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A soft and flexible biosensor using a phospholipid polymer for continuous glucose monitoring

MingXing Chu · Hiroyuki Kudo · Takayuki Shirai · Kumiko Miyajima · Hirokazu Saito · Nobuyuki Morimoto · Kazuyoshi Yano · Yasuhiko Iwasaki · Kazunari Akiyoshi · Kohji Mitsubayashi

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Abstract A flexible biosensor using a phospholipid polymer to immobilization of glucose oxidase (GOD) was fabricated and tested. At first, an enzyme membrane formed by immobilizing GOD onto a porous polytetrafluoroethylene (PTFE) membrane using the phospholipid polymer (2-methacryloyloxyethyl phosphorylcholine (MPC) copolymerized with 2-ethylhexylmethacrylate (EHMA) : PMEH) was evaluated. According to the result of amperometric measurement, average density of GOD to be immobilized was optimized to 38.9 units cm^{-2}. Temperature and pH dependences were also investigated. Then, a flexible glucose sensor was fabricated by immobilizing GOD onto a flexible hydrogen peroxide electrode using PMEH. The flexible glucose sensor showed a linear relationship between output currents and glucose concentration in 0.05–1.00 mmol L^{-1}, with a correlation coefficient of 0.999. The calibration range covered the normal tear glucose level of 0.14–0.23 mmol L^{-1}. This indicates that the flexible biosensor is considered to be useful for monitoring of glucose in tear fluids.

Keywords Biosensor · Phospholipid polymer · Flexible · Glucose sensor

1 Introduction

Diabetes mellitus is a major public health problem, affecting ~5% of the population in the world. The chronic complications of diabetes cause enormous human suffering

M. Chu
Graduate School of Medical and Dental Sciences,
Tokyo Medical and Dental University,
2-3-10 Kanda-Surugadai,
Chiyoda-ku, Tokyo 101-0062, Japan

H. Kudo · K. Miyajima · H. Saito · K. Mitsubayashi (✉)
Department of Biomedical Devices and Instrumentation,
Institute of Biomaterials and Bioengineering,
Tokyo Medical and Dental University,
2-3-10 Kanda-Surugadai,
Chiyoda-ku, Tokyo 101-0062, Japan
e-mail: m.bdi@tmd.ac.jp

T. Shirai
Graduate school, Tokyo University of Technology,
1401-1 Katakura,
Hachioji, Tokyo 192-0982, Japan

H. Saito
Department of Mechanical Engineering,
Tokyo National College of Technology,
Hachioji, Tokyo 193-0997, Japan

N. Morimoto · K. Akiyoshi
Department of Organic Materials, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University,
2-3-10 Kanda-Surugadai,
Chiyoda-ku, Tokyo 101-0062, Japan

N. Morimoto
Department of Materials Processing,
Graduate School of Engineering,
Tohoku University,
Sendai, Miyagi 980-8579, Japan

K. Yano
School of Bionics,
Tokyo University of Technology,
1401-1 Katakura,
Hachioji, Tokyo 192-0982, Japan

Y. Iwasaki
Department of Chemistry and Materials Engineering Faculty of Chemistry, Materials and Bioengineering Kansai University,
3-3-35 Yamatetyou,
Suitasi, Osakafu 564-8680, Japan

in the form of blindness, kidney failure, amputations, and increased risk of coronary artery disease and stroke (Taylor et al. 1999). The main characteristic of diabetes is a chronically raised blood glucose concentration (hyperglycemia) (Pasic et al. 2007). For this reason, to monitor blood glucose level is essential for diabetes mellitus, Thus, glucose sensor for continuous blood sugar monitoring is strongly requested become an intensively investigated research area (Borole et al. 2005).

Periodic finger prick tests, are often used by diabetic patients for measuring their own blood sugar level, due to the fact that glucose levels in blood can change rapidly (2.25 mg dL^{-1} min^{-1}), often failing to detect all hypoglycaemic and hyperglycaemic events (Ricci et al. 2007) as compared to other kinds of glucose sensors were reported (Sanz et al. 2007; Ahmed et al., 2005; Gerritsen et al. 2000; Gifford et al. 2006; Lam et al. 2002; Nielsen et al. 2005). These glucose sensors measure blood glucose level directly by invasive method. To remove discomfort, it is necessary to develop a non-invasive and biocompatible glucose sensor for continuous glucose monitoring. There reported that glucose is contained in tear fluid of human (Daum and Hill 1982). Moreover, there are some relationships between blood glucose and tear glucose (Sen and Sarin 1980). Based on this background, we developed a glucose biosensor to achieve continous monitoring of tear glucose levels, in order to compare to blood glucose levels. The fabrication of glucose sensor has two major steps enzyme immobilization and electrode formation.

Glucose oxidase (GOD) is inexpensive, stable and practically applied in clinical and chemical analysis (Shan et al. 2007). Enzyme immobilization is an important step and many methods such as adsorption, crosslinking, electrochemical polymerization covalent bonding and entrapment (Zheng et al. 2002; Dipali et al. 2007). In this work, a MPC polymer (PMEH) was utilized for enzyme immobilization. The PMEH is biocompatibile polymer, which has a similar molecular configuration to a cell membrane. Biomaterials are important for medical microdevices that can be applied clinically with efficiency and in safety. MPC polymer can minimize damage on tissues as hydrogel by lubricating between the surface of organs and devices, and it can reduce protein adsorption and improve blood compatibility (Ishihara et al. 1998). The first, characteristics of a biosensor using PMEH based enzyme membrane were tested. Current dependence on immobilizing enzyme density was then investigated. Temperature and pH dependences were also investigated. Then, about the electrode formation, we used advanced microelectromechanical systems (MEMS) technology; which is widely used for fabrication of microsensor, providing many advantages such as high precision, high functionality and mass-production. But, some inflexible or brittle materials such as glass, silicon and polymer wafers (Piechotta et al. 2005; Suzuki et al. 2001; Pedersen et al. 2004; Bang et al. 2004; Adrega et al., 2006) have typically been used as substrates for those glucose sensors. In this work, a flexible PDMS membrane was used as substrate for electrode formation. The last, a flexible glucose biosensor by using PMEH to immobilize GOD on a hydrogen peroxide electrode was fabricated. The fabrication and evaluation of the glucose biosensor will be detail present as follows.

2 Experimental

2.1 Materials

D(+)-Glucose (Wako Pure Chemical Industries Ltd., Japan) was used to prepare standard glucose solution, and hydrogen peroxide solution (Kanto Chemical Co. Inc., Japan) was diluted with distilled water for experiment. Glucose oxidase (GOD: EC 1.1.3.4, 146000 uints g^{-1}) was obtained from Sigma-Aldrich Company of USA. A porous PTFE membrane (Pore size: 0.2 μm Nihon. Millipore. K. K., Tokyo, Japan) was utilized for enzyme immobilization. Hydrophobic polydimethyl siloxane (PDMS: Silpot 184, Dow Corning Toray Co., Ltd., Japan) was used as major material of the flexible glucose sensor. PMEH was synthesized using free radical immobilization method. A phosphate buffer solution (PB: pH 7.4, 20 mmol L^{-1}) was used for experiments.

2.2 Enzyme immobilized membrane with PMEH

In the immobilization process, a mixture of GOD and PMEH solution with ethanol solvent was prepared. The mixture was coated onto the PTFE membrane and cured for 3 hours at 4°C. Thus, an enzyme membrane was obtained. The enzyme membrane was cut into pieces of 1x1 cm square, and fixed on a Clark-type dissolved oxygen electrode (MODEL BO-P Able & Biott Co., Ltd., Japan) (Fig. 1) and covered with a supporting nylon mesh net, and was secured with a silicone O-ring. To remove redundant enzyme at the surface of the enzyme membrane, the sensing area of the Clark-type dissolved oxygen electrode with the enzyme membrane was placed into a 20 mL measuring cell filled with PB (pH 7.4, 20 mmol L^{-1}), and was rinsed for 10 min. In the assessment section, constant voltage of −600 mV was applied to the Pt-working electrode of the dissolved oxygen sensor by a computer controlled potentiostat (MODEL 1112, BAS Inc Co., Japan). The enzyme membrane was tested in a 20 mL measuring cell filled with PB (pH 7.4, 20 mmol L^{-1}). The optimum density to be immobilized of GOD was investigated. Also, the temperature (15–40°C) and pH (5.5–9.0) dependences were

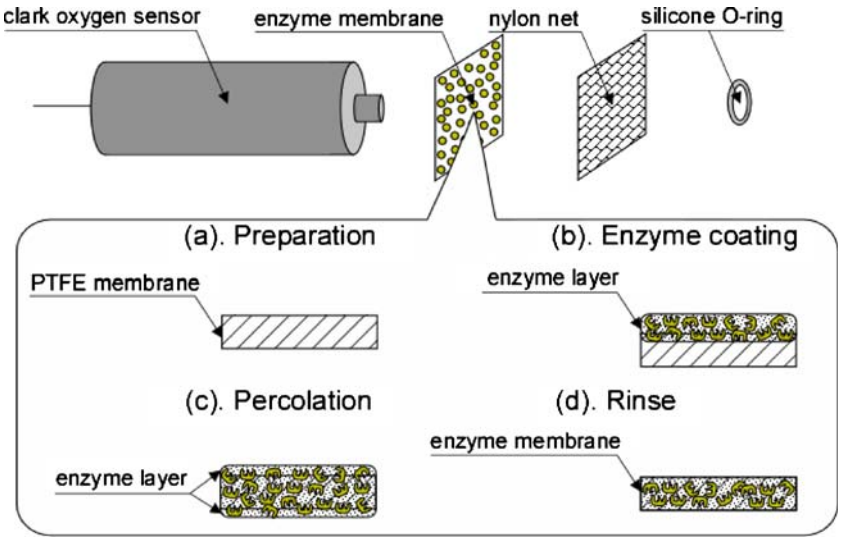

Fig. 1 Fabrication and wearing of enzyme membrane used hydrophilic PMEH (**a**).preparation of PTFE membrane; (**b**).coating of enzyme; (**c**).percolation of enzyme; (**d**).rinse

investigated. The changes of output currents were recorded using PC, which was connected to the potentiostat via 16 bit A/D converter (ADC-16, pico Technology Ltd., UK).

2.3 Construction of the flexible glucose sensor with PDMS and PMEH

Figure 2 shows the structure of the flexible glucose sensor. The glucose biosensor has a flexible hydrogen peroxide electrode (Pt-working electrode and Ag/AgCl-reference/counter electrode) formed on a 3 mm x 50 mm x 350 μm PDMS membrane. GOD was immobilized using PMEH onto the sensing area of the flexible sensor. The fabrication processes of the glucose biosensor are as follows. At first, a 350 μm thick PDMS membrane was formed on a 525 μm thick dummy silicon wafer by utilizing spin-coating system (1H-DXII, Mikasa Corp., Tokyo, Japan) and cured at room temperature for 24 hours. Then, a resistance layer was formed by spin-coating a positive photoresist (Shipley S1818, Rohm and Haas Electronic Materials Co., USA) onto the PDMS membrane, and deposited it on the hot plate and dried at 60°C for 60 min. Then the photoresist was patterned into a pattern of the electrode using a mask aligner (PEM-1000, Union Optical Co., Ltd., Japan). After that, a 200 nm thick Pt film was sputtered onto the PDMS membrane using a sputtering system (MODEL E-200, CANON ANELVA Co., Ltd., Japan). The pattern of the Pt electrode was covered using the positive photoresist. A 300 nm thick Ag film was sputtered onto the 200 nm thick Pt film. The photoresist was removed from the surface of the PDMS membrane using acetone. Thus, the PDMS membrane with the thin Pt and Ag electrode was obtained. The Ag electrode was electrochemically chloridized with 1.0 mmol L^{-1} hydrochloric acid solution at a constant voltage of−60 mV. Finally, a flexible hydrogen peroxide electrode was fabricated.

In the enzyme immobilization process, a mixture of GOD (2.67 mg) and a 10 μl of PMEH with ethanol solvent (10 wt%, ethanol: 90 wt%) was prepared, and cured under 4°C. The mixture was then coated onto the sensing region of the hydrogen peroxide electrode for 3 hours at 4°C. In order to avoid the enzyme leakage from enzyme membrane, 2.5 μL of

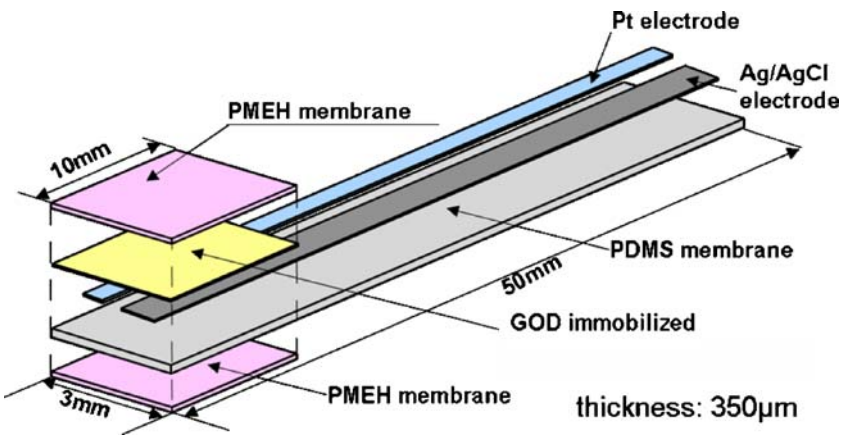

Fig. 2 Construction of flexible sensor used functional polymers

PMEH solution was coated onto enzyme membrane and cured at 4°C for 3 hours. Finally, the flexible glucose biosensor using phospholipid polymer was fabricated.

2.4 Assessment of the flexible glucose sensor

At first, response to hydrogen peroxide of the flexible biosensor was investigated. The measuring site of the glucose sensor was placed into a 20 ml beaker filled with a PB (pH 7.4, 20 mmol L^{-1}). The terminal electrodes of the glucose biosensor were connected to a computer controlled potentiostat. A constant voltage of 400 mV versus Ag/AgCl reference/counter electrode was applied to the Pt working electrode. Then, the changes of output current induced by increasing the hydrogen peroxide concentration in the cell were recorded by computer.

After that, glucose determination characteristics of the flexible biosensor were investigated. The measuring site of the biosensor was then placed into a 20 mL measuring cell filled with PB (pH 7.4, 20 mmol L^{-1}). A tip of the flexible biosensor was connected with the computer controlled potentiostat. A constant voltage of 400 mV versus Ag/AgCl reference/counter electrode was applied to the Pt working electrode. The output current for various concentrations of glucose were then investigated.

3 Results and discussion

3.1 Evaluation of enzyme membrane with PMEH

As shown in Fig. 3, the output currents were affected by the density of the enzyme. The output current of the glucose biosensor increased gradually by increasing the volumes of immobilized GOD enzyme. When the volumes of immobilized enzyme exceeded more than 38.9 units cm^{-2}, the output current was saturated as the figure indicates. The response time to 90% of the steady state for 1.0 mmoL L^{-1} glucose solution was approximately 36.7 s. The standard deviation was 0.021 μA and the coefficient of variation was 1.27% in. According to these results, the optimum density of GOD was determined to be 38.9 units cm^{-2}.

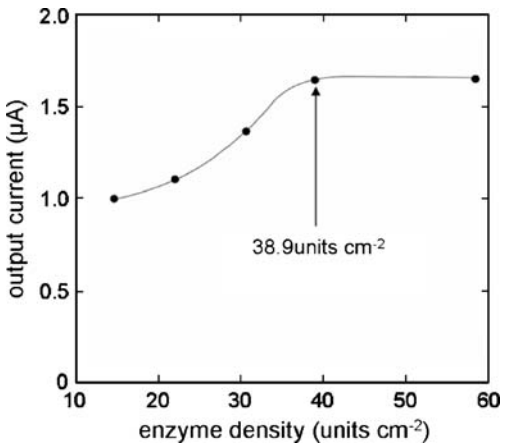

Fig. 3 Dependence on enzyme density used phospholipid polymer for enzyme immobilization. Average volumes of GOD and was optimized to 39 units cm^{-2}

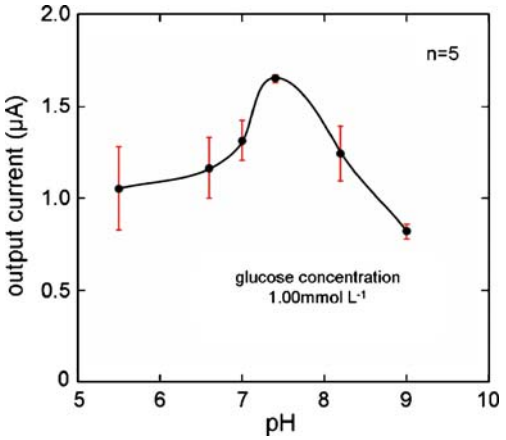

Fig. 4 Dependence on pH on the current of glucose sensor in PBS (20 mmol L^{-1}, pH 7.4) containing 1.00 mmol L^{-1}glucose at 25°C, applied potential,−600 mV

Figure 4 shows the current dependences on pH for 1.0 mmoL L^{-1} glucose solution. In the pH range from 5.5 to 7.4, the current increases as pH increases. The sensor showed a peak at pH 7.4. Then, the current showed a rapid decrease during pH 7.4–9.0. The optimal pH was 7.4 for the enzyme activity in the pH range from 5.5 to 9.0. The errors are also shown in Fig. 4. In this experiment, the calibration range of pH covers the standard range of tear pH in normal human (6.4–7.7). The pH of the human precorneal tear fluid depends on the time of equilibration of CO_2 with the surrounding medium. Under normal conditions, an oscillation of the pH value occurs in the precorneal tear (Fischer and Wiederholt 1982). Moreover, the pH of tear fluid easily changes in the ophthalmopathy patients.

The effect of temperature was also investigated. As shown in Fig. 5, temperature dependence on the current of the glucose sensor was evaluated between 15 and 40°C for glucose solution (0.60 mmol L^{-1}). The current of the glucose sensor increases as the temperature rises from 15 to 35°C. When the temperature rises further to 40°C, the current increase was not observed and became steady state. In other words, the activity of the GOD enzyme has achieved the highest level. Also, the error level is the lowest at 25°C in Fig. 5. The activity of GOD enzyme was affected in both the high and low temperature. To keep the stability of the glucose sensor, we chose the 25°C as the optimum temperature for the glucose biosensor in our experiments.

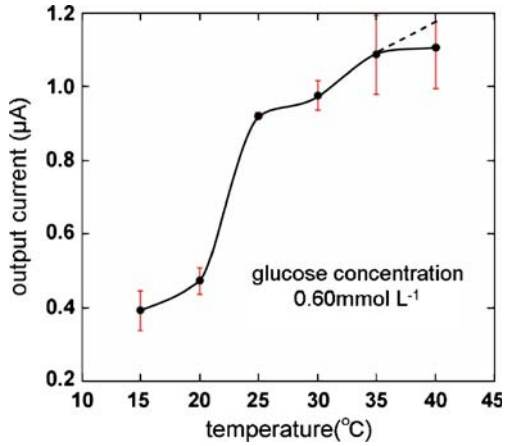

Fig. 5 Dependence on temperature on the current of glucose sensor in PBS (20 mmol L^{-1}, pH 7.4) containing 1.00 mmol L^{-1} glucose at 25°C, applied potential,−600 mV

3.2 Evaluation of the characteristics of the flexible biosensor

Figure 6 shows the calibration curve of hydrogen peroxide using the flexible electrode. In the small box of Fig. 6, the sensor outputs increase rapidly following the application of standard hydrogen peroxide solution to the measuring cell and reach the steady state. The response time of the transparent device to reach 90% of the steady-state current to hydrogen peroxide was approximately 5.0 s. The output current response of the hydrogen peroxide electrode was linearly related to the hydrogen peroxide concentration in a range of 0.10–2.50 mmol L^{-1}, with a correlation coefficient of 0.998. The following equation was obtained by analyzing the experiment result.

$$\text{output current}\,(\mu\text{A}) = 0.312 + 7.098[\text{hydrogen peroxide}\,(\text{mmol L}^{-1})]$$

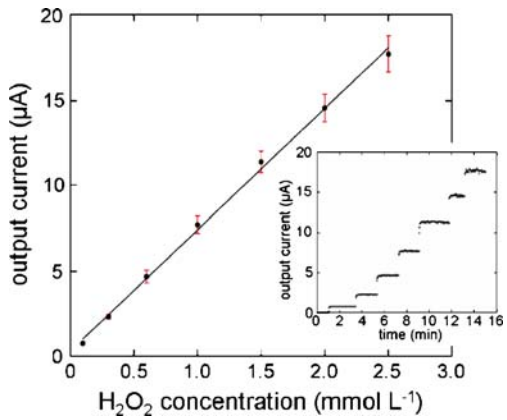

Fig. 6 Calibration curve of the biosensor using phospholipid polymer for hydrogen peroxide solution in PBS (20 mmol L^{-1}, pH 7.4)

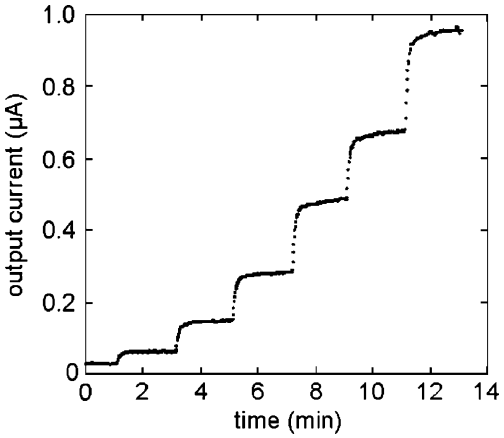

Fig. 7 Calibration curve of the glucose biosensor using phospholipid polymer for glucose solution

Also, the reproducibility of the output of the hydrogen peroxide electrode was investigated. The error level increase as the hydrogen peroxide concentration increased.

After that, the characteristics of the flexible glucose biosensor were investigated. As shown in Fig. 7, the biosensor showed an excellent response to glucose under the optimal experimental conditions (GOD: 38.9 units cm^{-2}, pH 7.4, 25°C). The response current immediately increased by dropping the standard glucose solution (high concentration) into the measuring cell filled with PBS (20 mmol L^{-1}, pH 7.4) and finally reached to a steady-state value, the output current response of the sensor was linearly related to the glucose concentration in a range of 0.05–1.00 mmol L^{-1}, with a correlation coefficient of 0.999. The average response time (T95) of the glucose biosensor was 41.6 s, the

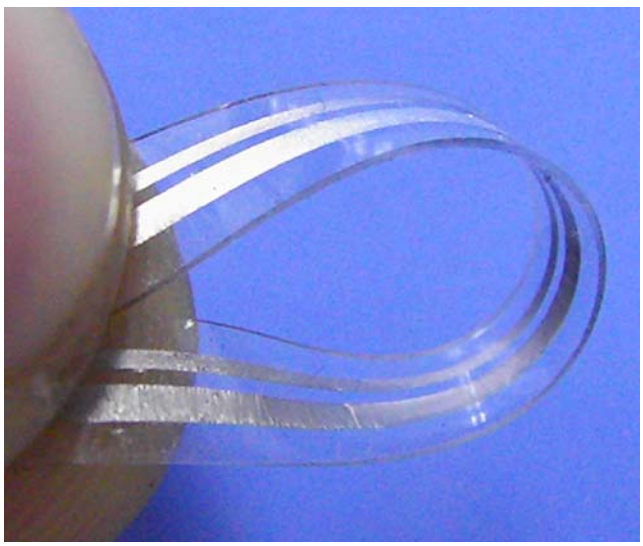

Fig. 8 Photograph of the flexible glucose sensor made of functional polymers (hydrophilic PMEH, GOD immobilized PMEH, hydrophobic PDMS) and electrodes (flexible Pt and Ag/AgCl film electrodes formed on the PDMS membrane)

sensitivities was 0.95 μA (mmol L^{-1})$^{-1}$. The follows relationship was confirmed by analyzing the experiment result.

$$\text{output current}\,(\mu\text{A}) = 0.006 + 0.947\left[\text{glucose}\left(\text{mmol}\,\text{L}^{-1}\right)\right]$$

The calibration range was 0.05–1.00 mmol L^{-1}, which contained the reported concentration of tear glucose is 0.14–0.23 mmol L^{-1} in normal human subject (Jin et al. 1997) and covered a higher glucose concentration.

As shown in Fig. 8. The glucose biosensor was successfully constructed using phospholipid polymer for enzyme membrane. As shown in the photograph, the flexible electrode was formed on PDMS membrane. PMEH was utilized for enzyme immobilization as biocompatible material. Because of flexibility, the sensor was able to fit the shape of eye. The characteristics of the glucose biosensor were considered to be useful for continuous monitoring of tear glucose level on the eye site.

4 Conclusions

In this work, a simple method using a phospholipid polymer to immobilize GOD was developed. Characteristics of the enzyme membrane were evaluated. The volumes of GOD were optimized to 38.9 units cm^{-2}. The response time to 90% of the steady state for glucose was approximately 36.7 s. In addition, temperature and pH dependence of the PMEH enzyme membrane were also investigated. After that, the flexible glucose biosensor was successfully fabricated, and the characteristics of the glucose biosensor were evaluated in optimal experimental conditions (GOD: 38.9 units cm^{-2}, pH 7.4, 25°C). At first, the output current of the hydrogen peroxide electrode was linearly related to the hydrogen peroxide concentration in a range of 0.10–2.50 mmol L^{-1}, with a correlation coefficient of 0.998. Then, the glucose biosensor showed an excellent response to glucose, the stability of the glucose biosensor was observed. The current output of the glucose sensor related to the glucose level over a range of 0.05–1.00 mmol L^{-1}, with a correlation coefficient of 0.999. The calibration range included the normal glucose concentration (0.14–0.23 mmol L^{-1}) in the tear fluids. In our future work, we will try to fabricate a non-invasive contact-lens biosensor for continuous monitoring of glucose on the eye site.

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