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Development of potentially implantable glucose sensors

Bindra, Dilbir Singh, Ph.D.
The University of Arizona, 1990
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DEVELOPMENT OF POTENTIALLY IMPLANTABLE
GLUCOSE SENSORS

by

Dilbir Singh Bindra

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF CHEMISTRY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1990
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Dilbir Singh Bindra entitled Development of Potentially Implantable Glucose Sensors and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

G.S. Wilson
M.F. Burke
M.J. Byers
W.B. Miller

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director
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SIGNED: [Signature]

Debhar Singh Bindra
To My Parents
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ABSTRACT

A novel glucose oxidase based needle-type glucose microsensor has been developed for subcutaneous glucose monitoring. The new configuration greatly facilitates the deposition of uniform enzyme and polymer films so that sensors with satisfactory in vitro characteristics (upper limit of linear range (>15 mM) and response time (<5 min) can be prepared in high yield (>60%). The sensor is equivalent in size to a 26 gauge needle (0.45 mm o.d.) and can be implanted with ease without any incision. The insertion of the sensor causes minimal trauma to the tissue and to the sensor itself. The multilayer structure of the sensor ensures satisfactory performance in subcutaneous tissue over extended periods of time (up to 20 days). The sensor response is largely independent of oxygen tension in the normal physiological range. It also exhibits the desired sensitivity and selectivity.

A two-point in vivo calibration procedure is adapted for in vivo evaluation of the sensors. Both short-term and long-term implantation experiments are described. The methods of cell culture toxicity testing are modified and applied to locate the source of toxicity in a multi-component glucose sensor. It is shown that a non-toxic sensor can be readily obtained by removing the leachable toxic substances through extraction in phosphate buffer.
A nonenzymatic glucose sensor that utilizes permselective membranes to achieve the selectivity required for screening glucose in biological fluids has been described. Interference from endogenous oxidizable substances such as amino acids, urea, ascorbic acid, and uric acid, as well as the effect of chloride and proteins on glucose response, is studied by using flow injection analysis. A set of membranes made of Nafion perfluorinated membrane and collagen, when arranged in front of the working electrode (gold), result in significant improvement in the system selectivity and sensitivity.
Chapter 1

INTRODUCTION

Undoubtedly, the most important unsolved problem in the medical biosensor area is a viable sensor for glucose capable of continuous monitoring of blood glucose levels. The successful development of a closed loop insulin delivery system (artificial pancreas) depends primarily on the availability of such a sensor. This system, consisting of an insulin pump that is responsive to a glucose sensor, will be able to continuously monitor blood glucose concentration and help regulate the insulin delivery in maintaining normal glucose levels in insulin-dependent type I diabetic patients. Although a subject of debate, close glycemic control still appears to be an important therapeutic approach. If easily attainable, this approach can prove to be a major step in the prevention of the late complications of diabetes.

Most parts of the closed loop insulin delivery system are already being employed. The insulin pump is very close to being perfected and the control system is also in a highly advanced stage of development. What is lacking at present is a reliable implantable sensor. Such a device must meet several strict requirements, and it has not been possible so far for a single sensor design to meet all
these requirements.

For a glucose sensor to be suitable for a closed loop insulin delivery system, it should have all of the following characteristics:

1. The sensor should be able to detect glucose with a high degree of specificity in the presence of other biological substances. A number of sensor designs, such as the sensors based on direct oxidation of glucose, suffer because of the lack of specificity.

2. The sensor should be able to provide a linear or a predictable response over the entire glucose concentration range likely to be found in a diabetic individual (usually between 2 and 30 mmol/L). However, a linear response from 1 to 15 mmol/L will be sufficient. This rather limited range is based on the findings of Harrison et al. (1985) who showed that the insulin secretion response of isolated human Islets of Langerhans to glucose stimulation has a plateau around 10 mmol/L.

3. At present, it is not clear how much of a delay between exposure and output would be clinically acceptable. Based on their theoretical physiological pharmacokinetic model of glucose homeostasis, Sorensen et al. (1982) have shown that a sensor response time of less than 10 min would not cause any loss in glucose regulation.
4. For a subcutaneously implantable sensor a lifetime of around a week could be acceptable because of the ease with which the sensor can be replaced or moved to a different site. This is a commonly used practice for subcutaneously implanted insulin pump catheter tips which are usually moved to a different site after 2 or 3 days to avoid serious inflammation of the tissue. A chronically implantable sensor must be inserted surgically and would obviously have to last much longer to be acceptable.

5. The sensor should be easily miniaturized, and the scaling down of the sensor size should not modify its performance characteristics. The desired size for a sensor will be a function of where and how it is implanted, and how often a replacement is required. Woodward (1982) suggested that a subcutaneous sensor, designed in form of needle, wire, or filament measuring less than 2 mm in diameter, would evoke a minimal inflammatory tissue response. The miniaturization, however, is a non-trivial process for most of the currently available sensors.

6. The sensor should be mechanically robust and should have a good shelf life.

7. The sensor should require minimal calibration. An easy one point or a two point in vivo calibration would have to suffice.
8. The sensor design should be amenable to mass production and should be cost effective.

9. All implanted parts of the sensor should be biocompatible. The biocompatibility of an implanted sensor is a two-way process that involves the time dependent effects of the tissue on the implanted sensor (host response) and the time dependent effects of the sensor on the tissue (material response). The interactions between the sensor and the surrounding tissue (inflammatory and immune responses) could result in severe disturbance of the tissue as well as in the sensor, so that the sensor is not able to function properly. Even if the effects are localized, they can still influence the sensor output because they occur in the sampling region. A variety of factors such as the size of the sensor, the site of its implantation, and the materials used in its fabrication determine the effectiveness of the sensor to survive in the physiological environment i.e. its biocompatibility.

Most of the glucose sensors are coated with an outer polymer membrane. Such polymers can interact with the tissue in two ways. One is commonly recognized as the polymer toxicity which is caused by the agents leached from the polymers such as residual solvents, monomers and various additives used in the polymer manufacturing process. The second is the direct interaction between the polymer surface and the tissue which is characterized by
the attachment of the cells and proteins to the surface. As
this membrane creates the interface between the tissue and
the sensor, the choice of the membrane material is very
crucial in determining the biocompatibility of the sensor.
Also, the sensor must not employ any potentially toxic
substances which might leach into the surrounding tissue
perturbing normal cell function.

The following is a brief review of various glucose
sensing devices with a special emphasis on enzyme-based
electrochemical sensors.

**ENZYME-BASED SENSORS**

The enzyme-based sensors, by far, have come the closest
to meeting the above mentioned requirements. For most such
sensors, the enzyme used is glucose oxidase (GOx) and the
mode of detection is electrochemical. The configuration of
these sensors is a function of whether the glucose oxidase
catalyzed oxidation of glucose is monitored by measuring
H$_2$O$_2$, O$_2$ or a redox-mediatior such as dimethyl ferrocene.
All configurations have their advantages and disadvantages.
The most successful oxygen detecting sensor (Scheme 1.1)
has been developed by Gough and coworkers (1986). As
oxygen is monitored, a low applied potential for
amperometric detector is required. The potential is in a
region where few endogenous electroactive species can
interfere. The signal of merit is the difference between
Scheme 1.1

Glucose + GOx(FAD) $\longrightarrow$ Gluconolactone + GOx(FADH$_2$)

GOx(FADH$_2$) + O$_2$ $\longrightarrow$ GOx(FAD) + H$_2$O$_2$

$\frac{1}{2} \text{O}_2 + \text{H}_2\text{O} + 2 \text{e}^- \quad \begin{array}{c} \text{-550 mV} \\ \longrightarrow \end{array} \quad 2 \text{OH}^-$

Scheme 1.2

Glucose + GOx(FAD) $\longrightarrow$ Gluconolactone + GOx(FADH$_2$)

GOx(FADH$_2$) + O$_2$ $\longrightarrow$ GOx(FAD) + H$_2$O$_2$

$\begin{array}{c} \text{H}_2\text{O}_2 \\ \text{+650 mV} \\ \longrightarrow \end{array} \quad 2\text{H}^+ + \text{O}_2 + 2\text{e}^-$

Scheme 1.3

Glucose + GOx(FAD) $\longrightarrow$ Gluconolactone + GOx(FADH$_2$)

GOx(FADH$_2$) + M$_{\text{ox}}$ $\longrightarrow$ M$_{\text{red}} + \text{GOx(FAD)}$

$\begin{array}{c} \text{M}_{\text{red}} \\ \text{+ 100 mV} \\ \longrightarrow \end{array} \quad \text{M}_{\text{ox}} + 2\text{e}^-$
the ambient oxygen level and the level attained as a result of oxygen depletion by the enzymatic reaction. This sensor, however, is difficult to miniaturize and exhibits long response times. An added complication is the need to use a differential measurement system to account for the fluctuation in basal oxygen levels. The hydrogen peroxide based sensor (Scheme 1.2) is the simplest and is easily fabricated. It suffers in principle from the poor electrochemical selectivity and the dependence of response on ambient oxygen. These problems can be largely corrected by using semipermeable membranes with specific transport properties. For example, if the ratio of glucose to oxygen flux can be decreased this can result in insensitivity of the sensor response to oxygen tension. Suitably designed membranes can also screen out substances that may interfere with the electrochemical response. Because the reduced enzyme is fairly easy to oxidize, mediators (M) such as ferrocene or quinone have been used in place of oxygen as the electron acceptor (Scheme 1.3) (Claremont et al., 1983; Cass et al., 1984). In principle, such a sensor should show almost complete insensitivity to variations in oxygen tension, but this is not always guaranteed. Oxygen can affect the response indirectly by competing with the mediator for the reduced enzyme. These sensors suffer from a lack of long-term stability and potential toxicity of the mediator.
Besides electrochemical, glucose oxidase-based sensors also include calorimetric (thermal) and optical sensors. The thermal sensors measure glucose levels through enthalpy changes that occur during the enzymatic reaction. These sensors suffer mainly because of non-specific heat interferences (Muehlbauer et al., 1989). Optical sensors, which are also referred to as optrodes, are based on the fluorescence variation of a dye (bromocresol green) with pH change as a result of glucose oxidation. These sensors are easily miniaturized but suffer because of slow response time, potential toxicity of the materials used and dependence of response on the buffer capacity of the medium. (Peterson, et al., 1984; Seitz, 1984).

NON-EZYMATIC SENSORS

Glucose sensors based on non-enzymatic approaches have also been known for several years. These sensors include electrocatalytic, affinity, and optical sensors. The electrocatalytic sensors are based on the direct oxidation of glucose on anodic noble metal (platinum or gold) surfaces (Richter et al., 1982; Gebhardt et al., 1978). Their specificity for glucose is less than optimal and they are also subjected to electrode poisoning by the products of oxidation and chloride (Marincic et al., 1979). These problems can be partially offset by the use of selective outer membranes and periodic regeneration of the working
electrode by electrochemical pulsing. The present status of the electrocatalytic sensor, however, does not favor its use as an in vivo sensor.

The affinity sensors (Schultz et al., 1982) exploit the fact that concanavalin A (Con-A) has a moderate affinity for glucose. The glucose can compete with another compound such as fluorescein-labeled dextran for the binding sites on Con-A. The extent of competitive displacement of the fluorescein-labeled dextran within a permselective polymer lumen reaction chamber is monitored by fiber optics and is related to glucose concentration. Such sensors suffer from relatively long response times (10-15 min) and material biocompatibility.

Optical sensors based on the measurement of absorption of infrared light at a specific wavelength or on degree of optical rotation of polarized light have been suggested for non-invasive glucose monitoring. The sensors based on the former principle are now commercially available for detecting body fat in humans and sugar content of vegetables and fruits. This appears to be a very promising approach for glucose monitoring but a lot of experimental details still need to be worked out.

IMPLANTATION SITE

Although the monitoring in the blood stream would allow the direct measurement of glycemia, the risk of serious
infection and the poisoning of the sensor surface by direct blood contact make such a site unsuitable for long-term in vivo glucose monitoring. From a practical point of view, extravascular fluid is a more suitable environment for glucose sensing because it is free from the clot forming elements of blood. Two different approaches to sampling of interstitial fluid namely, the wick technique in dogs (Fischer et al., 1987) and microdialysis in humans (Janle-Swain et al., 1987) have shown that under steady-state conditions, subcutaneous glucose concentration reflects blood glucose concentration. Therefore, subcutaneous tissue can be a valid alternative for sensor implantation. The subcutaneous tissue is easily accessible, and as an injection site, it is well tolerated by most diabetic patients. The peritoneal cavity has also been proposed as a site for continuous glucose monitoring. However, Velho et al., (1989) have shown that the peritoneal cavity presents slower and more heterogeneous glucose kinetics than the subcutaneous tissue, making it a less desirable implantation site. Finally, if feasible, non-invasive monitoring would obviously be the method of choice. Some of the optical sensors designed for such monitoring are still in the developmental stage as mentioned earlier.
GOALS OF RESEARCH

1. The main goal of this research is to develop an implantable electrochemical glucose sensor based on the use of glucose oxidase. The efforts will be devoted to obtaining better control of the sensor fabrication process, miniaturization of the sensor, development of new enzyme immobilization methods, and studies of various support and protective membrane materials. The effect of various factors such as oxygen tension, pH and hydrodynamics of the test solution, and endogenous interferences on the sensor output will be determined in vitro. These results along with in vivo results will be used to distinguish between the physiological response and the characteristics of the sensor itself.

2. A flow injection analysis scheme will be explored as a means of selectively detecting glucose in biological fluids. This method will make use of pulsed amperometry as the mode of detection.
Chapter 2

DESIGN, FABRICATION AND IN VITRO EVALUATION OF AN IMPLANTABLE GLUCOSE SENSOR

INTRODUCTION

Development of a glucose oxidase based implantable glucose sensor for continuous monitoring of physiological glucose levels was first proposed more than 20 years ago (Updike et al., 1967; Albisser et al., 1974). Since then, efforts have been devoted to the miniaturization of the sensor, finding ways to efficiently immobilize (stabilize) glucose oxidase, and to development of necessary polymer membrane coatings to improve sensor linearity, selectivity and stability. However, little attention has been paid to whether the sensor design is compatible with implantation and whether the sensor fabrication techniques are compatible with the size and shape of the desired sensor.

It is generally agreed within the medical community that subcutaneous monitoring may be the mode of choice for an implantable glucose sensor. Several enzymatic glucose sensors (Fischer et al., 1987; Shichiri et al., 1982; Velho et al., 1988; Pickup et al., 1989; Clark et al., 1988; Matthews et al., 1988; Gough, 1986 and Ege, 1989) which have been utilized with some success in the past for in
vivo monitoring of glucose are compared in Table 2.1. As noted, most of the sensors are too large to be suitable for in vivo monitoring if frequent changes of the sensor are required. For such applications, a miniaturized sensor (less than <0.5 mm in diameter) is needed that can be conveniently implanted by the patient. The fabrication of such devices usually requires the deposition of several layers of polymer and enzyme directly on the sensor. The miniaturization of the sensor, therefore is not trivial. There exists also a need to achieve a better reproducibility of the sensor's final characteristics by a better control of the fabrication process.

With these things in mind, we have designed a needle-type hydrogen peroxide-based amperometric sensor equivalent in shape and size to a 26 gauge needle (0.45 mm o.d. overall, 0.25 mm o.d. for sensing portion). A reference electrode of high surface area is also included in the sensor. The cylindrical and symmetrical shape provides an optimal compromise between sensor size and the sensing area. The miniature needle shape is an ideal design for an implantable sensor and is believed to minimize inflammation (Woodward, 1982). Another critical property of the stated geometry (Figure 2.1) is that it permits the reproducible deposition of the several polymeric films necessary for the satisfactory performance of the sensor.

This chapter deals primarily with the in vitro
Table 2.1 Needle-type glucose sensors used in in vivo studies.
<table>
<thead>
<tr>
<th>Outer Dia. (mm)</th>
<th>Implantation Site</th>
<th>Determinant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3</td>
<td>sc tissue</td>
<td>dimethyl ferrocene</td>
<td>Pickup et al. (1989)</td>
</tr>
<tr>
<td>1.0</td>
<td>sc tissue</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Shichiri et al. (1982)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Velho et al. (1988)</td>
</tr>
<tr>
<td>2.5</td>
<td>sc tissue</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Fischer et al. (1987)</td>
</tr>
<tr>
<td>1.2</td>
<td>pc</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Clark et al. (1988)</td>
</tr>
<tr>
<td>0.5</td>
<td>sc tissue</td>
<td>dimethyl ferrocene</td>
<td>Matthews et al. (1988)</td>
</tr>
<tr>
<td>2.0</td>
<td>sc tissue, blood vessel</td>
<td>$\text{O}_2$</td>
<td>Gough et al. (1986)</td>
</tr>
<tr>
<td>0.35</td>
<td>sc tissue</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Ege et al. (1989)</td>
</tr>
</tbody>
</table>

sc  subcutaneous
pc  peritoneal cavity
evaluation of these sensors. The various factors which can effect the *in vivo* performance of the sensor such as effect of temperature and $\text{H}_2\text{O}_2$ on enzyme stability, $\text{O}_2$ and pH dependence of the response and selectivity toward possible interferents are better studied *in vitro*. A thorough understanding of in vitro response is very important for the correct interpretation of the *in vivo* results.
EXPERIMENTAL SECTION

Equipment

Amperometry was performed using a Bioanalytical Systems, Inc. (W. Lafayette, IN) Model LC4A amperometric detector. Current-time curves were recorded on a Kipp and Zonen Model BD 40 strip-chart recorder.

Materials

High purity glucose oxidase (EC 1.1.3.4) (242,000 units/g) was obtained from Biozyme Laboratories International Ltd. (San Diego, CA). Bovine serum albumin (Fraction V, 98-99% albumin), bovine IgG and bovine fibrinogen (Type 1-S) were all products of Sigma. Bovine serum was purchased from Hyclone Laboratories, Inc. (Logan, UT) and was diluted to 80% with phosphate buffered saline (PBS), pH 7.4 before using.

All buffer solutions were prepared with water from a Barnstead Nanopure II System. PBS, pH 7.4 was prepared from phosphate salts (0.1 M) and sodium chloride (0.15 M) with 0.02% sodium azide (w/v) as preservative. A glucose stock solution (0.5 M) was prepared in PBS and was allowed to mutarotate overnight at room temperature. It was subsequently stored at 4°C. L-ascorbic acid (Fisher) and L-cysteine (Sigma) solutions were prepared just before use, as they are subject to oxidative decomposition in solution.
Polyurethane (SG 85A) was obtained from Thermedics, Inc. (Woburn, MA). Cellulose acetate (39.8% acetyl content) and soluble Nafion (5% solution in lower aliphatic alcohols) were obtained from Aldrich. All solvents used for polymer solutions were of analytical grade. The glutaraldehyde solution used in immobilization process was purchased from Aldrich as a 25% aqueous solution.

Fabrication of the Sensor

Indicating Electrode

One end of a 10 cm long Medwire Corporation (Mount Vernon, NY) Teflon-coated platinum iridium (Pt-Ir) wire (0.25 mm o.d.) was stripped to form a cavity as shown in Figure 2.1. This was achieved by first putting a circular cut on the Teflon coating (35 μm thickness) with a paper cutter 4 mm from the tip and then pulling the Teflon out to create a cavity of about 1 mm length. The excess Teflon at the tip was trimmed off with the cutter and the tip was sealed off with silicone rubber glue (Dow Corning Corporation, Midland, MI).

Reference Electrode

The reference electrode was formed on the Teflon surface, 1.5 mm above the exposed Pt-Ir surface. A thin silver wire (0.1 mm o.d., 15 cm length) was tightly wrapped around the Teflon surface covering about 4 mm of its length. A wire wrapping tool (Gardner-
Figure 2.1: Schematic diagram of the sensor, (a) Teflon-coated Pt-Ir wire (0.25 mm o.d.), (b): Teflon tip, (c): Sensing cavity (1 mm length), (d): Ag/AgCl reference electrode, (e): Heat-Shrinkable tubing, (f): Reference electrode terminal, (g): Working electrode terminal.
Figure 2.1 Schematic diagram of the sensor.
Denver) was utilized for this purpose. The straight section above the wrapped silver wire was covered with 5 cm long, 1.5 mm o.d. heat shrinkable Teflon tubing (Zeuss Industrial Products, Inc., Raritan, NJ). Protruding lengths of silver wire and Pt-Ir wire were left uncovered for electrical contact. A heat gun (Fisher) operating at 600 °C was employed for shrinking the Teflon tubing. A layer of silver chloride was formed by passing current (0.4 mA/cm²) for 60 min through the wrapped silver wire while it was dipped in a stirred 0.1 N HCl solution. The reference electrode was rinsed with de-ionized water for 6 h. The Ag/AgCl reference electrodes prepared in this manner showed potentials of 64±3 mV (n=10) in 0.15 M NaCl at 37 °C vs. Ag/AgCl (3M NaCl).

Enzyme Immobilization

The exposed portion of the Pt-Ir wire acted as the starting point for the construction of the sensing element. The bare surface was first degreased by washing with acetone. It was then rinsed with deionized water and dried in a cold air stream before polymer deposition.

The lower part of the sensor (Figure 2.1-from arrow x to the distal end of sensor) was dipped into 5% cellulose acetate (CA) solution in 50% acetone - 50% ethanol for 10 seconds and was withdrawn slowly. It was then exposed to the vapor above the CA solution for 5 seconds and was
Figure 2.2: Expanded view of the sensing cavity,

(a): cavity holding a drop of enzyme solution,
(b): multilayer structure.
Figure 2.2 Expanded view of the sensing cavity.
dipped again into the CA solution for 10 seconds. The sensor was removed and dried in air at room temperature (23 °C) for one minute and then placed in deionized water for 6 h to permit displacement by water of entrapped solvent in the membrane pores.

A small volume (1 μL) of GOx solution (20 mg/mL in 0.1 M PBS) was held at the tip of a wire (0.5 mm o.d.) in the form of a droplet and was transferred to the sensing element (cavity) simply by moving it through the droplet (Figure 2.2). The enzyme solution was allowed to dry for 30 min at room temperature while holding the sensor horizontally. To immobilize the enzyme the sensor was either exposed to the glutaraldehyde vapor generated from 0.5 mL of 25% glutaraldehyde solution placed at the bottom of an enclosed 4 mL glass bottle for 40 min at room temperature or a small volume (1 μL) of 2% glutaraldehyde solution was transferred to the cavity and was allowed to react with the adsorbed enzyme for 1 h at room temperature. The sensor was rinsed thoroughly in deionized water and dried in air for 1 h. With some care the enzyme deposition can be restricted only to the cavity (Figure 2.2).

Nafion was used as an alternative to CA. After cleaning the sensing portion of the sensor as above, it was electrocoated with Nafion using the method described by Brazell et al. (1987). One drop of Nafion (5% solution) was
placed in a 2 mm loop formed at one end of a copper wire. A DC potential of +3V was applied to the working electrode with respect to the loop for 10 seconds. The sensor was pulled out of the loop before turning off the potential and was dried in air for 2 h. The sensor was rinsed with PBS and the enzyme immobilization was carried out in the same fashion as above.

**Outer Protective Layer**

In order to complete the preparation of the sensor, the whole sensor (Figure 2.1- from arrow y to the distal end of sensor) was dip coated with 5% polyurethane (PU) solution in 98% tetrahydrofuran (THF) - 2% dimethylformamide (DMF). The PU solution (10 µL) was held in a wire loop (2 mm i.d.) and the sensor was passed through the loop lengthwise with the sensing end entering first (Figure 2.3) This left a uniform polymer film on the sensor. The sensor was dried in air for 6 h at room temperature and then left in 0.1 M phosphate buffered saline, pH 7.4 for 72 h to permit outer membrane conditioning. It was possible to increase the thickness of the PU membrane if the desired linear range was not obtained after the first coating. This was accomplished by repeating the above process.

**In Vitro Calibration of the Sensor**

The sensor was dipped into a temperature and oxygen
Figure 2.3 Outer membrane coating technique.
Figure 2.3 Outer membrane coating technique.
tension-controllable cell containing 10 mL of stirred PBS, pH 7.4 (37 °C, air-saturated) and a potential of +600 mV (for hydrogen peroxide detection) was applied between the working and the reference/counter electrodes. The background current was allowed to stabilize for at least 30 minutes. The calibration of the sensor was carried out by adding increasing amounts of glucose (or an interferent) to the stirred buffer. The current was measured at the plateau (steady state response) and was related to the concentration of the analyte.

The sensors were usually stored in 0.1 M PBS, pH=7.4 at room temperature during the stability studies. However, it was also possible to store sensors dry at room temperature. The dry sensors usually recovered their original characteristics within 2 h after they were put back into the buffer.

**Ethylene Oxide (EO) Sterilization**

The sensors were sealed into gas sterilization pouches and were exposed to ethylene oxide gas for 12 h in a gas sterilizer (Millipore Corporation, Bedford, MA). The sensors were left in the pouches until they were needed. In all cases the sensors were allowed to degas for at least 48 h to get rid of the adsorbed EO before use. This procedure is described in detail elsewhere (Zhang et al., submitted).
RESULTS AND DISCUSSION

Sensor Preparation

The enzyme sensors used in this study were composed of a hydrogen peroxide detecting electrode, a CA membrane carrying the immobilized glucose oxidase and an outer PU membrane. The CA membranes were formed using a wet process. Dip coated membranes were allowed to partially dry in air after which they were submerged in water to permit displacement of residual solvent. The end product was a water swollen membrane. The porosity of these membranes was controlled by their water content which in turn was a function of the concentration of ethanol (nonsolvent pore former) in the casting-solution containing CA and acetone (Kesting et al., 1965; Chawla et al., 1975). The membranes prepared from a CA solution in 50% acetone - 50% ethanol mixture with a 1 min drying period prior to their immersion in water showed high permeability and good selectivity against small anions. The hydrogen peroxide to ascorbate response ratio ranged between 3 and 10 (relative response corresponding to 0.1 mM solutions of each), compared to the ratio of unity for a membrane showing no permselectivity. The permselective nature of CA membranes is well known and has been utilized previously to selectively remove interference from anions such as ascorbate and urate (Sittampalam et al., 1983). The glucose oxidase was
physically adsorbed on the CA membrane followed by insolubilization with glutaraldehyde. This is by far the most commonly employed method for immobilization of GOx because of its simplicity. The glutaraldehyde treatment not only helps to keep the enzyme at the electrode surface but also improves its operational stability as we shall see later in this Chapter. In the absence of glutaraldehyde treatment, the sensors showed a gradual loss of sensitivity as shown in Figure 2.4. As the enzyme was entrapped behind the PU membrane, this was not merely a result of enzyme loss from the electrode surface. The soluble enzyme could have lost its activity via loss of its flavin prosthetic group.

One of the most crucial parts of the sensor fabrication is the design of the semi-permeable outer membrane. The outer membrane, if properly selected, prevents degradation of the sensor in the biological environment and also decreases the glucose mass transfer relative to oxygen, leading to increased linearity and insensitivity to fluctuations in oxygen tension (Ikeda et al., 1980). This membrane also protects the enzymatic reaction from endogenous catalase which breaks down hydrogen peroxide and could effect the sensor output. Considering the many functions this membrane performs, it is reasonable to assume that a barrier with stable and
Figure 2.4 Storage stability curves for glucose sensors prepared without glutaraldehyde treatment.
Figure 2.4 Storage stability of the sensor.
reproducible transport properties is essential for proper functioning of the sensor. A large variety of membranes has been suggested for this purpose, including polyurethane (Velho et al., 1988; Shichiri et al., 1982), cellulose acetate (Clark et al., 1988) and Nafion (Harrison et al., 1988). There is no general agreement on the optimal membrane material. PU was selected for the present work because of its high permeability to oxygen and because it has been widely used for implantable devices. A 5% PU solution was selected to prepare these membranes as it provided the possibility of adding an additional layer if the desired characteristics were not obtained after the first deposit. If the initial deposition results in a membrane which is too thick, the sensor must be discarded. The use of a wire loop to deposit PU as described in the experimental section provided good control over the amount of polymer which was applied to the sensor. Because of the rapidity of this deposition procedure it was possible to put down a PU layer without significantly disturbing the underlying enzyme and CA membranes. To a certain extent it was possible to control the thickness of the membrane by varying the volume of the polymer solution in the loop or by varying the length of the sensing cavity. The amount of polymer delivered per unit sensing area was more for a shorter cavity resulting in thicker but more uniform membranes as monitored by sensor microphotographs (Figure
2.5) Cavity lengths ranging between 0.7 mm and 1 mm provided a good compromise between sensitivity and selectivity and also produced very uniform membranes. As a direct consequence of the new geometry and the new polymer deposition procedure, it was possible to prepare these sensors at a success rate which was better than 60%.

**Multilayer Permeabilities**

One of the biggest challenges for a hydrogen peroxide based glucose sensor is the elimination of interferences resulting from the presence of electrochemically active physiological compounds. Unfortunately detection of hydrogen peroxide requires a rather high applied potential. Any substance which is capable of being oxidized at +600 mV at a platinum surface may possibly act as an interferent. The list of such compounds normally found in blood serum includes ascorbic acid, uric acid and cysteine (Wilson et al., 1990). Acetaminophen has also been shown to give significant interferences at physiological levels (Moatti et al., 1990). The subcutaneous levels are not generally known and may differ from those in the blood.

One of the functions of the inner membrane covering the platinum anode is to improve electrochemical selectivity of the sensor by discriminating against such interferents. Since both ascorbic acid and uric acid are negatively charged at physiological pH, the presence of a
Figure 2.5 Microphotographs of the sensor.

A. Implanted portion of the sensor versus a human hair.

B. Expanded view of the sensing cavity.
Figure 2.5 Microphotographs of the sensor.
negatively charged membrane such as CA on the electrode surface is very effective in retarding such interferents. Moreover, the presence of the highly negative enzyme layer and the outer PU layer further help to improve the selectivity.

The response for various interferents is reported as the bias introduced by their presence on the response of a glucose solution (Table 2.2). The resulting error was the percentage change of the sensor output in response to 5.5 mM glucose, when the concentration of interfering compound was varied from zero to its physiological maximum. This represents the maximum possible error since variation from zero to the maximum level is not normally expected. As seen in Table 2.2, the multilayer structure of the sensor was very effective in removing most of the endogenous interferences. Errors due to acetaminophen were sizeable.

Nafion, in the form of a cation exchange membrane, is also effective in selectively excluding anions from the electrode surface (Nagy et al., 1985; Bindra et al., 1989). A few sensors were prepared with Nafion as the inner membrane. These sensors showed even better discrimination against anions. For example, the error resulting from the presence of ascorbic acid was between 1% and 2% as compared to 2% to 4% for cellulose acetate-based sensors (Table 2.2).

All attempts to use Nafion as the outer membrane so
Table 2.2 Sensor response for various interferents.
<table>
<thead>
<tr>
<th>Interferent</th>
<th>Physiological Conc. (^a) (mM)</th>
<th>Bias (^b) (mM)</th>
<th>%Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acorbic acid</td>
<td>0.01-0.11</td>
<td>0.18±0.06</td>
<td>3.3±1.1</td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.27-0.48</td>
<td>0.35±0.10</td>
<td>6.4±1.8</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>0.03-0.10</td>
<td>0.04±0.03</td>
<td>0.73±0.55</td>
</tr>
<tr>
<td>Urea</td>
<td>1.3-4.3</td>
<td>ND(^c)</td>
<td>0</td>
</tr>
<tr>
<td>Fructose</td>
<td>&lt;0.4</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>&lt;0.17</td>
<td>2.5±0.6</td>
<td>45±10</td>
</tr>
</tbody>
</table>

Results are shown as mean ± SD for 20 sensors.

\(^a\) Serum levels

\(^b\) Apparent increase in glucose concentration when interferent added at physiological maximum.

\(^c\) ND, not detected.
far have resulted in sensors that show a loss of sensitivity and noisy signals when tested in solutions containing proteins. This is probably the result of protein adsorption on Nafion film. Nafion, however, has been used previously as a protective outer membrane in devices used for glucose analysis in whole blood (Harrison et al., 1988). This discrepancy in results may be due to the difference in thickness of the Nafion film used in the two studies.

**In Vitro Characteristics of Sensor**

The *in vitro* characteristics of the sensor are given in Table 4.3. Besides being specific for glucose as discussed in the previous section, the sensor response must also be linear from 1 to at least 15 mmol/L (Harrison et al., 1985). One of the functions of the outer polyurethane membrane is to reduce the flux of glucose to the electrode surface and in turn extend the linear dynamic range. The cost of improved linearity is an increase in response time and a decrease in sensitivity of the sensor. The sensors with an upper limit of linear range between 15 and 20 mM showed sensitivities between 1.2 and 3.4 nA mM⁻¹ mm⁻². The response time is not very critical to the *in vivo* performance of the sensor if it is less than 5 min. The absolute background currents were low (0.4 - 1 nA). Thus the signal to background ratio at normal glucose levels (5.5 mM) was between 7 and 10.
Table 2.3 *In vitro* characteristics of a needle-type glucose sensor.
Table 2.3 *In Vitro* Characteristics\(^a\) of a Needle-type Glucose Sensor

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background current ((\text{nA/\text{mm}^2})^b)</td>
<td>1.3(\pm)0.5</td>
</tr>
<tr>
<td>Normalized Sensitivity ((\text{nA/\text{mM/mm}^2}))</td>
<td>2.3(\pm)1.1</td>
</tr>
<tr>
<td>Response time ((T_{90%})) ((\text{min}))</td>
<td>3.5(\pm)1.0</td>
</tr>
</tbody>
</table>

Results are shown as mean \(\pm\) SD for 20 sensors.

\(^a\) Reported for the sensors with upper limit of linear range \(\geq 15\text{mM}\).

\(^b\) Background currents were recorded after one hour of polarization.
**Stability of Reference Electrode**

The stability of the reference electrode is usually overlooked during the in vitro evaluation of a sensor. For a two electrode amperometric system, a variation in reference potential could cause a change in sensor output depending, of course, on the extent of the variation. There is a possibility that the potential of the reference electrode could change due to flow of current through it.

The potentials of the Ag/AgCl electrodes were monitored during the operation of the sensor vs. an external standard reference electrode. The variation in potential under normal operating conditions (+600 mV, 37°C, 5.5 mM glucose) was found to be 3±2 mV (n=4) over a 12 h period. Considering the fact that the sensor output was almost independent of the applied potential over a wide potential range (Table 2.4), it was reasonable to assume that such a small variation in the reference potential would not affect the sensor response. As a matter of fact, the drift in sensor output, expressed as a percentage change in the sensor output to 5.5 mM glucose was found to be only 5±2% (n=4) over a 24 h period. The potential of the reference electrode was also found to be independent of output current in the 0-15 mM range.

**Operational and Storage Stability**

The storage stability of these sensors was excellent. The polyurethane coated sensors showed an increase in
Table 2.4 Effect of applied potential on sensor response and background current.
<table>
<thead>
<tr>
<th>Applied Pot. (vs. Ag/AgCl, 0.15 M Cl^-) (V)</th>
<th>Sensitivity to glucose (nA/mM)</th>
<th>Background current (nA)(^a)</th>
<th>Response to H(_2)O(_2) (nA/0.1mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.32</td>
<td>0.52</td>
<td>-0.1</td>
<td>2.5</td>
</tr>
<tr>
<td>0.45</td>
<td>0.59</td>
<td>0.2</td>
<td>3.9</td>
</tr>
<tr>
<td>0.50</td>
<td>0.65</td>
<td>0.2</td>
<td>4.0</td>
</tr>
<tr>
<td>0.60</td>
<td>0.65</td>
<td>0.4</td>
<td>4.0</td>
</tr>
<tr>
<td>0.72</td>
<td>0.69</td>
<td>1.5</td>
<td>3.8</td>
</tr>
</tbody>
</table>

\(^a\) Background currents were recorded after 30 min. of polarization.
response (and a corresponding decrease in linearity) for the first few days following preparation before reaching a stable value. This resulted from the swelling of PU membranes as the residual solvents were replaced with water during the conditioning process increasing their permeability to glucose (Figure 2.6). The stabilization period was usually between 3 and 7 days. The buffer treatment is also an important step in removing the leachable toxic substances such as residual organic solvents and excess glutaraldehyde from a freshly prepared sensor thus making the sensor non-toxic (Zhang et al., 1990).

In order to check the operational stability of the sensor, the sensor calibration was run daily over a seven day period. Between each calibration, the sensor was stored in a fresh solution containing 5.5 mmol/L glucose at 37°C. Owing to the storage procedure employed, the enzyme was turning over continuously during the seven day test. As no potential was applied during the test period, hydrogen peroxide was also allowed to accumulate in the storage buffer. The observed stability of the sensors (Table 2.5) under these conditions casts considerable doubt on the claims in the literature that hydrogen peroxide will deactivate glucose oxidase used in an enzyme sensor (Tse et al., 1985). Both the linearity of the sensor as well as its response to hydrogen peroxide were maintained (Table 2.5).
Figure 2.6 Storage stability curves for two different sensors. The sensors were stored in 0.1M PBS, pH 7.4 at room temperature between each calibration.
Figure 2.6 Storage stability of the sensor.
Table 2.5 Operational stability of sensors.
Table 2.5 Operational Stability of Sensors\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Day1</th>
<th>Day2</th>
<th>Day3</th>
<th>Day4</th>
<th>Day5</th>
<th>Day6</th>
<th>Day7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity to Glucose\textsuperscript{c} (nA/mM/mm\textsuperscript{2})</td>
<td>4.1</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.3</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Sensitivity to H\textsubscript{2}O\textsubscript{2}\textsuperscript{c} (nA/mM/mm\textsuperscript{2})</td>
<td>91</td>
<td>91</td>
<td>91</td>
<td>106</td>
<td>100</td>
<td>91</td>
<td>83</td>
</tr>
<tr>
<td>Linear range, upper limit (mM)</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Sensor stored continuously in 0.1 M PBS, pH 7.4 containing 5.5 mmol/L glucose at 37\textdegree C.
These results also suggest absence of thermal inactivation of the enzyme at 37°C. It has previously been shown that immobilization with glutaraldehyde increases the maximum stable temperature 8°C over that of free enzyme (Sasso et al., 1987).

**Effect of Temperature and pH**

The temperature coefficient was measured by changing the temperature of the test solution from 30°C to 42°C. The output current for 5.5 mM glucose increased by 2.5±0.6% (n=3) per 1°C increase in temperature. This suggests that the response of the sensor is defined by membrane diffusion and not enzyme kinetics as expected. A small variation in body temperature, therefore, during in vivo measurement is not expected to cause a big error in the measurement. The output of the sensor was also found to be stable in the pH range 7.2-7.8. In a separate study it was also shown that the pH at the implantation site (subcutaneous tissue of a rat) varied by only ±0.1 pH units (n=3) over a period of 5 h (Figure 2.7). A commercially available needle-type pH electrode (0.5 mm o.d.) (World Precision Instruments, Inc., New Haven, CT) was used for this purpose. The pH electrode was calibrated before and after the in vivo pH measurement in 0.1 M phosphate buffer, pH 7.0 and in 0.1 M phosphate buffer, pH 7.4 each containing 106 meq/L NaCl. A Ag/AgCl wire (0.5 mm o.d.) was used as a reference electrode for
Figure 2.7 In vivo pH measurement in subcutaneous tissue of a rat.
Figure 2.7  In vivo pH measurement.
both in vitro and in vivo measurements.

**Effect of Hydrodynamics and Oxygen Tension**

The effect of stirring on sensor output was checked by turning a magnetic stirrer on and off during the sensor calibration. The sensor response was found to be essentially independent of the stirring rate thus confirming the fact that diffusion of glucose through the PU membrane is the rate limiting step rather than the enzymatic reaction. This characteristic of the sensor makes it immune to the external depletion layer effects associated with making measurements in unstirred solutions.

A commonly expressed concern for a glucose oxidase-sensor is that changing $P_{O_2}$ levels in the subcutaneous tissue may effect the sensor output. The effect of oxygen tension on the sensor output was checked by equilibrating the test buffer with various air/nitrogen gas-mixtures. The oxygen partial pressures were adjusted between 8 and 160 mm Hg and were monitored by an oxygen electrode (Appendix 1).

For a linear sensor (> 15mM), the glucose response was found to be essentially independent of the oxygen partial pressure of the test solution in the above-mentioned range. Even at the 15 mM glucose level the difference in sensor output for the two extreme $P_{O_2}$ levels was $3\pm2\%$ (n=4). A non-linear sensor, on the other hand,
showed a strong dependence on oxygen tension (Figure 2.8). The advantage of using PU as the outer membrane because of its high oxygen permeability is clearly demonstrated. Our preliminary estimate of the subcutaneous pO$_2$ levels at the sensor sites in a rat is in the range of 8-18 mm Hg (Appendix 1). In a recent study (Fischer et al., 1989), the subcutaneous pO$_2$ levels in dogs are reported as 54±18 mm Hg (n=5). There is thus still some question as to what the exact subcutaneous oxygen values are. Nevertheless a carefully designed sensor has been demonstrated to be virtually insensitive to changes in oxygen tension as low as 8 mm Hg. The above-mentioned results, however, do not predict exactly how the sensor will respond to such low oxygen levels during an in vivo experiment.

**Sensor Testing in Serum**

Another important consideration for implantable sensors designed for long-term applications is the sensor stability in the presence of biological fluids.

The sensors were tested in the presence of three major plasma proteins namely albumin, gamma globulin and fibrinogen added to the test buffer at their physiological concentrations. Although the concentration of plasma proteins in interstitial (subcutaneous) fluid is supposed to be low, a higher concentration at the implantation site is possible as a result of tissue/cell damage or as a result of inflammatory processes. None of the proteins
Figure 2.8 Effect of oxygen tension on sensor output for a non-linear glucose sensor, (a): 160 mm Hg (●), (b): 40 mm Hg (+), (c): 8 mm Hg (◼).
Figure 2.8 Effect of oxygen-tension on sensor output.
showed any effect on the sensor output, even after an extended exposure of the sensor to these proteins for several hours. Though these studies with single proteins are not necessarily representative of the events that might occur in the dynamic physiological environment, we can still conclude that the outer membrane was effective in excluding protein from the electrode surface.

The sensors, however, retained only 70-75% of their sensitivities when they were tested in serum (bovine). The sensitivity values were stable even after several days of continuous contact with serum under sterile conditions. Also, the loss in sensitivity was reversible as the sensors recovered their original sensitivities when they were pulled out of the serum and washed with PBS. This rather large decrease in sensitivity could not be explained only on the basis of increased viscosity of the medium because sensor output was unaffected by the increase in viscosity when a large amount of albumin (6 g/dL) was added to the buffer. There are obviously components in the serum which alter the permeability of the outer membrane. It was quite possible that this adsorption process was initiated by some small compound present in serum, as there was no inhibition from single protein solutions. A recent study, in which an electrochemical sensor was exposed to various fractions of serum as obtained by ultrafiltration concludes
that the removal of large proteins from serum is not enough to completely prevent the poisoning of the sensor (Elbicki et al., 1989). These results indicate that a decrease in sensitivity can be expected upon implantation. This underlines the need for in vivo calibration of the sensor.

**Effect of Sterilization**

The sensors with stable characteristics were tested before and after EO sterilization in order to see if sterilization affected the sensor performance. The sensors showed no change in sensitivity, response time or linearity upon EO treatment (Table 2.6). There was no evidence also of any kind of residual EO or any bacterial contamination in sterilized sensors as confirmed by cell culture toxicity tests (Zhang et al., 1990).

In conclusion the performance characteristics of the sensor clearly meet the requirements of an implantable glucose sensor. In addition to the geometry it is believed that the sensor fabrication techniques also contribute significantly to its sensitivity and stability. A special technique involving using wire loop makes possible the application of the same amount of polymer to the sensor each time in what appears to be a film of uniform thickness. Fabrication success rates of greater than 60%
Table 2.6 Effect of ethylene oxide sterilization.
Table 2.6 Effect of Ethylene Oxide Sterilization

<table>
<thead>
<tr>
<th></th>
<th>Sensor #1</th>
<th></th>
<th>Sensor #2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Sensitivity to Glucose (nA/mM/mm²)</td>
<td>1.9</td>
<td>2.0</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Sensitivity to H₂O₂ (nA/mM/mm²)</td>
<td>44</td>
<td>47</td>
<td>56</td>
<td>50</td>
</tr>
</tbody>
</table>
are easily obtained. The \textit{in vivo} studies with this novel sensor are fully justified.
Chapter 3

**IN VIVO EVALUATION OF SENSORS**

**INTRODUCTION**

*In vivo* measurements are extremely important to the development of a viable sensor. Such measurements will not only tell if the sensor actually works while implanted but will also provide insight into such physiological phenomena as sensor encapsulation as a result of tissue reactions and depletion of oxygen and glucose at the implantation site.

Although blood glucose level is the parameter of clinical interest, the sensor monitors the subcutaneous glucose levels for various practical reasons described previously in the introduction (Chapter 1). For a subcutaneous sensor to be a part of a closed loop insulin delivery system, it is essential to establish a relationship between sensor output and the blood glycemia. Until recently, this was not a trivial exercise because a well defined relationship between subcutaneous glucose concentration and the blood glucose concentration was non-existent. But as a consequence some recent findings (Fischer et al., 1987; Janle-Swain et al., 1987) which concluded that under steady-state conditions, subcutaneous glucose levels and the blood glycemia were identical, it became possible to use steady state blood glucose levels to
calibrate a subcutaneously implanted sensor. On this basis, a two-point in vivo calibration procedure was described by Velho et al. (1988). This method has allowed the determination of in vivo characteristics of the sensor such as an in vivo sensitivity coefficient and an in vivo background current.

The aim of the present work is to study the in vivo characteristics of the new sensor described in Chapter 2 using the calibration protocols established previously. Both short-term and long-term implantation experiments are described.
EXPERIMENTAL SECTION

Short-term Implantation Protocol (Velho et al., 1988)

Overnight-fasted male Wistar rats (250-300 g body weight) were anesthetized with chloral hydrate (400 mg/kg, injected intraperitoneally) and polyethylene (Biotrol 3, Biotrol, France) and silicone (Silastic 602-135, Dow Corning, Midland, MI) catheters were inserted respectively into the left jugular vein and homolateral carotid artery, for glucose or insulin injection and for blood sampling. A sensor was implanted into the subcutaneous tissue at the back of the neck through the lumen of a 16 gauge catheter, which was subsequently pulled out, leaving the sensor in place. Animals were kept spontaneously breathing under a flow of carbogen (O₂:CO₂, 95:5) and warmed under a lamp.

After stabilization of the signal, glucose was infused intravenously to produce an increase in plasma glucose level. Blood samples were serially drawn at 5 min intervals. Samples were immediately centrifuged and plasma glucose concentration was determined with a glucose analyzer (Beckman Instruments, Fullerton, CA). The sensor output was recorded and transformed into a profile of subcutaneous glucose concentration according to a two-point *in vivo* calibration.
**In Vivo Calibration**

Using the pre-glucose perfusion and post-glucose perfusion steady states of the blood glucose and the sensor output, an *in vivo* sensitivity coefficient (S.C.) was calculated as the ratio between the change in sensor output and the change in glucose concentration (Figure 3.1). In the absence of infusion the blood level would rise momentarily in response to a glucose bolus and then begin a slow return to the basal level. The *in vivo* background current (I₀) was calculated by interpolation as shown in Figure 3.2. The apparent subcutaneous glucose concentration was obtained by subtracting from the sensor current (I) at any given time, the *in vivo* background current (I₀) and then dividing the difference by the sensitivity coefficient (S.C.).

**Long-term Implantation Protocol**

The rat was lightly anesthetized with halothane. The sensor was implanted subcutaneously through the lumen of a 16 gauge catheter as described in the previous section. In order to calibrate the sensor, the blood glucose was varied by administering either one of the following injections:

1. Glucose (30% by wt.) injected IP (1 gm/kg)
2. Insulin (1 U), injected subcutaneously.

The blood sampling was done at the tail vein with a heparinized pipette. The blood analysis was done either with a Beckman Analyzer or using an Accu-Chek IIIm Blood
Figure 3.1 Determination of in vivo sensitivity coefficient.

In vivo sensitivity coefficient (S.C.) = \( \Delta I / \Delta C \).
**Figure 3.1** Determination of *in vivo* sensitivity coefficient.
Figure 3.2  Two-point *in vivo* calibration curve.

*In vivo* background current = $I_o$

Apparent subcutaneous glucose concentration = $(I-I_o)/S.C.$
Figure 3.2  Two-point *in vivo* calibration curve.
Glucose Monitor (Boehringer Mannheim, Indianapolis, IN).

In between the calibrations, the sensor was disconnected from the amperometric unit and the rat was left freely moving in his cage with the sensor still implanted. This procedure was non-traumatic for the animal as it did not require any surgery. It was possible to test the sensor over a period of several days.
RESULTS AND DISCUSSION

**In Vivo Calibration**

A normal anesthetized rat was infused with glucose (20 mg/kg/min), in order to increase its plasma glucose concentration from 5.2 to a constant of 11.8 mmol/L (Figure 3.3). The output current from a sensor implanted in the subcutaneous tissue increased form 6.6 to 8.7 nA (Figure 3.4). From these values it was possible to calculate an in vivo sensitivity coefficient (S.C.) and an extrapolated in vivo background current (I₀), which were 0.32 nA/mM and 4.9 nA, respectively. By using these values it was possible to transform the sensor output at any given time into an apparent subcutaneous glucose concentration. Figure 3.4 shows that the variations of this "apparent subcutaneous glucose concentration" detected by the sensor closely follow those of blood glucose with a 5 min time lag. Under the steady-state conditions (subcutaneous and blood levels equilibrate) both values are identical. Finally, Figure 3.5 shows the in vitro calibration curves obtained before implantation, immediately after explantation and 12 h after explantation, indicating essentially no change in the characteristics of the sensor. These results clearly indicate that the sensor indeed is implantable. The end of the sensor is capped with Teflon which prevents it from penetrating into the tissue farther than is required. The insertion of the sensor causes minimal trauma to the tissue.
Figure 3.3 Change in (A) plasma glucose concentration (m) and (B) sensor output (■) during glucose infusion (20 mg/kg/min).
Figure 3.3 Change in glycemia and sensor output during glucose infusion.
Figure 3.4 Change in plasma glucose concentration (□) and apparent subcutaneous glucose concentration (*) during continuous glucose infusion.
Figure 3.4 Correlation between subcutaneous glucose and blood glycemia.
Figure 3.5 In vitro calibration curves, (a) before implantation ($\circ$), (b) immediately after explantation ($\star$), (c) 12 hours after explantation (■).
Figure 3.5 In vitro calibration curves.
and to the sensor itself.

Another interesting observation is the large difference between \textit{in vitro} and \textit{in vivo} sensitivity coefficients. The \textit{in vivo} sensitivities are usually between 20 and 40\% of the corresponding \textit{in vitro} values. Based on the results of sensor testing in pure serum (Chapter 2), these huge losses upon implantation cannot be solely attributed to the protein adsorption on the sensor surface. The factors such as tissue $O_2$ supply, local perfusion and implant orientation should also be considered as the possible reasons for this behavior.

\textbf{Long-term Implantation}

The long-term implantation study has been carried out with 4 sensors so far for periods ranging between 5 and 21 days. In all cases, the sensor was able to detect glucose for the entire implantation period. The correlation between subcutaneous glucose concentration and blood glycemia for 4 different sensors over a 5 day implantation period was excellent ($y = 0.52 + 0.91x$, $r = 0.96$). These results indicate that the sensor can be used for long term determination of blood glucose concentration.
Chapter 4

PT-BLACK BASED GLUCOSE SENSORS

INTRODUCTION

Recently, a combination of platinization and enzyme attachment has been used on platinum (Ikariyama et al., 1987, 1989), reticulated vitreous carbon (Sasso et al., 1990) and glassy carbon (Gunasingham and Tan, 1989) for fabrication of enzymatic glucose electrodes. The electrodes prepared in this fashion are stable and show very high sensitivity and fast response time toward glucose. The advantage of these enzyme electrodes has been realized so far only in flow injection analysis for development of a rapid and selective method for determination of glucose in blood and serum. The desired selectivity for the analysis is obtained either by using a differential system (Ikariyama et al., 1989) in which difference between an enzymatic and a non-enzymatic electrode is measured or by using permselective electrode coatings such as 1, 2-diaminobenzene (Sasso et al., 1990) or Nafion (Gunasingham, 1990). However, it still remains to be seen if these arrangements can also be applied to measurements in the batch mode when the selectivity requirements are considerably stricter.

The purpose of this work is to explore the possibility
of using Pt-black surfaces for the construction of glucose microelectrodes suitable for in vivo glucose monitoring. This technique is quite attractive because it offers great flexibility in the choice of electrode shape and size thus allowing easy miniaturization. The ease with which the enzyme can be immobilized and restricted only to the sensing portion are also very useful features. There are certain problems, however, inherent to the use of Pt-black surfaces which include large background currents, instability of the electrode surface, the complications arising due to direct electrochemical oxidation of glucose and the poor selectivity. All these issues must be addressed before a decision is made regarding the usefulness of Pt-black based glucose sensors.
EXPERIMENTAL SECTION

Materials  Hydrogen hexachloroplatinate (IV) hydrate and lead acetate were obtained from Aldrich. The other materials used in the study were described previously (Chapter 2).

Instrumentation  The platinum was electrodeposited with an ECO Model 551 potentiostat/galvanostat (ECO Instruments, Inc., Cambridge, MA) or a Hewlett Packard DC power supply Model 6204 B (Hewlett Packard, Berkeley Heights, NJ). All absorbance measurements were carried out on a Varian Cary 219 Scanning UV/VIS Spectophotometer (Varian Associates, Palo Alto, CA). The other instrumentation was described previously (Chapter 2).

Sensor Fabrication

The mechanical fabrication of the sensor was the same as described in Chapter 2.

Preparation of Pt-black

1. Galvanostatic Preparation  The platinization was carried out in a solution containing 3.3% (w/v) hexachloroplatinate hydrate and 0.03% lead acetate. Lead acetate acted as an inhibitor of grain growth and its concentration in the plating solution was crucial (lead acetate concentrations greater than 0.03% gave platinized surfaces which were greyish in color). The working electrode was subjected to a total of six alternate anodic and cathodic galvanostatic
pulses, each 1 min at a current density of about 100 mA/cm². The electrode was then thoroughly washed with distilled water.

2. Potentiostatic Preparation This method in which the Pt film was deposited from an ultrasonically-stirred solution followed the procedure of Marrese (1988). The Pt-black coatings prepared by this method were strongly adherent.

The plating apparatus is shown in Figure 4.1. The plating solution was same as above. The electrode to be plated was immersed in the solution and the ultrasonic cleaner (Fisher) was turned on. A potential of -2V was then applied between the working and counter electrodes for exactly 2 min. The power was then turned off followed by the ultrasonic bath. The electrode was rinsed thoroughly with distilled water.

Adsorption of Enzyme

The working electrode was immersed for 3 h in 1 mL of 0.1M phosphate buffer (PB), pH 7.4 containing 30 mg of glucose oxidase. This time was established by running a loading curve using radiolabeled enzyme. In most cases the enzyme incorporation was 90% complete within 3 h (Figure 4.2). The electrode was then dipped into bovine serum albumin (BSA) solution (20mg/mL) for 30 min. In order to form a film of BSA, the sensor was exposed to glutaraldehyde vapor for 40 min in the same fashion as
Figure 4.1  Schematic diagram of the ultrasonic plating apparatus;

A: ultrasonicator
B: plating solution
C: working electrode
D: counter electrode.
Figure 4.1 Schematic diagram of the ultrasonic plating apparatus.
Figure 4.2 Enzyme loading curve for a Pt-black electrode.
Figure 4.2 Enzyme loading curve.
described in Chapter 2. The enzyme incorporated electrode was then washed in freely stirred PB, pH 7.4 for 20 h.

The non-enzymatic electrode was prepared by dipping the working electrode in BSA for 3 h and then exposing it to the glutaraldehyde vapor for 40 min. The electrode was then washed with PB as above.

External Outer Layer

The polymer solutions used for dip coating the sensor were Nafion (5 wt% in lower aliphatic alcohols), Polyurethane (4 wt% in 98% tetrahydrofuran and 2% dimethylformamide) and cellulose acetate (6 wt% in 50% acetone and 50% ethanol). The dip coating procedure for each polymer was the same as described previously in Chapter 2.

Radiolabeled Glucose Oxidase Preparation

The Iodine monochloride method described by Helmkamp, Contreras and Bale (1967) was chosen to incorporate $^{125}$I into GOx. A 0.5 mM ICl reagent was prepared by adding 4 μL of pure ICl to 100 mL of 2 M NaCl. In a 1 mL conical vial was placed 80 μL of 0.5 mM ICl and the desired amount of Na$^{125}$I solution. Typically, 1.0 to 2.5 μCi of $^{125}$I was used to iodinate 1.0 mg of protein, depending upon the specific activity needed. The desired amount of glucose oxidase in a volume of 0.20 mL was added to the vessel with the room lights turned off. The contents of the vessel
were mixed by tapping and allowed to react for 2 min in the dark. The reaction mixture was applied to the top of a G-25 Sephadex column (25 x 1 cm) which had been equilibrated with PBS, pH 7.4. Elution was continued with PBS at a flow rate of 0.5 mL/min and 1 mL fractions were collected. The effluent was monitored with a LKB Wallac 80000 Automated Gamma counter and the first fraction, containing labeled protein, was collected. The pooled protein was concentrated to about 1.5 mL using pressure dialysis. The ratio of protein-bound iodide to total iodide was determined by the trichloracetic acid (TCA) precipitation method (Der-Balian, 1980). The value, expressed as efficiency of labeling, was usually 95-98%. The same data were used to calculate the specific activity of the iodinated glucose oxidase in mCi of $^{125}$I attached per mg of glucose oxidase. The spectrophotometric determination of enzymatic activity of radiolabeled glucose oxidase solution showed retention of 80-85% of the original activity (Sternberg et al., 1988).
RESULTS AND DISCUSSION

Scanning Electron-microscope Examination

Observation of Pt-black surfaces with the electron-microscope showed porous microparticles of platinum. As seen in Figure 4.3, the deposition of Pt particles was more uniform when the platinizing solution was ultrasonically stirred. The surface features were on the order of 1 µm.

Surface Area Measurements - Hydrogen Adsorption from Solution

The current-potential curve for a platinum electrode in aqueous solution shows peaks for the formation and oxidation of both adsorbed hydrogen and adsorbed oxygen. Measurements of the areas under these peaks for adsorbed hydrogen and oxygen, assuming they represent monolayer coverage, can be used as a means of determining the area of the electrode (Woods, 1974, Barna et al., 1982).

Figure 4.4 illustrates the characteristic surface voltammetry of platinum in 1M H₂SO₄ for an ultrasonically platinized electrode. Peaks Hc show formation of adsorbed hydrogen and Ha, oxidation of adsorbed hydrogen.

The surface areas were derived from

\[ A = \frac{Q_H}{Q_H^O} \]

where,

\[ Q_H^O = \text{charge associated with one-to-one H-M (hydrogen-metal) correspondence per unit area.} \]

For polycrystalline
Figure 4.3 Scanning electron micrographs of Pt-black surface:
A. prepared with ultrasonic stirring on.
B. prepared with ultrasonic stirring off.
Figure 4.4  Cyclic voltammetric current-potential curve for a Pt-black electrode in 1 M H₂SO₄.
Figure 4.4 Cyclic voltammogram for a Pt-black electrode in 1M $\text{H}_2\text{SO}_4$. 
Pt the accepted value is 210 \( \mu \text{C/cm}^2 \).

\( Q_H \) = charge under the voltammetric peak for hydrogen adsorption (\( H_c \)).

The roughness factors (actual area/geometric area) for the electrodes prepared by Method 1 were very high (300-600) but they fell off rather quickly upon continued washing. The roughness factors for the electrodes prepared by Method 2 were only in the range of 50-75, but these values were stable over a long period of time (several days) indicating that the Pt-black coating prepared by Method 2 was strongly adherent. Only the particles that could survive the ultrasonic agitation became part of the electrode. On basis of these results Method 2 was selected for further work.

**Enzyme Immobilization**

The efficiency of enzyme immobilization was checked by monitoring the loss of radiolabeled glucose oxidase from the electrode surface during the washing period. The enzyme loss was estimated at 12±4\% (n=4) for 20 h washing period for the cases when enzyme was not cross-linked with glutaraldehyde (Figure 4.5). This indicates a strong adherence of glucose oxidase to the electrode surface. The loss was minimal when the enzyme was crosslinked.

Table 4.1 shows the amount of total enzyme immobilized on the electrode surface, sensitivity and saturation
Figure 4.5 Enzyme retention versus washing time for 4 different Pt-black sensors.
Figure 4.5 Enzyme retention versus washing time.
Table 4.1 Correlation between amount of enzyme immobilized and sensor output.
Table 4.1 Correlation between Amount of Enzyme Immobilized and Sensor output

<table>
<thead>
<tr>
<th>Amount of Enzyme (µg)</th>
<th>Sensitivity (normalized) (arbitrary units)</th>
<th>Sat. Current (normalized) (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.95</td>
<td>2.4</td>
<td>4.5</td>
</tr>
<tr>
<td>2.2</td>
<td>3.1</td>
<td>15</td>
</tr>
<tr>
<td>0.90</td>
<td>2.4</td>
<td>2.3</td>
</tr>
<tr>
<td>2.2</td>
<td>10.7</td>
<td>13</td>
</tr>
<tr>
<td>1.4</td>
<td>14.4</td>
<td>10</td>
</tr>
</tbody>
</table>
current for five different sensors. The sensitivity and the saturation current values were normalized to that of hydrogen peroxide response in order to account for the difference in surface area. As seen in Table 4.1, there was not always a direct correlation between amount of enzyme immobilized and the sensitivity or the saturation current of the sensor. This is probably due to the fact that not all the immobilized enzyme is active or accessible to the glucose. At any rate, all of the sensors produced current response large enough to be useful.

**Effect of Applied Potential**

In order to select the optimum working potential, the effect of applied potential on background current and the sensitivity of the sensor was monitored. As seen in Table 4.2, it was possible to use potentials as low as 0.35 V vs. Ag/AgCl (3M NaCl) without losing much sensitivity. The advantages of working at lower potential were the lower background currents and the shorter background stabilization periods. A working potential of +0.35V was selected for the work.

**Direct Oxidation of Glucose vs. Enzymatic Oxidation**

An untreated Pt-black electrode initially shows a large current resulting from direct oxidation of glucose when it is exposed to glucose for the first time. This current, however, decays off rather quickly to reach a
Table 4.2 Effect of applied potential on sensitivity and background current.
Table 4.2 Effect of Applied Potential on Sensitivity and Background Current

<table>
<thead>
<tr>
<th>Applied Pot. (V)</th>
<th>Sensitivity to Glucose (nA/mM)</th>
<th>Background current (nA)(^a)</th>
<th>Sensitivity to H(_2)O(_2) (nA/25\uM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.35</td>
<td>75</td>
<td>6</td>
<td>900</td>
</tr>
<tr>
<td>0.40</td>
<td>75</td>
<td>10</td>
<td>900</td>
</tr>
<tr>
<td>0.50</td>
<td>84</td>
<td>16</td>
<td>950</td>
</tr>
<tr>
<td>0.65</td>
<td>86</td>
<td>50</td>
<td>925</td>
</tr>
</tbody>
</table>

\(^a\) Background currents were measured after 1 h of polarization.
much lower value due to electrode fouling by oxidation products. Chloride is also known to have a significant inhibitory effect on glucose oxidation kinetics (Marincic et al., 1979). It was interesting therefore, to find out what effect the enzyme immobilization had on such response.

For a sensor that had been completely saturated with glucose oxidase, response to glucose in an argon saturated solution was only 4% of the response obtained for the same sensor in an air-saturated solution. Therefore the contribution of direct oxidation to the observed signal could at the most be only 4%. The glucose response for a non-enzymatic sensor was unaffected by the argon bubbling, as expected. The sensor response to fructose was also used as an indicator for direct oxidation. The response ratio of glucose and fructose was between 1.8 and 2.0 for an non-enzymatic electrode. The glucose oxidase-based sensor, on the other hand, showed ratios as high as 80, indicating that for such a sensor selective enzymatic oxidation was the predominant reaction.

Cellulose Dialysis Membrane

In order to obtain sensor characteristics suitable for in vivo monitoring, it was necessary to use a perm-selective electrode coating. Several materials such as Nafion, PU and CA were tried, but CA proved most satisfactory. The sensors were prepared by dip-coating a
glucose oxidase and BSA coated electrode in a polymer solution, as described in the Experimental Section. When the sensors were dip-coated in 5% Nafion solution, the enzyme was almost completely denatured due to high proton activity of the Nafion solution (pH=2.0). The resulting sensors showed a dramatically large decrease in sensitivity and their response became mostly non-enzymatic. The direct deposition of PU also resulted in significant loss of enzyme activity probably because of the exposure to THF and DMF. The sensors again showed response which was mostly non-enzymatic. These results indicate that only a limited amount of enzyme is immobilized on Pt-black electrodes unlike the case of Pt based sensors (Chapter 2) where the enzyme was immobilized in excess. In this case it was possible for the sensor to lose part of its enzyme activity without losing a great deal of sensitivity.

Despite this, it was possible to retain some enzyme activity by coating the electrode with CA. The in vitro characteristics of such a sensor are given in Table 4.3. Sensor clearly has the sensitivity, linearity, response time and selectivity required for an implantable glucose sensor. The CA membrane was successful in discriminating against ascorbate. The resulting errors due to presence of ascorbate, however, were higher than those observed for Pt based electrodes. This indicates that the increase in glucose response due to increase in electrode area for Pt-
Table 4.3 *In vitro* characteristics of an enzymatic electrode.
Table 4.3 *In Vitro* Characteristics of an Enzymatic Electrode

<table>
<thead>
<tr>
<th></th>
<th>Bare electrode</th>
<th>CA-coated electrode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (nA/mM)</td>
<td>40</td>
<td>4.5</td>
</tr>
<tr>
<td>Linear range, upper limit (mM)</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>Response time (min)</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Drift (%/24 h)</td>
<td>-</td>
<td>±4</td>
</tr>
<tr>
<td>H₂O₂/Ascorbate</td>
<td>1.2</td>
<td>16</td>
</tr>
<tr>
<td>Error (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Error resulting from the presence of 0.1 mM ascorbate on sensor output for 5.5 mM glucose.
black sensors is not high enough to compensate for the simultaneous increase in the ascorbate response. The storage stability of the sensor was excellent (Figure 4.5). For the sake of comparison, the in vitro characteristics of a non-enzymatic sensor are given in Table 4.4. As noted, the selectivity remains extremely poor even after the sensor is coated with CA. Another interesting observation is that decrease in sensitivity upon CA deposition was much larger for an enzymatic sensor (Table 4.3) as compared to a non-enzymatic sensor (Table 4.4). This clearly indicates that the loss in sensitivity for the enzymatic electrode is not solely due to the diffusional constraints imposed by the cellulose acetate membrane but is also due to the loss of enzymatic activity.

In conclusion the Pt-black sensors are advantageous because of the higher sensitivities/unit geometric area and the ease with which the enzyme can be immobilized. These advantages, however, are counter balanced by the increased sensitivity of the sensor to oxidizable interferents and higher susceptibility of the immobilized enzyme to denaturation. Therefore, unless a way is found to retain most of the enzyme activity when the sensor is coated with a suitable polymer, the scope of these sensors for in vivo glucose monitoring will be fairly limited. A new polymer, Eastman AQ 29D which is available as an aqueous suspension
Figure 4.6 Storage stability curve for a Pt-black based glucose sensor.
Figure 4.6 Storage stability for a Pt-black based glucose sensor.
Table 4.4 In vitro characteristics of a non-enzymatic electrode.
<table>
<thead>
<tr>
<th></th>
<th>Bare electrode</th>
<th>CA-coated electrode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (nA/mM)</td>
<td>3.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Linear range, upper limit (mM)</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Glucose/Fructose</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2/\text{Ascorbate} )</td>
<td>1.5</td>
<td>15</td>
</tr>
<tr>
<td>Error (%)(^a)</td>
<td>730</td>
<td>53</td>
</tr>
<tr>
<td>Drift (%/24 h)</td>
<td>-</td>
<td>-22</td>
</tr>
</tbody>
</table>

\(^a\) Error resulting from the presence of 0.1 mM ascorbate on sensor output for 5.5 mM glucose.
and is known to have good permselectivity (Gorton et al., 1990; Wang et al., 1990), is a good candidate for future studies. The membrane coating out of an aqueous solution (pH=6.0) should not disturb the enzyme.
Chapter 5
COVALENT ENZYME COUPLING ON CELLULOSE ACETATE MEMBRANES

INTRODUCTION

It is quite evident that miniaturized enzyme sensors required for in vivo applications will yield high sensitivity, stability and fabrication success rate only if enzyme layer and associated polymer layers can be deposited in a reproducible fashion. Therefore there exists a need for optimization of procedures for both enzyme and protective layer preparation. The purpose of this work was to find a suitable method which allows efficient and reproducible coupling of GOx to a support material suitable for an in vivo glucose sensor.

Among the available methods of enzyme immobilization, three methods are currently used (Figure 5.1): physical adsorption or entrapment followed by intermolecular crosslinking with bifunctional reagents (Arnold et al., 1982), covalent coupling between enzyme and activated support (Thévenot et al., 1979, Wingard et al., 1984, Moody et al., 1986) or activated enzyme and support (Kulys et al., 1986, Mullen et al., 1986, Mascini et al., 1986), and reversible immunological coupling (de Alwis et al., 1987). The bifunctional reagents such as glutaraldehyde are widely used to stabilize the physically adsorbed enzymes by crosslinking. This method provides relatively low enzymatic
Figure 5.1 Various methods of enzyme immobilization:

a) physical adsorption
b) physical adsorption followed by crosslinking
c) covalent attachment
d) immunological coupling.
Figure 5.1 Various methods of enzyme immobilization.
activities because part of the immobilized enzyme merely acts as a support for the rest. Other disadvantages include lack of control over immobilization and diffusional limitations. In spite of all these problems, this method continues to maintain its popularity because of its simplicity and quickness. The methods involving covalent coupling to solid supports are of great interest since they generally yield the best activity stabilities (Thévenot, 1982) and also provide good control over immobilization. Nevertheless two difficulties may be encountered: low levels of activatable or activated surface groups on the support and denaturation of enzyme if covalent coupling is accomplished through functional groups of the enzyme which are essential to its catalytic activity. The diversity of this method, however, permits the avoidance of the latter problem (Barker et al., 1987). As cellulose acetate (CA) membranes of different thickness and permeability may easily be prepared by film casting or coating and because they exhibit significant permselectivity towards anions (Sittampalam et al., 1983), we have studied a method for covalently attaching glucose oxidase (GOx) to these membranes. These membranes are activated and coupled to bovine serum albumin (BSA) in order to increase the number of functional groups to which GOx may be coupled and to impart a proteic environment to the enzyme. GOx is coupled to CA-BSA membranes using parabenzoquinone (PBQ) as
bifunctional reagent (de Alwis et al., 1987, Ternynck et al., 1976, Webb, 1966) (Figure 5.2).
Figure 5.2 Chemical reactions involved in CA-BSA-PBQ-GOx membrane preparation.
Figure 5.2 Chemical reactions involved in CA-BSA-PBQ-GOx membrane preparation.
EXPERIMENTAL SECTION

Instrumentation

GOx activity of membranes was determined by continuous monitoring of enzymatically generated hydrogen peroxide on a combined platinum disc electrode (anode diameter 2 mm) (YSI Model 2150) covered with a general purpose cellulose acetate membrane. This electrode was connected to a YSI model 25 Oxidase Meter (Yellow Springs Instruments, Yellow Springs, OH) and to a potentiometric recorder (Curken). Determinations of solution and membrane radioactivity were performed on a LKB Wallac 80000 Automated Gamma Counter. GOx membranes were mounted on a rotated platinum disc electrode (anode diameter 3 mm) using a specially designed membrane holder tip (Figure 5.3). The rotation speed of the membrane and anode was controlled by a Solea-Tacussel (Lyon, France) EDI motor and Controvit power unit. A Ag/AgCl reference and a platinum wire auxiliary electrode were connected, together with the platinum disk anode, to a BAS LC4A amperometric detector (Bioanalytical Systems, W. Lafayette, IN.) and potentiometric recorder (Curken). All experiments were performed in thermostated cells at 37°C.

Chemicals

Pure p-benzoquinone (PBQ) was obtained from Merck and was recrystallized from petroleum ether. Sodium cyanoborohydride was a product of Sigma and Cerium (IV)
Figure 5.3 Schematic diagram of rotating membrane electrode (a) rotating disk electrode shaft and electrical contact, (b) Kel-F body, (c) threaded collar, (d) membrane support cap, (e) platinum electrode and (f) membrane.
Figure 5.3 Schematic diagram of the rotating membrane electrode.
sulfate $2\text{H}_2\text{SO}_4$ was obtained from Alfa Division (Ventron). The other materials used for the study were described previously (Chapter 2 and Chapter 4).

**Glucose Oxidase Membrane Preparation**

**Cellulose Acetate Membrane Preparation**

With stirring, 1.8 g of CA was dissolved in 15.8 g of acetone, and 2.8 mL of distilled water was added. After homogenization, this solution was cast on a glass plate and evaporated for 60 s at 22°C to form a thin membrane (0.015 mm in the wet state and 0.010 mm in the dry state). The CA membrane was removed from the glass plate by immersing the plate in distilled water. The membrane was cut into smaller pieces and stored in distilled water.

**Cellulose Acetate Membrane Activation**

The cellulose acetate membranes were suspended in 100 mL of 100 mM sodium periodate for 20 min. The membranes were washed in distilled water for 5 min and immersed in 10 mL of 10 mg/mL BSA solution in 0.1 M borate buffer pH 9.0. The Schiff Base formed was reduced for 2 hours by adding 4 mg sodium cyanoborohydride to the 1 mL of BSA solution containing the membrane. After another washing in distilled water, the membranes were stored in PBS, pH 7.4 at room temperature until the GOx coupling reaction was performed.
Glucose Oxidase Activation

Freshly prepared PBQ solution 0.1 mL (15 mg in 1 mL ethanol) was introduced in an aluminum foil-covered tube containing 0.5 mL of GOx solution (20 mg in 0.1 M PBS pH 7.4). After 30 minutes' incubation at 37°C, the mixture was filtered through a G-25 Sephadex column (1 x 10 cm) coupled with a peristaltic pump (20 mL/h) and equilibrated with 0.15 M sodium chloride. The first fraction, a pink-brown band of 2-3 mL, was collected and used as the enzyme coupling solution.

Coupling Reaction

The CA-BSA membranes were suspended in 2-3 mL of activated GOx solution, whose pH was adjusted to 8.0-9.0 with 0.25 mL of 1.0 M sodium carbonate solution. After 38 h of incubation at room temperature, the membranes were removed, washed in stirred 0.15 potassium chloride solution containing 1 M lysine for 12 h and finally stored in 0.1M PBS, pH 7.4 containing 1.5 mM sodium azide. The azide was added to all storage and experimental buffers to limit microbial contamination and catalase activity.

Radiolabeled GOx

Preparation and characterization of ¹²⁵I labeled glucose oxidase is described in chapter 4.
Glucose Oxidase Membrane Characterization

Enzymatic Surface Activity Determination

The hydrogen peroxide anodic sensor, covered with a non-enzymatic CA membrane, was immersed into a thermostated cell (37°C) containing 10 mL of PBS. When the background current was stabilized, i.e. after 10 min of polarization, 0.1 mL of 1 M glucose standard solution was added to the buffer solution. The incremental production of hydrogen peroxide was measured by placing the CA-BSA-PBQ-GOx membrane in the stirred buffer solution and then removing it. This process was repeated several times. The resulting increase in current, after suitable calibration with hydrogen peroxide standards, could be used to estimate the membrane surface enzymatic activity.

Sensor Response

The rotating membrane electrode (Figure 5.3) was dipped into a thermostated cell at 37°C containing 25 mL of PBS. When the background current was stabilized, i.e. after about 20 min polarization, several 0.025 to 0.125 mL additions of 1 M glucose standard solution were performed until a 15-20 mM final concentration was obtained. Then one or two 25 μL aliquots of 0.1 M hydrogen peroxide were added to the buffer solution. Response was calculated by comparing steady-state current either to the background current (I_b) prior to any glucose addition or to the
steady-state current corresponding to the previous addition.

**Immobilized Enzyme determination**

The mass of immobilized GOx was estimated from the gamma activity of the membranes prepared using radiolabeled GOx and the specific activity of radiolabeled enzyme solution. By taking into account the initial enzyme activity of lyophilized powder (U/g) and a 15-20% decrease of this activity due to the radiolabeling procedure (Chapter 4), a further estimation of membrane activity expressed in U/cm² was accomplished.
RESULTS AND DISCUSSION

Characteristics of Glucose Oxidase Membranes

Figure 5.4 shows the correlation between enzymatic activity, as monitored by surface activity, and the mass of immobilized enzyme. The mass of enzyme on the membrane of different membranes was controlled such that the amount of enzyme immobilized could be varied. All these data obtained just after coupling show an excellent correlation between surface activity and the mass of GOx immobilized. Furthermore when the enzyme activity of these membranes, estimated from the amount of GOx immobilized, and the specific activity of the initial lyophilized GOx powder, were compared to experimental activity values, an excellent correlation was obtained. In fact the measured / calculated activity ratio was equal to 0.84 ± 0.29 for 6 membranes and 22 experiments indicating that radiolabeling procedure allows a good evaluation not only of total GOx mass but also of its enzymatic activity.

Several experiments involving the use of radiolabeled GOx were carried out in order to understand the reasons for the loss of enzymatic activity with time, a phenomenon observed for most membranes prepared. Figure 5.5 compares the time dependent loss of enzymatic activity with the loss of enzyme mass from the membrane surface. As one can see, the removal of enzyme from the membrane is directly
Figure 5.4 Correlation of GOx surface activities with immobilized $^{125}$I-GOx amount in different CA-BSA-PBQ-GOx membranes.
Figure 5.4 Correlation between surface GOx activity and amount of immobilized GOx.
Figure 5.5 Relative evolution of surface GOx activity (o) and $^{125}$I-GOx amount (*) in a CA-BSA-PBQ-GOx membrane.
Figure 5.5 Relative evolution of surface GOx activity and GOx amount.
reflected in the loss of biological activity. However, in the absence of radiochemical measurements, membranes showed significant improvement on these stability values, probably because of decreased handling. A relative activity value as high as 80-85% was frequently observed after passage of 2-3 weeks (Figure 5.6). As the membranes were very fragile and unsupported, handling them on a routine basis must have caused their partial disintegration. These results demonstrate that enzyme inactivation is negligible after coupling and that any slight surface activity decrease, when observed, is probably related to immobilized enzyme loss from the membrane.

Table 5.1 presents pertinent analytical parameters for GOx membranes when mounted on a platinum anode. The sensor sensitivities, as referred to anode area, of the CA-BSA-PBQ-GOx membranes are very close to those for highly active acyl-azide activated collagen membranes (Thévenot et al., 1979). As CA membranes are significantly thinner than reconstituted collagen, especially when the latter are swollen, both transient and steady state response times for them are correspondingly much shorter. The stability of 37°C and anion permselectivity of CA membranes are also preferable to those of collagen for development of in vivo glucose sensors.

This study underlines the importance of careful control of coupling conditions in order to achieve
Figure 5.6 Evolution of surface GOx activity in a CA-BSA-PBQ-GOx membrane, not routinely handled for radiochemical measurements.
Figure 5.6 Evolution of surface GOx activity.
Table 5.1 Pertinent parameters for GOx membranes.
Table 5.1 Pertinent Parameters for GOx Membranes

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Collagen-GOx</th>
<th>CA+GOx+CA</th>
<th>CA+BSA-PBQ-GOx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane material</td>
<td>collagen + cellulose</td>
<td>cellulose</td>
<td>cellulose</td>
</tr>
<tr>
<td>Attachment procedure</td>
<td>acyl azide</td>
<td>acyl azide</td>
<td>entrapment covalent</td>
</tr>
<tr>
<td>Membrane thickness (um)</td>
<td>-dry</td>
<td>100</td>
<td>100 + 15</td>
</tr>
<tr>
<td></td>
<td>-swollen</td>
<td>200 - 400</td>
<td>300 + 15</td>
</tr>
<tr>
<td>Glucose responses:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-sensitivity&lt;sup&gt;a&lt;/sup&gt; (mA.M&lt;sup&gt;-1&lt;/sup&gt;.cm&lt;sup&gt;-2&lt;/sup&gt;)</td>
<td>8 - 10</td>
<td>0.01 - 0.1</td>
<td>0.01 - 0.1</td>
</tr>
<tr>
<td>-linear range (mM)</td>
<td>lower limit</td>
<td>0.001</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>upper limit</td>
<td>2 - 3</td>
<td>40</td>
</tr>
<tr>
<td>-response time transient (s)</td>
<td>20 - 50</td>
<td>50 - 90</td>
<td>10 - 60</td>
</tr>
<tr>
<td>steady-state (min)</td>
<td>2 - 4</td>
<td>3 - 6</td>
<td>2 - 4</td>
</tr>
<tr>
<td>-stability&lt;sup&gt;b&lt;/sup&gt; (days)</td>
<td>120 - 2000</td>
<td>4 - 8</td>
<td>10 - 150</td>
</tr>
</tbody>
</table>

(a) all sensor sensitivities are referred to anode area, i.e. 7.06 mm<sup>2</sup>.
(b) stabilities are evaluated for a 50 % decrease of sensitivity.
reproducible immobilization of enzyme with high activity and stability. The coupling methods described here are successfully adapted to needle-type microsensors (Sternberg et al., 1988). An extended linear range, necessary for subcutaneous implantation, is achieved by adding an external membrane (polyurethane) with concomitant sacrifice of sensitivity and to some extent response time.
DETERMINATION OF GLUCOSE IN BIOLOGICAL FLUIDS

INTRODUCTION

The detection of glucose in biological fluids has long been essential in bioanalysis. As a result of development of the enzyme electrode by Clark (1962) and by Updike (1971), glucose detection based on the highly specific glucose oxidase catalyzed reaction has been the method of choice. Millions of glucose measurements are made annually by enzyme electrodes in whole blood, plasma and other biological fluids. Despite the specificity of the enzyme reaction, sensor response is influenced by the partial pressure of oxygen (a co-substrate) in the medium and by the presence of electroactive interferents such as ascorbic acid and uric acid. By the use of permselective membranes it has been possible to eliminate most of these difficulties but at some cost in sensor complexity (Sternberg et al., 1988).

Glucose sensors based on direct oxidation of glucose at noble metal electrodes have been known for many years. Direct oxidation has been suggested as the basis for an implantable glucose sensor (Lewandowski et al., 1982) or generally as a means for the detection of glucose in
biological fluids. Such sensors should have a good long-term stability, because there is no enzyme to denature or escape from the electrode surface. These sensors, however, have several problems. Glucose is not well-behaved electrochemically and analysis based on the interpretation of current-voltage curves is complicated and time-consuming (Marincic et al., 1979). Response is subject to electrode fouling by oxidation products (Lerner et al., 1979) and to interference from amino acids, ascorbic acid, urea (Richter et al., 1982) and a variety of drugs such as acetaminophen. Moreover, chloride is known to have a significant inhibitory effect on glucose oxidation kinetics (Marincic et al., 1979).

In spite of the numerous difficulties mentioned above, direct oxidation of glucose can be made tractable by suitable conditioning or modification of the sensing electrode. For example, the application of multistep potential waveforms to the working electrode, which incorporates cleaning and activation steps along with detection, has made possible rapid, sensitive and reproducible measurements. This technique, known as pulsed amperometric detection (PAD), has proven useful for the measurement of a wide range of analytes separated by liquid chromatography (Neuburger et al., 1988). In PAD, the electrode is held at a detection potential (E₁) for a short time during which the signal is collected from
the oxidation of analyte (Figure 6.1). The potential of the electrode is then increased to E2 to completely oxidize the electrode surface and any other adsorbed materials. The potential is then reversed to an extreme reducing value (E3) to bring the electrode surface back to its native form. The potential is again brought back to the detection potential where signal is collected. Usually an appropriate delay period is provided before the current is sampled to permit the decay in the charging current (Figure 6.1). The potential of each step in the pulse and the duration of each pulse can be adjusted to fit the application. The PAD is shown to have greatest sensitivity at extremes of pH.

Flow injection analysis is well known for its rapidity, precision and ability to handle small sample volumes. Its analytical parameters can be well controlled and reproducibly maintained. The use of flow injection coupled to electrochemical detection for glucose determination in blood serum is well documented (de Alwis et al., 1987). Most of these methods employ immobilized enzyme reactors and amperometric detection with working electrode (mostly Pt) poised at 0.7V vs Ag/AgCl. The working electrode is covered with a dialysis membrane such as cellulose acetate to provide the required protection and the selectivity.
Figure 6.1 Triple-pulse potential waveform.

E1, detection potential
E2, oxidative cleaning potential
E3, reductive cleaning potential.
Figure 6.1 Triple-pulse potential waveform.
We have developed a flow injection system coupled with a pulsed amperometric detector which is able to reliably measure physiological glucose levels without any prior sample separation or cleanup. The interferences are limited to a large extent by using suitable membranes which not only selectively control the diffusion of certain species, but also protect the electrode from coming in direct contact with biological fluid.
EXPERIMENTAL SECTION

Apparatus. The flow injection system consisted of a Shimadzu Model LC-6A pump and SCL-6A controller, Waters Associates Model 710B Auto Sampler, a Princeton Applied Research Model 400 electrochemical detector and a flow-through thin-layer electrochemical cell consisting of single gold working electrode (MP1300), Pt counter electrode and Ag/AgCl (sat’d KCl) reference electrode. A schematic diagram of the flow injection system is shown in Figure 6.2. The detector output was processed by a Shimadzu CR 4A integrator, the peak area being used as the basis for analysis. The PARC Model 400 is designed to apply a repeating sequence of three applied potentials to the electrochemical cell according to a specified timing sequence.

Materials. All solutions were prepared from analytical grade chemicals using water from a Barnstead Nanopure II system. All buffers were refiltered through a 0.45 μm filter after preparation. L-ascorbic acid solutions were prepared just before use, as ascorbic acid is subject to oxidative decomposition in solution. Soluble Nafion (5% by wt. in 90% lower aliphatic alcohols and 10% water) was obtained from Aldrich Chemical Company, Milwaukee, WI. The collagen and cellulose acetate (M.W. cutoff 12000-
Figure 6.2 Schematic diagram of the flow injection system.
Figure 6.2 Schematic diagram of the flow injection system.
membranes were supplied by YSI, Yellow Springs, OH and Viscase Corporation, Chicago, IL., respectively. Normal control serum was obtained from Ortho Diagnostic Systems Inc., Raritan, NJ. Normal human serum was also used and was obtained from Lawrence Memorial Hospital, Lawrence, KS.

All measurements were performed in 0.1 M phosphate buffer (PB), pH 7.4 unless specified otherwise. Samples for the glucose recovery experiment were prepared by adding appropriate amounts of glucose (50-500 mg/dL) to control serum or human blood serum. These samples were diluted 1:5 with 0.1 M PBS, pH 7.4 (100 meq/L NaCl) before injecting.

Nafion coating. A Nafion membrane was cast over the working electrode by spreading a thin layer of Nafion solution on the clean and dry surface of the Kel-F block holding the electrode. Exactly 50 μL of the Nafion solution was placed over the electrode and was spread with a brush over the whole flow channel (2.5x0.8 cm) including the gold electrode surface. The electrode was then left to dry at room temperature overnight. The thickness of Nafion film was roughly estimated as 2-3 μm by using a density of 1.58 gm/cm³ for Nafion film (Harrison et al., 1988). A precast collagen (100 μm thick-dry state) or cellulose acetate (30 μm thick) membrane was used for the outer protective layer. The teflon
gasket (spacer, 0.5 mm thick) was replaced on top of the membranes before reassembling the cell.

The three step potential sequence for the detection of glucose at a gold electrode has been optimized elsewhere (Johnson et al., 1986, Neuburger et al., 1987) and was adopted without any major modifications. In general, the choice of potentials is determined from the current-potential response curve of the analyte of interest. A sequence consisting of a detection potential (+100 mV, 0.499 s), an oxidative cleaning potential (+650 mV, 0.249 s) and a cathodic reactivation potential (-800 mV, 0.166 s) was used for all experiments. As the time period for completion of one cycle is less than 1 s, the detection is essentially continuous as in DC amperometry.
RESULTS AND DISCUSSION

Glucose Calibration Curves

The choice of 0.1 M PB, pH=7.4 as the mobile phase was made to be consistent with physiological pH even though the optimal condition for measurement is pH=12-14 (11). The protective membranes are, moreover, not stable at such pH extremes. The function of these membranes is discussed in the next section.

The background current for a freshly polished gold electrode took around three hours to reach a steady state value after initial application of the pulse sequence. This coincides with the time it took for the sensor to produce a reproducible response to a given amount of glucose injected repeatedly (Figure 6.3). This phenomenon has been explained previously as being due to the continuous microscopic roughening of the electrode surface (until a constant area is obtained) by alternate formation and removal of surface oxide through electrochemical pulsing (Neuburger et al., 1987). However, reported times for background stabilization were only 5-10 min when 0.2 N NaOH was used as the mobile phase (Neuburger et al., 1987). Presumably the physiological medium conditions do not favor the formation and the subsequent reduction of the deposit formed.

The successful maintenance of electrode activity by
Figure 6.3 Stabilization of (a) glucose response (△), (b) background current (□) after initial application of the pulse. Sample injected, 30 μL of 25 mg/dL glucose in 0.1 M PB, pH=7.4.
Figure 6.3  Stabilization of sensor response and background current.
application of the triple-pulse waveform is illustrated in Figure 6.4 and 6.5 for flow injection and batch determination of glucose, respectively. For batch determinations, a needle-type gold electrode (Nafion-coated) was dipped into a cell containing 10 mL of stirred PB, pH 7.4. The current time curve (Figure 6.5) was obtained by adding increasing amounts of glucose to the stirred buffer. Direct current (DC amperometry) detection, on the other hand, resulted in a quick loss of sensitivity with no response observed after a few minutes (Figure 6.6 and 6.7). This apparently resulted from fouling of the electrode surface by adsorbed reaction products.

The glucose response was found to be linear within the physiological range. The detection limit was approximately 25 nmol in a 30 μL sample (4.5 μg of glucose) for S/N=10. The reproducibility of the sensor response was evaluated by repetitive injection of the 30 μL sample. Ten injections were used to calculate a relative standard deviation of 1%. A flow rate of 0.25 mL/min was selected as it provided a good compromise between sensitivity and speed of analysis. The sensor response was found to be stable for at least one week.

Effect of Chloride

Figure 6.8 shows the effect on the oxidation current of increasing chloride concentration in a glucose sample
Figure 6.4: Pulsed amperometric detection of glucose in flow injection mode: sample injected, 30 μL of 60 mg/dL glucose, mobile phase, 0.1 M PB, pH=7.4 at 0.25 mL/min; working electrode, gold covered with Nafion and collagen.
Figure 6.4 Pulsed amperometric detection of glucose in flow injection mode.
Figure 6.5: Pulsed amperometric detection of glucose in batch mode: each injection represents an increment of 0.25 mM glucose concentration in the test solution (0.1 M PB); working electrode, gold covered with Nafion.
Figure 6.5  Pulsed amperometric detection of glucose in batch mode.
Figure 6.6  DC amperometry of glucose in flow injection mode: Sample injected 30 μL of 60 mg/dL glucose; mobile phase, 0.1M PB, pH=7.4 at 0.25 mL/min; working electrode, gold covered with Nafion and collagen.
Figure 6.6: DC measurement of glucose in flow injection mode.
Figure 6.7: DC amperometry of glucose in batch mode: G1, G2 and G3 represent three successive injections of glucose into the test solution (0.1 M PB, pH=7.4), each representing an increase of 1 mM glucose concentration; working electrode (8 mm$^2$), gold covered with Nafion.
Figure 6.7  DC measurement of glucose in batch mode.
Figure 6.8 Effect of chloride on glucose response for (a) bare gold electrode (□), (b) Nafion-coated gold electrode (△). The glucose concentration is 100 mg/dL. Sample size, 30 μL.
Figure 6.8 Effect of chloride on glucose response.
using a bare gold electrode or a Nafion coated gold electrode. About 80% of the response was lost for the bare gold electrode at physiological concentrations of chloride. Even though the variation in chloride ion concentration within the normal physiological range (100-106 meq/L) had little effect on the glucose oxidation current, the overall loss in sensitivity was too significant to be overlooked. Nafion, in the form of a cation exchange polymer membrane, is effective in selectively excluding anions from the electrode surface. This property of Nafion has previously been exploited for elimination of a chloride interference effect on cupric ion-selective electrodes (Hoyer et al., 1988) and ascorbic acid interference in the determination of dopamine (Nagy et al., 1985). Nafion also has been successfully employed as a dialysis membrane to provide a protein and interferent-free environment near the electrode for the determination of glucose in whole blood (Harrison et al., 1988). As seen in Figure 8, a Nafion coated electrode preserves most of its response in the presence of chloride. The effect of chloride ion was far less pronounced when 0.2 N NaOH (pH=13) was used as the mobile phase. This was primarily due to the greatly enhanced sensitivity for glucose at high pH as the absolute magnitude of the effect is the same.
Effect of Proteins

The effect of proteins on glucose response was studied by adding Bovine serum albumin (BSA) to the glucose standards. For a bare electrode the response was largely suppressed due to the surface poisoning by BSA adsorption. But unlike the case of DC amperometry, where protein adsorption tends to be irreversible, the electrode here regained its original activity within 10 minutes of protein injection as the pulsing slowly removed all of the surface adsorbed protein. At pH 13, though the poisoning was less severe (probably because denatured protein did not adsorb well on the electrode surface) it was still sufficient to affect the results in a glucose recovery experiment (Table 6.1).

The Nafion-coated electrode also did not offer complete protection against protein fouling, resulting in poor recovery of glucose from biological samples. It was, however, possible to put a collagen membrane in front of the Nafion to prohibit protein from reaching the inner Nafion layer. This bilayer membrane structure completely eliminated the inhibitory effect resulting from the protein adsorption while maintaining the desired permselective characteristics (Figures 6.9 and 6.10). A similar bilayer coating, with a cellulose acetate film covering the Nafion layer, has been utilized previously by Wang and coworkers (Wang et al., 1987) for selective detection of cationic
Table 6.1 Results of glucose recovery from control serum for different working electrode conditions.
<table>
<thead>
<tr>
<th>conditions</th>
<th>% recoveryb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare gold (pH = 13)</td>
<td>~ 50</td>
</tr>
<tr>
<td>Bare gold</td>
<td>~ 24</td>
</tr>
<tr>
<td>Nafion-coated gold</td>
<td>~ 60</td>
</tr>
<tr>
<td>Collagen on Nafion-coated gold</td>
<td>95±3 ( n=4 )</td>
</tr>
</tbody>
</table>

aAll measurements made at pH=7.4, unless specified.

bSamples were prepared by adding 100 mg/dL glucose to Control Serum. Samples were diluted 1:5 before injecting. The recovery results were calculated by comparing the signals from glucose-spiked serum samples (corrected for the serum blank) and glucose samples in 0.1 M PBS.
Figure 6.9 Effect of BSA on glucose response for (a) bare gold electrode, pH 7.4 (○), (b) bare gold electrode, pH 13 (△), (c) collagen on Nafion-coated gold electrode, pH 7.4 (□). The glucose concentration is 100 mg/dL. Sample size, 30 μL.
Figure 6.9 Effect of BSA on glucose response.
Figure 6.10 Glucose response curves (a) aqueous glucose samples (○), (b) glucose-spiked human blood serum samples (1:5 diluted) (△)
Sample size, 30 μL; working electrode, gold covered with Nafion and collagen.
Figure 6.10 Glucose response curves.
neurotransmitters in urine samples.

Interference studies

All interfering compounds were studied at their physiological minimum and maximum levels while the glucose concentration was kept constant at 100 mg/dL. As expected, the glucose measurement was strongly influenced by the presence of ascorbic acid, uric acid, amino acids and acetaminophen.

Under the conditions of measurement, the bare sensor was found to be far more sensitive towards ascorbic acid and uric acid than towards glucose. Therefore, even though both ascorbic acid (0.2 - 2 mg/dL) and uric acid (4.0 - 8.5 mg/dL) are present in body fluids at concentrations that are significantly lower than that of glucose (60-110 mg/dL), the errors caused by their presence were fairly significant. For example, the apparent response for a glucose sample (100 mg/dL) on a bare electrode was increased by 140% when ascorbic acid was added to the sample at its physiological maximum concentration (2 mg/dL). On the other hand, as both ascorbic acid and uric acid are present as anions at physiological pH, their transport to the electrode surface can easily be restricted by putting a Nafion coating on the electrode surface. The presence of ascorbic acid and uric acid at their physiological maximum values, caused an error of less than 5% apparent glucose concentration for a Nafion coated
electrode.

As the molar concentrations of urea are comparable to that of glucose in body fluids, its presence has always created difficulties in the development of the electrocatalytic glucose sensor. This is especially true for Pt black-based sensors, where the reported errors are as high as 20% resulting from the influence of urea (Richter et al., 1982). As urea is uncharged in neutral solution and is smaller in size than glucose, it is not trivial to realize a membrane which will selectively discard urea while allowing glucose to pass through. Fortunately, under the measurement conditions of the present experiment, urea was almost inactive, causing an error of less than 2% (Figure 6.11) at its physiological maximum (26 mg/dL).

Amino acids are also potential physiological interferences and a considerable amount of work has been done previously dealing with the elimination of their influence. A wide variety of membranes such as polysulfone (Lewandowski et al., 1982) and Permion 1025 (Richter et al., 1982) have been employed for this purpose. In the pulsed amperometric mode, the potentials required for amino acid oxidation are much higher (+500 mV) than the one used for glucose oxidation. It is believed that the amino acid oxidation is catalyzed by the metal
Figure 6.11 Effect of urea and amino acids on glucose response. Response curves (a) glucose only (□), (b) glucose in presence of all amino acids at physiological maximum (◇), (c) glucose in presence of urea at physiological maximum (▲). Sample size, 30 µL.
Figure 6.11 Effect of urea and amino acids on glucose response.
oxide formation (Welch et al., 1989) unlike the glucose oxidation which is inhibited by such a process. Despite this, interference from a mixture of all amino acids (present at their physiological maximum levels) for a bare electrode was so great that the response towards glucose was almost obscured. However, the transport of these amino acids was greatly restricted by the Nafion membrane, probably because of their zwitterionic character at neutral pH. For a modified electrode with a collagen membrane over the Nafion film, an error of approximately 19% resulted in the response of a 100 mg/dL glucose solution (Figure 6.11) when all amino acids were added at their physiological maximum levels (Giner et al., 1981). However, the response for all glucose concentrations fluctuated within ± 3% when amino acid concentrations were varied from physiological average to the physiological extremes. Therefore, the error resulting from amino acid interference can be cut down to less than 3% by adding amino acids at their average physiological concentrations to the calibration standards.

A few other carbohydrates besides glucose are also found in body fluids, but at comparatively much lower concentrations. Sensor response to their presence is summarized in Table 6.2. As one can see, only galactose causes some interference at its physiological concentration level.

Recently, acetaminophen (sold as an over-the-counter
Table 6.2 Sensor response for various carbohydrates.
Table 6.2. Sensor Response for Various Carbohydrates

<table>
<thead>
<tr>
<th>Compound</th>
<th>Response at equal conc. (relative)</th>
<th>Physiological conc. (mg/dL)</th>
<th>Response at physiological max. (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>60-110</td>
<td>1.0</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.7</td>
<td>&lt; 4.5</td>
<td>0.06</td>
</tr>
<tr>
<td>D(-)Fructose</td>
<td>N.D.</td>
<td>&lt; 7.5</td>
<td>0</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.54</td>
<td>&lt; 5</td>
<td>0.02</td>
</tr>
</tbody>
</table>

N.D. - not detected
drug) has generated some interest as a possible interferent (Palleski et al., 1986). Under the conditions of the present experiment, acetaminophen was found to be mostly inactive, but it caused inhibition of the glucose response when injected with glucose (Figure 6.12). Acetaminophen may have been preferentially adsorbed resulting in a blocked surface and decreased glucose oxidation. The normal physiological levels for acetaminophen are believed to be around 2 mg/dL. At this level, the resulting error in the response for 100 mg/dL glucose was less than 5%. Higher errors have been reported for an enzyme-based sensor under similar conditions (Bindra et al., 1990).

**Effect of Membrane Permeability on Glucose Sensitivity**

In a recent study (Larew et al., 1989), it has been pointed out that a condition of transient alkalinity is generated in the vicinity of the working electrode surface during the cathodic reactivation step of the pulse sequence as both stripping of the oxide layer as well as the reduction of dissolved oxygen accompany the formation of OH\(^-\) as described by eqns. (1) and (2), respectively. As a result, the determination of

\[
\text{Au}_2\text{O}_3 + 3 \text{H}_2\text{O} + 6 \text{e}^- \longrightarrow 2 \text{Au} + 6 \text{OH}^- \quad (1)
\]

\[
\text{O}_2 + 2 \text{H}_2\text{O} + 4 \text{e}^- \longrightarrow 4 \text{OH}^- \quad (2)
\]
Figure 6.12 Effect of acetaminophen on glucose response. The concentration of glucose is 100 mg/dL. Sample size, 30 μL.
Figure 6.12 Effect of acetaminophen on glucose response.
glucose is possible by pulsed amperometric detection in neutral and acidic media of low buffer capacity if the timing of the pulse sequence is suitably modified. Our results are consistent with this behavior (Bindra and Wilson, 1989). As expected, a bare gold electrode showed an increase in sensitivity with a decrease in mobile phase and sample buffer concentration (Figure 6.13). This effect was less pronounced when the electrode was covered with protective membranes. The glucose sensitivities for different working electrode membranes are summarized in Table 6.3. As can be seen, the Nafion-coated electrode showed a higher sensitivity as compared to the bare electrode. This is probably due to the fact that Nafion, being anionic is able to restrict the diffusion of OH\(^-\) away from the electrode thus generating higher local pH at the electrode surface. The enhancement of the sensitivity by partitioning of glucose into Nafion also cannot be ruled out. For the Nafion/Collagen system, the loss in sensitivity due to the diffusional constraints imposed by the collagen membrane was compensated by the sensitivity increase caused by the underlying Nafion layer (Table 6.3).

Effect of Variation in Timing Sequence of the Pulse

The effect of variation in \(t_2\) (oxidative cleaning step) and \(t_3\) (cathodic reactivation step) upon glucose signal is shown in Table 6.4. As seen in the table, the
Figure 6.13 Effect of mobile phase buffer concentration on glucose response for (a) bare gold electrode (□), (b) collagen and Nafion covered gold electrode (○). Sample injected, 30 μL of 100 mg/dL glucose prepared in mobile phase buffer.
Figure 6.13 Effect of mobile phase buffer concentration.
Table 6.3 Effect of membrane properties on electrode sensitivity.
<table>
<thead>
<tr>
<th>conditions</th>
<th>relative sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare gold</td>
<td>1.0</td>
</tr>
<tr>
<td>Nafion-coated gold</td>
<td>2.4</td>
</tr>
<tr>
<td>Collagen on Nafion-coated gold</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Sample injected, 30 μL of 100 mg/dL glucose in 0.1 M PB, pH=7.4. Mobile phase, 0.1 M PB, pH=7.4.
Table 6.4 Sensor response (arbitrary units) as a function of pulse timing sequence.
<table>
<thead>
<tr>
<th></th>
<th>166 ms</th>
<th>416 ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>249 ms</td>
<td>14.9</td>
<td>19.5</td>
</tr>
<tr>
<td>416 ms</td>
<td>22.4</td>
<td>22.5</td>
</tr>
</tbody>
</table>
sensor response decreased when \( t_2 \) was made smaller, irrespective of the value of \( t_3 \). This was due to the fact that at smaller \( t_2 \) there was less available oxide for generation of \( \text{OH}^- \) during the subsequent reduction step (eqn 1). At long \( t_2 \), the response was independent of the value of \( t_3 \). Thus reduction of oxygen (eqn 2) was not a significant source of \( \text{OH}^- \) at long \( t_2 \). However, at short \( t_2 \), the sensor response was higher at longer \( t_3 \) implying that the reduction of oxygen was a significant source of \( \text{OH}^- \). This explanation is based on the discussion by Larew and Johnson (1989). As the sensitivity was not a limiting factor, a shorter value for \( t_2 \) (249 ms) was used because it provided better signal stability.

**Continuous Glucose Monitoring**

Attempts were made to use PAD for continuous monitoring of glucose. A needle-type gold electrode was used for this purpose. For a bare gold electrode, the response toward glucose in phosphate buffered saline, pH 7.4 was very low and unstable. This was primarily due to the inhibitory effect of chloride present in the test solution. It was possible to obtain a stable, sensitive and linear response to glucose by using gold electrode which was covered with Nafion and CA or PU. The selectivity of these electrodes against various amino acids, however, was extremely poor. Tryptophan, cysteine and histidine showed large responses
which made glucose monitoring in their presence meaningless. As the option such as the addition of various interferents to the calibration standards is not available during the continuous in vivo monitoring, the use of PAD electrode in its present form for such an application is not feasible.

In conclusion this work has demonstrated that by suitably modifying the working electrode, pulsed amperometric detection can be made to selectively detect glucose in biological samples. The separation steps carried out at the electrode surface help remove interferences to a large extent and avoid unnecessary sample cleanup. It has also been shown how the membrane permeabilities can be manipulated to yield increased sensitivity. As mentioned earlier, a reasonable estimate of physiological glucose levels is possible provided the calibration standards are prepared with chloride and various amino acids added at their average physiological concentrations. Though the results seem satisfactory, further improvement in the system selectivity is possible through a better choice of inner permselective membrane.
Chapter 7

FUTURE DIRECTIONS

SENSOR DESIGN

We have demonstrated a level of reliability of our sensors in both *in vitro* and *in vivo* measurements. However, we feel that further improvements in the sensor design are needed with respect to its implantability. At present, the sensor can only be implanted with the help of a catheter. Ultimately, a sharp preparation of the sensor which can be implanted by itself will be desired. A possible solution will be to put the sensor inside a 25 gauge needle (preferably made of a non-conducting material) after its preparation. Further miniaturization of the sensor is, however, required for it to fit inside a 25 gauge needle. Side windows can be cut near the needle tip to expose the sensing cavity. If the location of reference electrode turns out to be a problem, a separate reference electrode can always be used. A separately implanted reference electrode or a transcutaneous reference electrode has already been employed successfully during *in vivo* measurements.
IN VIVO PERFORMANCE

The in vivo sensitivities ranging between 20% and 40% of the corresponding in vitro values were commonly observed. The reasons for this large difference in sensitivities are still unclear. The in vivo parameters are calculated based on subcutaneous glucose levels being identical to blood glucose levels. Provided that this assumption is valid, we can consider several factors which might be responsible for the sensitivity loss on implantation.

a. Protein Adsorption

A good start will be to first understand why the sensor loses sensitivity when tested in serum (Chapter 2). The sensor can be tested in various fractions of serum obtained after ultrafiltration. The individual serum components can then be added to the various serum fractions to possibly pinpoint the problem. Initial experiments along these lines have indicated that the extent of sensitivity loss goes down with a decrease in the molecular weight cut off of the membrane used for ultrafiltration.

b. Oxygen availability

We have already shown in vitro that at clinically relevant glucose concentrations essentially constant response can be obtained from the glucose sensor over a wide range of oxygen tensions (Appendix 1). However, we
are still concerned about the question of how the sensor output would be affected by changes in the availability of oxygen at the implantation site.

The experiments should be designed in which both oxygen and glucose sensors of same shape and size are implanted in the same animal. The tissue oxygen can be varied by varying the composition of the breathing mixture as suggested by Fisher et al. (1989), and its effect on the glucose sensor output can be studied. There is also a need to get a better estimate of the subcutaneous oxygen levels. A large number of experiments are necessary to arrive at a statistically relevant range for sub-Q oxygen tension, which will probably prove to be dependent on the sensor orientation and the site of subcutaneous implantation.

Another point of concern is the high background currents seen for the oxygen sensor during in vivo measurements (Appendix 1). It will be interesting to see if the protective membrane (polyurethane), which has a high affinity for oxygen, is causing this high background.

c. Sensor Orientation

An easy way to study the effect of sensor orientation on its in vivo characteristics is to employ sensors with different geometries. Initial results with the sensors in which the sensing element was located at the tip of the sensor in the form of a cavity (0.2 to 0.3 m.m. deep) rather than at the side (Chapter 2) indicate a much smaller
loss of sensitivity on implantation. However, it is not yet clear whether it is the location of the sensing cavity, the size of the sensing area, or the difference in the membrane structure/thickness which is responsible for this behavior. A further exploration of these factors will help understand the possible reasons for the sensitivity loss and will also help to further improve the sensor design.

ALTERNATE MEMBRANE MATERIALS

The Eastman Kodak Company has recently introduced a series of polymers (poly(ester-sulfonic acid)) which are sold under the trade name of "Eastman-AQ". Coating sensors with polymers from this series such as Eastman AQ 55D (Wang et al., 1990) or Eastman AQ 29 D (Gorton et al., 1990) has improved their ability to exclude anionic interferences and to prevent against fouling in presence of proteins. Moreover, these polymers can be coated out of an aqueous solution and therefore can be easily put down without disturbing the underlying enzyme or polymer layers. As these features are extremely desirable for an implantable glucose sensor, it will be interesting to use these polymers by themselves or in combination with other polymers for sensor fabrication.
Appendix 1

**IN VIVO OXYGEN MEASUREMENTS**

In vivo oxygen measurements in the vicinity of an implanted glucose sensor are extremely important for accurately interpreting the in vivo response of such a sensor.

**Micro Oxygen Electrode**

A 10 cm long Teflon-coated Pt-Ir wire (0.25 mm o.d.) was stripped to form a cavity of about 0.5 mm length as described previously in chapter 2. The wire was dip-coated with 5% polyurethane solution in 98% THF - 2% DMF. After drying the sensor in air for 6 h, it was left in PBS, pH 7.4 to condition for 3 days. A separate Ag/AgCl wire (0.5 mm o.d.) was used as the reference/counter electrodes during in vivo oxygen monitoring.

**In Vitro Calibration of O₂ electrode**

The sensor was dipped into a temperature and oxygen tension-controllable cell containing 10 mL of stirred PBS, pH 7.4 (37°C) and a potential of -550 mV (for oxygen reduction) was applied between the working and the reference electrodes. The oxygen tension in the test buffer was varied by equilibrating the buffer with various air/nitrogen gas mixtures. The sensor output was recorded for several oxygen partial pressures between 0 and 160 mm Hg. The sensors which were linear up to at least 40-50 mm
Hg were used for *in vivo* \( \text{O}_2 \) monitoring. On the average, the *in vitro* characteristics were identical before and after the implantation. The sensor output showed a strong dependence on temperature.

**In Vivo Oxygen Monitoring**

The micro oxygen electrode was implanted in the subcutaneous tissue of an anesthetized rat in the same fashion as described previously for the glucose sensor. The electrode output was monitored for a 6 h period. At the end of this period, the animal was sacrificed by an overdose of anesthesia. The electrode output in the dead animal was taken as the *in vivo* background current for the implanted electrode. The tissue oxygen concentrations were calculated by dividing the net electrode output by the *in vitro* sensitivity coefficient obtained after the sensor was explanted. A trace for one such measurement is shown in Figure A.1.

The preliminary estimate of the subcutaneous oxygen levels in subcutaneous tissue of a rat is in the range of 8-18 mm Hg (n=4). These measurements however, are based on the assumptions that *in vivo* and *in vitro* sensitivities are same and the oxygen tension in the subcutaneous tissue of a dead rat is zero, which may or may not be valid. Further studies are necessary to establish the validity of these assumptions.
Figure A.1 A typical trace of \textit{in vivo} oxygen monitoring.
Figure A.1 A typical trace of *in vivo* oxygen monitoring.
Appendix 2

CELL CULTURE TOXICITY TESTS

One aspect of the sensor development is to study the biological performance of the sensor i.e. its biocompatibility. Toxicity evaluation of an implantable device is usually addressed first as one of the fundamental screening processes for biocompatibility.

The tissue culture method is an effective way for evaluating the toxicity of biomaterials. It has been well established that the tissue culture methods show very good correlation with animal assays and are frequently more sensitive to toxic moieties (ASTM, 1988; Brown, 1983; Johnson et al., 1985). The conventional test procedures (ASTM, 1988) were found not to be sensitive enough to test our needle-type glucose microsensors. These procedures were therefore, modified to fit our needs (Zhang et al., 1990). The positive and negative control materials were also established. The location, source and degree of toxic effect in a multi-component glucose sensor was tracked by microphotographing the cell cultures and measuring the spatial distribution or morphological change of the lysed cells. A freshly prepared sensor showed moderate toxicity, mainly due to the presence of glutaraldehyde and the residual solvents in the polymer layers. It was, however, possible to reduce the toxicity by removing the leachable
toxic substances through extraction in phosphate buffer for a week and a non-toxic sensor was readily obtained. The buffer treatment is also an important step in stabilizing the polymer structure and the sensor sensitivity as described in Chapter 2.

Since the sensitivity of this \textit{in vitro} test has generally proven to be higher than the \textit{in vivo} test, a negative result does increase the likelihood that \textit{in vivo} applications are feasible. Long term implantation tests for biocompatibility are necessary and intensive \textit{in vivo} investigations are needed.
Appendix 3

The University of Kansas

Dean, Graduate College
Administration Building
Room 322
Degree Checking

August 10, 1990

Dear Sir or Madam:

This letter is to certify that the following application involving Dilbir S. Bindra was reviewed and approved by the Institutional Animal Care and Use Committee:

Title: Studies of Potentially Implantable Glucose Sensors.
Principal Investigator: George Wilson, Ph.D.
Department: Chemistry
Date of Approval: February 01, 1990
Animal Involved: Rat

This Institution has an Animal Welfare Assurance on file in the Office for Protection from Research Risks. The Assurance Number is A-3339-01.

Sincerely,

[Signature]

Dr. James F. Bresnahan
Director
The IACUC has reviewed your Statement of Animal Use dated January 11, 1999 for the project titled: Studies of Potentially Implantable Glucose Sensors.

and has taken the following action:

    Project approved

    Project disapproved (see below)

If disapproved: more information is needed requires changes in protocol(s)

Explanation:

Please submit revisions as an addendum to your original statement. Do not resubmit the entire document. Three copies of the revision and a copy of the original statement should be forwarded to the Animal Care Unit Secretary, B054 Malott.
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Zhang, Y.; Bindra, D.S.; Barrau, M.B.; Wilson, G.S. *Biosensors*, Submitted.