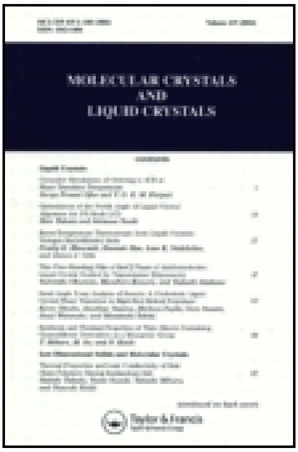

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Immobilization of Glucose Oxidase within Chitosan and Sol-Gel Matrix for Biosensors

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*Enzyme-modified electrodes were fabricated by entrapping glucose oxidase (GOx) and ferrocene (Fc) using chitosan membrane and sol-gel matrix. In chitosan membrane entrapment method, the ferrocene and glucose oxidase were coated sequentially on a glassy carbon electrode and entrapped by chitosan. In sol-gel matrix method, the ferrocene and glucose oxidase were embedded into sol-gel matrix without using any complicated linking processes. The prepared electrode was characterized by cyclic voltammetry (CV). Both Fc/GOx/CHI electrode and Fc/GOx/Sol-gel exhibited a good electrochemical performance for the glucose analysis. The response current of the both Fc/GOx/CHI and Fc/GOx/Sol-gel electrodes increased linearly with the increase of glucose concentration in the range of 0–10 mM glucose. The apparent Michaelis-Menten constants (*Km*) for the enzymes immobilized in the chitosan membrane and sol-gel matrix were observed to be 1.67 mM and 12.59 mM, respectively. The maximum current of the Fc/GOx/CHI and Fc/GOx/Sol-gel electrodes were 5.85 μA and 7.52 μA, respectively.*

Keywords Ferrocene; Glucose oxidase; Chitosan; Sol-gel; Glucose sensor

Introduction

Enzymatic glucose sensors have been intensively investigated due to the increasing demand for blood glucose monitoring. Glucose oxidase (GOx) is the most widely used in the development of glucose sensors [1]. The GOx catalyzes the electrochemical oxidation of glucose that can be amperometrically detected at electrodes. However, the lack of efficient electron transfer between redox enzymes and electrode surfaces is the major obstacle for the development of a practical enzymatic glucose sensor. Generally, active sites for the glucose oxidation are deeply embedded inside the enzyme. Enzyme deactivation and poor stability are also significant drawbacks in the development of practical enzymatic glucose sensors. In order to overcome these problems, electron-shuttling mediators as well as enzyme immobilization onto the appropriate matrix have been used.

A variety of electron mediators such as ferrocene, $Os^{2+/3+}$ complexes, ferricyanide, benzoquinone, and their derivatives have been reported to significantly improve the electron transfer between the redox enzymes and the electrodes and thus remarkably enhance the electrochemical performance of the bioelectrodes [2–7].

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Among the electron mediators, ferrocene and its derivatives have been reported to be the most efficient [8]. Ferrocene has good electrochemical reversibility and stability at low potential and can transfer electrons from the enzyme active site to the electrode surface.

An electron mediator can be used in solution or entrapped into the polymer or sol–gel matrix [9–11]. Metal-oxide gels produced by sol-gel processes are more frequently used for enzyme immobilization in biosensors. Recently, chitosan has been shown to be a promising candidate for enzyme immobilization in bioelectrode applications due to its membrane forming ability, good biocompatibility, non-toxicity, and high mechanical strength [12–15].

In this study, two entrapment methods, polymer and sol–gel matrix, were tested and compared. In chitosan entrapment method, the ferrocene and glucose oxidase were coated sequentially on a glassy carbon electrode and entrapped by chitosan. In sol-gel matrix method, the ferrocene and glucose oxidase were embedded into sol-gel matrix without using any complicated linking processes. The simply integrated ferrocene/glucose oxidase electrodes were characterized for the application in glucose sensors.

Experimental

Materials

Glucose oxidase (GOx) from *Aspergillus niger* (Type II-S, 21000 units/g solid) and D-glucose (99.5%) were purchased from Sigma (USA). Ferrocene (Fc), chitosan (CHI) (high molecular weight, >75% deacetylated), and tetramethyl orthosilicate (TMOS) were supplied from Aldrich (USA).

Preparation of enzyme-immobilized electrodes

The ferrocene/glucose oxidase electrode (Fc/GOx electrode) was fabricated by two methods; chitosan and sol-gel entrapment. First, a ferrocene solution (10 μL, 15 mM in ethanol) and GOx solution (10 μL, 30 mg/mL) were sequentially dripped onto the electrode and dried at room temperature. And then, the electrode was coated with a chitosan solution (10 μL, 5 mg/mL), which was prepared by dissolving chitosan in a sodium acetate buffer (50 mM, pH 4), and dried at room temperature. In sol-gel method, 30 mM ferrocene was mixed with a sol-gel solution. The homogeneous sol-gel solution was prepared by mixing TMOS 10 mL, 0.04 M HCL 0.3 mL, and D.I. water 2 mL. The GOx solution (60 mg/mL) and the ferrocene/sol-gel mixture were mixed at 1:1 ratio. The resulting solution was pipetted onto the electrode and then dried at room temperature. The glucose sensor was stored in a phosphate buffer at 4°C.

Analysis

The electrochemical properties were examined by cyclic voltammetry (CV) using a modular potentiostat (VSP, Bio-Logic) with a conventional three-electrode system: a glassy carbon electrode as a working electrode, a platinum wire as a counter electrode, and an Ag/AgCl (saturated KCl) electrode as a reference. The glassy carbon electrode was polished using 1 μm diamond and 0.05 μm alumina slurry and then washed with distilled water prior to use. All the CVs were obtained in sodium phosphate buffer (50 mM, pH 7) at room temperature under ambient conditions.

Results and Discussion

In order to determine the effect of the electron mediator (i.e., ferrocene in this study) in the electrochemical oxidation of glucose catalyzed by GOx, the GOx-immobilized electrode not containing ferrocene was first tested. As shown in Fig. 1, there were no detectable redox peaks in the presence of glucose in the CV data. This result indicated that the active site of the GOx was not electrically connected with the electrode. Oxidoreductases on electrode surfaces are known to achieve no significant electron transfer between their redox center and the electrode because of the electrical insulation by the surrounding protein matrixes [16]. In the case of GOx, flavine adenine dinucleotide (FAD), which is responsible for redox reactions catalyzed by GOx, is buried within the protein shell (diameter about 13 Å). Based on this preliminary experiment, the electron mediator was observed to be crucial for efficient electron transfer in the enzyme based electrochemical reactions.

Therefore, ferrocene was incorporated into the GOx immobilized electrode system. In this work, the ferrocene was physically embedded into the chitosan membrane without using a linking process. That is, ferrocene solution in ethanol and GOx solution were simply coated using layer-by-layer steps onto a glassy carbon electrode. The electrode was then covered with chitosan, resulting in the formation of a layered film of Fc, GOx, and CHI on the electrode. The electrochemical performance of the prepared Fc/GOx/CHI electrode was examined by CV, as shown in Fig. 2. The typical oxidation and reduction peaks of glucose oxidation were observed, indicating that the embedded ferrocene mediator functioned properly relative to the electrode not containing ferrocene. The CV measurements were carried out in 50 mM PBS containing glucose of different concentrations. The CV measurements were carried out in 50 mM PBS containing glucose of different concentrations. The peak current appeared at around 0.25 V. The peak current at each glucose concentration was presented in inset of Fig. 2. The current increased with the increase of glucose concentration in the range of 0–60 mM. The linear range was about 0–10 mM.

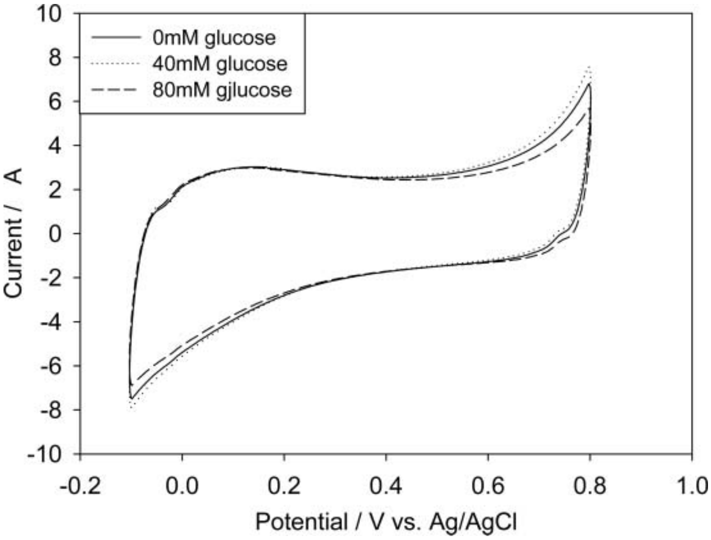

Figure 1. Cyclic voltammograms of a GOx immobilized glassy carbon electrode in the absence and presence glucose in pH 7.0 phosphate buffer at a scan rate of 100 mV/s.

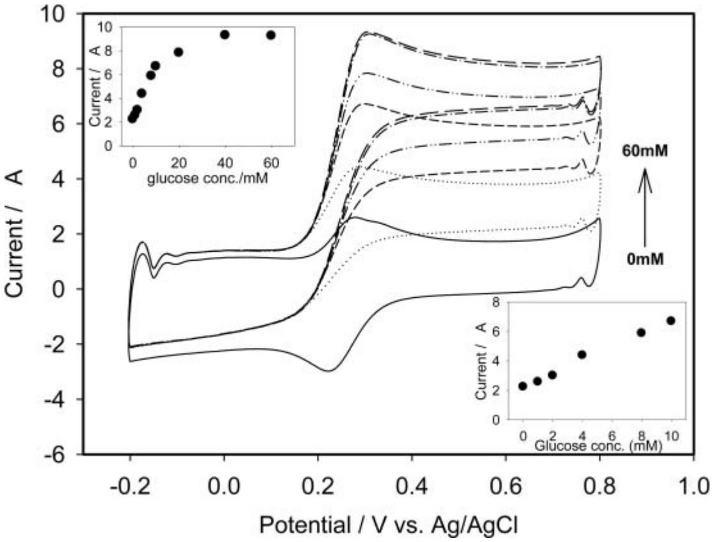

Figure 2. Cyclic voltammograms of the Fc/GOx/CHI electrode at various glucose concentrations in pH 7.0 phosphate buffer at a scan rate of 100 mV/s. Inset: plot of current vs glucose concentration.

The CV analysis of the Fc/GOx electrode was carried out by varying the scan rate from 1 to 200 mV/s. In this analysis, the redox peak currents were shown to be linearly proportional to the root mean square of the scan rate in the range of 1–200 mV/s, as shown in Fig. 3. According to Randles–Sevick equation [17], this is a typical characteristic of non-surface controlled electrochemical behavior, demonstrating that the electrochemical reaction of the Fc/GOx/CHI electrode was a diffusion-controlled process.

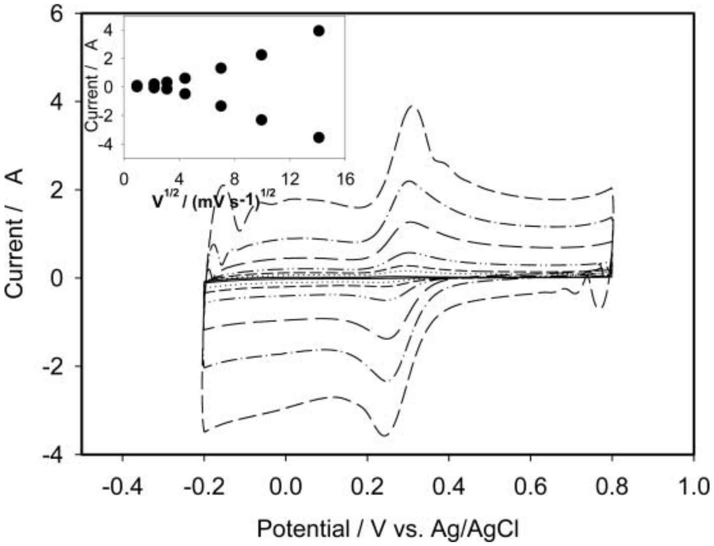

Figure 3. Cyclic voltammograms of the Fc/GOx/CHI electrode in pH 7.0 phosphate buffer at various scan rates. Inset: plot of anodic and cathodic peak currents vs scan rate (V).

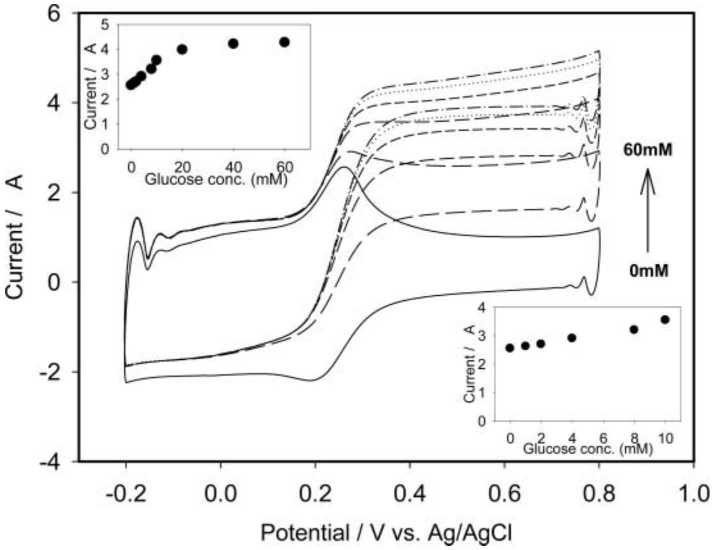

Figure 4. Cyclic voltammograms of the Fc/GOx/Sol-gel electrode at various glucose concentrations in pH 7.0 phosphate buffer at a scan rate of 100 mV/s. Inset: plot of current vs glucose concentration.

The ferrocene and GOx were also physically embedded into the sol-gel matrix as described earlier. The electrochemical performance of the prepared Fc/GOx/Sol-gel electrode was examined by CV, as shown in Fig. 4. The typical oxidation and reduction peaks of glucose oxidation were observed. The similar CV patterns were observed in the Fc/GOx/CHI electrode as discussed earlier. The change in the anodic peak current response of the Fc/GOx electrode at different glucose concentrations is shown in the inset of Fig. 2. In the range of 0–60 mM glucose, the anodic peak current increased with the increase of glucose concentration and leveled off at a higher concentration (>10 mM), representing a characteristic of Michaelis–Menten kinetic mechanism [18]. In order to compare the reaction parameters on the Fc/GOx electrodes prepared by the two methods (chiotosan and sol-gel entrapment), the apparent Michaelis–Menten constants for the immobilized enzymes and maximum currents were calculated based on the Lineweaver–Burk plot. The apparent Michaelis-Menten constants (Km) for the enzymes immobilized in the chitosan membrane and sol-gel matrix were 1.64 mM and 12.59 mM, respectively. The maximum current of the Fc/GOx/CHI and Fc/GOx/Sol-gel electrodes were 5.52 μA and 7.52 μA, respectively. The smaller Km value of Fc/GOx/CHI electrode than that of Fc/GOx/Sol-gel electrode can be attributed to greater glucose affinity of the enzyme immobilized in the chitosan membrane than the enzyme immobilized in sol-gel matrix. However, the maximum current of the Fc/GOx/Sol-gel electrode was higher than that of Fc/GOx/CHI electrode, probably due to the porous structure of the sol-gel matrix allowing relatively fast diffusion of the glucose towards the electrode surface for the redox reaction to occur.

The CV analysis of the Fc/GOx/sol-gel electrode was also carried out by varying the scan rate from 1 to 200 mV/s. In this analysis, the redox peak currents were shown to be linearly proportional to the root mean square of the scan rate in the range of 1–200 mV/s, as shown in Fig. 5. As discussed earlier in Fc/GOx/CHI electrode, the electrochemical reaction of the Fc/GOx/Sol-gel electrode was found to be a diffusion-controlled process.

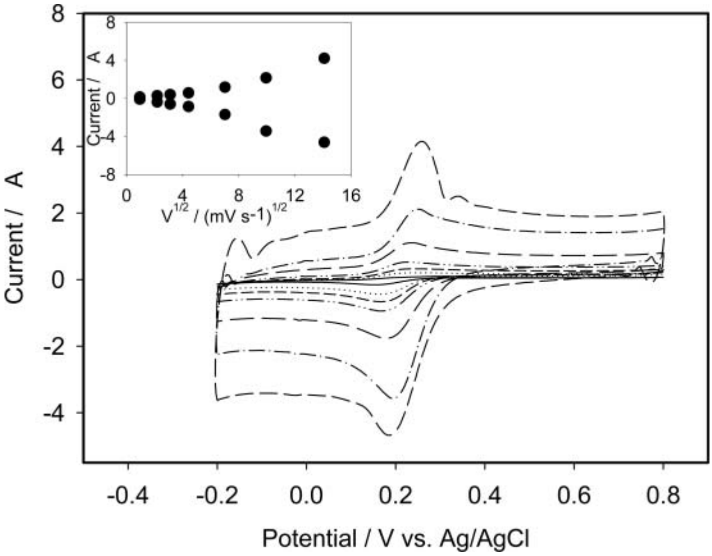

Figure 5. Cyclic voltammograms of the Fc/GOx/Sol-gel electrode in pH 7.0 phosphate buffer at various scan rates. Inset: plot of anodic and cathodic peak currents vs scan rate (V).

These results indicated that the ferrocene molecules were efficiently connected on the electrode for electron transfer from the enzyme to the electrode, and well embedded within the chitosan membrane or sol-gel matrix and did not leach into the aqueous buffer solution. Although further systematic leaching tests are needed, it was thought that ferrocene was stably retained within the chitosan as well as sol-gel matrix because of its insolubility in water and/or its incorporation into the matrix

Conclusions

We have demonstrated that ferrocene and glucose oxidase could be effectively entrapped using a chitosan membrane as well as sol-gel matrix for the fabrication of a glucose sensor. Both of the Fc/GOx/CHI electrode prepared by sequential deposition of each compound and the Fc/GOx/Sol-gel electrode prepared by simple mixing of each compound without using any complicated linking processes exhibited a good electrochemical performance for the glucose analysis. The apparent Michaelis-Menten constants (Km) for the enzymes immobilized in the chitosan membrane and sol-gel matrix were 1.64 mM and 12.59 mM, respectively. The maximum current of the Fc/GOx/CHI and Fc/GOx/Sol-gel electrodes were 5.53 μA and 7.52 μA, respectively. The response current of the both Fc/GOx/CHI and Fc/GOx/Sol-gel electrodes increased with the increase of glucose concentration in the range of 0–60 mM glucose.

Acknowledgments

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