***In vitro* and Short-term *in vivo* Characteristics of a Kel-F Thin Film Modified Glucose Sensor**

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A new outer layer composition, consisting of polytetrafluoroethylene (PTFE), Kel-F oil, and Nafion, is suggested to minimize the detrimental effect of dissolved oxygen and to extend the linear response range of a glucose oxidase (GOx)-based sensor using nonconducting polymer. The morphology of Kel-F/PTFE/Kel-F/Nafion polymeric laminate was followed during fabrication by SEM. When Kel-F film was formed on the PTFE outer layer, the linear response was extended to 21 mM, at a sensitivity of 2.8 ± 0.8 nA/mM mm². We demonstrate that a sensor without Kel-F/PTFE/Kel-F/Nafion outer layer is relatively oxygen dependent, whereas by comparison a sensor with Kel-F/PTFE/Kel-F/Nafion outer layer is oxygen independent. The current of such a glucose sensor implanted in the subcutaneous tissue stabilized within 60 min, and the lag between blood glucose changes and sensor output was within 1 min. The *in vivo* characteristics of the glucose sensor described show great promise for one-point *in vivo* calibration.

(Received June 2, 2003; Accepted July 25, 2003)

Introduction

The enzyme-based biosensors for continuous glucose monitoring have long been investigated as a promising method of improving glycemic control in persons with diabetes. However, *in vitro* and *in vivo* functions of sensors still remain to be further improved to allow wide-spread use of an implantable real-time sensor.

First of all, as for a GOx-based sensor, which requires oxygen as a co-substrate to carry out glucose oxidation, it is important to examine the so-called "oxygen effect", which means the dependence of the sensor performance on the oxygen supply. Zhang *et al.*¹ demonstrated that the demand for oxygen by the sensor is primarily determined by its sensitivity, because the absolute current output is a direct measure of the rate of oxygen consumption. Non-linear sensors exhibit a strong oxygen dependence because the overall process depends on oxygen and glucose fluxes. Many efforts to minimize the oxygen effect have included the use of proper membrane coverage that improves the surface availability of oxygen relative to glucose. Gough *et al.*² reported a novel sensor configuration in which oxygen diffuses into the membrane from two directions while glucose diffuses from only one. Wang and Lu³ used Kel-F oil, which has very high oxygen solubility, as an internal supply of oxygen for a carbon paste electrode. However, this type of sensor has not yet been successfully implanted in a body to monitor glucose concentration in diabetes. Zhang *et al.* constructed a practically useful implantable glucose sensor, which was essentially free of oxygen fluctuation, by controlling

the sensor's sensitivity and linearity. Sensitivity was controlled by the thickness of an outer polyurethane membrane. The outer layer served to increase the relative oxygen supply because it functioned only as a diffusion barrier to glucose, while it imposed little or no effect on oxygen diffusion.

The second issue is to develop a reproducible and predictable method for sensor fabrication. When an enzyme layer is prepared on a miniaturized/microfabricated needle or a microfabricated electrode, electropolymerization has valuable advantages over solvent casting because it is not limited in terms of the geometry and area of the electrode. The method also offers a site-selectivity that makes it suitable for the development of a multianalyte-sensing system, and the uniformity of the polymer film on the electrode surface can cope with more complex geometries.^{4,5} Yang *et al.* fabricated a miniaturized glucose sensor using microfabrication and electropolymerization techniques.⁶ Glucose oxidase (GOx) was entrapped during the electropolymerization of a 1,3-diaminobenzene (1,3-DAB) on a recessed rectangular microfabricated platinum electrode. On the other hand, many GOx-based sensors prepared by electropolymerization have suffered from problems due to a restricted linear response range, though they have shown high sensitivities and fast responses. As was demonstrated in the study reported by Zhang *et al.*, the restricted linear response range results from a stoichiometric limitation of the enzyme reaction by oxygen. Therefore, the glucose sensitivity of a sensor should be adjusted by applying an outer membrane to extend the linear response range of a sensor.⁷ Yang *et al.* extended the range of linear response to higher concentration by using Teflon and polyurethane film on a recessed rectangular microfabricated platinum electrode as an outer layer.

As the third issue of biocompatibility, in spite of the very high

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number of papers concerning enzyme immobilization in electrogenerated polymeric films, relatively few have successfully addressed the fabrication and characterization of implantable needle-type glucose sensors for practical *in vivo* applications.⁸ In a previous study,^{6,9} we used PTFE as a permselective diffusion-limiting biocompatible outer layer on the enzyme layer, consisting of poly(1,3-DAB), glutaraldehyde, poly-L-lysine, and GOx. It was demonstrated that PTFE dispersed in aqueous media could be applied as an outer membrane on the electropolymerized enzyme layer. In spite of the improved linear response range of the sensor, however, it had unsatisfactory linearity, mechanical stability, and biocompatibility, which prevented it from being implanted *in vivo*.

We report here upon studies on the construction of a new outer layer, based on a Kel-F thin film. The film was used to extend the linear response range as well as to minimize the oxygen effect of the glucose sensor. To enhance the biocompatibility of the sensor, Nafion, which has been widely used as a biocompatible layer, was coated as an outer film in this study. Finally, the *in vivo* performance was evaluated using the finished sensor. Morphological changes of the electrode surface during sensor fabrication and the cross sections of Pt/poly(1,3-DAB), glutaraldehyde, poly-L-lysine, and GOx/Kel-F/PTFE/Kel-F/Nafion were determined by SEM.

Experimental

Materials

Glucose oxidase (E.C. 1.1.3.4, high purity, 250000 unit/g, without O₂ type II-S, *Argellius niger*), D-(+)-glucose, and acetaminophen (AP) were used as supplied by Sigma. L-Ascorbic acid, 1,3-diaminobenzene (1,3-DAB, 99+%), glutaraldehyde (25wt% solution in water), dispersed polytetrafluoroethylene in water (60%, PTFE solution), and Nafion were purchased from Aldrich and used without further purification. Poly-L-lysine (hydrobromide salt with molecular weight, 23400) was used after it had been dialyzed in 0.1 M NaCl. Poly-L-lysine solution was stored at 4°C when not in use. Kel-F oil was generously donated by Powderchek (Fremont, CA), for which we are very grateful.

Instrumentation

Electrochemical experiments were performed with a Model cDAQ-1604 multichannel potentiostat (ELBIO Co., Seoul). Ag/AgCl (3 M KCl) and platinum wire were used as the reference and counter electrodes, respectively. Acquired data were transferred to a personal computer using USB protocol. The control and data acquisition program was written in LabVIEW software (National Instrument, version 5.0). When the outer membrane was formed, it was dehydrated in a vacuum oven (Lab-line, 3606-1CE). The surface morphologies of fabricated electrodes were examined under the scanning electron microscope (SEM) (Model JSM 840-A).

Fabrication of sensors

The glucose sensor was fabricated as reported previously by Chung *et al.*⁹ Teflon-coated platinum-iridium (Pt/Ir) alloy wires were employed as an electrode body for the needle-type of sensor. The Teflon skin of the wire was removed from the end so that the area of the exposed bare Pt/Ir surface was 1.72 mm² (3 mm in length). The electropolymerization of the 1,3-DAB was carried out using five consecutive cycles between +0.2 and +1.0 V at 2 mV/s. After rinsing with deionized water, the

enzyme electrode was dipped in an ethanol solution containing 20% Kel-F oil and then in a PTFE dispersion diluted to 30%. The electrode was again dipped into an ethanol solution containing 20% Kel-F oil. Finally, the Nafion film was coated using a 1.5% Nafion solution. The electrode was dried under vacuum at 30°C for 1 h after each coating process.

In vitro and *in vivo* evaluations of the sensor

Experiments were performed in 0.1 M phosphate buffered saline (PBS) solution containing 0.15 M NaCl and 0.1 g/L NaN₃, and also in a temperature-controllable cell containing 15 ml of PBS (pH 7.4, 37°C). The effect of oxygen tension was investigated by bubbling oxygen-nitrogen mixtures at various partial pressures into the test solution. During the experiment, pO₂ was maintained at a constant levels throughout the solution and the cell atmosphere. pO₂ was measured using a DO meter (Pentagon 777, BioTel Co.). A serum test in undiluted serum and animal experiments were carried out to examine the biocompatibility of the glucose sensor. Ten male Sprague Dawley rats weighing 400 – 500 g were used in the *in vivo* studies. Each animal was anaesthetized with 50 mg of ketamine given intramuscularly. After the neck was shaved, indwelling catheters were inserted into the jugular vein and carotid artery. To insert the glucose sensor subcutaneously, a 1.5 cm incision was made in the dorsal area, and the sensor was then inserted into the tunnel created by the blunt dissection. Care was taken to avoid any bleeding and no blood was observed in any tunnel during implantation. Immediately and the day after the sensor insertion, a continuous sensor recording was performed. During the first 60 min, the sensor output was allowed to stabilize. Although initially the output was unstable, current outputs at *ca.* 1 h after insertion were stable. An intravenous glucose tolerance test was then performed in the awake, unstressed, and fasting state. Wires in the sensor were connected to a potentiostat, the sensor was held at a standing potential of 0.4 V. Currents were recorded digitally. A 50% glucose solution (300 mg/kg body weight) was injected into a jugular vein catheter in 20 to 30 s, and blood samples were collected from the carotid artery. All blood samples were analyzed in duplicate using a Glucose Analyzer II (Beckman).

Results and Discussion

In vitro test and scanning electron microscopy

Figure 1a shows a schematic diagram of the glucose sensor. As reported in a previous study,⁹ the enzyme layer was deposited by cycling the potential between 0.2 and 1.0 V in a 1,3-DAB solution containing GOx, glutaraldehyde, and poly-L-lysine, whereas the outer layer was formed by dip-coating in a PTFE, Kel-F oil, and Nafion solution. Unlike the previous layer structure, the Kel-F and Nafion films were added as a glucose diffusion limiting membrane and a biocompatible layer, respectively. Although the SEM cross sectional image (Fig. 1b) does not discriminate between the individual layers, it shows that the total thickness of the layers is *ca.* 3 µm. As indicated in Fig. 1(b), the dark layer in the lower part is PTFE of thickness *ca.* 2 µm and the bright layer on the top of the PTFE layer is assigned to be the Kel-F plus Nafion layer.

The effect of the Kel-F coating as a glucose diffusion barrier is shown in Fig. 2. The linear response range was extended to 21 mM whereas the sensitivity became much lower when the Kel-F film was coated onto the PTFE outer layer, which is porous and has some defects. This result corresponds to the thinking of Zhang *et al.*, who adjusted glucose sensitivity by

Fig. 1 (a) A schematic diagram of the constructed glucose sensor. (b) A SEM image of the cross section of Pt-Ir/poly(1,3-DAB), GOx, glutaraldehyde, poly-L-lysine/Kel-F/PTFE/Kel-F, the surface of which is shown in Fig. 3(e).

using a polyurethane membrane in a GOx-based glucose sensor. The Kel-F coating decreased the diffusion rate of glucose into the enzyme layer, but left the oxygen diffusion unaffected, resulting in an increased relative oxygen supply. Perfluorocarbon polymer layers have recently been reported to serve as a glucose diffusion limiting membrane and offer the prospect of enzyme-based biosensors with excellent longevities.¹⁰

Figure 3 shows morphology changes of the electrode surface during the sensor fabrication. Electrodeposition of 1,3-DAB gives a particulate film immobilizing GOx (Fig. 3b). The enzyme layer was so thin that surface cracks of several tens of nanometers on the bare Pt surface (Fig. 3a) remained visible even after the layer had been formed. In a previous report,⁹ the film thickness appeared to be 54 nm from an EQCM study. When Kel-F oil was cast on a poly(1,3-DAB) layer, it appeared as particulates (300 – 400 nm in diameter) leaving the poly(1,3-DAB) surface uncovered (Fig. 3c). However, when PTFE was cast in the same situation, it enhanced the homogeneity of the film (Fig. 3d). The PTFE layer homogeneously covers the previous layer with very fine particles, and results in a flat morphology. The PTFE layer also plays a role as a good substrate for Kel-F deposition. The SEM image in Fig. 3e shows that the Kel-F layer cast onto a PTFE layer forms a more compact layer compared to the first Kel-F layer on poly(1,3-DAB) (Fig. 3c). It is expected that the Kel-F layer covering the sensor over its entire surface range will reduce the oxygen effect.

Nafion has been widely used as an outer layer to reduce glucose diffusion and to improve biocompatibility in implantable miniaturized glucose sensors.^{11–14} When Nafion, as a biocompatible membrane, is cast on the Kel-F layer, it is

Fig. 2 The effect of outer layer as a diffusion-limiting barrier. Current responses for sensors with (a) PTFE, (b) Kel-F/PTFE/Kel-F, and (c) Kel-F/PTFE/Kel-F/Nafion in PBS. Steps correspond to the current responses of sensors on adding 1, 3, 6, 9, 12, 15, 18, and 21 mM of glucose. Ascorbic acid (AA) and acetaminophen (AP) were added to assess interference.

deposited more compactly with smaller particle size than Kel-F (Figs. 3e and 3f). The PTFE on the enzyme membrane are from the aqueous phase, in which clusters of PTFE particles are physically dispersed. It is no wonder that the clusters of PTFE particles deposited form the coarse PTFE membrane, if one takes account of the facts that the size of the PTFE particles is quite large and they readily aggregate to make assemblies. The Kel-F oil supposedly does not easily fill the large crevices because of its low viscosity. As a result, more analytes penetrate the PTFE membrane. It is believed that the polymer backbone of Nafion is basically hydrophobic enough to coat the PTFE as well as Kel-F. The interstitial space among the particle assemblies can be effectively stuffed by exposure to the Nafion solution. The more compact outer membrane structure is expected to slow down the rate of mass transfer, and thereby decrease the oxygen effect. Table 1 summarizes the sensitivities, currents, and response times of the two types of outer layer compositions, that is Kel-F/PTFE/Kel-F, and Kel-F/PTFE/Kel-F/Nafion. Each background current stabilizes within 30 min and this proved to be very reproducible, to within 1.0 nA. Sensitivities for Kel-F/PTFE/Kel-F, and Kel-F/PTFE/Kel-F/Nafion were 2.8 ± 0.8 and 1.6 ± 0.6 nA/mM mm², respectively, where the response times (t_{90}) of both sensors were less than 60 s in quiescent solution and less than 5 s in constantly stirred solution. Nafion has another advantage as an outermost membrane. It is soluble in a small quantity of alcohol and can be dispersed in water. Coating processes with Nafion cause minimal damage of previously immobilized enzymes, while most biocompatible hydrophobic polymers including polyurethane require organic solvents that are fatal to enzymes.

Oxygen effect

The glucose oxidase-based sensor requires oxygen as a co-substrate to carry out the glucose oxidation. Therefore, it is important to examine the effect of oxygen upon the performance of the sensor.¹⁵ Zhang *et al.* investigated the relationship between sensitivity and linearity at several oxygen partial pressures, and concluded that it is essential that the sensitivity should be suppressed to such a degree that the rate of oxygen consumption by the sensor is negligible compared to the rate of tissue oxygen supply. As already mentioned, many

Fig. 3 SEM images showing morphological changes of the electrode surface during sensor fabrication. (a) Bare Pt; (b) after poly(1,3-DAB) was electrodeposited with immobilized GO_x; (c) after coating with Kel-F; (d) after coating with PTFE; (e) after re-coating with Kel-F; (f) after a final coating with Nafion.

Fig. 4 Calibration curves under various oxygen tensions (air: circles, 40 mmHg: squares, 15 mmHg: triangles) for sensors with (filled symbols) and without (open symbols) an outer layer of PTFE/Kel-F/Nafion. *In vitro* measurements were performed in 0.1 M PBS (pH 7.4, 37°C) with constant stirring.

Fig. 5 Current-time responses of a glucose sensor with a Nafion outer layer (a) in PBS (at glucose concentrations of 1, 3, 6, 9, 12, 15, 18, and 21 mM) and (b) in serum (at glucose concentrations of 4.6, 6, 9, 12, 15, 18, and 21 mM). Currents during stabilization are omitted. Continuous monitoring data in serum containing 6 mM glucose (inset).

GO_x-based sensors prepared by electropolymerization have suffered from problems due to a restricted linear response range.

To solve this problem, we used Kel-F oil, which has remarkable oxygen solubility. Taking advantage of this property, Wang and Lu³ constructed a first-generation enzyme electrode, which consisted of a graphite powder and Kel-F oil, operating under severely depleted oxygen conditions. Based on the suppositions that the Kel-F thin film plays an important role in the oxygen-rich membrane and also provides a glucose diffusion-limiting barrier, the Kel-F thin film was formed on the PTFE outer layer (Fig. 3e). Figure 4 shows the calibration

curves obtained under various oxygen tensions for the sensors with and without an outer layer of Kel-F/PTFE/Kel-F/Nafion. The figure shows that a sensor without an outer layer is oxygen-dependent, whereas a sensor with an outer layer is relatively oxygen-independent. In terms of sensitivity and linearity, this result is consistent with that of Zhang *et al.*, rather than with that of Wang *et al.* Zhang *et al.* achieved a sensor with good linearity at very low oxygen partial pressure by lowering its sensitivity by thickening the outer layer. They suggested that optimal sensitivity for sensor preparation should be in the range

Table 1 *In vitro* characteristics of glucose sensors

Outer layer	Background current ^a / nA mm ⁻²	Sensitivity ^b / nA mM mm ⁻²	Response time (<i>T</i> _{90%})	Interference ^c /nA mm ⁻²	
				Ascorbic acid (0.11 mM)	Acetaminophen (0.17 mM)
Kel-F/PTFE/Kel-F	0.8 ± 0.4 (<i>n</i> = 7)	2.8 ± 0.8 (<i>n</i> = 7)	< 5 s ^c < 60 s ^d	0.5	0.0
Kel-F/PTFE/Kel-F/Nafion	0.5 ± 0.2 (<i>n</i> = 7)	1.6 ± 0.6 (<i>n</i> = 7)	< 5 s ^c < 60 s ^d	0.2	0.0

a. Background current is stabilized within 30 min. b. Over the concentration range 1 – 6 mM. c. In stirred solution. d. In quiescent solution. e. Interference is added at the maximum physiological concentration in blood.

Fig. 6 SEM images of different electrode surfaces after testing in serum. (a) Pt-Ir/poly(1,3-DAB), GOx, glutaraldehyde, poly-L-lysine/Kel-F/PTFE/Kel-F (Fig. 3e) after test. (b) Pt-Ir/poly(1,3-DAB), GOx, glutaraldehyde, poly-L-lysine/Kel-F/PTFE/Kel-F/Nafion (Fig. 3f).

of 1.5 – 3.6 nA/mM for *in vivo* sensor. Considering the sensitivities of sensors with a Kel-F and a Kel-F/Nafion outer membrane in Table 1, one can produce a practically useful glucose sensor that is free of oxygen fluctuations.

Serum and *in vivo* tests

In order to develop a reliable implantable glucose sensor, one must study and control the effects of the biological environment on the sensor. Therefore, the performance of the sensor with the outer layer of Kel-F/PTFE/Kel-F/Nafion was investigated in serum. In Fig. 5, current-time responses in PBS and undiluted serum are compared. The background currents were obtained from sensors stabilized for 30 min. Longer stabilization times were required in serum than in PBS. Although the sensitivity decreased a little, the current increased in proportion to the glucose concentration as concentrated glucose solution was added, and this was stable during the serum test. After the sensors were tested in serum, their surface morphologies were examined under an SEM (Fig. 6). It was evident that decreased sensitivity was due to the adsorption of biocomponents on the sensor surface.¹³ The SEM images that were obtained after dipping in serum samples do not show any significant

Fig. 7 Sensor signal obtained during an *in vivo* test (line) and glucose concentrations as determined using a glucometer (open circle).

difference between the outer membranes with and without Nafion. The observation indicates that the biocompatibility of the outer membrane is apparently not improved by coating with Nafion. After the serum test, the stability of the sensor during *in vitro* continuous monitoring was examined in serum containing 6 mM glucose. The current response was stable after 15 h (Fig. 5 inset).

For the *in vivo* evaluation, a glucose sensor was implanted into the subcutaneous tissue on the back of the neck of a male Sprague Dawley rat and the output of the glucose sensor was monitored. Plasma glucose was measured independently using a Glucose Analyzer II (Beckman). In Fig. 7, the sensor current had been allowed to stabilize for 60 min and the figure shows the sensor response to an injection of 1 ml of 50% glucose, and the following gradual signal decrease. It is noticeable that the lag time between changes in the blood glucose levels and the sensor response was within 1 min and the linearity extended to about 20 mM. In spite of the assumption of no lag time, the sensor output current correlated very well with blood glucose concentration. The sensitivity in subcutaneous tissue (*in vivo* sensitivity) was about 1 nA/mM and this was comparable to *in vitro* value. *In vivo* characteristics of the present glucose sensor are very promising for a one point calibration, considering that the apparent maximal rate of change of glucose in blood is 0.25 mM/min.¹⁶ More *in vivo* results will be presented in future papers.

Conclusion

We report here upon a method used to prepare a new outer layer of Kel-F film, to reduce the oxygen effect and to extend the

linear response range of a glucose sensor prepared by electropolymerization. Output from the finished sensor was almost independent of variations in the oxygen level. The sensitivity of the fabricated sensor was 1.6 ± 0.6 nA/mM mm², which is consistent with the optimal sensitivity for an *in vivo* glucose sensor as demonstrated by Zhang *et al.* That the Nafion coating enhanced biocompatibility was demonstrated by a serum test and short-term animal studies. The morphology of Kel-F/PTFE/Kel-F/Nafion was also characterized by SEM images. We believe that the Kel-F/PTFE/Kel-F/Nafion coating laminate structure shows great promise as the basis for the development of *in vivo* sensors based on the microfabricated electrode.

Acknowledgements

This work was supported by the Korea Science and Engineering Foundation through the Advanced Biometric Research Center.

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