Wearable biosensor for monitoring rabbit tear glucose

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Abstract: - A wearable amperometric glucose sensor was fabricated and tested. Also, the sensor was utilized to tear glucose monitoring. The sensor was constructed by immobilizing GOD onto a flexible oxygen electrode, which was fabricated using "Soft-MEMS" techniques onto a functional polymer membrane. In purpose of bioinstrumentation, adhesive agents were not used for constructing the flexible biosensor. Linear relationship between glucose concentration and output current was obtained in a range of 0.039 – 0.537 mmol/l. Current dependences on pH and temperature were also evaluated. The current was largest at pH 7.0 and the current increased when temperature increased. This indicates that the output current depends on enzyme activity. Based on the basic characteristics investigation, the glucose sensor was applied to measurement of glucose in tear fluids on an eye site of a Japan white rabbit. The change of tear glucose level induced by oral-administration of glucose was monitored as a current change of the sensor attached on the eye site. In this investigation, the tear glucose level varied from 0.2 mmol/l to 0.5 mmol/l. Although there was a delay of several tens of minutes towards blood sugar level, it is considered to be possible that non-invasive continuous glucose monitoring can be realized using the flexible biosensor.

Key-Words: - **wearable biosensor, tear glucose, rabbit, diabetes, GOD, MEMS, flexible electrode**

1 Introduction

Rapid increasing of diabetes mellitus is now global problem and development of a safe and convenient blood sugar level monitoring technology is strongly required. Generally, continuous glucose level monitoring do not measure blood glucose directly, but rely instead on measurement of the glucose levels in other biological fluids. Particularly, correlation with glucose level in interstitial fluid of subcutaneous tissue to blood glucose level is often used [1]. Relationships between other biochemical substances of body fluids (tears, airway mucus, sweat and saliva) and personal physical conditions are also reported [2] and expected to be used for continuous bioinstrumentations. We paid attention to the relationship between the tear glucose level and the blood sugar level which is previously reported using discrete monitoring method such as capillary electrophoresis [3].

In this study, we have developed a flexible amperometeric biosensor towards potential use within eyes for continuous tear glucose monitoring. The biosensor was fabricated using "Soft-MEMS" techniques [4]. The sensor has an 84 μm thick laminar structure, which contains gas-permeable membrane, non-permeable membrane, electrolytes, electrodes and glucose immobilized membrane. The electrodes were formed using microfabrication process. Also, the biosensor was utilized to continuous glucose monitoring in tear fluids on rabbit's eye site. The advantage of the flexible sensor is that the sensor has thinner and flexible structure suitable for biomedical instrumentation because they do not cause discomfort. The sensor consists of reed-shaped functional polymers and film electrodes.

In this paper, details of the design, fabrication and evaluation of the flexible biosensor are presented. Also, the result of continuous and non-invasive tear glucose monitoring using the flexible biosensor is reported.

2 Experimental section

2.1 Fabrication of a flexible biosensor

A schematic structure of the flexible biosensor is illustrated in Fig. 1. The glucose sensor has a 3mm x 50mm x 84μm laminar structure, which consists of an enzyme immobilized membrane and film-like oxygen electrode (Pt working electrode and Ag/AgCl reference/counter electrode). Owing to continuous tear glucose measurement in mind, whole structural members were constructed with flexible polymers. Adhesive agents were not used for constructing the wearable glucose sensor. The

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sensor was constructed by immobilizing the enzyme membrane onto the sensitive area of the oxygen electrode.

The film-type oxygen electrodes has four layers; (i) a flexible gas-permeable membrane (polypropylene, thickness: 25 μm), (ii) a 200 nm thick Pt electrode and a 300 nm thick Ag/AgCl electrode, (iii) a membrane filter with dimensions of 1 mm x 1.5 mm (IsoporeTM TKTP04700, Millipore Corp., USA) containing electrolytic solution (0.1 mol/l KCl 198-03545, Wako Pure Chemical Industries, Ltd., Japan) and (iv) a non-permeable membrane (Ionomer, film thickness: 50μm).

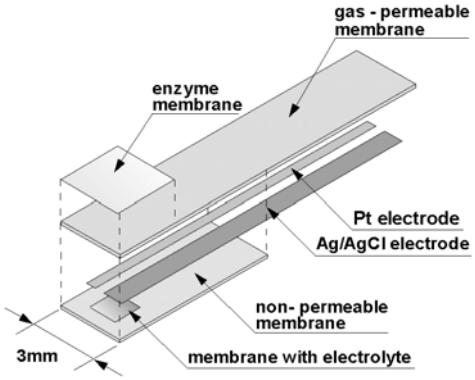

Figure 1. Structure of a wearable sensor for tear glucose monitoring.

The electrode reaction is given as following equations:

Cathode (Pt) : $O_2+2H_2O+4e^- \rightarrow 4OH^-$
Anode (Ag/AgCl) : $4Ag+4Cl^- \rightarrow 4AgCl+4e^-$

The oxygen electrode was fabricated using MEMS (Micro Electro Mechanical Systems) techniques. At first, the gas-permeable membrane was fixed on a dummy silicon wafer using polyimide tape. Positive photoresist (Shipley S1818, Rohm and Haas Electronic Materials Co., USA) was then spin-coated. In order to prevent the gas-permeable membrane from thermal damage, the positive resist was cured at 60 °C. The photoresist was patterned into the shape of electrodes using UV exposure system (MA-10, Mikasa CO., LTD., Japan). Then, a 200 nm thick Pt was sputtered (CFS-4ES-231, SHIBAURA ENGINEERING WORKS CO., LTD., Japan) onto the patterned surface with a background pressure of 3.0 x 10-3 Pa and the sputtering pressure of 3.0 x 10-1 Pa. The pattern of the Pt working electrode was covered by positive photoresist (RP-2, San-hayato Co., Japan). And a 300nm Ag was deposited directly on Pt. The gas-permeable membrane was then released from the dummy Si wafer and the Pt and Ag electrodes were formed using lift-off process. The Ag/AgCl electrode was formed by controlled-potential electrolysis. A constant voltage of 110 mV was applied to the Ag film in a 0.1 mmol/l HCl solution.

Then, the flexible oxygen electrode was constructed in a sandwich configuration with a membrane filter (IsoporeTM) containing electrolyte solution between the gas-permeable membrane with Pt and Ag/AgCl electrode and the non-permeable sheet. All the edges of the layer-built cell with 0.1 mmol/l KCl were fastened by heat-seal system (SURE Sealer, NL-201P, Ishizaki Electricity Manufacturing Co. Ltd., Japan). The temperature was 150 °C. Thus, the flexible Clark-type oxygen electrode with a bag-like electrolyte cell was obtained.

The wearable glucose sensor was fabricated by immobilizing glucose oxidase (GOD: EC1.1.3.4, G-7141, Sigma Chemical Co., USA) onto the sensing region of the flexible oxygen electrode. GOD was immobilized using water-soluble photosensitive resin (AWP: Azide-unit pendant Water-soluble Photopolymer, Toyo Gosei Kogyo Co., Ltd., Japan) as shown in the upper part of Fig. 1. In order to improve the contact between the gas-permeable membrane and the enzyme immobilized membrane, an aminopropylsilane monolayer was prepared on the surface of the gas-permeable membrane.The sensing region of the oxygen electrode was coated with 1-3 aminopropylsilane monolayer. Then, it was rinsed and dried for 30 min at room temperature. 15 mg of GOD was mixed into 500 μl phosphate buffer solution (PBS: pH 7.0, 20 mmol/l). The 40μl GOD and PBS mixture was mixed with a 40μl AWP. The GOD/PBS and AWP mixture was applied to the monolayer formed on the oxygen sensor as an enzyme membrane. AWP was cured under 5 °C. After that, the enzyme membrane was cured using UV light, thus obtained the flexible biosensor for tear glucose measurement as shown in Fig 2.

Figure 2. Photograph of the flexible glucose sensor.

2.2 Evaluation of the flexible biosensor

The sensor was calibrated using a batch measurement system using a 50 ml measuring cell

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filled with PBS (pH 7.4, 20 mmol/l, containing glucose solution). Oxygen consumption induced by enzyme reaction of the enzyme immobilized membrane was measured by two-electrode electrochemical method. The sensor was connected to the measurement system and the reduction potential of -550 mV versus Ag/AgCl reference/counter electrode was applied to the Pt working electrode using PC-controlled potentiostat (HAB-151, HOKUTO DENKO Co., Japan). The output currents were recorded by PC, which was connected to the potentiostat via A/D converter (ADC-16, pico Technology Ltd., UK), within the glucose concentration ranging from 0.025 – 1.475 mmol/l.

The current dependences on various pH and temperatures at defined glucose concentration (1mmol/l) were also investigated. The constant potential of - 550 mV versus Ag/AgCl electrode was applied to the Pt electrode in solutions of various pH (6.0 – 12.0) and various temperature (15 – 60 °C). The current change induced by varying operating condition was recorded using PC. In the experiment, mixtures of disodium hydrogen phosphate (196-02835, Wako Pure Chemical Industries, LTD., Japan) and potassium dihydrogenphosphate (169-04245, Wako Pure Chemical Industries, LTD., Japan), which pH was controlled using pH meter (D-25, Horiba Korea LTD., Korea) to measure pH dependence.

2.3 Tear glucose measurement

Using the flexible glucose sensor, tear glucose monitoring on eye site of a rabbit (Japan white rabbit, sex: female, age: 18 month, weight: 2kg) was carried out. The rabbit was placed into a cylindrical fixation device. In order to reduce physical load, the animal experiment was carried out without use of an anesthetic. The experimental method of glucose monitoring on eye site is shown in the left box of Fig. 3. The sensing region of the sensor (glucose immobilized membrane) was attached on the pupil and the other end of the sensor was fixed using fixing tape. The sensor was operated using the PC-controlled potentiostat and the output current was continuously monitored using PC, which was also used for evaluation of basic characteristics.

The monitoring test on eye site was carried out within two steps. At first, the sensor output during steady state was monitored and the stability of the output current was investigated. After that, the change of tear glucose level induced by oral glucose load was measured using the glucose sensor. Fig. 3 shows the experimental method of continuous tear glucose measurement. The sensor was attached on the rabbit's eye site as well as the preceding test. Glucose solution was then orally-administrated to the rabbit. Quantity of the glucose solution was determined to 2g (1g of glucose per 1kg of weight). Blood glucose level was also measured using a commercially produced monitoring kit (MEDISAFE, TERUMO Co., Japan) as a controlled study. Blood sample was taken from the rabbit's ear.

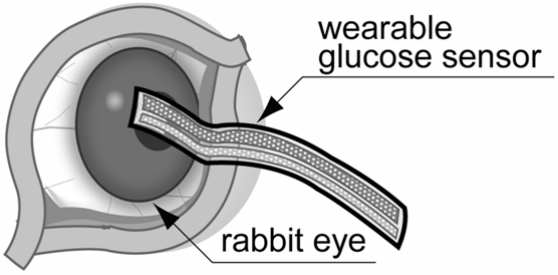

Figure 3. Measurement method of glucose concentrations in tear fluids using the flexible glucose sensor.

3 Results and discussion

3.1 Characteristics of the biosensor

The calibration plot of the flexible biosensor is shown in Fig. 4. In this figure, the linear relationship between glucose concentration and output current was confirmed in a range of 0.039 – 0.537 mmol/l, deduced by regression analysis, as shown by following equation;

output (μA) = -0.06 + 1.822 [glucose (mmol/l)]

The calibration range of the glucose sensor covers the glucose concentration of normal tear fluid (0.14 mmol/l) and the result indicates that the flexible biosensor has an appropriate measurement range for tear glucose measurements in diabetic patients. The typical response curve of the sensor is also shown in the inset figure of Fig. 4.

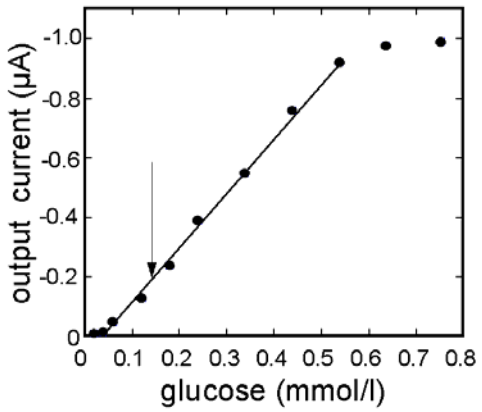

Figure 4. Calibration curve of the glucose sensor.

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The current change induced by various pH showed a peek at pH 7.0, which is the optimal pH of the enzyme activity. In the pH ranging from pH 5.0 to 7.0, the current increases as pH increases. The current rapidly decreased during pH 7.0 – 7.5. In case of pH > 7.5, the output current showed a slow decrease. This is because the activity of immobilized enzyme degrades significantly in alkaline pH. The current increases as the temperature increase less than 45 °C. This indicates that the reaction rate is the dominant factor in this temperature range. On the other hand, the current decreases over 45 °C. In this temperature range, thermal deactivation of immobilized enzyme determines the output current rather than reaction rate.

3.2 Tear glucose monitoring the biosensor

Prior to continuous tear glucose measurement, the sensor was attached on the rabbit eye as mentioned before and tear glucose level of the rabbit eye was monitored repeatedly. The average value of tear glucose level was 0.124 mmol/l. Those glucose concentrations in tear fluids were estimated from the calibration plots. Those values were fixed by pH and temperature dependence.

The estimated tear glucose level and blood sugar level during the oral administration of glucose to the Japan white rabbit were compared. We confirmed the stability of the sensor output for 10 minutes and then oral glucose tolerant test was carried out. The initial estimated glucose level in tear fluids was 0.2 mmol/l and increased up to 0.5 mmol/l by the experimentation. As the figure indicates, the glucose level in tear fluids increases by 3-fold while blood glucose increases by 2-fold. This might be considered that the output current includes an effect of interferences. Blood glucose level increased immediately when glucose load was applied to the rabbit. On the other hand, tear glucose level increased within a delay of 10 - 20 minutes in compare with blood glucose level. The initial current change started 10 minutes after glucose administration. The tear glucose level significantly increased from 40 to 60 minutes after oral administration. Although the effect of interferences and a delay of tens of minutes, the output current, which has relationship with the tear glucose level, tracked the blood glucose level with a considerable correlation during this investigation. Significant variation of the output current was not observed except when the sensor displacement was occurred by blinking during 5 minutes. During the investigation, the sensor output was not degraded or drifted. Thus, continuous tear glucose monitoring was successfully demonstrated. We have to improve a novel wearable sensor like a contact lens for long term analysis of tear glucose level because the strip-style flexible sensor is easy to come off from the rabbit eye.

The flexible biosensor is not only useful for continuous measurement at the ophthalmic site and the skin surface, but also useful for the chemical analysis in the biological fluids secreted from the internal organs, tissues, etc.

4 Conclusion

A flexible and wearable amperometric glucose sensor was developed and utilized to tear glucose monitoring. The sensor was constructed by immobilizing GOD onto a flexible oxygen electrode, which was fabricated using MEMS techniques onto a functional polymer membrane. Linear relationship between glucose concentration and output current was obtained in a range of 0.039 – 0.537 mmol/l.
Owing to unique features of flexibility, biocompatibility and thinner structure, continuous tear glucose monitoring was carried out. The sensor was sufficiently stable and sensitive when it is attached on rabbit eye. Thus, change of tear glucose level induced by oral administration of glucose was measured using the biosensor. Blood sugar level was also measured by a commercially produced monitoring kit as a controlled study. As a result, the tear glucose level was increased from 0.2 to 0.5 mmol/l. Although the change of tear glucose level delayed in tens of minutes from that of blood sugar level, it is considered to be possible that non-invasive continuous glucose monitoring can be realized using the glucose sensor.

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