

Diffusion-controlled Detection of Glucose with Microelectrodes in Mediated Bioelectrocatalytic Oxidation

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This paper describes a diffusion-controlled electrolysis of glucose with mediated bioelectrocatalysis at microdisk-electrodes. Under conditions of an extremely fast enzyme reaction, compared with the diffusion of glucose, the current in potential-step chronoamperometry reaches an almost steady state within 10 s, and is proportional to the glucose concentration. The current can be detected at +0.1 V (vs. Ag|AgCl) with 9,10-phenanthrenequinone as a mediator. The addition of carboxymethylcellulose increased the linear range up to 10 mM.

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Introduction

Electrochemical biosensors, which allow affordable, simple, selective and sensitive detection inherent with biological functions, have emerged as commonly utilized sensors for monitoring specific substances and diagnosis tests in clinical analysis. Hence, numerical efforts have been devoted to improve their performances.¹ Recently, the demand for miniaturized biosensors has been increasing, and had led to the development of portable or *in vivo* sensing systems. Along this line, many researchers have focused on applying microelectrodes to electrochemical biosensors.²⁻⁴

One of the benefits of utilizing microelectrodes, however, is the fact that microelectrodes produce steady-state currents within a short time to detect analytes, owing to spherical diffusion properties. A microdisk-electrode, for example, is an expedient tool to obtain steady-state currents (i_{ss}) due to stable spherical-diffusion under quiescent conditions.^{5,6} The i_{ss} can be expressed as

$$i_{ss} = 4nFrDc, \quad (1)$$

where n , F , r and D denote the number of electrons, the Faraday constant, the electrode radius and the diffusion coefficient. This means that, once the conditional parameter rD is estimated, the bulk concentration of the analyte can be easily determined from i_{ss} within a few seconds. Therefore, microelectrodes are suitable for not only miniaturization, but also as highly reproducible sensors under diffusion-controlled conditions.

On the other hand, bioelectrocatalytic systems also produce steady-state currents under kinetic-controlled conditions. The

response is, however, very sensitive to any change in the enzyme activity, including changes of the temperature and the pH. Therefore, the utilization of enzyme kinetic-controlled steady-state currents is not a good idea for analytical purposes, compared with diffusion-controlled steady-state currents. In addition, the above two properties used to generate steady-state currents compete with each other. Therefore, it has been believed that the detection of bioelectrocatalytic currents at microelectrode is not appropriate for analytical purposes.

However, the utilization of a bioelectrocatalytic system is very useful for the electrochemical detection of biologically related substances, including glucose. To address these problems, we tried to realize a diffusion-controlled electrolysis of enzyme substrate (glucose in this case) in a mediated bioelectrochemical system at microelectrodes. Our hypothesis is that under the conditions where the enzyme reaction rate is much faster than the rate of the diffusion process upon increased activity of the enzyme with a suitable mediator, the current will apparently be controlled by the mass transfer of glucose. When such electrolysis proceeds on microelectrodes, we may obtain highly reproducible steady-state currents of glucose, which are almost independent of any change in the enzyme activity, and are very useful for analytical purposes.

In this work, we utilized FAD-dependent glucose dehydrogenase (FAD-GDH), which catalyzes the oxidation of glucose with some electron acceptors, including artificial ones, but not with oxygen, and exhibits thermostability, high substrate specificity, and a large Michaelis constant for glucose.^{7,8} Therefore, FAD-GDH is now frequently utilized in affordable blood glucose sensors. To achieve fast mediated enzyme-kinetics, we selected *p*-benzoquinone (BQ) and 9,10-phenanthrenequinone (PQ) as highly reactive mediators for FAD-GDH in our previous study (data not shown). Metabolic disorders, like diabetes mellitus, are reflected by human blood glucose concentrations higher or lower than the normal range of 4–6 mM; hence,

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numerous efforts have been devoted to detect such high concentrations of glucose.^{9,10} Therefore, we also tried to extend the detection upper limit up by the addition of carboxymethylcellulose (CMC) so as to decrease the diffusion coefficient.

Experimental

Reagents and chemicals

FAD-GDH from *Aspergillus terreus* was kindly donated by Ikeda Food Research Co., Ltd. (Japan), and used without further purifications. The concentration of the FAD-GDH stock solution was determined spectrophotometrically using the molar extinction coefficient of free FAD of $11.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 465 nm.¹¹ CMC sodium salt (high viscosity) was purchased from Sigma Aldrich Inc. (USA). All other chemicals were of analytical reagent grade, and were used without further purification. A D-glucose stock solution was prepared with 0.1 M phosphate buffer (pH 7.0), and stored overnight to achieve the mutarotative equilibrium. Epoxy resin (Alardite 2020) and silver-epoxy resin (Dotite D-753) were obtained from Huntsman (USA) and Fujikura Kasei (Japan), respectively.

Electrochemical measurements

Potential-step chronoamperometry was carried out on a three-electrode system with an ALS CHI 611B electrochemical analyzer (BAS Inc.) at 25°C. A platinum (Pt) wire and Ag/AgCl/KCl (sat.) electrode were used as a counter and reference electrodes, respectively. All potentials are referred to the reference electrode in this work. The measurements with microdisk-electrodes were performed in a laboratory-made electrolysis cell at a total volume of 20 μL in a Faraday cage.

The microdisk-electrode was prepared with a 50- μm diameter Pt wire (Nilaco, Japan). Firstly, the Pt wire was connected to a 0.5-mm diameter titanium wire with silver-epoxy resin (4 h, 55°C). The connected wire was inserted into a ceramic tube (i.d. 1 mm; Wilco, Japan) and filled with epoxy resin (overnight, 55°C). The surface of the Pt microelectrode was polished to flat and a mirror finish with sandpaper (#1500) and alumina slurry (1, 0.3 and 0.05 μm) in turn.

In amperometric glucose detection, 1 μL of a glucose sample solution was added to 19 μL of 0.1 M phosphate buffer (pH 7.0) containing 0.21 mM FAD-GDH and a mediator (1 mM BQ or 0.2 mM PQ). After mixing the enzyme solution by pipetting with careful attention to evaporation, the mixed solution was immediately transferred to the electrolysis cell. Amperometric detection was performed under quiescent conditions in the potential step from -0.4 V to $+0.85 \text{ V}$ for BQ, or from -0.4 V to $+0.1 \text{ V}$ for PQ.

Results and Discussion

Steady-state current for glucose with BQ as mediator

FAD-GDH obeys a ping-pong bi-bi mechanism. To achieve diffusion-controlled electrolysis, an extremely fast enzyme reaction is required. From a kinetic view point, we first selected BQ as a highly reactive mediator. The overall reaction is written as



Figure 1 shows the time dependence of the currents at several total bulk concentrations of glucose (c_{glc}). The charging current

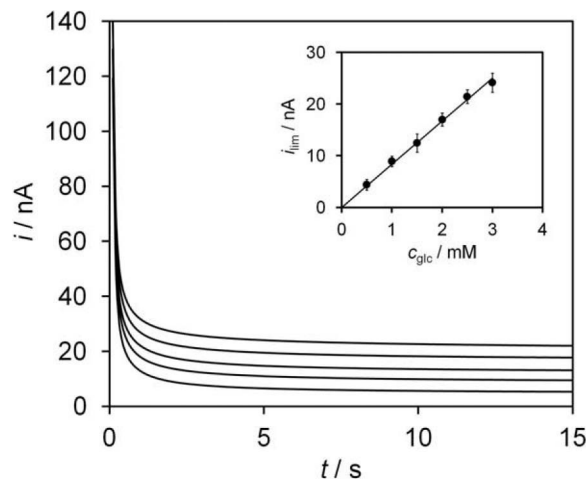


Fig. 1 Chronoamperometric responses for glucose at a microdisk-electrode in a 0.1 M phosphate buffer (pH 7.0) containing of 0.21 mM FAD-GDH and 1 mM BQ in a potential step from -0.4 V to $+0.85 \text{ V}$. The curves were obtained at glucose concentrations (c_{glc}) of 0.5, 1, 1.5, 2 and 2.5 mM from bottom to up. Inset: The current value after 10 s (i_{lim}) as a function of c_{glc} . The error bars were evaluated by the Student t -distribution with a 99%-confidence level.

disappeared within 3 s after the potential step, and the diffusion current became almost steady state after 5 s. Aoki and Osteryoung derived an equation to characterize the current (i)-time (t) curve at microdisk-electrodes with a dimensionless parameter, $\tau = 4Dt/r^2$:

$$i = i_{\text{ss}} (1 + 0.71835 \tau^{-1/2} + 0.05626 \tau^{-3/2} - 0.00646 \tau^{-5/2} \dots). \quad (3)$$

The i value approaches i_{ss} when the diffusion field grows to a size much larger than r . For example, for the conditions $n = 2$, $D > 1.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, and $r = 25 \mu\text{m}$, the i/i_{ss} ratio becomes smaller than 1.3 at $t = 10 \text{ s}$. Therefore, in the following, the currents after 10 s were taken as i_{lim} as practically steady-state values, as judged from Fig. 1.

The i_{lim} values (at $+0.85 \text{ V}$) exhibited a linear response against c_{glc} in the range from 0.5 to 3 mM with a correlation coefficient of 0.998, as shown in the inset of Fig. 1. When $c_{\text{glc}} < c_{\text{BQ}}$, the enzyme reaction is completed immediately after mixing the enzyme, glucose and the mediator, because an extremely fast enzyme reaction proceeds at high concentrations of FAD-GDH. The reducing equivalent of glucose is completely transferred to that of the mediator in solution before electrolysis. This is a typical endpoint assay as a highly reproducible amperometric detection method with microelectrodes in mediated bioelectrocatalysis, where the current is controlled by the diffusion of reduced mediator.¹³

On the other hand, even when $c_{\text{glc}} > c_{\text{BQ}}$, the i_{lim} increased linearly with c_{glc} . The steady-state current at microdisk-electrodes in homogeneous mediated enzyme reaction has been numerically calculated.¹⁴ However, the authors have focused on curved characteristics between the steady-state current and c_{glc} at relatively low activity of the enzyme. A numerical calculation of the current for EC' reactions (but not enzyme reaction) at microdisk-electrodes has also been studied.¹⁵ According to the report, when the concentrations of the reactants are controlled by diffusion, the reaction plane (or zone) where a sharp change in the concentration of the reactants occurs, due to the fast reaction, is constructed near the electrode surface.

In our case, most probably in the steady state the concentration of D- β -glucose at the outside of the reaction plane is decreased down to $0.62c_{\text{glc}}-c_{\text{BQ}}$. Any D- β -glucose diffused to the reaction plane is oxidized inside of the reaction plane, and its concentration becomes zero at the electrode surface. On the other hand, BQ is completely reduced outside of the reaction plane, and the reduced BQ diffuses to the electrode surface, where the reduced BQ is oxidized. The regenerated BQ is enzymatically re-reduced inside of the reaction plane. The reaction plane draws close to the electrode surface with an increase in c_{glc} occurs. As a result, i_{lim} increases with c_{glc} , analytical expression based on a simplified model, and the details of a simulation, will be reported elsewhere.

The sensitivity ($= 4nFD$) was evaluated to be $1.3 \times 10^{-5} \text{ A M}^{-1}$ by considering 62% as a concentration ratio of β -D-glucose against c_{glc} in mutarotative equilibrium. This value is close to that of hydroquinone ($1.3 \times 10^{-5} \text{ A M}^{-1}$) with a similar D value. These results indicate that the diffusion-controlled electrolysis of glucose is almost realized under the stated conditions. The relative error was evaluated as 4.5% at $c_{\text{glc}} = 1 \text{ mM}$ and 5.5% at $c_{\text{glc}} = 2.5 \text{ mM}$, based on the Student t -distribution with a 99%-confidence level.

Practical glucose analysis with 9,10-PheQ and CMC

Electrochemical interferences in blood, such as ascorbate and urate, can cause false glucose detection by non-glucose-derived electrons. In a considerable number of studies aimed to overcome the drawback of their electrochemical oxidation, polymeric membranes have often been utilized to eliminate them by size exclusion or electrostatic repulsion.¹⁶ However, it is difficult to strictly control the permeability of membranes on miniaturized biosensors, and to realize high reproducibility. One of the alternatives to reduce the interfering signals is to operate at a low electrode potential of around 0 V. However, the use of such mediators with relatively low formal potentials leads to a decrease in the electron-transfer kinetics from the enzyme to the mediator. Fortunately, we found that PQ has high reactivity with FAD-GDH comparable to BQ, even though the formal potential is about -0.2 V . In addition, the oxidation of the reduced form of PQ reached to the diffusion-control state at around 0 V due to fast electrode kinetics. This means that the use of PQ can avoid the signals of electroactive species, such as ascorbate and urate.

In addition, we tried to extend the upper limit of the linearity. Since the linear range seems to be strongly related to the ratio of the enzyme reaction rate and the diffusion, one simple way to extend the upper limit is to decrease the D values by increasing the viscosity. In this work, 1% CMC was added into the measurement solution. The i_{lim} value (at $+0.1 \text{ V}$) showed a linear relationship against c_{glc} in the range from $50 \mu\text{M}$ to 10 mM with a correlation coefficient of 0.994. The upper limit of the linear range of c_{glc} is 50-times higher than c_{PQ} . The sensitivity ($4nFD$) was decreased down to $1.7 \times 10^{-6} \text{ A M}^{-1}$, due to the increased viscosity. The relative error was evaluated to be 4.3% at $c_{\text{glc}} = 6.0 \text{ mM}$ by the Student t -distribution with a 99%-confidence level. Other polymers, such as polyethylene glycol, may also be utilized to increase the viscosity.

Conclusions

As far as we know, we were the first to realize a bioelectrocatalytic assay with microelectrodes under diffusion-controlled conditions. The i_{lim} value is highly reproducible, easy in measurements, and practically independent of any change in the enzyme activity. Therefore, this concept may be utilized especially in disposable glucose sensors. On the other hand, however, in order to realize such diffusion-controlled electrolysis at enzyme/mediator co-immobilized microelectrodes, a very thin immobilized layer with very high enzyme-activity may be required. Such enzyme/mediator co-immobilized microelectrodes would be useful for low-concentration detection. Details will be described elsewhere.

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