

IMMOBILIZATION OF GLUCOSE OXIDASE AND FERROCENE REDOX POLYMER IN CROSS-LINKED POLY (VINYL ALCOHOL) WITH BOVINE SERUM ALBUMIN AS PROTEIN STABILIZER

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Abstract

A method of tethering a mediator to an enzymatic membrane was studied. Glucose oxidase (GOD) and ferrocene redox polymer were immobilized in cross-linked poly (vinyl alcohol) (CLPVA) with bovine serum albumin (BSA) added as a protein stabilizer. Redox hydrogel polyallylamine ferrocene was prepared by cross-linking polyallylamine hydrochloride with glutaraldehyde and attaching the ferrocene covalently. The biosensor response to glucose was evaluated amperometrically at 0.363V. High BSA concentration resulted in improved current response but lower K_m^{app} . CLPVA, which had been proven to be an excellent retainer of GOD could not retain both GOD and ferrocene redox polymer effectively, thus affecting the stability of the enzymatic membrane.

Keywords

Biosensor, Glucose Oxidase, Redox Polymer, Cross-linked PVA, Amperometric.

1. Introduction

A biosensor is a sensor that is based on the use of a biological material for its sensing function. The bio-component specifically reacts or interacts with the analyte of interest resulting in a detectable chemical or physical change. Three general strategies are used for the electrochemical sensing of glucose. All methods employ glucose oxidase (GOD), an enzyme that catalyzes the oxidation of glucose to gluconic acid with the production of hydrogen peroxide. The first detection scheme measures oxygen consumption; the second measures the concentration of hydrogen peroxide produced by the enzyme reaction; and the third uses a diffusible or immobilized mediator to transfer the electrons from glucose oxidase to the electrode.

The use of an artificial electron acceptor or mediator to replace the natural acceptor oxygen in the oxidation of glucose by glucose oxidase is a preferable approach that has been explored to overcome tissue oxygen dependence. In addition, the oxidation of the reduced mediator occurs at low potential thus reducing the sensitivity of the sensor to interfering substances such as uric acid, ascorbic acid and acetaminophen. These substances will break down electrochemically and thus give interfering signals. In addition, mediated biosensors offer other advantages such as increased linear response and perhaps an extended biosensor lifetime, because hydrogen peroxide, which can contribute to the deactivation of the enzyme, is not being generated, [1].

After years of research, progress is finally being made toward implantable continuous glucose monitors. Most implantable glucose sensors are amperometric enzymatic biosensors. The first report on an implantable amperometric

ferrocene-modified glucose sensor was in 1986 [2]. However, the initial promise exhibited by mediator based glucose sensors for in vivo applications, has failed to materialize. The main problem remains the limited long-time-use stability of mediated glucose sensors, which has been attributed to the leaching of the mediator. In addition, the loss of mediator is a particularly important issue for implantable sensors because of the inherent toxic effect of the mediators used. Therefore, in order to develop a stable implantable mediated glucose sensor, a suitable immobilization method should be investigated to avoid the leaking of mediator as well as the enzyme.

A promising strategy in biosensor design is the immobilization of both enzyme and mediator, which generally require polymeric material. Different redox polymers have been used as mediators including osmium polymers [3,4], ferrocene polymers [5] and Nafion-N-methyl phenazium [6]. The advantages of using the redox polymer are several with the main advantage is more stable biosensors since leaking of mediator from the electrode is minimized and higher and faster responses are observed due to proximity between the enzyme and the mediator [7].

In this study, the enzyme and the ferrocene redox polymer (Fc) were immobilized in cross-linked PVA (CLPVA). Bovine serum albumin (BSA) was added to stabilize the enzyme. CLPVA was applied as a solid support due to the ability to form very homogenous films with very high quality. Redox hydrogel polyallylamine ferrocene was prepared by crosslinking polyallylamine hydrochloride with glutaraldehyde and attaching the ferrocene covalently [8]. Amino group of cross-linked polyallylamine and carboxyl group of ferrocene carboxylic acid were activated using carbodiimide reagents. Larger response currents were expected with a polycationic redox

hydrogel such as derivatized polyallylamine [9]. The effect of BSA loading also was investigated.

2. Approach and methods

2.1 Chemicals

Glucose oxidase (E.C. 1.1.3.4) from *Aspergillus niger* were purchased from Sigma (England). Ferrocene carboxylic acid (97%) was purchased from Aldrich (Germany). Cystamine dihydrochloride (98%) were purchased from Aldrich (China). Peroxidase horseradish (E.C. 1.11.1.7, type VI from Horseradish), glucose (corn sugar, 99.5%), poly (allylamine hydrochloride) (Average MW CA:70 000), HEPES (99.5%, pH 6.8-8.2), bovine serum albumin (BSA), polyvinyl alcohol (PVA, Average MW 70 000-100 000), glutaraldehyde and *N*-cyclohexyl-*N'*-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate were purchased from Sigma (USA). Tetramethylorthosilicate (TMOS), kalium di-hydrogen phosphate, di-kalium hydrogen phosphate, kalium chloride, acetic acid, methanol, sulfuric acid and hydrochloric acid were purchased from Merck (Germany). All chemicals were used as received.

2.2 Synthesis of poly(allylamine) ferrocene (PAA-Fc).

Preparation of ferrocene-containing redox polymer was done according to a previous work [8]. 581 mg of polyallylamine hydrochloride and 5 mL of 20% glutaraldehyde solution were dissolved in a HEPES buffer (50mM, pH6.8) to a total volume of 25mL in a beaker and was then left to gelate. The cross linked gel was crushed through a sieve and freeze dried. 60mg of this polymer was suspended in 50mL of HEPES buffer (50mM, pH6.8) containing 115mg of ferrocene carboxylic acid.

Water soluble carbodiimide was added drop wise during the first hour. The reaction was allowed to proceed for 4 days. Small particles of the ferrocene modified polyallylamine hydrogel were rinsed with a phosphate buffer solution. These particles were enclosed in dialysis tubes containing phosphate buffer. The dialysis was carried out for 3 days.

2.3 Cross linking with PVA and BSA addition

5% PVA stock solution was prepared by dissolving PVA in water and heating the solution to 80–90 °C under stirring for about 30 minutes. Then, the 5% PVA stock solution was mixed with 10% acetic acid as a buffer, 50% methanol as a quencher, and 10% sulphuric acid as a catalyst in the volume ratio of 5: 3: 2: 1. Appropriate amount of 2% glutaraldehyde was added to the solution in order to obtain a cross-linking ratio of 0.06. Cross-linking ratio is defined as the ratio of the moles of glutaraldehyde per moles of PVA repeat unit. Then, polyallylamine ferrocene, BSA and GOD were added to the CLPVA solution and an aliquot of the mixture was pipetted on a glass slide and air-dried for 20 minutes. Then, it was covered with another glass slide and the two glass slides were clamped together and left for

24hr at 4°C. The membranes obtained were swollen in phosphate buffer at 4°C.

2.4 Enzyme and mediator leakage detection

Leakage of enzyme was measured colorimetrically. The chromogen solution was prepared by diluting 0.1 mL of 1% O-dianisidine in 12 mL of 0.1 M phosphate buffer, pH 6.7. Then, 150µL of 18% aqueous glucose solution and 50µL of 200µg/mL peroxidase solution were added to 1.25 mL of the chromogen solution. The mixture was then placed in a water bath at 25°C for temperature equilibration. Then, 50µL of the washing solution was added to the mixture. The reaction was allowed to proceed for 5 minutes before 100 µL of 4 M HCL was added to stop the reaction. The amount of colour formed was measured by reading the absorbance value at 450nm [10].

Leakage of ferrocene derivatives mediator was measured electrochemically. The washing solution was subjected to cyclic potentials from 600mV to -100mV with scan rate 10mVs⁻¹. The concentration of the mediator was determined using a calibration curve.

2.5 Electrochemical measurement

Electrochemical measurements were carried out using a potentiostat with a three-electrode configuration (Metrohm µAutolab Type 111). The working electrode (WE) used was a platinum electrode. A platinum auxiliary electrode was used as the counter electrode (CE). An Ag/AgCl/ KCl was employed as the reference electrode (RE).

Before use, the enzyme electrode was rinsed with doubly distilled water, and immersed in 0.1M phosphate buffer (pH7.0) until a stable electrochemical response was produced. Glucose stock solutions was allowed to mutarotate at room temperature overnight before use. All solutions were deoxygenated before each amperometric run. For kinetics, response time and stability studies, the amperometric experiments were run at 363 mV vs Ag/AgCl. All experiments were performed at a temperature of 25±1 °C and under deoxygenated condition, unless otherwise specified.

3. Results

3.1 Retention of enzyme and mediator in membranes

To investigate the ability of the membranes to retain GOD and ferrocene mediator, the washing solutions for the CLPVA-GOD/Fc membranes were assayed for any sign of enzyme activity and also leakage of the mediator. Figure 1(a) and 1(b) show the leaking profiles of GOD and ferrocene for the CLPVA-GOD/Fc membranes.

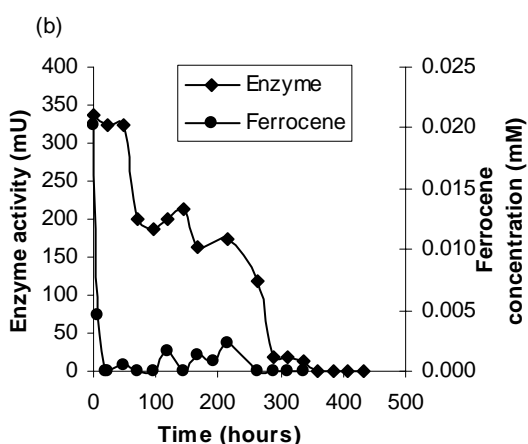
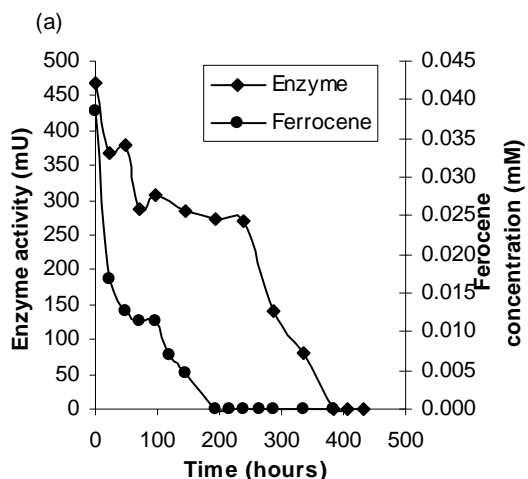


Figure 1. Leaking profile for CLPVA-GOD/Fc membrane with different GOD and BSA loading a) 1:1 b) 1:3 (weight ratio of GOD: BSA).

As shown in figure 1(a) and 1(b), the leaking of enzyme as well as mediator decreased with time. No sign of enzyme activity was observed in the washing solutions after 15 days for membranes with the weight ratio of 1:3 (GOD: BSA), which was 1 day earlier compared to membranes with the weight ratio of 1:1 (GOD: BSA). Meanwhile, leakage of ferrocene from membranes with the weight ratio 1:3 (GOD: BSA) stopped after 11 days, which was 2 days later than the membranes with the weight ratio of 1:1 (GOD: BSA).

3.2 Kinetics properties of the membranes

Current response vs glucose concentration curves for both CLPVA-GOD/Fc membranes are shown in Figure 2. The kinetic properties of the membranes were determined from the modified electrochemical Lineweaver-Burke plots (Figure 3).

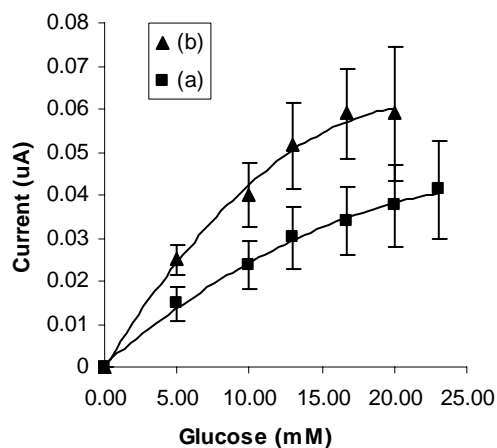


Figure 2. Current response vs glucose concentrations curves for CLPVA-GOD/Fc membranes with different GOD and BSA loading a) 1:1 b) 1:3 (weight ratio of GOD: BSA).

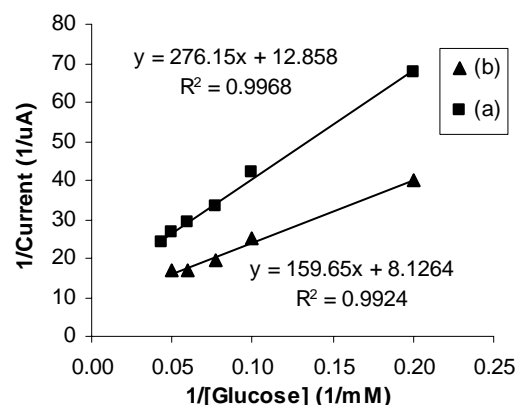


Figure 3. Double-reciprocal (Lineweaver-Burke) plots of CLPVA-GOD/Fc membranes with different GOD and BSA loading a) 1:1 b) 1:3 (weight ratio of GOD: BSA in mg).

The apparent Michaelis-Menten constant, K_m^{app} for membranes with weight ratio (GOD: BSA) 1:1 and 1:3 were approximately, 21.48mM and 19.66mM, respectively. Meanwhile, the corresponding maximums current, I_{max} for both cases were 0.08 μ A and 0.12 μ A, respectively.

3.3 Stability of CLPVA-GOD/Fc membranes

Stability of CLPVA-GOD/Fc membranes was investigated to determine the shelf life of the sensors. The current outputs of the membranes to 5mM glucose at certain period were measured. Figure 4 shows the effect of storage time on stability of CLPVA-GOD/Fc membranes.

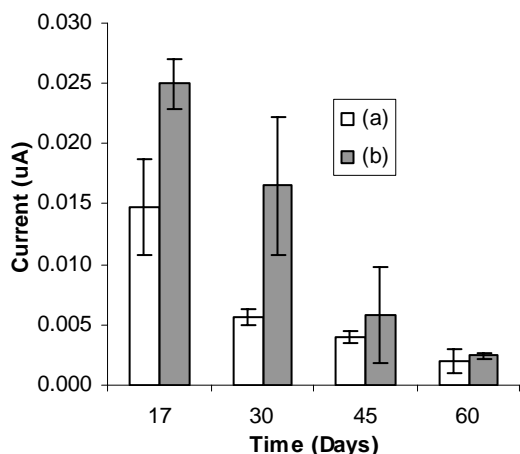


Figure 4. Stability of CLPVA-GOD/Fc membranes with different GOD and BSA loading a) 1:1 b) 1:3 (weight ratio of GOD: BSA).

As shown in figure 4, the membranes retained approximately only 38.87% and 66.00% of the initial current after 1 month, for membranes with weight ratio (GOD: BSA) 1:1 and 1:3, respectively. Then, after 2 month, only 3.5% and 9.7% of the initial current remained, respectively for both membranes.

4. Discussion

The retention of enzyme and mediator in the membranes were very poor although CLPVA was applied as a solid support. For both membranes, the leakage of ferrocene stopped earlier compared to the enzyme. However, the leaking of ferrocene should not have occurred since ferrocene was covalently attached to the polyallylamine hydrogel. The leaking might be due to high concentration of enzyme as well as ferrocene redox polymer that might have exceeded the immobilization capacity of the membranes. The excess enzymes and mediator were not immobilized within the solid support and leached out easily from the membrane.

The membranes with higher BSA gave higher current response towards glucose. BSA stabilized the enzymes, creating a 'biological like' environment. Albumin improves enzymatic activity because of better mass distribution of the various proteins without altering the mechanical properties of the membrane. BSA could also prevent the polymer matrix from over-swelling [8], which could extend the distance between the redox sites of the polymer. As the distance increased the electron transfer rate among neighbouring redox sites would decrease.

The apparent Michaelis-Menten constants, K_m^{app} were 21.48mM and 19.66mM respectively for membranes with weight ratio (GOD: BSA) of 1:1 and 1:3. These values were larger than the K_m^{app} of glucose oxidase in solution that has been reported to be approximately 12.43mM and 15.94mM at temperature 25°C and 30°C, respectively [11].

Generally, the K_m^{app} of an immobilized enzyme will be larger than that of the free enzyme in solution due to the effect of the diffusion of substrate to the active sites [10]. In this work, membranes with high loading of BSA had lower K_m^{app} . The low K_m^{app} suggested that the enzyme had a high affinity for the substrate [12].

As shown in figure 4, the stability of CLPVA-GOD/Fc membranes was not good. This could be due to the deterioration of the immobilized GOD or problems with the mediator. Brooks et al., however, reported that the loss of activity of ferrocene glucose sensors was more strongly influenced by the loss of enzyme by denaturation or detachment [13]. The addition of extra ferrocene to spent electrodes did not affect activity but the addition of more glucose oxidase rejuvenated the sensitivity to glucose. Thus, stability of CLPVA-GOD/Fc membranes could be improved if the immobilization process was more effective.

5. Conclusions

In this work, immobilization of glucose oxidase and ferrocene redox polymer in CLPVA with the addition of BSA has been done. A membrane with greater BSA content gave higher current response but had a smaller K_m^{app} , which might eventually decrease the detection limit of the biosensor. Extensive study must be done to improve the retention of enzyme and mediator as the CLPVA which had been shown to be an excellent retainer of GOD [10] was not able to retain both GOD and ferrocene redox polymer effectively. This would ultimately influence the stability of the membranes.

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