[Frontiers in Bioscience 10, 512--520, January 1, 2005]

COIL-TYPE IMPLANTABLE GLUCOSE BIOSENSOR WITH EXCESS ENZYME LOADING

Bazhang Yu [1], Yvonne Moussy [2] and Francis Moussy [1]

University of South Florida, [1] Department of Chemical Engineering, [2] Department of Mechanical Engineering, 4202 E. Fowler Avenue, ENB 118, Tampa, FL 33620-5350

TABLE OF CONTENTS

1. ABSTRACT

As part of our overall long-term objective of designing a glucose sensor for long-term subcutaneous implantation, a coil-type implantable glucose sensor loaded with excess glucose oxidase (GOD) inside the coils of a 0.125mm diameter coiled platinum-iridium wire has been developed. The excess GOD was immobilized in a glutaraldehyde/bovine serum albumin (BSA) gel reinforced with cotton and located inside the coils chamber of the sensor. The excess GOD increased the lifetime of the sensor. Based on this coil-type design, various coil-type glucose sensors with cellulose acetate (CA), poly(vinyl chloride)(PVC), polyurethane (PU), poly(bisphenol A carbonate) (PC) and Nafion outer membranes were investigated and compared. Comparatively, Nafion based biosensors provided the best long-term response stability. However, Nafion can still not meet the lifetime requirement of the coil-type sensor with high enzyme loading because the observed function failure of these sensors was indeed caused by outer membrane damage rather than loss of enzyme activity. Additional experiments also revealed that hydrogen peroxide accumulation occurred in the GOD impregnated cotton when the sensors were not polarized which could cause a small false positive measurement. However, this artifact can be easily avoided by using an appropriate measurement technique.

2. INTRODUCTION

The lifetime of an implantable glucose biosensor based on amperometric detection of H_2O_2 greatly depends on enzyme loading inside the sensing element. In most cases, the enzyme cannot be replaced. In theory, glucose oxidase only catalyses the oxidation of glucose and is not consumed in the reaction. In practice, the activity of the enzyme may decrease with the repeated/continuous use of the sensor. Enzyme immobilized in a sensing element can slowly leach out through the outer membrane and gradually lose its activity with time. Degradation of glucose oxidase by hydrogen peroxide has also been demonstrated (1). Loss of enzyme activity may be accelerated by the attack of various aggressive chemical substances found in the tissue or in blood, for example as a result of inflammation. Therefore, excess enzyme is required in implantable glucose sensors to minimize the effect of progressive loss of enzyme activity and maintain the response sensitivity at a high level.

Excess enzyme loading may be obtained by 1) increasing the thickness of the enzyme layer, 2) employing an immobilization method with high enzyme loading and 3) improving the sensor construct. Increasing the thickness can not only lengthen the response time but also produce a high outward tension when the enzyme layer swells, thus cracking the polymeric outer membrane. The usefulness of this approach is therefore limited. Methods used for immobilized enzyme in biosensors include (1) adsorption; (2) physical entrapment; (3) chemical cross-linking; (4) covalent coupling; and (5) electrochemical co-deposition. Among various methods, electrochemical co-deposition is frequently used in implantable biosensors because it can

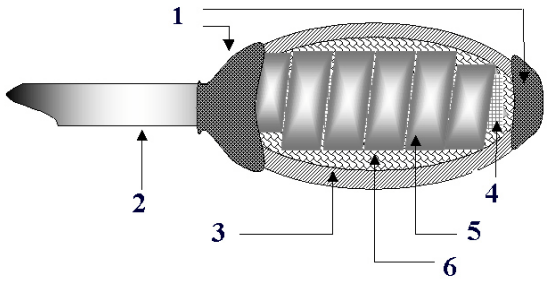

Figure 1. Schematic diagram of sensing element based on a coiled Pt-Ir wire. 1- electrically-insulating sealant; 2- Teflon-covered platinum wire; 3- outer membrane; 4- cotton fiber with enzyme gel; 5- stripped platinum wire; 6- enzyme layer.

provide reproducible and site-selective immobilization of enzyme. Enzyme entrapped in the electropolymerized film is stable but the quantity is limited by the self-limiting characteristic of the polymerization reaction (2). It is easier to increase enzyme loading by using chemical cross-linking agents such as glutaraldehyde. The chemical cross-linking layer may be much thicker than the electropolymerized layer, which is usually 100nm thick, and has a large enzyme capacity. However, as previously mentioned, a thick enzyme layer is not desirable for implantable biosensors since this will significantly increase the response time of the sensor and also induce cracks in the outer membrane because of swelling.

Therefore, in order to increase the enzyme loading to a desired level, a different approach needs to be considered, e.g. modification of the sensor construct. Implantable glucose biosensors commonly utilize a needle-type construct where the sensing layer is formed on the platinum wire (2-5) or in the recess of a needle-type sensor (6,7). Catheter-type(8) and button-type(9) are seldom used for implantation.

This paper describes a new method for loading extra enzyme inside a coil-type electrode. Various sensors constructed by this method have been investigated and compared with sensors using more traditional electrodeposited or cross-linked enzyme loading methods on the working electrode.

3. MATERIALS AND METHODS

3.1. Apparatus

Electrochemical experiments were performed with a computer-controlled Model 263A potentiostat (Princeton Applied Research). The glucose sensor, the reference electrode (Calomel, Sigma-Aldrich; or flexible Ag/AgCl, World Precision Instruments, Inc.), and platinum wire (ϕ1mm) counter electrode were inserted into a 10-ml glass beaker. A magnetic stirrer (Isotemp, Fisher Scientific) was used to provide the convective transport for electrochemical measurements as desired. Membrane morphometrics were observed and photographed using a Leica S6D stereomicroscope (Leica Microsystems Ltd.) and Philips 515 Scanning Electron Microscope (SEM).

3.2. Reagents

Dextrose, BSA and glutaraldehyde (50%) were obtained from Fisher Scientific. Cellulose acetate (CA) (average M_w ca 30,000), poly(bisphenol A carbonate) (PC), poly(tetrafluoroethylene-co-vinylidene fluoride-co-propylene) (PF), polyurethane (PU), poly(vinyl chloride) (PVC), poly(4-vinylpyridine-co-styrene) (PVS), Brij 30, isopropyl myristate (IMP), Aliquat 336 (AL) and dibutyl phthalate (DBP), potassium ferricyanide, bovine adult serum, 1,2-phenylenediamine, GOD (EC 1.1.3.4, 157,500U/g), L-ascorbic acid, acetaminophen, uric acid, creatinine, acetone, chloroform, tetrahydrofunan were obtained from Sigma-Aldrich-Fluka. All glucose solutions were prepared from a phosphate buffer (PBS) with the ionic strength of ca 0.16 M (0.025M Na_2HPO_4, 0.025M KH_2PO_4 and 0.15M NaCl). All measurements were implemented at room temperature (24±1°C).

3.3. Preparation of glucose biosensors

3.3.1. Pt wire coiling

The top 10mm of a 40-50mm long Teflon-covered platinum-iridium wire (ϕ0.125mm, Pt:Ir=9:1, World Precision Instruments, Inc.) was wound up along a 30-gauge needle to form a coil-like cylinder. The cylinder unit had an outer diameter of 0.55mm and an inner diameter of 0.3mm and a length of ca. 1mm. This design dramatically increases the sensing area of a flexible implantable biosensor within an acceptable length range and creates a ca. 0.07mm^3 inner chamber for extra enzyme storage (Figure 1). A cotton thread was inserted inside the chamber of some of the sensors to retain the enzyme solution during enzyme coating of the electrodes. The cotton fibers also reinforced the cross-linked gel stability and reduced formation of air bubbles in the chamber.

3.3.2. Enzyme immobilization

GOD was added to the sensors through three different approaches: 1) cross-linked to the cotton-reinforced glutaraldehyde/BSA gel inside the coil chamber, 2) electrochemically co-deposited to the Pt wire and 3) cross-linked to the outer of the coil electrodes by glutaraldehyde. The chemical cross-linked method used in 1 and 3 was achieved simply by coating an aqueous solution containing 1% (wt.) GOD, 4% BSA and 0.6% glutaraldehyde on the working electrode. When the cotton was involved, the enzyme solution also impregnated the cotton fibers within the coil chamber during coating and formed a cotton-reinforced cross-linked gel after drying. Thus, in this case, GOD was present in both the coils chamber within the cotton and also as a coating over the coiled electrode. The enzyme loading in the chamber can be 2-6 times of that of the enzyme layer surrounding the coil cylinder. Electrochemical co-deposition was carried out at 0.7 V vs SCE for 5 min in a PBS solution containing 5mM phenylenediamine, 20U/ml GOD, and 1μl/ml 0.25% glutaraldehyde. The resulting GOD-entrapped polyphenylenediamine film (*o*-PPD) was overoxidized at 0.9 V for at least 3 min. Various combinations of these entrapment methods were used and evaluated *in vitro*.

3.3.3. Outer membrane coating

A 2% (w/v) solution of polymer such as CA, PC, PF, PU or PVC was used as coating of outer membrane.

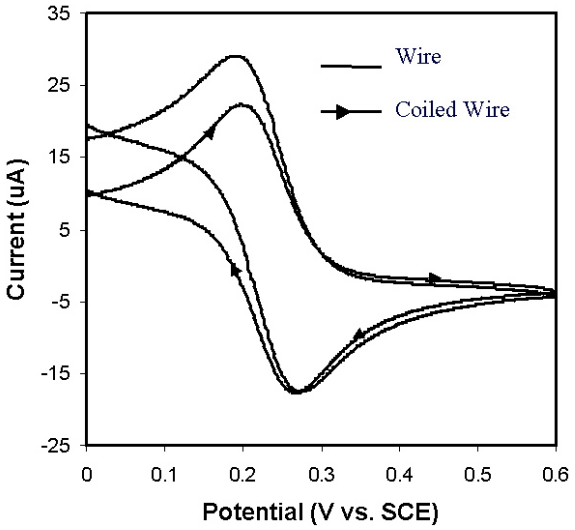

Figure 2. Cyclic voltammograms of $K_3Fe(CN)_6$ from a platinum wire and a coiled-wire electrode. Solution: 1M KCl+4mM $K_3Fe(CN)_6$; scan rate =50mV/s.

Acetone, chloroform or tetrahydrofunan was used as the solvent for CA, PC or PF, PU and PVC, respectively. Brij 30, IMP, AL and DBP may be selectively added to improve the film extensibility as desired. A Nafion layer was prepared by dip-coating 3 times in a 5% Nafion solution of lower aliphatic alcohols and water, and then annealed at 120°C for 30 min as previously reported (10). All new-prepared sensors were dried at room temperature for at least 24 hours. Finally, the two ends of the sensing element were sealed by electrically-insulating sealant (Brush-On electrical tape, North American Oil Company).

3.3.4. Measurement methods

Newly prepared glucose biosensors were conditioned for at least 24 hours in a 5mM glucose PBS solution and then continuously polarized at +0.7V vs. SCE until the minimum background current was reached in PBS. For long-term investigations, the sensors were stored in a 5mM glucose PBS solution that was renewed every 2 days and the response sensitivity (S) was repeatedly assessed by 1) measuring the response current (I_1) of a 5mM glucose solution, 2) adding a concentrated glucose solution into the measured solution to increase the glucose concentration to 15mM and 3) measuring the response current (I_2) of the resulting solution. The sensitivity was expressed as the current increase caused by a 1mM glucose increase, i.e. $S = (I_2 - I_1)/10$. 5mM and 15 mM glucose concentrations were selected because these concentrations were located in the linear response region (ca. 1-25mM) of the studied sensors. Similarly, the response time was also obtained when glucose concentration changed from 5mM to 15mM and represented as $T_{95\%}$, i.e. the time needed to reach 95% maximum current. Calibration plots were obtained by measuring the response currents at different glucose concentration steps. Chemical interference tests were performed by determining the anodic current variation when adding 1mM interferant to a 5mM glucose PBS solution. When not specified in the legends, the applied potential during amperometric measurements was 0.7V vs SCE.

4. RESULTS AND DISCUSSION

4.1. Influence of the coil geometry on the sensor function

A Teflon-covered platinum wire was used to examine potential electrode property changes caused by coiling. The top 10mm of the wire was stripped by removing the Teflon tube, then polished, cleaned and sonicated sequentially. To prevent leakage through the junction between the Teflon tube and the platinum wire, a sealant was applied before surface processing (see Figure 1). A series of cyclic voltammograms (CV) were recorded in a solution of 4mM potassium ferricyanide and 1mM KCl. The wire was further prepared into a coiled electrode and the same measurement was carried out once again. Figure 2 shows the typical CV curves from the straight platinum wire and after coils were made. The peak potential separation of the straight wire electrode was approximately 80mV, compared to 65mV when coiled. These values were a little larger than the theoretical value of 59mV for a reversible electrochemical reaction. This may be attributed to higher solution resistance due to the low concentration of supporting electrolytes and surface roughness of the platinum wire due to the difficulties of polishing. Other electrochemical behavior such as the peak current ratio of approximately one and the proportional relationship of I_p vs $\nu^{1/2}$ were in the expected range for a reversible one-electron oxidation-reduction process. This verified that the coiling of the wire did not change the electrochemical property of the electrode except for the reduction of the electrochemically effective surface area. The electroactive area of the electrode may be estimated by using CV peak currents and the reported diffusion coefficient(11). Based on the peak currents, the measured area of 10mm long wire (ϕ0.125mm) was 0.056±0.002cm^2 before coiling and 0.041 ±0.001cm^2 after coiling (n=5).

4.2. Function of GOD impregnated cotton and *o*-PPD Film in Sensors

Cotton fibers used in our sensor design play an important role by: (1) eliminating air bubbles that may be entrapped in the chamber during coating, (2) stabilizing the enzyme gel inside the chamber and (3) making the enzyme solution easier to remain in the coils. In this study, we attempted to find out if the additional enzyme in the cotton fibers would affect the performance of the sensors. We also investigated if an additional *o*-PPD film to the GOD impregnated cotton would improve the response stability. For these studies, Nafion was selected to serve as the outer diffusion-limiting membrane of the sensors because of its excellent permeability and durability. It should be noted that a thinner Nafion layer was used in these sensors than for the sensors described in the later sections in order to minimize the influence of membrane swelling. Four kinds of coil-type sensors with or without cotton fibers were fabricated by using different enzyme immobilization methods as described in Table 1, and then tested after being conditioned in PBS for three days. In Table 1, Method 1 refers to the use of a GOD-impregnated cotton inside the

Table 1. Influence of enzyme immobilization on response properties

Nafion-based glucose sensor		**1**	**2**	**3**	**4**
Electrode Area (A, cm^2)		0.030	0.031	0.028	0.028
Enzyme immobilization	Method 1	Absent	Absent	Present	Present
	Method 2	Present	Absent	Present	Absent
	Method 3	Present	Present	Present	Present
Nafion Outer Layer		Present	Present	Present	Present
Sensitivity (nA/mM)		1.3	2.6	6.1	8.8
Response Time (95%, s)		41	30	73	47

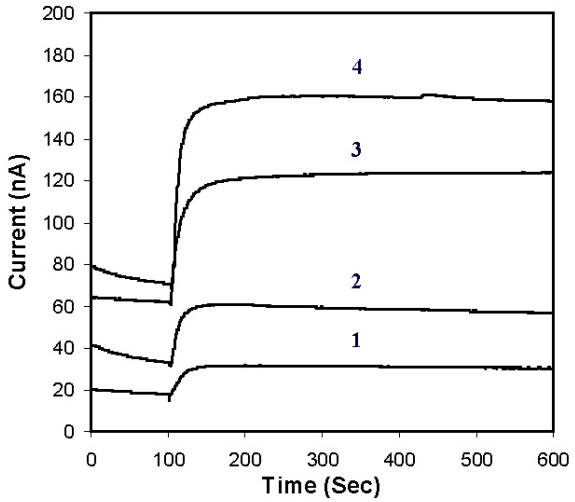

Figure 3. Amperometric response curves of the various electrodes listed in Table 1. Glucose concentration varied from 5mM to 15mM, without stirring.

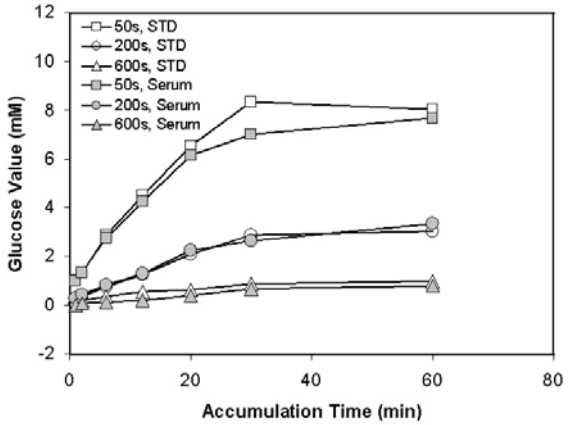

Figure 4. Calculated glucose concentration reflecting H_2O_2 accumulation in sensor. Measurements were carried in PBS after the sensor had been placed in a 15mM glucose solution or serum, without stirring for up to 60 minutes. The sampling times were 50, 200 and 600 sec; sensor: Pt/*o*-PPD/GOx/Nafion.

coil chamber. Method 2 refers to the use of an electrochemical GOD-co-deposited film and Method 3 to the use of a GOD cross-linked layer around the outer of the coil cylinder. Method 1 was always used in combination with Method 3 since the enzyme solution that impregnated the cotton in the coil chamber also coated the coiled electrode. In this case, GOD was present in both the coil chamber within the cotton and also as a coating over the coiled electrode. Our experimental set-up did not allow us to use Method 1 without Method 3. The *o*-PPD glucose biosensors have been extensively reported and commonly showed a short lifetime, typically 10 days (12,13). The short lifetime can be attributed to the limited enzyme loading in the electropolymerized film. Therefore, the *o*-PPD film (Method 2) was always thickened by a GOD cross-linked layer around the outer of the coil cylinder (Method 3) as described in our previous work(3).

The response time characteristics of these sensors were obtained by varying the glucose concentration from 5mM to 15mM as shown in Figure 3. The response curves 1-4 were obtained from the sensors 1-4 (see Table 1), respectively. Before testing, these sensors were polarized at +0.7V vs. SCE to completely eliminate the influence of background currents in PBS as well as the influence of H_2O_2 accumulation. The results showed that the sensors with additional enzyme in the coil chamber (sensors 3 & 4) had a larger response current than those without (sensors 1 & 2). No significant response delay was observed when the additional GOD in the coil chamber was used. Comparing sensors 1 to 2, and 3 to 4, respectively, the involvement of the *o*-PPD film resulted in a slight increase in the response time and a slight decrease in the response sensitivity. The decrease of sensitivity was probably caused by non-conductive polymer occupying electroactive sites at the platinum surface while the increase of response time can be attributed to the increase of the overall thickness of the enzyme layer. The linearity of the sensors is in the range of 1-25mM and no obvious difference can be observed among these sensors.

4.3. H_2O_2 accumulation occurring inside the cotton in the coil chamber

Even when the sensor is not polarized, the glucose oxidase continues to convert glucose to hydrogen peroxide and gluconic acid. Thus, accumulation of H_2O_2 in the coil chamber of the sensor can occur. When the polarized potential is applied, the H_2O_2 accumulated gradually during non-polarization periods can be oxidized at the inner surface of the coiled electrode and result in an apparent increased glucose level. In implantation applications, measurements are usually carried out in an intermittent manner. Therefore, it is very important to understand how the accumulated H_2O_2 can affect the measured results.

Figure 4 was obtained by measuring the response of the sensor in PBS after the sensor had been kept in a 15mM glucose solution or in 15mM glucose bovine adult serum

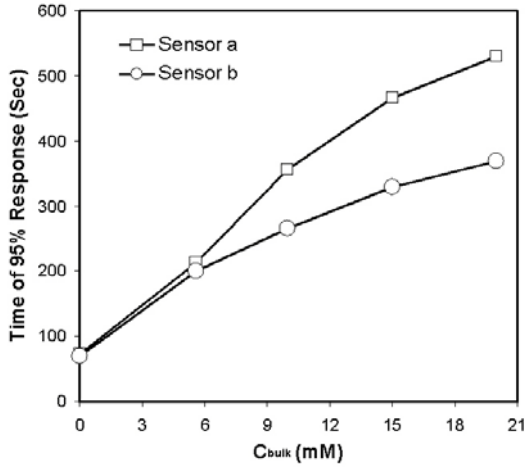

Figure 5. Influence of bulk glucose concentration on response time in accumulation process. Sensor a: Pt/*o*-PPD/GOx/Nafion with GOD impregnated cotton; Sensor b: Pt/*o*-PPD/GOx/Nafion without GOD impregnated cotton; accumulation time in bulk solutions: 30 min; measurements were carried in 5.6mM glucose/PBS without stirring.

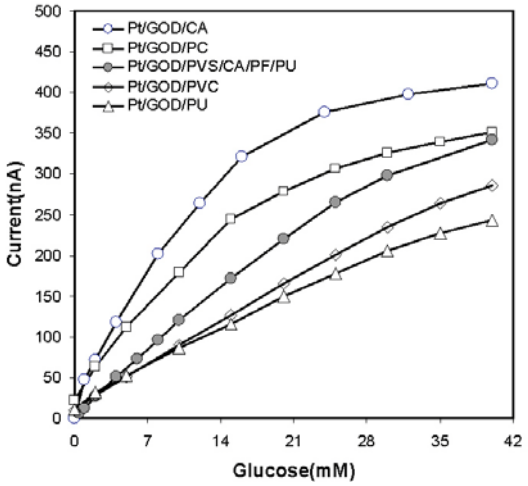

Figure 6. Calibration plots of coil-type glucose sensors with various polymer outer membranes. without stirring.

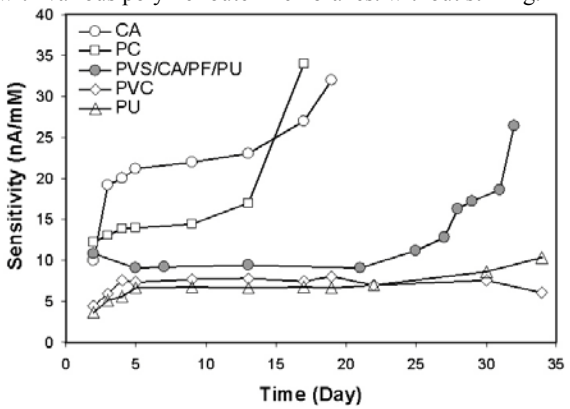

Figure 7. Long-term sensitivity variation of glucose sensors with different outer-membranes**.** All sensors were stored in 5mM Glucose/PBS solutions at room temperature.

for the indicated time (up to 60 min). The resulting currents were converted into glucose concentrations based on the current-concentration relationship that was obtained by pre-calibration in order to facilitate discussion.

The signals in the first 20s involved a component of charging current and the converted concentration might exceed bulk concentration (15mM), therefore, these data before 50s were not used for discussion. Data were collected at time 50, 200 and 600 seconds. The calculated glucose values for each sampling times (at 50, 200 and 600 sec.) were plotted against the accumulation time (up to 60 min.) (Figure 4). Comparing the two groups of results obtained from solutions (empty marks) and serum (solid marks), respectively, no significant difference in H_2O_2 accumulation was found. It implied that the amount of glucose diffusion inward the sensor was identical when the sensor was placed in a glucose solution and in serum. The longer the sensor was kept in glucose solutions or serum, the higher the H_2O_2 accumulation was found.

H_2O_2 accumulation also depended on glucose bulk concentration. Obviously, a higher glucose concentration gradient will force more glucose (which leads to more H_2O_2) into the sensor. Therefore, when the sensor is on standby in a higher glucose concentration and then exposed to a lower glucose concentration, a longer sampling time is needed to eliminate the influence of accumulated H_2O_2. Figure 5 gave the dependence of 95% response time on bulk concentration. The sensor (a) was kept in various glucose solutions (0-20mM) for 30 min and then measured in a 5.6mM glucose solution (the same as physiological concentration), where the 95% response time denoted the time at 5.88mM for the decreasing response variation (C_{bulk}≥5.6mM) or at 5.32mM for the increasing response variation (C_{bulk}<5.6mM). For comparison, the same measurements were implemented using another sensor (b) without GOD impregnated cotton. The response delay existed not only in sensor (a) but also in sensor (b) although the latter was less significant. This was because H_2O_2 accumulation in sensor (b) only occurred in the enzyme layer over the coiled platinum wire. The accumulation in the enzyme layer (6, Figure 1) can become as significant as with the inner gel (4, Figure 1) if a thick enzyme layer is used. When a thin enzyme layer was used, the accumulation in the inner gel was dominant (sensor (a)). It should be noted that although the H_2O_2 accumulation in the inner gel caused a response delay, the concentration conditions in Figure 5 were extreme and seldom occur in physiological status. Even so, the influence of accumulated H_2O_2 may be minimized by properly extending the sampling time and shortening the interval between two measurements.

4.4. Evaluation of several castable polymers in coil-type glucose sensors

Various castable membranes have been used as the outer membrane for glucose sensors, typically CA (14,15), PC(16,17), PU (15,18,19), and PVC (20,21). These various membranes were evaluated as possible outer membranes for coil-type glucose sensors with excess enzyme loading. A composite polymer membrane (PVC/CA/PF/PU) was also used to combine the various properties of the individual membranes, where PVC and PF provided interference inhibition, PU improved biocompatibility, and the CA layer was used as structural support. The various polymer films used in Figures 6 and 7 were formed using the same

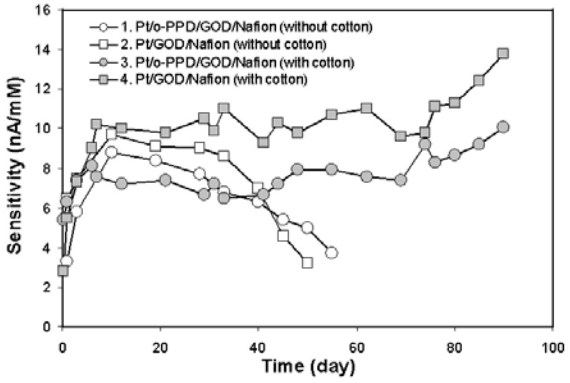

Figure 8. Long-term sensitivity variation of Nafion glucose sensors. All sensors were stored in 5mM Glucose/PBS solutions at room temperature. Sensors are described in

numbers of dip-coatings with a 2% w/v polymer solution and therefore had a similar thickness (ca. 10-20µm). All sensors were dried for 2 days at room temperature before testing.

The currents produced by sensors with different membrane configurations in response to varying glucose concentrations are showed in Figure 6. CA and PC provided higher response signals than PU, PVC and the composite membrane, indicating these two membranes have lower diffusion resistance for glucose. However they showed a narrower linearity range (1-15mM). Despite the diffusion properties of PU and PVC being similar (linearity up to 30mM), PU showed better and more stable response characteristics. The composite membrane showed an excellent response to glucose in the range of 1-20mM and could be used for at least 20 days in aqueous solutions. The long-term sensitivity variation of these sensors can be found in Figure 7. A common phenomenon was that the sensitivity increased rapidly when the sensor started to fail to respond. The sensitivity rise implied that structural defects had appeared in the polymer membranes and therefore the membranes became less resistive to glucose molecular. Membrane defects can be created during the membrane coating or swelling process when the membrane material lacks structural strength. The swelling of enzyme gel imposed an extra tension upon the outer polymer, and as a result, defects could form. These defects could be observed using SEM. Defects can develop into cracks in days and ultimately result in the enzyme gel gradual dissolution or degradation through the cracks.

Although CA and PC have better membrane permeability, their poor mechanical durability make them unsuitable to be used as the outer membrane of implantable glucose sensors. In contrast, PVC and PU have better membrane strength. PVC is widely used for a support matrix of ion-selective electrodes under heterogeneous and water swelling conditions (22) but seldom used in amperometric sensors (20) due to its hydrophobic nature. In fact, PVC-based glucose sensors were experimentally found to be unstable and drift was observed all the time.

The above polymers can be ranked based on their hydrophilic properties according to the following sequence: CA>PU>PC>PVC. It was reported that more hydrophobic materials have a higher risk of adsorbing proteins (23). PU can meet the requirements of implantable applications and has been frequently used as the outmost layer of the sensing element (15, 18, 19). We found that our coil-type sensor with PU can work stably for about 40 days in solutions. The sensor based on the composite membrane showed a somewhat longer lifetime than CA or PC (but shorter than PU), implying that improvement in membrane characteristics over CA or PC alone may be realized by combining several layers of different polymers. The present composite membrane may indeed be a mixture membrane without apparent layer boundaries because of intersolubility in mutual solvents. Although the improvement from the composite membrane is not large, influences of polymer blend and additives on membrane structure and property are worth further investigations.

4.5. Long-term function resulting from excess enzyme loading

The best method to evaluate the long-term stability of a glucose sensor is to trace the sensitivity rather than the response current as reported (24,25) because the response current can be readily affected by background current or accumulated H_2O_2 in the enzyme layer. Background currents can be different in different swelling stages of membrane layers while H_2O_2 accumulation may result in positive measurement errors. The sensitivity can directly reflect enzyme activity changes if no significant changes occur in the properties of the outer polymeric membrane (15). Figure 8 repeatedly recorded the response sensitivities of four coil-type Nafion glucose sensors over 90 days. Nafion, a negatively charged copolymer of tetrafluorosulfonated and sulfonyl fluoride vinyl ether (26), has an excellent stability after high-temperature curing (10). Because both hydrophobic fluorinated groups and strong hydrophilic sulfonyl groups appear at the polymer chains, Nafion has been extensively used as the outer membrane of glucose sensors (27, 28). The sensors with cotton in the coil chamber showed a much longer lifetime than those without cotton, indicating that the cotton played the enzyme reservoir role. The sensitivity of the cotton-filled sensors increased in the first week then maintained a stable level over 60 days at least. The initial increase of sensitivity commonly occurred for the relatively thick membrane including both enzyme and Nafion layers. When the membrane was thinner, the rising time to reach a stable sensitivity was shorter. Fluctuations for the sensitivity values measured at different days may result from contamination or experimental inaccuracy. Most sensors started to show an increasing sensitivity after 90 days, indicating that the Nafion membrane started to deteriorate. In addition, no strong evidence supported that the involvement of the electropolymerized film can bring improvement to the lifetime of the sensor.

4.6. Interference investigation

Some substances in physiological fluids such as L-ascorbic acid, acetaminophen and uric acid can be oxidized at the applied potential for hydrogen peroxide

Table 2. ΔC_{glu} values caused by 1mM interferants

Electrode and Internal Layer		Pt/GOx/						Pt/ o-PPD	Pt/o-PPD/GOx/
Outer Membrane		CA	PC	PU	PVC	Multi	Nafion		Nafion
ΔC_{glu} (mM)	L-Ascorbic Acid	+5.0±0.9	+7.6±1.4	+1.1±0.5	+1.7±0.6	+3.0±1.1	+3.6±0.5	+1.7±0.7	+0.2±0.1
	Acetaminophen	+21±4	+22±3	+34±4	+14±2	+10±2	+9.0±1.0	+18±3	+10.0±0.5
	Uric Acid	+9.0±2.1	+5.2±1.5	+1.5±0.9	+3.2±1.2	+2.0±1.0	+1.3±0.8	+3.4±1.6	+0.5±0.2
	Creatinine	-5.0±1.3	-6.2±1.7	-2.4±0.5	-1.1±0.3	-1.6±0.4	-0.8±0.3	-0.8±0.2	-0.9±0.2

Table 3. Serum glucose measurement correlation of various sensors vs. FreeStyle strips. Applied potential=0.7V (vs. Ag/AgCl)

Glucose Sensor	Measured Values Serum Glucose (mmol/L)					Correlation Equation (vs FreeStyle)	r^2
	No.1	No.2	No.3	No.4	No.5		
Pt/GOx/CA	1.53	4.89	7.63	11.62	13.07	$C=0.959C_F - 0.103$	0.997
Pt/GOx/PC	1.53	4.62	7.54	10.63	12.06	$C=0.871C_F + 0.142$	0.999
Pt/GOx/PU	1.52	5.43	8.80	12.53	14.30	$C=1.052C_F - 0.093$	0.999
Pt/GOx/PVC	1.53	4.34	6.71	9.81	11.19	$C=0.797C_F + 0.184$	0.998
Pt/GOx/PVS/CA/PF/PU	1.44	4.81	8.20	11.30	13.03	$C=0.955C_F - 0.060$	0.999
Pt/GOx/Nafion	1.54	4.46	7.45	9.93	11.36	$C=0.809C_F + 0.329$	0.999
Pt/PPG/GOx/Nafion	1.73	4.10	7.04	9.95	11.10	$C=0.793C_F + 0.290$	0.999
FreeStyle test strips	1.67	5.00	8.56	12.00	13.70	$C= C_F$	1

oxidation and result in erroneous glucose values. The interference effect of several substances on various sensors has been widely examined, but reported in different ways (2, 29). The objective of this interference experiment was to compare interference inhibition function of various polymer membranes. The sensors with various inner membranes and outer membranes as described in Table 2 were pre-calibrated with 5 and 15mM glucose solutions before interference measurements. The experiment was carried out in two steps. We first recorded the response current (I_{S1}) of the sensor in 5mM glucose solution until it stabilized and we then added C_{IN} mM interferant to the solution and recorded the interference current (I_{IN}). The measured glucose concentration difference (ΔC_{glu}) caused by 1mM interferant was calculated by

$$\Delta C_{glu} = \frac{(I_{IN} - I_{S1})}{S.C_{IN}} \quad (3)$$

where S was sensitivity of the sensor.

Most investigations on interference have been focused on L-ascorbic acid, acetaminophen and uric acid because they have relatively high physiological concentration (0.11mM, 0.17mM and 0.48mM, respectively) (29). Creatinine showed a negative interference to glucose measurements but this interference was indeed negligible in its physiological concentration (20μM).

The tabulated ΔC_{glu} values have verified that the material and nature of the polymer outer membranes had profound influences on the interference species. Highly permeable polymers such as CA and PC also allowed the passage of various interferants. Conversely, PU and PVC showed high resistance to L-ascorbic acid, uric acid and creatinine. The interference effect of acetaminophen was significant and could be partially inhibited by Nafion and PVC. The multi membrane (PVS/CA/PF/PU) was apparently better than CA, PC and PU in preventing interference. It was also found that the *o*-PPD-based Nafion sensor offered more protection against interference from L-ascorbic acid and uric acid.

4.7. Performance of various coil-type sensors in bovine adult serum

Serum samples (No.1- No.4, Table 3) with different glucose concentrations were prepared by diluting original bovine adult serum (No. 5) with PBS solutions. All sensors were pre-calibrated by using 0, 2, 5, 10, 15mM glucose standard solutions at 0.7V vs. a flexible Ag/AgCl reference electrode. To eliminate the influence of accumulation, the sensors were polarized in the PBS solution until a stable background current was reached before serum measurements. Serum measurements were implemented one by one in the sequence of No.1 to No. 5 and the sensors were deproteinized after each test by simply dipping in a 5% NaClO solution and then by rinsing with PBS solutions. Correlations between the measured values obtained from each coil-type sensor and FreeStyle glucose meter in bovine adult serum have been given in Table 3. It was observed that the measured value (C) was closer to the assigned value (C_F) when the polymer was more hydrophilic. This meant that the behavior of CA and PU in serum was similar to that in aqueous solutions and less affected by protein. PC, PVC and Nafion could also perform well although the measured values were commonly lower than C_F.

5. CONCLUSION

This study demonstrates that the introduction of the inner enzyme gel in the coil-type glucose sensor can significantly improve the long-term stability of the sensor. The excess enzyme in the inner gel can maintain constant response sensitivity for at least 60 days when the outer polymer membrane is not damaged. In the above long-term observation, function failure of the sensor was mainly attributed to outer membrane deterioration rather than enzyme function loss. Nafion exhibited the longest lifetime in contrast to CA, PC, PU and PVC, but it still not long enough for the goal of long-term implantation applications.

Further efforts must be focused on improving the outer membrane or seeking new membrane material which has better durability as well as interference-eliminating nature. H_2O_2 accumulation in the inner gel can influence the measurements to some extent but can be solved technically by improving the measurement procedures. Therefore, the concept introducing excess enzyme by structure design can be potentially used in other sorts of sensors.

6. ACKNOWLEDGMENTS

This work was supported in part by NIH/NIBIB grant #1R01 EB01640-01 to Dr. F. Moussy and Co-Investigators.

7. RERFERENCES

1. Valdes T.I. & F. Moussy: A ferric chloride pre-treatment to prevent calcification of Nafion membrane used for implantable biosensors. *Biosens Bioelectron* 14(6), 579-85 (1999)
2. Yang Q., P. Atanasov & E. Wilkins: Development of needle-type glucose sensor with high selectivity. *Sens Actuators* B 46, 249-256 (1998)
3. Moussy F., S. Jakeway, D.J. Harrison & R.V. Rajotte: In vitro and in vivo performance and lifetime of perfluorinated ionomer-coated glucose sensors after high-temperature curing. *Anal Chem* 66, 3882-3888 (1994)
4. Moussy F. & D.J. Harrison: Prevention of the rapid degradation of Ag/AgCl reference electrode after subcutaneous implantation in rats. *Anal Chem* 66, 674-679 (1994)
5. F. Moussy, D.J. Harrison, D.W.O'Brien & R.V. Rajotte: Performance of subcutaneously implanted needle-type glucose sensors employing a novel tri-layer coating. *Anal Chem* 65, 2072-2077 (1993)
6. Bindra D.S., Y. Zhang, G.S. Wilson, R. Sternberg, D.R. Thevenot, D. Motti & G. Reach: Design and in vitro studies of a needle-type glucose sensor for subcutaneous monitoring. *Anal Chem* 63, 1692-1696 (1991)
7. Heller A.: Subcutaneous glucose electrode. U.S. Pat. 6,514,718 (2002)
8. Abel P.U., T. von Woedtke, B. Schulz, T. Bergann & A. Schwock: Stability of immobilized enzymes as biosensors for continuous applications in vitro and in vivo. *J Mol Catal B: Enzymatic* 7, 93-100 (1999).
9. Clark L.C.JR.: Implantable glucose sensor. U.S. Pat. 6,343,225 (2002)
10. Mercado R.C . & F. Moussy: In vitro and in vivo mineralization of Nafion membrane used for implantable glucose sensors. *Biosens Bioelectron* 13, 133-145 (1998)
11. Arvia A.J., J.C. Bazan & J.S.W. Carrozza: The diffusion of ferrocyanide and ferricyanide ions in aqueous solutions of potassium hydroxide. *Electrochimica Acta* 13, 81-90 (1968)
12. Malitesta C., F. Palmisano, L. Torsi & P.G. Zambonin: Glucose fast-response amperometric sensor based on glucose oxidase immobilized in an electropolymerized poly(o-phenylenediamine) film. *Anal Chem* 62, 2735-2740 (1990)
13. Vidal J.C., S. Mendez & J. R. Castillo: Electropolymerization of pyrrole and phenylenediamine over an organic conducting salt based amperometric sensor of increased selectivity for glucose determination. *Anal Chim Acta* 385, 203-211 (1999)
14. Choi S.H., S.D. Lee, J.H. Shin, J. Ha, H. Nam & G.S. Cha: Amperometric biosensors employing an insoluble oxidant as an interference-removing agent. *Anal Chim Acta* 461, 251-260 (2002)
15. Ward W.K., L.B. Jansen, E. Anderson, G. Reach, J.C. Klein & G.S. Wilson: A new amperometric glucose microsensor: in vitro and short-term in vivo evaluation. *Biosens Bioelectron* 17(3), 181-189 (2002)
16. Linke B., W. Kerner, M. Kiwit, M. Pishko & A. Heller: Amperometric biosensor for in vivo glucose sensing based on glucose oxidase immobilized in a redox hydrogel. *Biosens Bioelectron* 9, 151-158 (1994)
17. Faridnia M.H., G. Palleschi, G.J. Lubrano & G.G. Guilbault: Amperometric biosensor for determination of lactate in sweat. *Anal Chim Acta* 278, 35-40 (1993)
18. Madaras M.B., I.C. Popescu, S. Ufer & R.P. Buck: Microfabricated amperometric creatine and creatinine biosensors. *Anal Chim Acta* 319, 335-345 (1996)
19. Jaffari S. A. & J. C. Pickup: Novel hexacyanoferrate (III)-modified carbon electrodes: application in miniaturized biosensors with potential for in vivo glucose sensing. *Biosens Bioelectron* 11, 1167-1175 (1996)
20. Reddy S. M. & P. Vadgama: Amperometric biosensors employing an insoluble oxidant as an interference-removing agent. *Anal Chim Acta* 461(2), 57-64 (2002)
21. Reddy S.M., B. Gangadharan & J. Manini: Amperometric sensor study of the selectivity of poly(vinyl chloride) membranes plasticised with surfactants and liquid crystals. *Anal Chim Acta* 508, 71-78 (2004)
22. Hirose S., E. Yasukawa, M. Hayashi & W.R. Vieth: Wet poly(vinyl chloride) membrane as a support: Sorption and transport of low-molecular-weight organic compounds and proteins. *J Membr Sci* 11, 177-185 (1982),
23. Uyen H.M.W., J.M., Schakenraad, J. Sjollema, J. Noordmans, W.L. Jongebloed, I. Stokroos & H.J. Busscher: Amount and surface structure of albumin adsorbed to solid substrata with different wettabilities in a parallel plate flow cell. *J Biomed Mater Res* 24, 1599-1614 (1990)
24. Van Os P.J.H.J., A. Bult & W. P. van Bennekom: A glucose sensor, interference free for ascorbic acid. *Anal Chim Acta* 305, 18-25 (1995)
25. Uang Y.M. & T.C. Chou: Fabrication of glucose oxidase/polypyrrole biosensor by galvanostatic method in various PH aqueous solutions. *Biosens Bioelectron* 19, 141-147 (2003)
26. Tsonos C., L. Apekis & P. Pissis: Dielectric properties of hydrated Nafion-(SO3K) membranes: thermally stimulated depolarization currents. *J Mater Sci* 33, 2221-2226 (1998)
27. Gogol E. V., G. A. Evtugyn, J. L. Marty, H. C. Budnikov & V. G. Winter: Amperometric biosensors based on nafion coated screen-printed electrodes for the determination of cholinesterase inhibitors. *Talanta* 53, 379-389 (2000)
28. Nam J., N. Kim & V. Volotovsky: Urease-based biosensor for mercuric ions determination. *Sens Actuators* B 42, 233-237 (1997)
29. Yasuzawa M. & A. Kunugi: Properties of glucose sensors prepared by the electropolymerization of a

positively charged pyrrole derivative. *Electrochem Com* 1, 459-462 (1999)

Key words: implantable glucose sensor, enzyme loading, glucose oxidase, biosensor, lifetime improvement, electrochemical sensor, Review

Send correspondence to: Francis Moussy, Ph.D, Department of Chemical Engineering, University of South Florida, 4202 E. Fowler Avenue, ENB 118, Tampa, FL 33620-5350, Tel: 813 974 5840, Fax: 813 974 3651, Email: fmoussy@eng.usf.edu

http://www.bioscience.org/current/vol10.htm