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AN INTERFERENCE-FREE IMPLANTABLE GLUCOSE MICROBIOSENSOR BASED ON USE OF A POLYMERIC ANALYTE-REGULATING MEMBRANE

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1. ABSTRACT

An implantable glucose microbiosensor that utilizes an electrodeposited redox polymer/glucose oxidase (GOX) sensing membrane and a highly hydrophilic poly (4-vinyl pyridine-co-acrylic acid) (PVP-PAC) overcoating as an analyte-regulating membrane has been developed. A gold microelectrode was electrodeposited with the redox polymer/GOX composite sensing membrane. Two polymers, a hydrophobic poly (4-vinyl pyridine) (PVP) and a hydrophilic PVP-PAC were investigated as possible analyte-regulating membranes. Each was mounted on the sensing layer via chemical cross-linking with poly(ethylene glycol) diglycidyl ether (PEGDGE). High current sensitivities of ~220 nA/mM glucose were obtained in the presence of PVP-PAC. In the case of PVP, much lower sensitivities of ~30 nA/mM were observed. However, only the biosensor with PVP-PAC showed excellent selectivity to glucose against interferants like oxygen and ascorbic acid (AA). The dynamic range is from 0 to 30 mM. The response time in amperometric measurements was less than 10 sec. Clinical trials showed good correlation between the readings of the subcutaneously implanted biosensor and blood sugar levels.

2. INTRODUCTION

Biosensors have been a long-standing field of research, dating back to the 1960s when Clark and Lyons pioneered the development of oxygen electrode based enzyme electrodes (1). To date, there are many applications of biosensors in clinical work, agriculture, food analysis and environmental monitoring (2, 3). Here, we shall focus on the development of enzyme-based biosensors for the monitoring of physiologically important molecules, specifically glucose. According to the World Health Organization, the number of people with diabetes mellitus worldwide will reach approximately 300 million by the year 2025. Hence there is an increasing need to develop sensitive and implantable biosensors that require minute procedures and are able to block out external interferants present in body fluids. The significant element in the design of a biosensor is the immobilization of the specific biological recognition layer, e.g. enzyme, onto a transducer surface, which is usually gold or carbon electrodes. Many methods of immobilization have been proposed, such as electropolymerization, chemical cross-linking, screen-printing and dip-coating using sol-gel matrixes (4-6). Of the above mentioned, electropolymerization is the most advantageous as it can generate sensing membranes on microelectrodes of complex geometry efficiently in a few simple steps (7). This allows the achievement of cylindrical geometry, gives control over the preparation of the electrodes and enables further similar applications on sensor arrays (8). Another prime concern is the selectivity and stability of the biosensor. The two main interferants found in blood sugar monitoring are oxygen and AA. They are present in higher concentrations in blood, as compared to other possible interferants such as uric acid and acetaminophen. If we are able to remove this major bulk of the interferants, the minority present will pose little threat to the accuracy of the biosensor.

GOX, one of the most widely used oxidases, selectively catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide. The concentration of glucose in solution can be determined by measuring either the concentration of hydrogen peroxide or partial pressure of oxygen gas. However, the amperometric measurement of hydrogen peroxide is compromised by electroactive interferants such as ascorbic acid and uric acid in blood. High concentrations of hydrogen peroxide may also affect GOX activity, and in turn the performance of the biosensors. Furthermore, oxygen is the limiting factor in this reaction, which is undesirable in the development of highly sensitive glucose biosensors. One solution to this problem is the use of an electron mediator, like ferrocene (10, 11), osmium complex (12) and hydroquinone (13). It electrically "wires" the reaction centres of GOX to the electrode and transfers electrons between them. As a result, the current detected in amperometry and cyclic voltammetry increases linearly with the glucose concentration in the test sample as long as the entire glucose influx is consumed in the electrode reaction. The dynamic range of glucose detection is also widened, the oxygen dependence largely removed and the effect of interferants reduced as the mediator lowers the polarized potential for amperometric detection. But the natural enzymatic oxidation of glucose with oxygen as a co-reactant, now considered to be a side reaction, still proceeds to some extent, depending on the kinetics of the mediated reaction, and in turn affects the accuracy and sensitivity of the biosensor. With such limitations, the analyte-regulating membrane offers a new alternative. The application of a polyurethane film introduced by Wilson's group increased the oxygen/glucose permeability ratio and optimized the linearity of the sensor response (14, 15). Another experimental study revealed that the application of a micromembrane alleviated the disproportionately large interferences by AA and uric acid (16). Furthermore, Heller et al. reported that the more blocking the membrane is, the better the apparent stability of the biosensor(16).

In this paper, we proposed the use of a highly hydrophilic membrane in the development of a practically interference-free glucose biosensor for continuously in vivo glucose monitoring. The analyte-regulating membrane was achieved through cross-linking between PVP-PAC and sensing membrane using PEGDGE. It formed a thin permselective polymeric layer, which yielded a rapid response and had an excellent selectivity for glucose over interferants such as AA and oxygen. The current detected was directly proportional to the concentration of glucose in the sample solution. The biosensor was then evaluated with respect to sensitivity, dynamic range and selectivity. Successful attempts were also made in clinical trails.

3. EXPERIMENTAL SECTION

3.1. Reagents and apparatus

GOX (EC 1.1.3.4, from *Aspergillus niger,* 191 units/mg) was purchased from Fluka. The electron-conducting redox polymer was synthesized from poly (4-vinyl pyridine-co-acrylamide), (PVP-PAA) and $Os(bpy)_2Cl_2$ (17). Glucose (from Sigma) solutions were allowed to mutarotate for 24 hours before use. AA solutions were prepared immediately before testing. Phosphate-buffered saline (PBS, pH 7.4) was prepared from phosphate salts (0.10 M) and sodium chloride (0.15 M). 4-(2-hydroxyethyl)-1-piperazineethanesulfonate) (HEPES, Aldrich), PVP-PAC and PEGDGE (M.W. 400, Polysciences, Warrington, PA) were used in the preparation of the membrane. All other chemicals used were of certified analytical grade and all solutions were prepared with millipore water.

Electrochemical experiments were performed with an AutoLab potentiostat/galvanostat running under the General Purpose Electrochemical System (GPES) Manager Version 4.9. A conventional three-electrode system cell, housed in the Faraday Cage, was employed with an Ag/AgCl reference electrode, a platinum wire counter electrode and a gold working electrode. In amperometry, the biosensor was operated at a potential of 0.45 V (vs. Ag/AgCl) and performed in 5.0 ml of PBS solutions, pH 7.4 at room temperature (~25˚C). The background current was allowed to stabilise first before readings were taken. Small aliquots of a 2.0 M glucose stock solution were added sequentially using a microlitre pipette to reach the desired glucose concentration in the solution. To test the effect of oxygen interference on the system after application of PVP-PAC, solutions were purged by bubbling nitrogen, air or oxygen gas into PBS solution for 30 min and a gas blanket was kept above the solution during the experiment.

3.2. Preparation of the biosensor

A gold microelectrode was sandpapered, polished with 0.3 μm and 0.05 μm alumina slurry (CH instruments, Inc., Austin, TX) sequentially and sonicated for 5 minutes in water between each polishing step. There are two methods to deposit the redox polymer: (a) applying a constant reducing potential of usually –1.4 V for 2 min; or (b) potential cycling between 0 and -1.45 V for 10 cycles at a scan rate of 0.1 V s^{-1}. Both methods gave similar amounts of deposits. In this work, the deposition was done using the later method at room temperature. The redox polymer/enzyme film was irreversibly co-electrodeposited from PBS containing 5.0 mg mL^{-1} redox polymer and 20 mg mL^{-1} GOX. The biosensor was kept at 4˚C when not in use. After the sensing layer was formed on the working electrode, 20 mg mL^{-1} of PVP-PAC and 0.40 M PEGDGE each dissolved in HEPES, were mixed and applied over the sensing layer. The electrode was kept at 4˚C for at least 24 h for the cross-linking reaction between the 2 layers.

4. RESULTS AND DISCUSSION

4.1. Immobilization of redox polymer and GOX on the gold electrode

The electrodeposition was carried out by applying a sequence of potential cyclings (17). The conditions of electrodeposition were optimized. Since the ligand exchange (cross-linking) occurred in the reduction process of the redox polymer, the surface density of the adsorbed redox polymer must be high for the fraction of cross-linked polymer to be large (17). The optimal potentials of 0 to –1.45 V (Figure 1) gave the maximum polymer deposition and GOX loading. The optimal concentrations of the redox polymer and GOX used were found to be 5.0 and 20 mg mL^{-1} respectively. In PBS

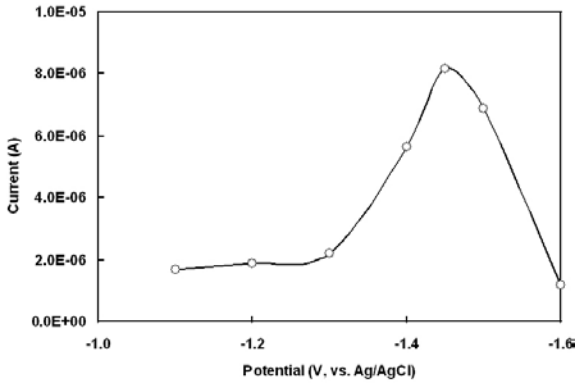

Figure 1. Effect of deposition potential on redox polymer/enzyme loading. 5.0 mg mL^{-1} redox polymer and 20 mg mL^{-1} GOX in PBS.

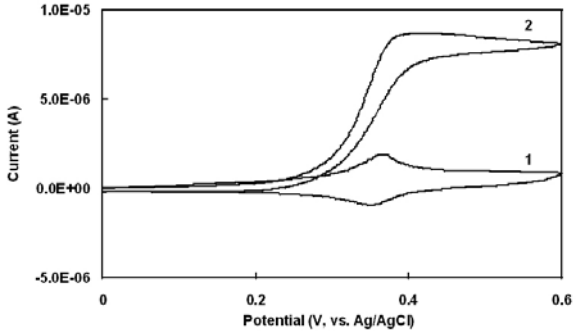

Figure 2. Cyclic voltammograms of the redox polymer/GOX composite membrane in PBS (pH 7.4) containing (1) 0 mM and (2) 10 mM glucose. Scan rate 10 mV s^{-1}.

solution, the surface of the electrode was negatively charged. The redox polymer used in this work is polycationic at neutral pH and can thus be electrostatically bound to the negatively charged surface. Subsequent electrostatic binding and cross-linking of anionic GOX to the positively charged redox polymer layer was then possible. The process was repeated as desired to deposit a redox polymer-GOX composite film.

4.2. Electrochemical characterization of the polymer/enzyme film

In PBS with the absence of glucose, the biosensor gave no catalytic response. Cyclic voltammetric tests showed only the Os^{2+}/Os^{3+} redox couple electrochemistry and indicated the amount of polymer deposited on the electrode surface (Figure 2 trace a). At a scan rate of 100 mV s^{-1}, the difference in the reduction and oxidation peak potential was less than 25 mV, showing that charge transfer from the film to the electrode is rapid (18). It can also be inferred from the rapid kinetics and symmetry of the reduction and oxidation curves that GOX does not appreciably affect the electrochemistry of the Os^{2+}/Os^{3+} redox couple.

In the catalytic oxidation of glucose, glucose first reacts with the oxidized form of GOX to form gluconic acid, leaving behind 2 electrons and 2 protons, thus reducing GOX. Os^{3+} reacts with the reduced GOX, accepting the aforementioned electrons to become Os^{2+} and regenerating the oxidized GOX. The electrons are then transferred from Os^{2+} to the electrode surface, generating a current while Os^{2+} oxidizes back to Os^{3+}. In a 10 mM of glucose in PBS, a typical catalytic i-E curve was obtained with a greatly increased oxidation current and a diminished reduction current (Figure 2 trace b). This meant that the sensing layer was homogenously maintained in the reduced state by the transfer of electrons from the reduced GOX to the Os^{3+} sites. For amperometric measurements, the potential was poised at 0.45 V. This potential was chosen due to the voltammograms peaking off around 0.35 V onwards. Response time is the time needed to reach 90% of the maximum response (T_{90}). Examples of factors affecting T_{90} are thickness of the sensing layer and temperature. Here, the value of T_{90} was 5 seconds. The linearity of the biosensor was in the range of 0 to 15 mM (Figure 3). In this concentration range, glucose is the limiting factor in the reaction and GOX was in excess. From 15 to 40 mM, the current increased at a slower rate. This is due to the partial saturation of the active sites on GOX. From 40 mM onwards, the current leveled off at a limiting current of 9.65 μA. As all the active sites on the enzyme were fully saturated and operating at a maximum speed, any increase in glucose concentration would not increase the catalytic oxidation current.

4.3. Effect of oxygen on catalytic oxidation of glucose

As mentioned earlier, oxygen normally affects the sensitivity of the biosensor because the reaction between glucose and oxygen occurs simultaneously as a side reaction. As illustrated in Figure 3, the current detected was higher in the absence of oxygen than that with dissolved oxygen in PBS solution. At low concentrations of glucose, e.g. 5.0 mM, the competition with oxygen caused a significant decrease (~22% drop) in catalytic current and at high concentrations of glucose, e.g. 40 mM, there is negligible effect (<2%). Since physiologically relevant blood sugar levels are between 3.0 and 20 mM, there is a need to suppress the oxygen interference in the system to achieve practical glucose biosensor, which leads us to exploiting the analyte-regulating membranes.

4.5. Effect of polymer overcoating on the performance of the biosensor

In the absence of an analyte-regulating membrane, the electrooxidation of anionic interferants was disproportionately high since the redox polymer used was polycationic. This is due to the accumulation of anionic interferants within the redox polymer scaling with the density of cationic sites (16). The advantage of the application of a hydrophobic PVP membrane was the extension of the dynamic range of the biosensor up to 80 mM. On the other hand, it lowered the current sensitivities drastically from 460 to 30 nA/mM glucose and the response time increased to 20 sec. The oxygen interference was more severe, as seen from Figure 4. At 5.0 mM

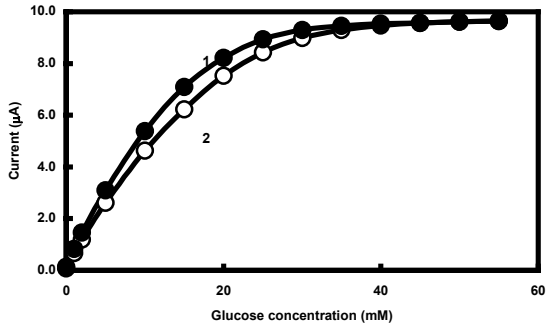

Figure 3 Amperometric response to the sequential addition of glucose to 5.0 ml of PBS solution (pH 7.4); (1) bubbled with nitrogen and (2) bubbled with air. Poised potential 0.45 V.

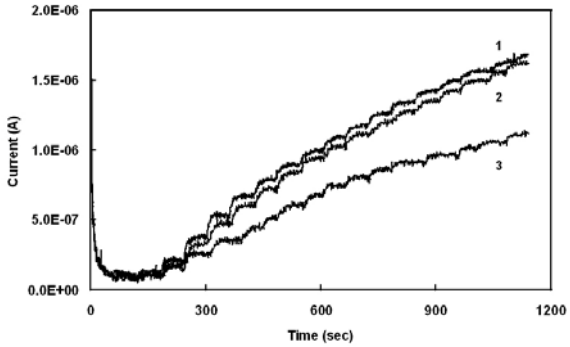

Figure 4. Amperometric responses of PVP coated biosensor to the sequential addition of glucose (1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 mM) to 5.0 ml of PBS solution (pH 7.4); (1) bubbled with nitrogen, (2) bubbled with air and (3) bubbled with oxygen. Poised potential 0.45 V.

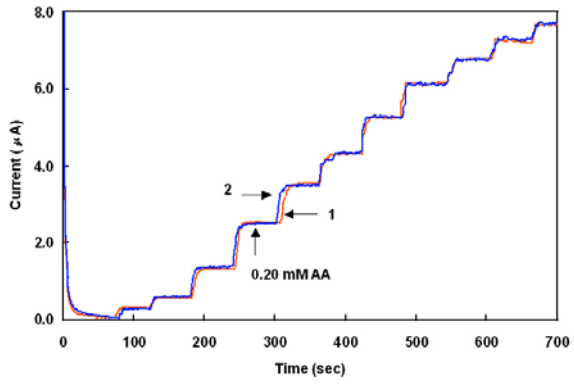

Figure 5. Amperometric responses of PVP-PAC coated biosensor to the sequential addition of glucose (1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45 mM) to 5.0 ml of PBS solution (pH 7.4), (1) bubbled with nitrogen and (2) bubbled with oxygen. Poised potential 0.45 V.

glucose concentration, the percentage drop in current due to oxygen in the absence of a membrane was ~22%, while the percentage drop in current due to oxygen in the presence of the PVP membrane was ~50%, due largely to the hydrophobic nature of both oxygen and PVP. It was evident that PVP is not a suitable polymer for regulating the analyte.

The introduction of acrylic acid into the hydrophobic polymer PVP resulted in a marked improvement of the performance of the biosensor. The resulting polymer PVP-PAC was highly hydrophilic in nature. As shown in Figure 5, the two amperometric graphs, bubbled with oxygen and bubbled with nitrogen, overlaid with a difference of <2% at 5.0 mM glucose and <1% at 10 mM glucose. This shows that the biosensor was insensitive to oxygen in the system. In the case of ascorbic acid, the increase in current caused by the addition of 0.2 mM AA to 10 mM glucose was negligible (Figure 5). Furthermore, the linearity extended considerably from 15 to 30 mM and the response time was kept under 10 sec. The only drawback of this membrane was the drop in current sensitivity to ~220 nA/mM glucose. Nevertheless, since normal blood sugar levels are between 4.0 to 10 mM, the biosensor is good enough for practical applications.

4.6. Performance of the biosensor *in vivo*

The objective of this experiment was to establish the validity of the biosensor in vivo. The biosensor was subcutaneously implanted in the abdomen area of a healthy person. Blood glucose levels were periodically measured in withdrawn samples, while the biosensor was calibrated 15 min after implantation, one blood glucose level determination and one current measurement to establish the scales, and the current output was continuously monitored afterwards. The results of the first 13 h are shown in Figure 6. As seen in Figure 6, the readings from blood glucose measurements and from the implanted biosensor correlated well and administration of AA during the trail showed little effect to the biosensor, demonstrating that the readings of the subcutaneous glucose biosensor provided, without any correction, clinically useful estimates of blood glucose levels. However, current sensitivity of the biosensor started to drop precipitously after ~72 h of implantation, presumably due to the slow encapsulation (fouling) of the biosensor by body fluids. When the failed biosensor was withdrawn and retested in PBS, most of their original sensitivity was recovered.

5. CONCLUSION

In this report it has been demonstrated that the adoption of PVP-PAC as the analyte-regulating membrane successfully blocks out interferants like oxygen and ascorbic acid in the measurement of glucose concentrations. In addition, the analytical signal of this biosensor is directly generated from the catalytic oxidation of glucose in the test sample. This contributes to the high sensitivity of this biosensor and sets it apart from other sensors that relying on indirect measurements of glucose through oxygen or hydrogen peroxide. The application of the analyte-regulating membrane over the sensing layer extended the dynamic range of the biosensor up to 30 mM. Response times of less than 10 sec were maintained throughout the experiments.

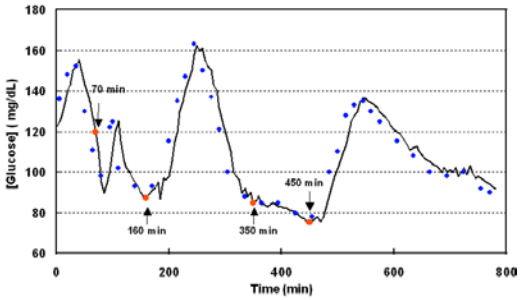

Figure 6. A typical *in vivo* trail in which the glucose concentration was (—) tracked by the subcutaneously implanted biosensors and (●) concentrations of glucose in blood samples measured with a YSI glucose analyzer. 0 Min: 15 Min After Breakfast; 70 Min: 1 Coke; 160 Min: Lunch (Pizza, organge juice, 1 Coke); 350 Min, 500 mg AA; 450 Min: Dinner (Steak, Bread, Salad, Wine).

6. ACKNOWLEDGEMENT

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