electrodes with $w = 10$ $\mu$m were fabricated with gold leaf and insulating tape using a novel and simple technique.

Figure 3 shows cyclic voltammograms of Fc oxidation at various concentrations using (a) an ultrathin-ring electrode with $w = 100$ nm and (b) a gold leaf electrode with $w = 10$ $\mu$m. The scan rate was 20 mV s$^{-1}$ and the Fc concentrations were 0.8 (curve 1), 0.4 (curve 2), 0.2 (curve 3), and 0 (curve 4) mM. The insets show the concentration dependence of the current at 600 mV.
overlap in these voltammograms. The gap between the forward and reverse scans arises from the pseudo-steady-state characteristics at the microband electrode. A comparison between the voltammograms in Figs. 3a and 3b demonstrates that the steady-state characteristics of the microband electrodes decreased with increasing $w$.

**MET-type bioelectrocatalysis using microband electrodes**

An extremely fast enzyme reaction is required to achieve diffusion-controlled electrolysis. The dashed line in Fig. 4a shows a cyclic voltammogram of reduction of 1,2-NQ. The solid line in Fig. 4a shows a cyclic voltammogram of catalytic oxidation of glucose in the presence of 1,2-NQ and FAD-GDH at $c_{\text{glucose}} = 1$ mM. Since the 1,2-NQ-reduction current decreased, 1,2-NQ was reduced in the solution. The increase in the oxidation current clearly shows the catalytic reduction of 1,2-NQ in the solution. The oxidation current displays steady-state characteristics in the forward scan.

Although the ultrathin-ring electrode does not provide a true steady-state current, the pseudo-steady-state current is useful in practical applications. Figure 4b shows a chronoamperogram recorded at 0.6 V increasing glucose concentrations by adding the glucose stock solution into the buffer solution containing 1,2-NQ and FAD-GDH. Upon adding glucose to the solution and mixing, the oxidation current increased immediately and then decayed over time to a pseudo-steady-state current. Interestingly, the time taken to reach the pseudo-steady-state current decreased with increasing glucose concentration. The decreasing time taken to reach the pseudo-steady-state current demonstrates the reaction plane coming closer to the electrode surface. The open circles in Fig. 4b indicate the currents used to construct the calibration curve (dashed line in Fig. 4e). The error bars show 90%-confidence levels ($n = 4$ for ultrathin-ring electrode and $n = 3$ for gold leaf electrode). The lines in e show the line of best fit for the data.
The pseudo-steady-state current is linearly correlated to $c_{\text{glucose}}$ over a 0 - 1.0 mM range; the calibration curve has a slope of $23 \pm 1 \, \text{nA} \, \text{mM}^{-1}$. This sensitivity was larger than that of previous micro-disk electrode system. The upper limit of $c_{\text{glucose}}$ for a linear dependence of $f_{\text{lim}}$ on $c_{\text{glucose}}$ was about five times larger than $c_{\text{redox}}$. This indicates that the enzymatic reaction is sufficiently fast compared with the diffusion of the substrate to the electrode. At the reaction plane near the electrode surface, the substrate flow is completely converted to the mediator flow by the enzymatic reaction. At $c_{\text{glucose}} > 1.0 \, \text{mM}$, $f_{\text{lim}}$ reached an upper limit. The current limitation may be attributed to the reaction plane coming very close the electrode surface at high glucose concentrations.

According to the numerical simulations presented previously, the upper limit of the calibration curve will increase with increasing $w$ because of its decreasing current density at the microband electrode. Bioelectrocatalysis at thick microband electrodes was examined using a gold leaf electrode with $w = 10 \, \mu\text{m}$. Figure 4c shows voltammograms of catalytic glucose oxidation at the gold leaf electrode when $c_{\text{glucose}} = 0$ (dashed line) and 1 (solid line) mM. At the gold leaf electrode, clear catalytic current was recorded. The voltammograms display a sigmoidal shape and the catalytic oxidation current displays pseudo-steady-state characteristics.

Figure 4d shows a typical chronoamperogram recorded at 0.6 V upon the addition of increasing glucose concentrations into the solution containing 1,2-NQ and FAD-GDH. The circles indicate the sampled pseudo-steady-state current when the variation of current in one second was less than 0.3% of the current. The sampled current displays a linear relationship with $c_{\text{glucose}}$ (circles in Fig. 4e); the slope is $43 \pm 1 \, \text{nA} \, \text{mM}^{-1}$ and the upper limit of the linear range is about 4 mM. The sensitivity of the gold leaf electrode is about twice that of the ultrathin-ring electrode. The difference of the sensitivities demonstrates that the current is controlled by the diffusion of glucose around the microband electrodes. The upper limit of the linear range of the calibration curve was about 20 times larger than $c_{\text{redox}}$. The increment of the upper limit with increasing value of $w$ agrees with a prediction of the numerical-simulation. These results indicate the advantages of the gold leaf electrode. However, the decline in the steady-state characteristics that appears in Fig. 3b predicts that the increment of $w$ any more is unattractive.

**MET-type bioelectrocatalysis using an enzyme-modified gold leaf electrode**

A substrate-diffusion-controlled MET-type bioelectrocatalytic reaction at the microelectrode is realized when the enzyme is densely packed in the reaction layer, because an extremely fast enzymatic reaction is required to convert the substrate flow into the mediator flow. Therefore, immobilization of the enzyme on the electrode surface appears to be a good approach, since the enzyme will be concentrated at the modified-electrode surface. In this section, the conversion of the substrate flow to the mediator flow by immobilized enzyme is examined at the gold leaf electrode, because its low current density will contribute to the ease of conversion. Figure 5 shows a calibration curve prepared from chronoamperometry at 0.6 V using an FAD-GDH-immobilized gold leaf electrode upon the addition of increasing glucose concentrations into a solution containing 1,2-NQ. The upper limit for the linear range in the calibration curve is about 1 mM. Additionally, a plateau appears in the curve at higher substrate concentrations. The plateau clearly demonstrates that the current is controlled by the enzyme reaction kinetics. Although a decrement of the current density is required to avoid any limitation of the enzyme kinetics, the increment of the electrode surface area spoils the steady-state characteristics. The discovery of enzymes with higher activity is necessary to resolve of this dilemma.

**Conclusions**

Novel amperometric glucose sensors were constructed using microband electrodes based on a MET type bioelectrocatalytic reaction. The combination of a microband electrode and an extremely fast enzyme reaction provided a pseudo-steady-state current response controlled by the substrate diffusion and the linear range of the calibration curve exceeded the mediator concentration. Numerical simulations revealed that increasing the electrode thickness improved the detection upper limit. Indeed, the detection upper limit of the calibration curve for the gold leaf electrode with a thickness of 10 μm (4 mM) exceeded that for the ultrathin-ring electrode with a thickness of 100 nm (1 mM). The upper limit of the calibration curve on the gold leaf electrode was 20 times higher than the employed mediator concentration.

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**References**