

IMMOBILIZATION OF GLUCOSE OXIDASE/FERROCENE CARBOXYLIC ACID IN COMPOSITE SILICA SOL-GEL/CROSS-LINKED POLY (VINYL ALCOHOL)/NAFION MEMBRANE

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ABSTRACT

A method of tethering a mediator to an enzymatic membrane was studied to construct a non-leaking mediated glucose biosensor. Ferrocene carboxylic acid and glucose oxidase were immobilized in a sol gel derived silica (SGS) matrix containing cross-linked poly (vinyl alcohol) (CLPVA) and Nafion. CLPVA was applied as a solid support due to the ability to form very homogenous films with high quality. SGS was used to increase the encapsulation capacity for the enzyme and mediator. The presence of Nafion, a negatively charged polymer, not only prevented the cracking of pure sol-gel derived silica film but also improved the sensitivity and stability of the enzyme/mediator membrane by minimizing the leaching of the mediator. The biosensor response to glucose was evaluated amperometrically at 0.363V. The immobilization technique resulted in an enzyme/mediator membrane that was simple to cast, had minimal mediator and enzyme losses, worked under lower operating potentials and provided good responses over a wide range of concentrations.

Keywords: Biosensor, Glucose Oxidase, Silica sol gel, Cross-linked PVA, Amperometric.

1 INTRODUCTION

A biosensor is an analytical device that converts information on biochemical substances into a quantifiable electronic signal. The area of biosensors, particularly enzyme-based amperometric electrodes, has received great attention in these last years. Amperometric biosensors combine the advantages of electrochemical technique with the high substrate specificity of enzymes (Sulak *et al.*, 2005). Glucose electrochemical biosensors based on enzymatic oxidation mediated by glucose oxidase (GOD) have generated considerable interest. This enzyme catalyzes the oxidation of glucose to gluconolactone in the presence of oxygen.

However, the use of a mediator to replace the natural acceptor oxygen is a preferable approach that has been explored to overcome tissue oxygen dependence. The oxidation of the reduced mediator occurs at low potential thus reducing the sensitivity of the sensor to interfering substances such as uric acid and ascorbic acid. In addition, mediated biosensors offer other advantages such as increased linear responses and perhaps extended biosensor lifetime, because hydrogen peroxide, which can contribute to the deactivation of the enzyme, is not generated (Reynolds *et al.*, 1992). However, the disadvantage of the mediated glucose sensor is the leaching of the mediator itself, which can reduce the stability and performance of the sensor. The loss of mediator will also result in an inherent toxic effect. Therefore, to develop a stable mediated glucose sensor, a suitable immobilization method should be investigated to avoid the leaking of mediator as well as the enzyme.

An interesting recent entrapment procedure used is the sol gel method. Sol gels are chemically inert, can resist swelling, are processed at low-temperatures, and have tuneable porosity. Over 80% of GOD remained active in sol-gels and the amperometric response agreed well with theoretical predictions (Audebert, 1993). Most of the sol-gel modified

biosensors are based on enzymes trapped in a silica matrix. Using a ferrocene mediator, the leaching problem is less severe if electroactive or ion exchange polymers, such as Nafion, are used to contain the mediator. In a simple Nafion–ferrocene film, where entrapment is provided by Nafion only, the oxidized and the reduced forms of ferrocene are believed to interact differently with the hydrophilic and hydrophobic phases of Nafion (Niu and Lee, 2002).

Thus, in this study, ferrocene carboxylic acid and glucose oxidase were immobilized in a composite sol gel-derived silica (SGS) matrix containing cross-linked polyvinyl alcohol (CLPVA) with Nafion. SGS is an excellent matrix for the entrapment of biomolecules without affecting their activity and stability. SGS-CLPVA will prevent the mediators from leaking out from the inner Nafion layer and effectively stop their leakage from the composite membrane. In addition, the enzyme may also interact favourably with a polyhydroxyl compound like PVA, leading to activity stabilization. This is because the hydroxyl groups in PVA may substitute for the bound water that is essential for the retention of protein tertiary structures, which is the basis of molecular activities (Niu and Lee, 2002).

2 METHODOLOGY

2.1 MATERIALS

Glucose oxidase (E.C. 1.1.3.4) from *Aspergillus niger* was purchased from Sigma (England). Ferrocene carboxylic acid (97%) was purchased from Aldrich (Germany). Peroxidase horseradish (E.C. 1.11.1.7, type VI from Horseradish), glucose (corn sugar, 99.5%), polyvinyl alcohol (PVA, Average MW 70,000-100,000units), glutaraldehyde were purchased from Sigma (USA). The Nafion solution (5% in a mixture of lower aliphatic alcohols and water) was bought from Fluka (USA). Tetramethylorthosilicate (TMOS) was purchased from Merck (Germany). All chemicals were used as received.

2.2 PROCEDURES

2.2.1 Preparation of Nafion–ferrocene Carboxylic Acid (Nafion-FcA) and SGS-CLPVA Solutions.

Ferrocene carboxylic acid solution in absolute alcohol was mixed with 2% Nafion solution in the volume ratio of 5:1 (Niu and Lee, 2002). For preparation of CLPVA solution, 10% PVA stock solution was mixed with 10% acetic acid, 50% methanol, and 10% sulphuric acid in the volume ratio of 5:3:2:1 (Abdul-Aziz, 2001). Later 2% glutaraldehyde was added in such a way that the cross-linking ratio (ratio of the moles of glutaraldehyde per moles of PVA repeat unit) was 0.06. The TMOS stock sol gel was prepared by mixing TMOS, 50% methanol, hydrochloric acid (HCl) and water in the mole ratio of (1:3:0.0013:3.7) at 4°C for 2 hours, based on a water/silicate mole ratio of 1:3.7. Then, the TMOS sol gel solution was mixed with the CLPVA solution in a volume ratio of 1:4 (Cajlakovic *et al.*, 2001). Finally, Nafion was added to the mixed silica sol solution based on 1:1 of optimal weight ratio of Nafion and PVA (Shao *et al.*, 2002).

2.2.2 Casting of SGS-CLPVA/Nafion Membranes

Two types of membranes with different GOD concentrations, 40mg/mL and 20mg/mL, were fabricated separately by casting the following solutions in sequence: 36µL Nafion–FcA solution, 54µL of respective GOD aqueous solution and 36µL SGS-CLPVA solution. Each layer was dried under ambient conditions after each casting before storage in a refrigerator at 4°C overnight. The enzymatic membranes were kept at 4°C in the refrigerator when not in use.

2.2.3 Detecting of Enzyme and Mediator Leakage.

Enzyme leakage was measured colorimetrically. 150µL of 18% aqueous glucose solution and 50µL of 200µg/mL peroxidase solution were added to 1.25mL of chromogen solution at 25°C. The chromogen solution was prepared by diluting 0.1mL of 1% o-Dianisidine in

12mL of 0.1M phosphate buffer, pH6.7. Then, 50 μ L of the washing solution was added to the mixture for 5 minutes at 25°C before 100 μ L of 4M HCl was added to stop the reaction. The absorbance value was read at 450nm. Leakage of ferrocene derivatives mediator was measured electrochemically by subjecting the washing solution to cyclic potentials from 100-600mV with a scan rate of 10mVs⁻¹.

2.2.4 Electrochemical Measurements

Electrochemical measurements were carried out using a potentiostat with a three-electrode configuration (Metrohm μ Autolab Type 111), where a platinum electrode was used as the working electrode (WE), a platinum auxiliary electrode was used as the counter electrode (CE) and an Ag/AgCl/ KCl electrode was utilized as the reference electrode (RE). All amperometric experiments were performed at a temperature of 25 \pm 1°C and under deoxygenated conditions.

3 RESULTS

3.1 RETENTION OF ENZYME AND MEDIATOR IN MEMBRANES

Two types of enzymatic membranes were prepared. One contained 40mg/mL GOD and the other contained 20mg/mL GOD. To investigate the ability of the membranes to retain GOD and ferrocene mediator, the washing solutions for the SGS-CLPVA/Nafion membranes were assayed for any sign of enzyme activity and also leakage of the mediator.

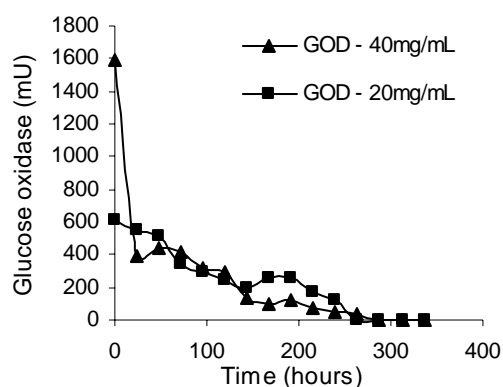


FIGURE 1. Enzyme leaking profile for SGS-CLPVA/Nafion membranes

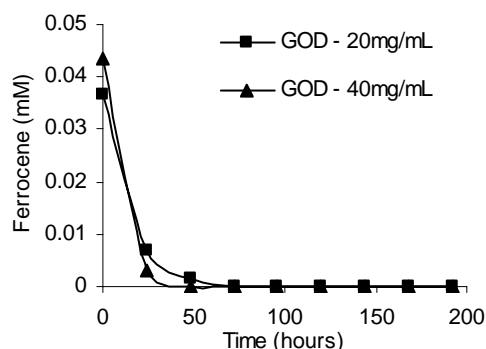


FIGURE 2. Ferrocene leaking profile for SGS-CLPVA/Nafion membranes

As shown in figures 1 and 2, the leaking of enzyme as well as mediator decreased with time for the two types of membranes with different GOD concentrations. No sign of enzyme activity was observed in the washing solutions after 12 days for both types of membranes. Meanwhile, leakage of ferrocene from membranes with 40mg/mL of GOD

stopped after 2 days, which was 1 day earlier than the membranes with 20mg/mL of GOD.

3.2 KINETICS PROPERTIES OF THE MEMBRANES

Figures 3 and 4 show the typical current response towards 5mM glucose solution and typical calibration curves for both types of membrane for kinetics study.

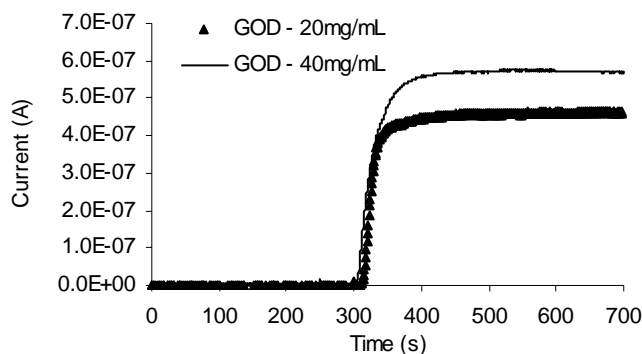


FIGURE 3. Typical current response of SGS-CLPVA/Nafion membranes

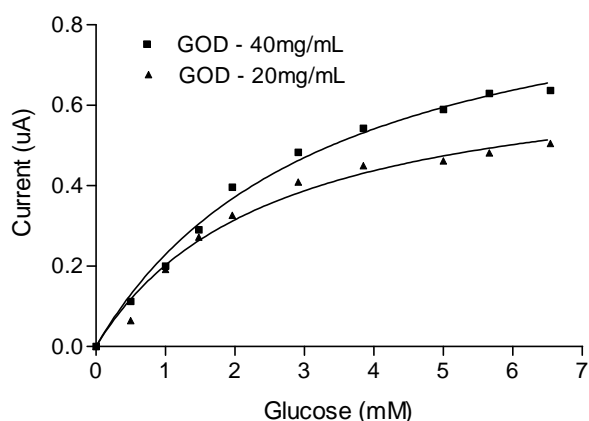


FIGURE 4. Typical calibration curves for SGS-CLPVA/Nafion membranes.

As shown in figure 3, the response time to arrive at 95% of the steady state current for membranes with GOD concentration of 40mg/mL and 20mg/mL were approximately, 87s and 73s, respectively. The kinetic properties of the membranes were determined from the modified electrochemical Lineweaver-Burke plots. The corresponding maximum current, I_{max} , for both cases was $1.23\mu A$ and $0.72\mu A$, respectively. The apparent Michaelis-Menten constant, K_m^{app} , for membranes with GOD concentration of 40mg/mL and 20mg/mL was approximately, 3.80mM and 3.08mM, respectively.

3.3 STABILITY OF SGS-CLPVA/NAFION MEMBRANES

The stability of SGS-CLPVA/Nafion membranes was investigated to determine the shelf life of the sensors. The current outputs of the membranes when subjected to 5mM glucose at certain periods were measured. As shown in figure 5, after 1 month, the membranes containing 40mg/mL and 20mg/mL GOD retained approximately 82.30% and 95.50% of the initial activities, respectively. After 2 months, only 59.50% of the activities of the membranes with 40mg/mL of GOD remained. On the other hand, the membranes with 20mg/mL GOD were still quite stable with 83.60% of the initial activity remained.

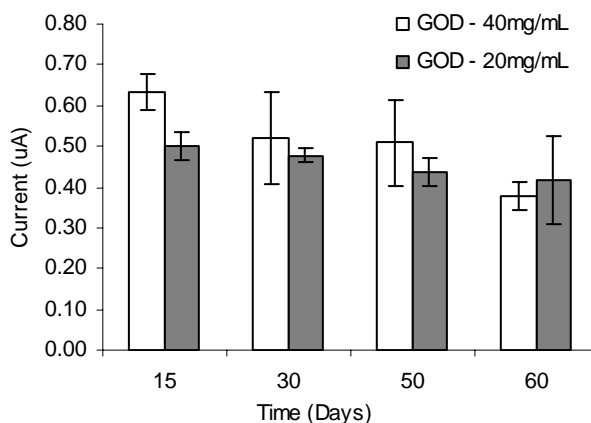


FIGURE 5. Stability of SGS-CLPVA/Nafion membranes

4 DISCUSSION

For both membranes, the leakage of ferrocene stopped earlier compared to the enzyme. With high ethanol content, the Nafion film cast should be stable and capable of good mediator retention (Niu and Lee, 2002). However, if there were weakly held species as well as leached ferrocene derivatives from the inner Nafion mediator layer, they will be retained by the outer SGS-CLPVA network layer. However, the leaking of the enzyme still occurred for a long period for both membranes. As shown in figure 1, by reducing the enzyme concentration, the amount of leached enzyme was reduced instead of the leaking period. The leaking of enzyme might be due to the possibility that the enzyme concentration might have exceeded the immobilization capacity of the membranes. The excess enzymes were not immobilized within the solid support and leached out easily from the membrane.

The response time for the two membranes was almost identical. Both membranes were quite thin thus the distance between the electrode and the reaction center of the enzyme was small. As a result, the time required to reach 95% of the steady state current was relatively short. However, the contact between the redox site and reaction center of enzyme must be improved to get a shorter response time of around 10s-20s. I_{max} is the current at very high and saturated concentrations of substrate. Under these conditions, every enzyme molecule will have the substrate attached to it and will be interacting with it to convert it to product as quickly as possible. I_{max} for the membrane with 40mg/mL GOD was $0.51\mu A$ higher than I_{max} for membrane with 20mg/mL GOD. It shows that in this case I_{max} depended on enzyme concentration. Sato and Okuma (2006) reported that current response was found to increase with the amount of enzyme, but it would be constant after reaching a maximum unit of GOD. This effectively says that in the presence of sufficient amounts of GOD, the response current is independent of the amount of GOD.

The K_m^{app} obtained for both types of membranes were quite low and with only a 0.72mM difference between them. K_m^{app} is independent of enzyme concentration. The K_m^{app} value depends on the strength of the bonds between the enzyme and substrate. If these bonds are strong, the K_m^{app} will be low, indicating that the immobilized enzyme retained its bioactivity and possessed high biological affinity to glucose. The high degree of affinity of the enzyme to the substrate may be explained by a favorable change in the structural organization of the enzyme due to the immobilization procedure (Arica *et al.*, 1995). Consequently, the active sites of the enzymes could be more readily available for enzymatic interactions.

As shown in figure 5, the stability of membranes was quite good. This could be due to the excellent SGS-CLPVA/Nafion matrix. CLPVA was applied as a solid support due to its ability to form very homogenous films of high quality. The presence of hydrophilic PVA and the relatively hydrophobic network of sol gel silica will modify the environment for ferrocene carboxylic acid retention. SGS was used to increase the encapsulation capacity for the enzyme and mediator. The presence of Nafion, a negatively charged polymer, not only prevented the cracking of pure sol-gel derived silica film but also

improved the sensitivity and stability of the enzyme/mediator membrane by minimizing the leaching of the mediator. The result is a consolidation of the effects of polymer, ionomer and sol gel network.

5 CONCLUSIONS

In this work, immobilization of glucose oxidase and ferrocene carboxylic acid in SGS-CLPVA/Nafion has been performed. The immobilization technique resulted in an enzyme/mediator membrane that was simple to cast, resulted in minimal mediator losses and is very stable at lower operating potentials. A membrane with greater GOD concentration gave higher current response. However, the K_m^{app} for SGS-CLPVA/Nafion membrane is independent of enzyme concentration. SGS-CLPVA/Nafion is a good matrix for the immobilization of mediator as well as an enzyme.

6 RECOMMENDATIONS

Extensive studies must be performed in order to improve the retention of enzyme and also the kinetic properties, especially K_m^{app} , to provide good responses over a wide range of concentrations. This will ultimately influence and improve membrane performance.

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