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Fabrication of an Implantable Fine Needle-Type Glucose Sensor Using γ-Polyglutamic Acid

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Implantable fine needle-type glucose sensors with an outer diameter of less that 0.2 mm were fabricated using a low-cost and non-animal origin polyamide, γ-polyglutamic acid (PGA) as a glucose oxidase (GOx) immobilizing material. Two types of PGA, γ-polyglutamic acid (PGAH) and γ-polyglutamic acid sodium salt (PGANa), were employed to prepare GOx immobilized film by the covalent attachment of GOx using water-soluble carbodiimide (EDC). Nafion/cellulose acetate composite film and polyurethane/polydimethylsiloxane composite film were employed as a permselective inner film and a biocompatible outer film, respectively. The procedure of enzyme-immobilized film fabrication affected the stability of the sensor; that is, GOx immobilized film prepared by pouring a mixture solution of GOx and EDC on a PGA precoated surface showed higher sensor stability than that prepared by pouring a mixture solution of GOx, PGA and EDC. Although, obvious differences in the sensor properties were not observed between the use of PGANa and PGAH, the electrode prepared with PGAH had a lower swelling degree. The glucose sensors prepared with both PGANa and PGAH were practically not affected by the existence of electroactive compounds, such as uric acid, and provided long-term stability for approximately 5 weeks. These sensors also showed good performance in horse serum.

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Introduction

In the last two decades, the number of diabetics has rapidly increased around the world, and the rising diabetes rates are still increasing, especially in the developing countries. The changes in lifestyle, diet and aging are the main cause, in addition to genetic predisposition. However, by managing the blood glucose level, it is possible to prevent serious diabetes complications. Therefore, it is clear that monitoring the blood glucose level closely is extremely important for the diabetes to maintain their health. Although, self monitoring of blood glucose concentration with a finger prick is a generally used method,[1] the development of implantable glucose sensors for continuous glucose monitoring is of great importance, since patients with insulin-dependent (type 1) diabetes have always feared low blood glucose concentrations,[2] especially during the night. Therefore, many methods of enzyme immobilization were proposed for the preparation of a glucose sensor. Conventional methods of enzyme immobilization are covalent attachment,[3-5] cross-linking,[3,6-14] hydrogel entrapment,[15,16] electropolymerized polymer entrapment,[8,17] and the combination of two or more methods.[18-21] Among the variety of procedures for the immobilization of enzyme, cross-linking is the most employed method for implantable glucose sensor fabrication,[7,9,11,14] since the enzymes are firmly immobilized with covalent binding. Moreover, the cross-linking of enzyme and bovine serum albumin (BSA) with glutaraldehyde is the most commonly used procedure. Since BSA is an animal-origin material, its use contains risks of unknown infections and prion diseases, which happened in the case of viral hepatitis, Creutzfeldt–Jakob disease (CJD), and bovine spongiform encephalopathy (BSE). The evaluation of the safety of the medical products is inspected based on ISO10993 of the global standard by Ministry of Health, Labour and Welfare in Japan or Food and Drug Administration (FDA). Although, the safety evaluations of animal origin material are also included, and the risk might be significantly low, the risk cannot be completely removed unless animal-origin materials are used.[22-25] γ-Polyglutamic acid (PGA) is a non-animal origin biodegradable polyamino acid, which is known as a sticky paste formed on the surface of fermented soybeans, "Natto". Since PGA is a low-cost, non-toxic, water-retentive material, it has been applied as a material in various fields, such as cosmetics, food, plastics and a flocculant for water treatment.[26-29] Recently, PGA has attracted a great deal of attention owing to its good biocompatibility,[30] and the research of PGA for clinical applications, such as bioglue,[31-34] drug delivery system (DDS)[33,35,36] and tissue engineering[37-39] has been prosperous. Considering that PGA is a polyamide with numerous carboxyl groups on the side chain, it seems to be suitable as a material of enzyme immobilization, since the carboxyl group can easily form covalent binding with a lysine residue of the enzyme in the presence of a condensation agent. Although there are several reports on the application of non-animal origin polysaccharides, such as chitin,[40] chitosan,[10,12,15] and agar,[16] for the fabrication of enzyme-immobilized film, just few are on non-animal origin polyamides, such as α-polyglutamic acid and poly-L-lysine;[18-21] above all, these materials are somewhat costly. To the best of

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our knowledge, an inexpensive non-animal origin polyamide, γ-PGA, has never been adopted to fabricate an enzyme sensor.

In this study, non-animal origin PGA was used as an enzyme-immobilizing material to fabricate an implantable fine needle-type glucose sensor; also, the sensor properties of the obtained electrodes were investigated. Two types of PGA, water-soluble γ-polyglutamic acid sodium salt (PGANa) and poorly water-soluble γ-polyglutamic acid (PGAH) were employed. The glucose sensors consisted of three layers: a permselective inner layer, an enzyme layer, and a biocompatible outer layer. Well-known Nafion/cellulose acetate composite film and polyurethane/polydimethylsiloxane composite film were introduced for the inner layer and the outer layer, respectively.[6,9] The enzyme layer was prepared by the covalent attachment of GOx and PGA with an amide bond, generated by the reaction of a lysine residue of GOx and carboxyl group of PGA in the presence of a water-soluble condensation agent, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). The fabrication of enzyme-immobilized film was performed by a one-step method and a two-step method, while the former was prepared by pouring a mixture solution of PGA, GOx and EDC; the latter was prepared by coating PGA film first, and then pouring a mixture solution of GOx and EDC. The properties of the obtained sensors were examined in a phosphate buffer solution (pH 7.4) and horse serum at 40°C, and the utility of non-animal origin PGA as an enzyme sensor preparation material was evaluated.

Experimental

Reagents and chemicals

Glucose oxidase (GOx) (265 U/mg, purified from *Aspergillus niger*) was purchased from Biozyme laboratories. γ-Polyglutamic acid sodium salt (M_w = 200000 – 400000) and γ-polyglutamic acid (M_w = 800000 – 1000000) were kindly supplied from Nippon Poly-Glu. A water-soluble condensing agent, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), was obtained from Toyo Kasei Kogyo. Cellulose acetate was obtained from Kishida Chemical. Nafion (perfluoronated ion exchange powder 5 wt% in mixture of lower aliphatic alcohols and water) was purchased from Aldrich. Polyurethane (Tecoflex) was purchased from Thermedics Inc. (Woburn, MA). Polydimethylsiloxane (MED-4211) was purchased from Nusil (Carpenteria, CA). Horse serum containing 0.55 mmol dm^{-3} glucose was purchased from Tissue Culture Biologicals (Los Alamitos, CA). Platinum-iridium (0.10 mm in diameter, Pt 90%-Ir 10%) wire was purchased from Nilaco. The insulation tube of polyimide (0.12 mm in inner diameter, 0.16 mm in outer diameter) was purchased from Furukawa Electric. All other reagents were of analytical grade, and were used without further purification.

Preparation of fine needle-type glucose sensor

A schematic illustration of fine needle-type glucose sensor is shown in Fig. 1. The sensor fabrication consists of three main steps: permselective inner-film preparation, enzyme immobilization, and biocompatible outer-film construction. The preparations of the permselective inner film and the biocompatible outer film were carried out according to methods of Wilson *et al*.[6,9] That is, the inner film was prepared by alternate five-times coating of both Nafion and cellulose acetate using a 5% Nafion solution and a 5% cellulose acetate solution, respectively. The outer film was prepared using a tetrahydrofuran solution containing 4.3 wt% polyurethane and 1.3 wt% polydimethylsiloxane.

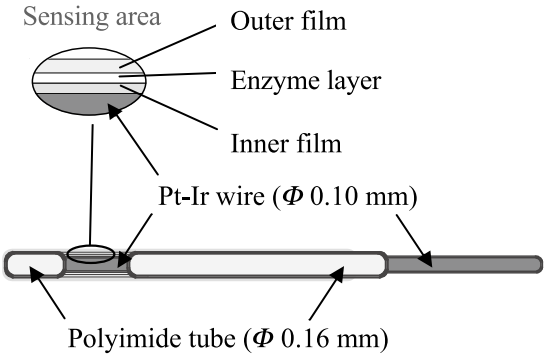

Fig. 1 Schematic illustration of a fine needle-type glucose sensor.

Immobilization of the enzyme was performed by two methods: 1) one-step method, in which the mixture solution of PGA, GOx and EDC was poured, and 2) two-step method, in which PGA coating was first performed, and then the mixture solution of GOx and EDC was poured. The enzyme-immobilized electrode using PGANa was prepared by both methods, while that using PGAH was prepared by only a two-step method, since the water solubility of PGAH was low, and GOx are generally unstable in organic solvents. For the one-step method preparation, 0.5 μL of a mixture solution of 0.25% (v/v) PGANa, 10 mg mL^{-1} GOx and 0.125% (v/v) EDC was poured on the inner film-coated electrode surface and dried for 30 min at room temperature. This operation was performed twice, and was followed by outer film preparation. For the two-step method preparation using PGANa, a wire loop was employed for a PGANa thin-film coating on the inner film-coated electrode. A wire loop was dipped in a 5.0% PGANa aqueous solution and a film was formed in the wire loop. The inner film-coated electrode was passed through the loop in order to form a thin PGANa film, and dried for 10 min at room temperature. PGANa coating was repeated 5 times in the same order. Subsequently, 0.5 μL of a 10 mg mL^{-1} GOx aqueous solution containing 0.125% (v/v) EDC was placed on the PGA-coated electrode and dried for 30 min at room temperature. This operation was performed twice, and was followed by outer-film preparation. The electrode using PGAH was prepared in a similar manner, except that a 5.0% PGAH dimethyl sulfoxide (DMSO) solution was used instead of a 5.0% PGANa aqueous solution. After construction of outer film, the electrode was dried at room temperature for 3 days and immersed in 0.1 mol dm^{-3} PBS for at least 2 days, before use. The outer diameter of the thickest part of the sensors was less than 0.2 mm.

Sensor measurement procedure

The principle of determining the current response is based on the formation of hydrogen peroxide during the enzyme catalytic reaction.

The amperometric responses of the prepared electrodes to glucose were examined at 40°C in a 0.1 mol dm^{-3} phosphate buffer solution of pH 7.4 containing 0.1 mol dm^{-3} NaCl by measuring the electrooxidation current at a potential of 0.6 V (*vs*. Ag/AgCl) for hydrogen peroxide detection. Amperometric measurements were performed with a Potentiostat Model 3104 (Pinnacle Technology Inc.).

The background current was stabilized within 15 min. The calibration of the sensor was carried out by adding increasing amounts of glucose to the stirred buffer solution. The current

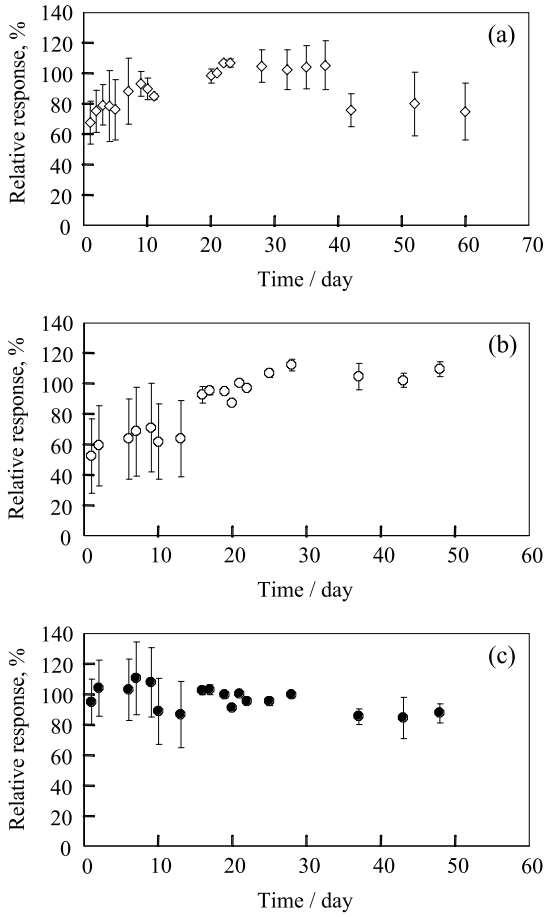

Fig. 2 Variation of sensor sensitivity with time for sensors prepared by the one-step method with PGANa (a), and by the two-step method with PGANa (b) and PGAH (c). Measurements were performed in a 0.1 mol dm^{-3} phosphate buffer solution (pH 7.4) at 40°C. The response current of 5.6 mmol dm^{-3} glucose obtained on each sensor on the 21st day was defined as the 100% relative response.

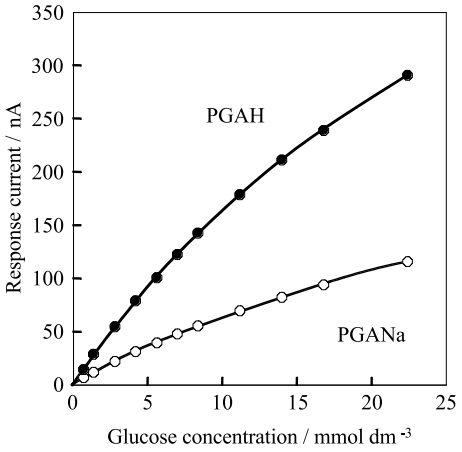

Fig. 3 Typical calibration curves of electrodes prepared by the two-step method with PGANa (open circle) and PGAH (closed circle) measured in a 0.1 mol dm^{-3} phosphate buffer solution (pH 7.4) at 40°C.

was measured at the plateau (steady-state response), and was related to the concentration of the analyte. The steady-state response currents were obtained within 60 s for all electrodes. The sensor response was also measured in horse serum containing 0.55 mmol dm^{-3} glucose at 40°C in order to evaluate the performance of the sensor in biological media.

Results and Discussion

Sensor response in phosphate buffer saline

Figure 2 shows the variation of the relative response in time on the electrode prepared by three different procedures. The response of the electrode was tested in 5.6 mmol dm^{-3} glucose at different intervals at 40°C and stored in phosphate buffer at 4°C when not in use. The response current of 5.6 mmol dm^{-3} glucose obtained on each electrode on the 21st day was defined as the 100% relative response, since the response of the sensor maintained approximately stable at that time. The response current of 5.6 mmol dm^{-3} glucose on the 21st day on sensors prepared by the one-step method with PGANa, the two-step method with PGANa and with PGAH, were 76.0, 70.8, and 106.3 nA, respectively. The electrode prepared by the one-step method with PGANa presented a stable response for about one month after an initial increase in the response for ten days, while some decrease in the response was observed latter (Fig. 2a). On the other hand, the electrode prepared by the two-step method with PGANa provided a stable response for more than a month and reliable decrease of response was not obtained, while initial increase of response was also observed (Fig. 2b). Moreover, the electrode prepared by two-step method with PGAH showed fairly unchanged sensor sensitivity for more than one month, while the dispersion of the response was observed owing the first two weeks (Fig. 2c). The difference in the initial time behavior of the sensor sensitivity between electrodes prepared from PGANa and PGAH may due to a difference of the swelling property of the obtained electrodes. The swelling degree of the electrode prepared with PGAH was lower than that prepared with PGANa. It is conceivable that the difference in the swelling capacity between the electrode sprepared with PGANa and PGAH were caused by differences in the swelling degree of the enzyme layer when hydrophobic outer-layer preparation was performed. Since an aqueous solution was used in the operation with PGANa, some content of water remained in the enzyme layer when the process of outer-layer fabrication was performed. Therefore, it is convenient to imagine that the outer layer was coated on a somewhat swelled enzyme layer when PGANa was employed. On the other hand, the swelling degree of the enzyme layer prepared with PGAH was significantly low when the outer layer was prepared, since DMSO was used as a solvent for PGAH.

The swelling of the enzyme-immobilized film facilitated the transportation of glucose within the film, which reached to increase of the hydrogen peroxide production. Although this phenomenon increases the response current of the sensor, a high degree of enzyme-immobilized film swelling causes a reduction of the film strength and the film disruption. The enzyme-immobilized film prepared from PGANa using the one-step method, in which the swelling degree was significantly higher than that using the two-step method, happened to have cracks and partial peeling off of the sensor film after 60 days of use. This partial loss of the sensor film led to a decrease in the response observed after 40 days of use (Fig. 2a). Therefore, the two-step method, which offers a sensor film with a certain swelling degree, was appropriate for enzyme immobilization.

Figure 3 shows typical calibration curves of the electrodes

Table 1 Influence of interferents on the glucose response current in a 0.1 mol dm^{-3} phosphate buffer solution (pH 7.4) containing 0.1 mol dm^{-3} NaCl

Interferent	Physiological conc./ mmol dm^{-3}	i_{G+i}/i_G[a]	
		PGANa	PGAH
Ascorbic acid	0.11	0.97 ± 0.03[b]	0.88 ± 0.02[b]
Uric acid	0.48	0.97 ± 0.01	0.97 ± 0.03
Urea	4.30	0.97 ± 0.01	0.95 ± 0.02
D-(−)-Fructose	0.40	0.98 ± 0.00	0.97 ± 0.00
Acetaminophen			

a. i_G, Response current of glucose (5.6 mmol dm^{-3}); i_{G+i}, response current of glucose (5.6 mmol dm^{-3}) in the presence of interferent at physiological maximum.
b. Mean values ± standard deviation were estimated from 3 separately prepared electrodes.

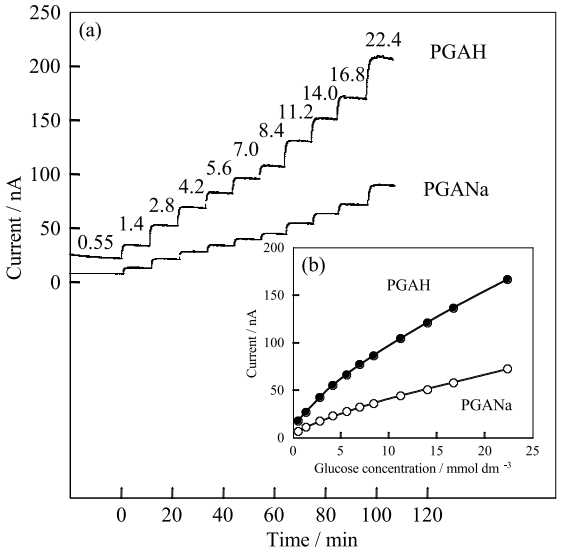

Fig. 4 (a) Typical current-time response of the sensors with increasing concentration of glucose in horse serum containing 0.55 mmol dm^{-3} glucose at 40°C. The numbers in the chart represent the corresponding glucose concentrations of the solution. (b) Variation of current with the glucose concentration for sensors prepared with PGANa (open circle) and PGAH (closed circle) measured in horse serum containing 0.55 mmol dm^{-3} glucose at 40°C.

prepared by the two-step method with PGANa and PGAH. The results described here were measured after 9 days of use. The response current increased with increasing concentration of glucose up to 22.4 mmol dm^{-3}, while the linear relationship between the glucose concentration and the response current was within the range lower than 10 mmol dm^{-3} for both electrodes. Considering the application of the sensor to *in vivo* measurements, it is desired to monitor the physiological glucose concentrations in the 2 – 25 mmol dm^{-3} range. Therefore, further improvements in order to expand the linear response are essential.

Influence of electroactive compounds

The interferences of electroactive compounds (interferents) existing in biological fluids to the glucose response were examined in the presence of their physiological maximum levels with the glucose concentration at 5.6 mmol dm^{-3} (Table 1). The level of interference is expressed in Table 1 as i_{G+I}/i_G: the ratio of the response current of glucose to the response current of glucose in the presence of an interferant. The glucose sensors prepared with both PGANa and PGAH using two steps were approximately not affected by the existence of electroactive compounds, except that the addition of ascorbic acid slightly influenced the response of a sensor prepared with PGAH. The reason for a decrease in the glucose response that occurred upon the addition of L-ascorbic acid was not clear.

Measurement in horse serum

Typical current-time curves with increasing concentration of glucose in horse serum containing 0.55 mmol dm^{-3} glucose at 40°C, for the sensor prepared from both PGANa and PGAH using two-step method are illustrated in Fig. 4(a). The numbers in the chart represent the corresponding glucose concentrations of the solution. Obviously, both sensors were shown to also work well in horse serum, while the response to glucose was 20 to 30% lower in horse serum compared with that in PBS. The oxidation current increased immediately after the addition of glucose, and reached 90% of the steady-state current within 50 s. The variation of current with the glucose concentration for sensors prepared with PGANa (open circle) and PGAH (closed circle) were measured in horse serum containing 0.55 mmol dm^{-3} glucose at 40°C. Although the response current of both electrodes increased with increasing concentration of glucose up to 22.4 mmol dm^{-3}, a linear relationship was obtained within the range of 6.0 mmol dm^{-3}.

Conclusions

In this work, a non-animal origin polyamide, PGA, was successfully applied for the fabrication of an implantable fine needle-type glucose sensor by covalently binding glucose oxidase and PGA using EDC. The glucose sensors consisted with three layers, where the permselective Nafion/cellulose acetate film layer and the biocompatible polyurethane/ polydimethylsiloxane film layer were formed inside and outside of the enzyme-immobilized film layer, respectively. The preparation of GOx-immobilized film by pouring a mixture solution of GOx and EDC on a PGA precoated surface was an admirable procedure, since the film obtained from this procedure had a lower swelling degree in water and a higher sensor stability compared with that prepared by pouring a mixture solution of PGA, GOx and EDC. Although some difference in the enzyme immobilization procedure occurred between PGANa and PGAH, due to a difference in the water solubility, the sensor with both PGA presented similar sensor properties, except that the sensor prepared from PGAH was slightly influence by the existence of ascorbic acid, and the swelling degree was low. Considering that the obtained sensors presented a long-term stability of approximately 5 weeks, a good response of glucose also in horse serum, and practically was not influenced by the existence of electroactive compounds, the sensors prepared by PGA were comparable with the sensor prepared by the most commonly used method, cross-linking of GOx and bovine serum albumin with glutardehyde.

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