Biocompatible Membranes and Coatings for Glucose Sensor

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Summary: This paper describes the preparation as well as evaluation of biocompatible membranes and coatings, for an amperometric glucose sensor in order to extend the linear range of the sensor characteristics. For this purpose, both Nafion and polyurethanes are highly suitable, as they increase the upper limit of linearity up to a factor of five. Nafion membranes underwent optimization of their diffusion behavior by temperature-controlled pressing. Sensors with coatings showed much smaller response times (one minute) than with membranes. In both cases, we achieved detection limits of 0.3 mmol/l glucose with a linear sensor characteristic of up to 10 mmol/l compared to 1.4 mmol/l for the unmodified electrodes. Furthermore, polymer coatings and membranes prevent clogging of the sensors by the bioanalytes.

Keywords: Amperometric sensing, Glucose, Oxygen, Nafion, Polyurethane.

Introduction

Diabetes mellitus is a chronic disease, while its treatment can be carried out on daily basis by self-monitoring of blood glucose followed by insulin injection. However, rough adjustment of insulin requirement or discontinuing its intake can cause severe hypoglycaemic episodes. Thus, there is a dire need to develop a continuous glucose monitoring system capable to access the blood glucose of patient. One of the major tasks in biosensor research for diagnostic purposes is the design of *in-vivo* glucose sensors [1-4] for patients suffering from diabetes mellitus. A variety of methods has been proposed including e.g. non-invasive [5] sensors or electrocatalytic methods with implanted flow-through cells [6] or based on nanoparticles [7-9]. However, most techniques rely on encymatic tests with glucose oxidase [10-13], as it is a very straightforward biochemical approach based on the following enzymatic conversion mention in the reaction below:

Several different ways are possible for electrochemical transducers to sense this reaction, such as measuring concentration changes of H_2O_2. However, a high potential (> +600 mV) of electrode may leads to cross-sensitivity towards other electro active substances. Mediators can help solving this problem, but they are often either unstable or toxic. To overcome these limitations, our sensor focuses on electrochemically detecting the oxygen rather than the peroxide - i.e. one of the starting materials.

For medical use in diabetes mellitus therapy, an implantable glucose sensor must comply with several requirements. Besides mechanical and electronic stability, these include miniaturization, short response times and a sensor characteristic with a linear calibration range at the physiologically relevant concentrations (3-10 mmol/l glucose in blood) being nearly insensitive towards other substances. Implantable biosensors should be more robust in comparison to *in-vitro* conditions because of aggressive biological environment and immune response. These requirements can be met by applying durable and biocompatible protective layers on sensitive sensors. Different materials are applied as protective membranes such as phosphorylcholines [14], polyurethane with phospholipids [15], hydrogels [16] to minimize biological reactions. But these materials are lack behind due to their mechanical strength and durability. Castable polymers and electropolymerized layers cannot be applied as protective layers because of their instability and rapid degradation, respectively [17]. Within this work, we have developed biocompatible Nafion, and polyurethanes [18, 19] protective layers and their evaluation is described in detail.

$$\mathrm{D-(+)-Glucose + O_2 + H_2O \xrightarrow{Glucose\text{-}oxidase} Gluconolactone + H_2O_2}$$

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Results and Discussion

Glucose oxidase retains its biological functionality during the immobilizing procedure, as can be seen in Fig. 1 depicting the electrode signal for solutions with different glucose concentrations. Amperometric sensor response is expressed in mV due to a feedback loop which compensates for current by a counter (balancing) voltage. Evidently, the sensor rapidly responds to glucose with reaction times being as low as about 1-5 seconds. It is already mentioned that electrode itself detects only oxygen; however, the signals obtained can be calibrated for glucose and validated by determining the respective glucose concentration with an auto-analyzer. Fig. 1 shows the actual electrode responses, whereas, the sensor characteristic for the interesting range is given in Fig. 2: evidently, the unmodified enzyme sensor responds linearly to an increase in glucose concentration up to 1.4 mmol/l and reaches saturation at about 25 mmol/l. Such a behavior is typical for a complete kinetically controlled reaction. Our glucose electrode follows the Michaelis-Menten equation with an apparent K_m^{app} of 4.5-5.5 mmol/l. As the glucose oxidase (GOD) is covalently bound to the immobilization matrix, the electrodes show excellent long time stability. Overall, the sensor response characteristic is very appreciable; however, for clinical purposes a larger linear range would be preferable to ease calibration and reliability of the system. One of the possibilities to reach this aim is to apply either a separate polymeric membrane or direct polymer coating to the electrode. Nafion is often used for biosensor preparation because of its excellent biocompatibility [20]. In addition, thin coatings are straightforwardly accessible by dipping the respective enzyme electrode into a commercially available diluted solution (5% Aldrich). Increase in thickness lower the permeability of coatings, but the ratio of diffusion coefficients for oxygen and glucose remains nearly constant with thickness. However, it is very difficult to control the dipping procedure sufficiently to obtain reproducible coatings on the sensing electrodes. Furthermore, Nafion also exhibits another limitation: membranes and layers may crack after some time in biological environments due to the deposition of calcium phosphates, which, however, can be prevented by pre-incubating with ferric chloride solution [21] or annealing the membranes at elevated temperatures [22]. To eliminate these problems, we developed a variety of membranes with different tailor-made permeabilities for glucose and oxygen. One key aspect for this is the thermal behavior of Nafion [23], as it releases sulphur dioxide in a temperature range 280-355°C and thus, increases its hydrophobicity as a consequence of losing sulfonium acid groups in the side chains. The amount of release depends on temperature, pressure and reaction time during membrane preparation.

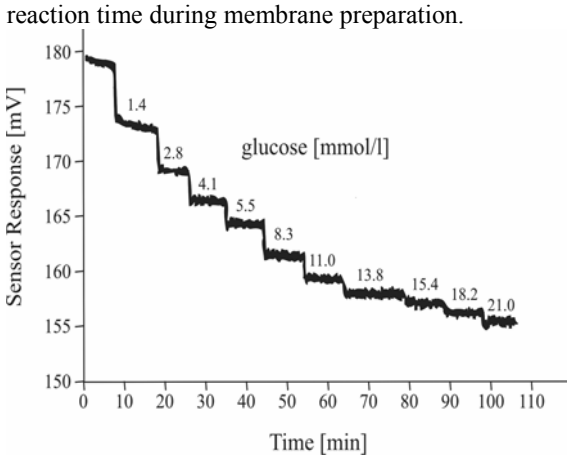

Fig. 1: Sensor response of the amperometric electrode towards different glucose concentrations.

Our thermic-pressing process resulted in 5 and 15µm thick Nafion membranes. Measurements of glucose diffusion coefficients showed that lower permeability always corresponds to higher temperature during the pressing process, whereas, diffusion coefficients of oxygen are only weakly influenced. With Nafion 1100 and 1500 different ranges of hydrophobic behavior are available. All these parameters (including variable conditions during membrane production) allow producing a large set of membranes with different permeabilities. During our sensor measurements, we compared all sensor responses obtained with the measurements without an additional polymer membrane on the electrode. Fig. 2 depicts the overall sensor responses decrease due to somewhat reduced accessibility of the sensor surface, the linear range for glucose measurements is substantially larger for the devices containing a membrane. We computed the respective upper limits of the linear part of the calibration curves via Eadie-Hofstee-Plots being one of the strategies to linearize Michaelis-Menten kinetics. Table-1 summarizes the resulting values of sensor linearity compared to membrane permeability. Evidently, the membrane increases the linear range by a factor of up to five, however, in all cases the maximum response time of the sensor did not exceed eight minutes. Both the linear range and the response properties make these sensor systems highly suitable for clinical purposes. Depending on the desired application, one can thus either optimize towards

short response times or towards larger linear ranges by carefully adjusting the permeability of the respective Nafion membranes.

Table-1: Upper limits of the linear range of glucose sensors without an outer diffusion control membrane and with differently permeable Nafion membranes, respectively.

Permeability	[10^{-5} cm^{-1}]	∞*	4.5	4.4	4.3
Linearity	[mmol/l]	1.4	6.2	6.9	9.6

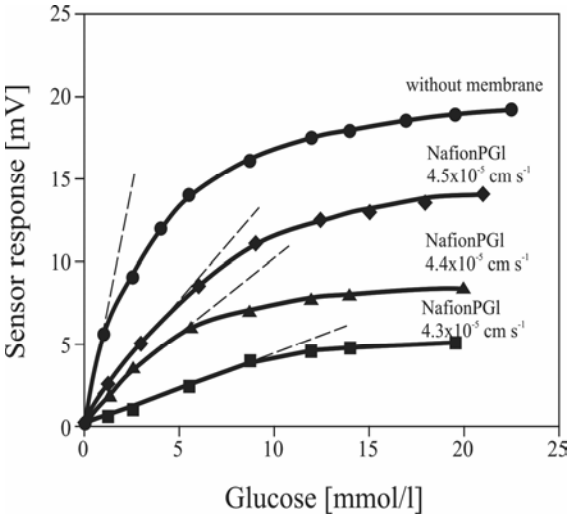

Fig. 2: Sensor characteristics without an outer controlling membrane (●) and with Nafion membranes of different glucose permeabilities (♦ PGl = $4.5x10^{-5}$ cm s^{-1}, ▲ PGl = $4.4x10^{-5}$ cm s^{-1}, ■ PGl = $4.3x10^{-5}$ cm s^{-1}).

However, directly depositing a protective polymer coating on the device surface is more advantageous than mounting an "external" Nafion membrane because of following reasons: it makes the system simpler and more compatible to industrial production processes and the sensor responses become faster. As already mentioned above, conventional drop-coating approaches, where the device is immersed into a precursor, hardly allow depositing polymers directly on the electrodes in a reproducible manner. To overcome this problem, we placed 5µl of the respective Nafion solution directly onto the electrode. The exact thickness of the coating could be calculated from the known density of the material. To estimate reproducibility, we cast coatings produced out of the same volume on a silicon wafer and measured the respective average thickness. From these measurements, we found that the values only slightly vary from each other, which means that layer thickness only depends on solution concentration. Compared to the "external" membranes, Nafion coatings show somewhat reduced linearity, which however is still by far sufficient, if the layer height exceeds 250 nm. Another major advantage of the coating is that the response times are very short, namely one minute or less.

Polyurethanes are among the most pervasive used polymer materials for coating enzyme electrodes [24, 25] because of their good biocompatibility and the appreciable hydrophobicity; one commercially available material for this purpose is e.g. Estane 5714 F1. Typical sensor responses of polyurethane coatings are shown in Fig. 3; the corresponding values of linearity as a function of layer thickness are given in Table-2. As we could already see in the case of Nafion, polyurethane reduces the overall sensitivity of the devices; however, it extends the linear range to the physiologically relevant concentration window. Our measurements revealed that sufficient linear behavior can be achieved if layers are thicker than approximately 600 nm. Thus, polyurethane coatings in principle show a similar potential in increasing the linear range of the glucose sensor in a similar way as Nafion coatings do and also has the advantage of a technologically very straightforward synthetic procedure and fast response. The latter is caused by the absence of the small buffer zone between membrane and electrode in the case of the separately mounted membranes.

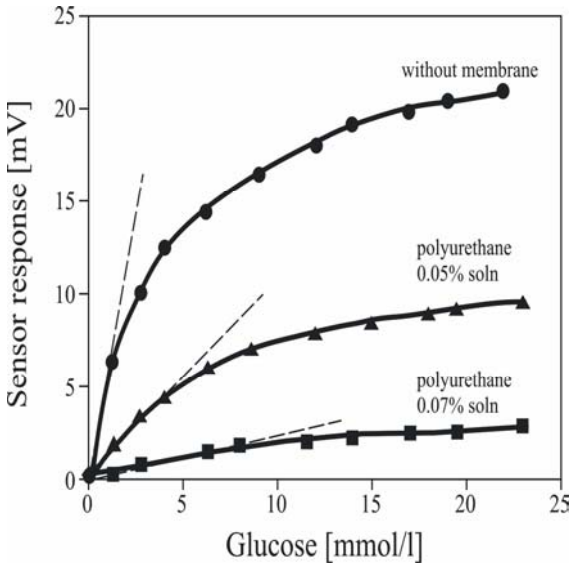

Fig. 3: Sensor characteristics without an outer diffusion control coating (●) and with polyurethane coating, casted from 0.05% (▲) – 0.07% (■) solution.

Table-2: Upper limits of the linear range of glucose sensors without an outer diffusion control membrane and with differently thick polyurethane membranes, respectively.

Concentration of casting solution	[%]	-*	0.05	0.07
Thickness of coating	[µm]	-	0.14	0.20
Linearity	[mmol/l]	1.4	4.9	7.2

At the working conditions of an in-situ biosensor in a human body, the ratio between oxygen and glucose is often non-stoichiometric. In this case it is fundamentally important that the membrane selectively lets oxygen pass through to avoid negative influence on the electrode. To assess the influence of this parameter, we tested both the systems with the Nafion membrane and the polyurethane coating, respectively at reduced oxygen levels by providing a gas mixture containing only five percent oxygen. As the effect for both materials and technologies is in principle the same, we only show the data for the polyurethane coating in Fig. 4. Evidently, also in this case introducing the coating decreases the absolute sensor output but in the same time increases the upper limit of the linear range in the sensor system. However, reducing the oxygen amount from 20% to 5% decreases the absolute sensor signals of the uncoated glucose sensor (denoted in Fig. 4 by the filled and the empty circles, respectively) by a factor of 3-10 depending on the glucose concentration. The polyurethane-coated system on the other hand is only marginally influenced by the change in oxygen concentration (less than 10%), especially in the physiologically relevant range below 10 mmol/l glucose. So, in this case the additional coating on the sensor does not only influence the linear range of the sensors in a positive way, but it also ensures that the signal remains unaffected even by large changes in oxygen concentration. According to our measurements, this effect is independent of the way the layer has been deposited, as the Nafion membranes show exactly the same effects as the polyurethane layers. Finally, clinical sensing requires testing of the systems directly in blood [26] to ensure proper functionality and to assess possible cross-reactions with other components of this very complex matrix. In our measurements, we used sheep blood as it is very similar to human blood and thus, work as standard testing environment for many clinical sensor tests. Carrying out measurements with our sensor at 37 °C, we observed that the device with a Nafion 1100 membrane is only very slightly influenced. The linearity of the sensor characteristic is somewhat reduced because of the lower solubility of oxygen in blood and the sensitivity is increased due to the higher enzyme activity at elevated temperatures. The main improvement is long-term stability, whereas, the sensor without membrane was clotted with blood protein within 3 hours, the sensor with the Nafion membrane produces steady signal over several day and prove the versatility of these polymers.

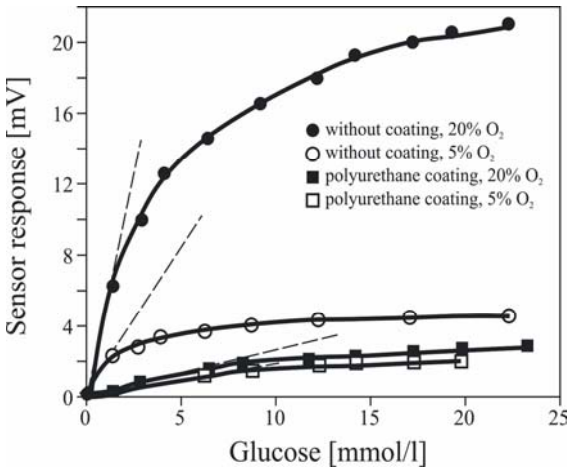

Fig. 4: Calibration curves of sensor at different oxygen concentrations without coating (● 20%, ○ 5% oxygen) and with polyurethane coating (■ 20%, ϒ 5% oxygen), respectively.

Experimental

Equipment

The blood glucose sensor system was fabricated on the basis of an amperometric oxygen electrode developed by Mund *et al*. [27] using a feedback loop to compensate for current. It consists of a flow-through cell containing the three electrodes, namely a working, a counter and a reference electrode (Ag/AgCl). This cell is a part of closed-loop system with 70 ml volume and circulated with a tube pump (Watson Malrox 101U) at 25°C. The main flask of the system contained the in-situ produced solutions with variable glucose concentration and gas composition within the electrolyte solution (gas mixer Super Mix 4000, CJT, Germany). We fixed external glucose diffusion limiting membranes on the working electrode with o-rings, whereas, the diffusion limiting layers were directly generated on the electrode material. We transferred all amperometric oxygen sensor signals into a computer and determined the respective corresponding glucose concentrations with an enzymatic auto analyzer (Specific Supra, Kone, Sweden) to calibrate the glucose sensor output.

Reagents

Polyepoxysilane EPS 150, an in-house development of company, was obtained from Siemens AG [28]. We purchased (3-Aminopropyl)-triethoxysilane HTR AP-3 from Merck (Darmstadt, Germany), oligotriacrylate OTA 480 (the reactive dilutor) from UCB S. A. (Drogenbos, Belgium), Darocure 1173 from Merck and 1,4-Diazabicyclo-[2.2.2]-octane (DABCO) from Aldrich. Glucose oxidase (GOD) (E. C. 1.1.3.4) (200 U mg^{-1}) was obtained from Sigma. As electrolyte, we applied an isotonic solution of NaCl (0.9%) in a buffered aqueous solution at pH=7.2. All other reagents were purchased from Merck and Fluka in analytical grade and used as received.

Preparation of Electrode System

First, we treated the surface of the oxygen-selective electrode surface with aminosilane HTR AP-3 at 150°C for 15 min to deposit a covalent anchoring spacer on the surface. Afterwards, we spread 5 µl of 1% solution containing EPS 150 (100 parts), HTR AP-3 (8 parts) and OTA 480 (2 parts) in dioxane over the surface and dried at 70°C. After hardening of the resin in nitrogen atmosphere using UV-light and 24 hours extraction in dioxane, we dipped the electrode head into a solution of proline and DABCO in a mixture of dioxane and water (2+1 parts) and incubated for 9 h at 60°C. Finally, we immobilized the GOD in the proline-containing polysiloxane-resin by first immersing the sensing surface into dioxane/water (1:1) for 18 h. After repeated extraction in the same solvent, we stored the electrode in electrolyte at 4°C.

Preparation of Diffusion Control Membranes

For preparing the Nafion membranes, we heated 1-3 g of Nafion granulate (Nafion 1100, DuPont) between two aluminum sheets and processed it with a plate press (polystat 200 S, Schwabenthan, Germany) for 1-10 min. The hydrophobicity of membrane is directly proportional to operating temperature. A touch press device (Frank, Germany) determined the respective membrane thickness. Aside of external membranes, we also directly cast polymer layers (Nafion 1100 and polyurethane Estane 5714 (Goodrich)) onto the glucose electrode surface by dropping 5 µl of the solution and drying at room temperature for 1 hour. The thickness of the layer was estimated by casting the same solution on a silicon wafer. After drying, we measured the average thickness with an alpha-step device and compared it with the thickness computed from a given density.

Diffusion Measuring Device

All diffusion coefficients of glucose through Nafion membranes were assessed at 25°C using a specially designed dialysis apparatus consisting of a membrane-separated system with two differently sized cells. We filled 25 ml of electrolyte into the smaller and 2500 ml into the larger compartment. After adding 25g of glucose to the large volume, thus, leading to an initial concentration of 10 g/l, we measured the time-dependent increase of glucose concentration in the small compartment by the means of an auto-analyzer. According to the following equation (eq. 1), the diffusion co-efficient can be calculated by linear regression [29]:

$$-\ln\left[\frac{c_t}{c_0}+\frac{v_1}{v_2}\left(\frac{c_t}{c_0}-1\right)\right]\bullet\left(\frac{1}{v_1}+\frac{1}{v_2}\right)^{-1}\bullet\frac{d}{A}=D\bullet t \qquad (1)$$

c_t is equivalent to the measured glucose concentration in the small compartment at a reaction time t, whereas c_0 represents the initial concentration in the large volume. A and d are abbreviations for the area and the thickness of the membrane. For determining the oxygen diffusion coefficient, we used the same equations. In this case, the system consisted of air-tight compartments, where the electrolyte in one cell had been saturated with nitrogen, and the other one with oxygen. At the beginning of the measurements we stopped the respective gas flows and monitored the rise of oxygen content by an oxygen electrode (Orion 97-08, recorder Orion EA 940).

Conclusion

Amperometric sensors based on glucose oxidase are a highly powerful tool for detecting glucose. However, for actual clinical use the linear range has to be improved. We reached this by covering the sensor with membranes or coatings based on both Nafion and polyurethane. This increases the linear range of the calibration curve by up to a factor of five and improves signal stability. In this way, it was possible to generate a sensor with a detection limit of 0.3 mmol/l glucose and a dynamic range up to 10 mmol/l, which is suitable for medical applications. The modified Nafion membranes offer excellent adaptation to all glucose sensor systems and are suitable for use in biotechnology. We could also

show that polyurethane coatings directly on the sensor surface are also capable of increasing the linearity of the sensor characteristic. Such coatings exhibit some advantages over membranes, as the response times can be reduced to less than a minute and the sensors become nearly independent of an insufficient supply of oxygen as occurring in blood and tissue. Finally, the polymer layers prevent clogging of the sensor surface by proteins while exposing to blood.

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