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Biosensor system for continuous glucose monitoring in fish

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ABSTRACT

A biosensor system was developed for continuous estimation of blood glucose in fish. Because it is difficult to measure blood components in real-time due to decreased sensor output resulting from blood coagulation and coalescing blood proteins at the sensor placement site, we used the eyeball scleral interstitial fluid (EISF) as the site of sensor implantation. Evaluation of the relationship between EISF and blood glucose concentrations revealed that the blood glucose concentration correlated closely with the EISF glucose concentration ($y = 2.2996 + 0.9438x$, $R = 0.960$, $n = 112$). To take advantage of the close correlation between blood and EISF glucose, we prepared a needle-type enzyme sensor for implantation in the fish sclera using a flexible wire electrode. The sensor provided a rapid response, good linearity, and reproducibility. Continuous glucose monitoring could be carried out by implanting this needle-type glucose sensor onto the eye. The findings indicated that the glucose concentration increased with sensor output current over time, and that changes in the blood glucose were continuously reflected in the EISF. The glucose concentration was estimated based on the one-point or two-point calibration methods. The two-point calibration method yielded the most accurate glucose monitoring (blood glucose range of 70–420 mg dL^{-1}) over 160 min. Sensor-estimated glucose and whole blood glucose values were highly correlated ($y = 0.4401 + 0.8656x$, $R = 0.958$).

1. Introduction

The presence of harmful chemical drug residues in the tissues of cultured fish fed antibiotics and antimicrobial drugs has recently become a serious concern. In an attempt to improve production efficiencies in fish farms, fish are often cultured in overcrowded conditions. The resulting poor water quality and various physical stressors may lead to mass outbreaks of infectious disease. In general, antibiotics and antimicrobial agents are used to prevent bacterial infections in most fish farms. From a food safety perspective, the impact of these drugs on consumers of cultured fish is not negligible. Furthermore, the release of these chemicals into the environment may contaminate the environment and lead to the emergence of drug-resistant strains of bacteria. Therefore, a farming technique that has minimal dependence on antibiotics and antimicrobial agents for farming safe and high-quality cultured fish is required.

To address this problem, fish physiologists have focused on developing hemanalysis methods for cultured fish. In fish, variations in blood glucose concentrations may represent respiratory and nutritional disturbances. Additionally, stress is a generalized response of fish that represents a complex set of adaptive reactions in response to stressors. Blood glucose concentrations are elevated by various stressors, such as sampling, netting, handling, or other acute stressors [1,2]. A decrease in total cholesterol levels is a useful indicator of reduced resistance to bacterial infection [3,4]. Thus, determination of blood glucose and cholesterol concentrations in fish is very important for evaluating and managing fish health. To prevent the spread of bacterial infections and to develop an early prevention method, these parameters are currently determined in fish using clinical laboratory test kits designed for humans. These tests require separate analysis of each blood sample, and are therefore not practical, except at some small fish farms, because most farms cannot afford to absorb the associated costs.

On the other hand, enzyme sensor systems have been rigorously developed for measuring blood components such as glucose [5,6], cholesterol [7], and lactic acid [8]. We developed enzyme sensor systems for measuring total cholesterol and glucose concentrations in fish blood. These sensor systems are based on flow injection analysis (FIA) [9], which allows for rapid and convenient measurement of the total cholesterol and glucose, but still requires a technique for obtaining blood samples. To miniaturize and simplify the sensor system for detecting blood components in fish, we developed a needle-type enzyme sensor consisting of a needle-type hollow container, an immobilized enzyme membrane, and a fiber optic probe coated with ruthenium complexes that produce fluorescence depending on the dissolved oxygen content [10]. Direct

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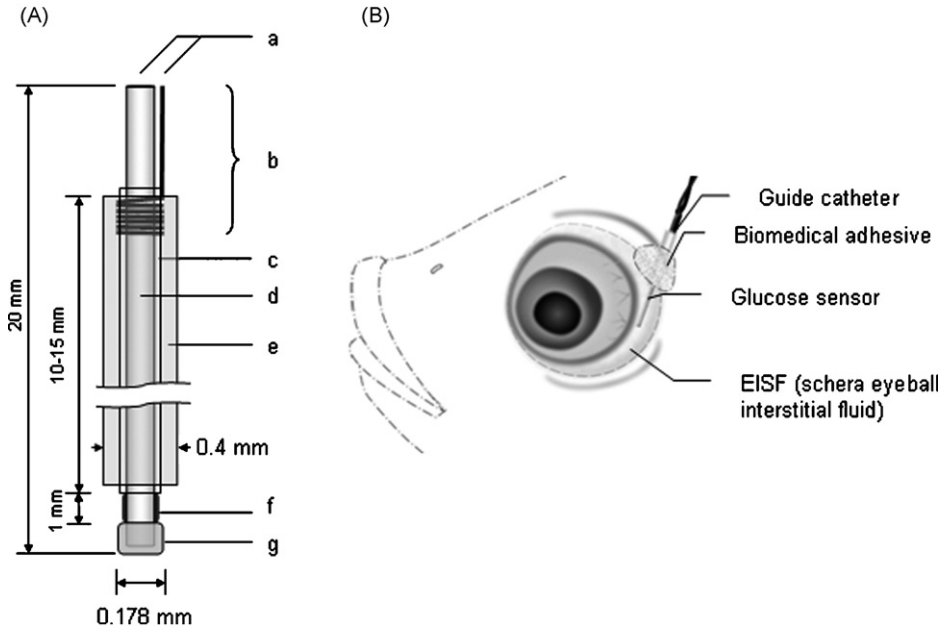

Fig. 1. (A) Diagram of needle-type glucose sensor: (a) Connecting point, (b) Copper wire, (c) Teflon coating, (d) Pt–Ir anode, (e) Ag/AgCl cathode, (f) glucose oxidase layer, (g) Epoxy resin. (B) Needle-type glucose sensor implantation into the interstitial fluid of the sclera (Nile tilapia; *Oreochromis niloticus*).

insertion of the sensor into the caudal vein also allows for rapid and convenient measurements of blood glucose concentrations, but measuring blood components in real-time is difficult because the sensor output is decreased by blood coagulation and coalescing protein (e.g., albumin, γ-globulin) on the sensor. In addition, it is not practical to implant enzyme sensors into fish blood vessels because of their fine vascular anatomy.

A continuous glucose monitoring system (CGMS) developed was for precise measurement of glucose in ISF, which mostly reflects that in blood [11]. This glucose sensor does not directly measure blood glucose concentrations, however, but rather glucose levels in the interstitial fluid (ISF) of the subcutaneous tissue surrounding the sensor. The CGMS is based on the principle that ISF glucose concentrations in the subcutaneous tissue reflect blood glucose levels. The dynamics and gradient relationship between blood and ISF glucose can be described by a two-compartment model of the separated capillary blood glucose and tissue beds [12]. The model demonstrates that the change in ISF glucose is related to changes in blood glucose by the rate of glucose diffusion across the capillaries and by the rate of glucose clearance from the ISF surrounding the tissue bed. According to this model, if the diffusion rate is constant, the relationship between blood and ISF glucose can be estimated using the mass balance equation. Currently, most continuous glucose monitoring methods, such as reverse iontophoresis [13], hypodermic needles, electrochemical enzyme sensors [14], and optical approaches [15], estimate glucose in the ISF compartment of the skin rather than in the blood. A sampling method to evaluate the correlation between glucose concentrations in the blood and ISF is desirable. There are various approaches for obtaining ISF, such as ultrasound [16], microdialysis [17], microporation using a laser [18], and penetration using a hollow microneedle array [19]. Results of studies using these approaches indicate that ISF glucose concentrations range from 87 to 101% of the blood glucose concentrations [20].

In our study, for the ISF sampling site we focused on the fluid-filled inner scleral surface of the eyeball (referred to hereafter as eyeball scleral ISF; EISF) and hypothesized that if the glucose diffusion rate is fixed in the EISF as in the ISF of the two compartment model, the sclera can be used as a sensor implantation site in fish (Fig. 1B). For continuous in vivo glucose monitoring, it is essential to develop an implantable and minimally invasive glucose sensor [21–26]. However, almost glucose sensors have been designed for humans and livestock, not fish. Moreover, fish scarfskin is very hard and susceptible to infection during the implantation of needle-type sensors. Therefore, more convenient fish health assessment requires a specialized sensor with high mechanical strength, minimal invasiveness, as well as the ability to be easily inserted and fixated. Here, we describe the sensor implantation and fixation method for our needle-type glucose sensor. Short-term continuous blood glucose monitoring was conducted under light anesthesia to determine whether blood glucose concentrations can be estimated from the EISF.

2. Materials and methods

2.1. Reagents

Glucose oxidase from *Aspergillus niger*; E.C. 1.1.3.4, type VII-S; 197000 unit g^{-1} was purchased from Sigma–Aldrich (St. Louis, MO). Bovine serum albumin (BSA) and 2-phenoxy ethanol were purchased from Wako Pure Chemical Industries (Tokyo, Japan). D-Glucose was dissolved in 0.1 M phosphate buffered saline (PBS) and mixed by rotating for 24 h before use. All other reagents were of analytical grade.

2.2. Glucose analyses for evaluating the relationship between blood and EISF glucose concentrations

2.2.1. Sampling and analytical procedure

Blood and EISF glucose concentrations were measured in Nile tilapias *Oreochromis niloticus*. We used Nile tilapias as the test fish cultured at Tokyo University of Marine Science & Technology. Each fish (body weight; ca. 100–300 g, body length; ca. 15–25 cm) was netted from the preserve and individually anesthetized with 2 ppm 2-phenoxy ethanol by bath exposure. Blood samples were then collected from the caudal vein using a heparinized syringe fitted with a 20G needle. The blood samples (100–500 μL) were centrifuged (650 × *g*, 10 min) to separate the plasma. EISF sampling was per-

formed as follows; pushing slightly on one eye caused a blister-like membrane to rise up to the surface; a heparinized syringe fitted with a 27G needle was inserted into the blister-like membrane. The EISF sample (50–500 μL) was obtained from the ISF in the eyeball sclera. To minimize measurement error induced by stress, the sampling procedure never lasted more than 5 min per fish. Each collected sample was transferred to a test tube and stored at −80 °C before use.

Fish blood and EISF glucose concentrations were determined using enzymatic colorimetric methods. Although the methods were intended for humans use, it can also be used for fish in the fields of fish physiology and fish pathology [27–33]. We used the commercial test kit (glucose test; Wako Pure Chemical Industries) according to the method of Tsuzuki et al. [29]. When each sample (20 μL) was mixed with 3 mL PBS (pH 7.1) containing enzymes (glucose oxidase, horseradish peroxidase) and color-producing reagent, the glucose was oxidized to produce gluconolactone and hydrogen peroxide. The hydrogen peroxide reacted with 4-aminoantipyrine and phenol to give a red color. Glucose concentrations were measured using a UV/Bis spectrophotometer (JASCO Co., Tokyo, Japan).

2.2.2. Correlation between blood and EISF glucose concentrations in randomly selected individuals

The purpose of this test was to confirm whether there are individual differences in the relationship between the blood and EISF glucose concentrations in Nile tilapia, *O. niloticus* for our model. Nile tilapia is widely cultured in many tropical and subtropical regions of the world and constitutes the third largest group of farmed finfish, right after carp and salmonids. The fish were reared in a plastic tank (80 mm × 60 mm × 50 mm) with a 240-L capacity. Our stock population consisted of 112 adult Nile tilapia (ca. 100–300 g), the fish were randomly selected, netted from the tank, and blood and EISF were sampled using the methods described above.

2.3. Needle-type glucose sensor preparation

We developed a needle-type enzyme sensor (diameter × length; 0.4 mm × 20 mm) for subcutaneous glucose monitoring in fish. The sensor was made using a 20-mm length of Teflon-coated platinum iridium wire (Fig. 1A). The Teflon was stripped at one end to expose 1.0 mm of the Pt–Ir wire as the sensing cavity (working electrode). Copper wire was wound around the Teflon-coated surface to connect to the potentiostat. Then, Ag/AgCl paste (BAS, Tokyo, Japan) was applied around the copper wire and used as the reference electrode/counter electrode). The tip of the wire was sealed with epoxy resin, leaving a 0.7-mm long sensing cavity. An enzyme solution, containing 2 mg (352 U mL^{-1}) glucose oxidase and 10 mg bovine serum albumin (BSA) dissolved in 1 mL of 0.1 M PBS (pH 7.8), was prepared. The sensing cavity was dipped and coated with a thin layer of the enzyme solution and air-dried for 10 min; this procedure was repeated twice. The wire tip was held in a vertical position in a vial, and 100 μL glutaraldehyde (50%) was added to induce cross-linking between the glucose oxidase and bovine serum albumin and the electrode was stored at 30 °C for 6 h. The sensor was then soaked and stored in PBS (pH 6.5) overnight at 4 °C. Once the cross-linking was complete, the sensor was thoroughly rinsed with PBS to remove glucose oxidase and glutaraldehyde residues (pH 6.5).

2.4. Evaluation of needle-type glucose biosensor

A two-electrode electrochemical system with the Pt–Ir wire working electrode and Ag/AgCl reference/counter electrode was used. A 650-mV potential (*vs.* Ag/AgCl) was applied to the Pt–Ir working electrode for the amperometric glucose measurement.

The sensor was connected to a multi-channel potentiostat (Pinnacle Technology Inc., Lawrence, KS) attached to a personal computer (DELL Dimension 4700C, Round Rock, TX) via either a USB 2.0 or RS232C port. The output current was recorded via computer-controlled data acquisition software (Pinnacle Acquisition Laboratory). For the glucose measurements, the calibration curve was prepared as follows. First, 2.0 mL of EISF was extracted using a heparinized syringe fitted with a 27G needle from Nile tilapia. The initial glucose concentration in the EISF was 51.02 mg dL^{-1} determined by the enzymatic colorimetric method. The EISF was transferred into a small vial (outer diameter × height; 19 mm × 55 mm). The sensor was then place into the vial to soak with stirring for 1 h to allow the background output current to stabilize. Then, 20 μL of standard glucose solution (5000 mg dL^{-1}) was added to the vial. When the sensor output had stabilized, the procedure was repeated until the sensor output no longer increased.

2.5. The continuous glucose monitoring system

2.5.1. Glucose sensor implantation into the EISF

Sensor implantation and fixation were performed according to the following procedure (Fig. 1B). Nile tilapia was not fed for 24 h before continuous glucose monitoring. At first, the fish were anesthetized in a water bath containing 2 ppm 2-phenoxy ethanol. A 22G catheter consisting of an outer Teflon layer and inner puncture needle (22G, 23 mm, SerflowTM, Terumo, Tokyo, Japan) was inserted into the EISF for sensor implantation. The inner puncture needle was then removed, and the excess exposed catheter on the skin was removed. Next, the sensor was inserted into the outer Teflon layer, and then immobilized using biomedical adhesive (Aronalpha-A Sankyo, Toagosei, Tokyo, Japan), the major ingredient of which was ethylcyanoacrylate monomer.

2.5.2. Sensor calibration

Continuous estimation of blood glucose was performed using both one-point calibration and two-point in vivo calibration methods [34–36]. The one-point calibration method is represented by the following formula:

$$G_{(t)} = \frac{I_{(t)}}{S}.$$

The sensor sensitivity (S), expressed in nA/(mg dL^{-1}), was determined from a single blood glucose determination as the ratio between the concomitant sensor output current (I; expressed in nA) and the blood glucose concentration (G). Subsequently, the glucose concentration was estimated based on the sensor output current (I).

First, the sensor output current was measured until it stabilized after the sensor was implanted into the sclera of the eye. A blood sample was obtained from the caudal vein using a heparinized syringe fitted to a 23G needle. Blood glucose (G) was determined using a Glucocard (Arkray KDK Co. GT 1640, Kyoto, Japan). Although the glucocard is intended for human use, a good correlation was observed between glucose concentrations in the fish blood sample determined by Glucocard and that by commercial enzymatic colorimetric test kit in our pre-experiment (R = 0.988, data not shown). Therefore, the parameter (G) was used as the calibration reference.

The two-point calibration method is represented by the following formula:

$$G_{(t)} = \frac{(I_{(t)} - I_0)}{S}.$$

The formula consists of the sensor sensitivity (S) and the intercept I_0 with the sensor output, i.e., the theoretical sensor output that would be observed in the absence of glucose in ISF. Parameters (S) and (I_0) were calculated from the change in blood glucose concentration G from G_1 to G_2, the change in the sensor current I from I_1 to I_2: $S = (I_2 - I_1)/(G_2 - G_1)$, followed by calculation of I_0 using the following formula: $I_0 = I_1 - (S \times G_1)$.

2.5.3. Experimental conditions

Nile tilapia were transferred to a water tank (40 cm × 24 cm × 50 cm) containing of 1 ppm phenoxy ethanol as a light-anesthetic agent to prevent the sensor signal from becoming unstable due to fish movement. Dissolved oxygen in the water tank was saturated using an air pump. To compare between absolute blood glucose concentrations and sensor-estimated glucose concentrations, blood was collected for blood glucose concentration measurement using the glucocard at regular intervals. Continuous glucose monitoring of EISF was performed under the above experimental conditions using our glucose sensor.

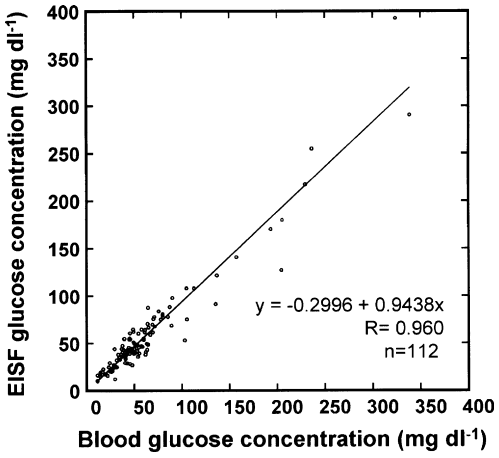

Fig. 2. Correlation between blood and EISF glucose concentrations in randomly selected fish (20–340 mg dL^{-1}).

3. Results and discussion

3.1. Comparisons between blood glucose and EISF

We investigated the correlation between glucose concentrations in the blood and in the EISF. Nile tilapia was captured randomly from the water tank, and both blood and EISF samples were obtained. The correlation between glucose concentrations in blood and EISF was investigated.

The observed blood glucose and EISF glucose concentration values for all subjects are shown graphically in Fig. 2. As shown in the figure, the glucose concentration in EISF was closely correlated with that in the blood ($y = 0.2996 + 0.9438x$, $R = 0.960$, $n = 112$). Eighty-four percent of EISF glucose concentrations were within 30% of the blood glucose concentrations. The glucose concentrations in EISF were slightly lower than those in blood. Glucose concentrations in EISF are approximately 87–101% those in the blood in humans and other mammals [20]. In Nile tilapia, EISF glucose concentrations were approximately 94% ($n = 112$) that of the blood. The glucose concentrations in the EISF and blood tended to be range between 30 and 100 mg dL^{-1}, consistent with the average normal blood glucose concentration in Nile tilapia of 50 mg dL^{-1} [34]. The results indicate that glucose concentration of EISF reflects that in blood.

3.2. Characteristics of the needle-type glucose biosensor

To evaluate the potential use of the sensors for in vivo applications, the linearity of the sensor was investigated in EISF. A typical biosensor calibration plot showing the relationship between the response current and glucose concentration is shown in Fig. 3(A). After the baseline stabilized in EISF, an aliquot of a standard glucose solution was added to the solution. A rapid and stable response was confirmed prior to each addition of glucose. The calibration curve of the needle-type glucose sensor is shown in Fig. 3(B). The results indicated that this biosensor was linear in the glucose concentration range of 51.02–459.60 mg dL^{-1}, with a correlation coefficient of 0.995 calculated using linear regression analyses.

The dependency of the sensor on pH and temperature for an 18-mg dL^{-1} glucose-induced current is shown in Fig. 4. The glucose oxidase activity strongly depended on the pH of the sample solutions or body fluid (Fig. 4A). Sensor output current increased rapidly in the range of pH 5.0–6.5. For pH greater than 6.5, the sensor output current gradually decreased. When the pH of EISF was measured, the value was almost 7.9–8.0, but an adequate sensor response was still obtained at pH 9.0. Moreover, as shown in Fig. 3, the sensor can be used to measure glucose in an EISF sample. Temperature is an important factor that has a significant effect on enzyme activity. For temperatures ranging from 10 to 36 °C, the current increased and

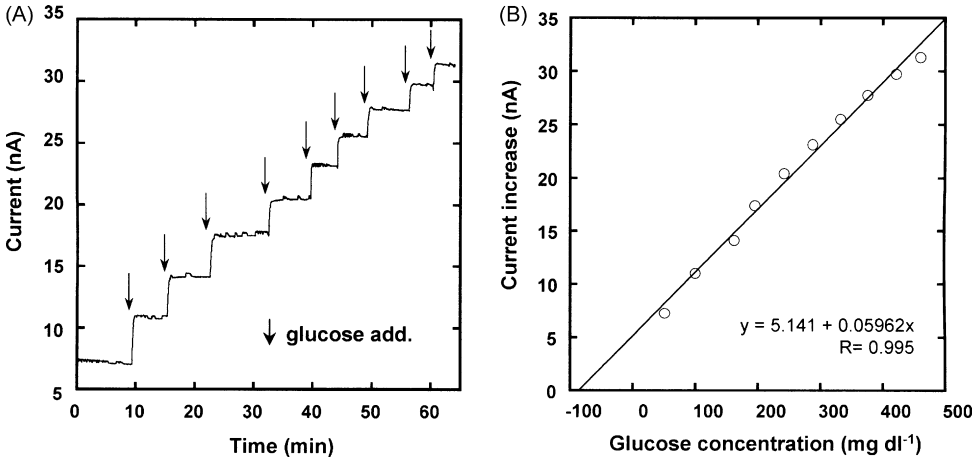

Fig. 3. (A) Typical response curve for the glucose sensor on sequential addition of standard glucose solution in EISF under a +650 mV potential (*vs.* Ag/AgCl). (B) Glucose calibration plots of the glucose biosensor (range of glucose concentrations 51.02–459.63 mg dL^{-1}). Sample solution was added in pH 6.5 PBS for 25 °C.

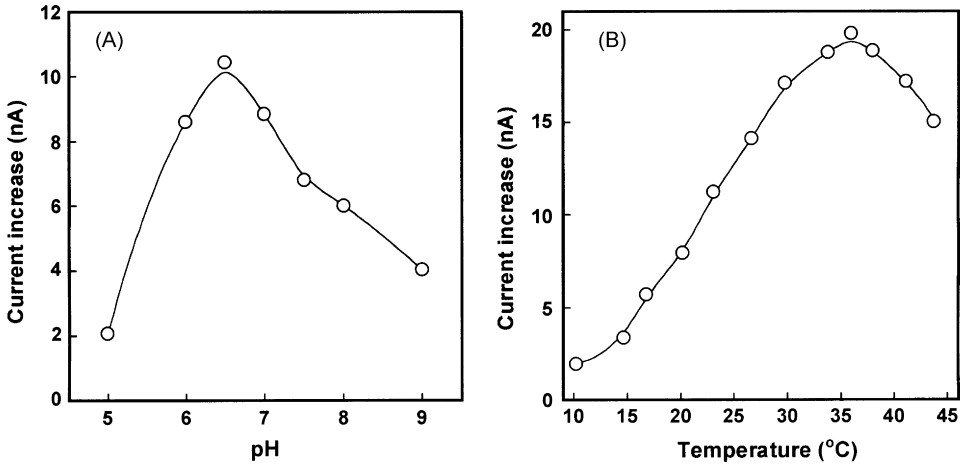

Fig. 4. Typical effects of pH (A) and temperature (B) on the glucose sensor response in 0.1 M PBS. In (A) the pH 6.5, in (B) the temperature was 25 °C. The measurements were performed using 18 mg dL^{-1} glucose standard solution under the potential of +650 mV (*vs.* Ag/AgCl).

reached maximum output (Fig. 4B). Above 36 °C, the current gradually decreased. In our experiments, Nile tilapias were cultured at 30 °C, and therefore the sensor response could be obtained.

3.3. Blood glucose monitoring using the needle-type glucose sensor

A typical response curve for in vivo continuous glucose measurement is shown in Fig. 5(A). Blood glucose was periodically measured using a glucocard, and the sensor current output was continuously monitored for 160 min. The glucose concentration and sensor output current both continuously increased. Thus, the change in blood glucose concentration was reflected in EISF glucose concentrations. The cause of the blood glucose increase was probably due to stress induced by the experimental conditions. For example, in this experiment, sample fish were captured using a spoon-net and transferred to a water tank under anesthesia. After sensor implantation into the eye, there was a rapid increase in blood glucose in response to physical stressors that trigger the release of cortisol, a glucocorticoid hormone. As in mammals, cortisol stimulates gluconeogenesis and increases blood glucose concentrations.

In this study, glucose concentration was estimated using two different calibration methods. To obtain an estimate of the precision and accuracy of the sensor readings, we compared the one-point and two-point calibration methods (Fig. 5B). One-point calibration was used as calibration value (G_1, I_1) in which G_1 was the initial blood glucose concentration (70 mg dL^{-1}) and I_1 was the subsequently obtained sensor output current (23.41 nA). Two-point calibration was calculated using the first parameter (I_1: 23.41 nA, G_1: 70 mg dL^{-1}) and glucose concentration and sensor output current obtained 45 min after sensor implantation (I_2: 38.94 nA, G_2; 196 mg dL^{-1}). As shown in Fig. 5(B), the two-point calibration method was more accurate than the one-point calibration method. Sensor values analyzed using the one-point calibration method produced low blood glucose values compared to the actual glucose value. The linear regression analysis between sensor-estimated glucose in EISF (X, mg dL^{-1}) and whole blood glucose concentrations measured by the glucocard (Y, mg dL^{-1}) is shown in Fig. 6. A good correlation was observed between sensor-estimated glucose and whole blood glucose (one-point

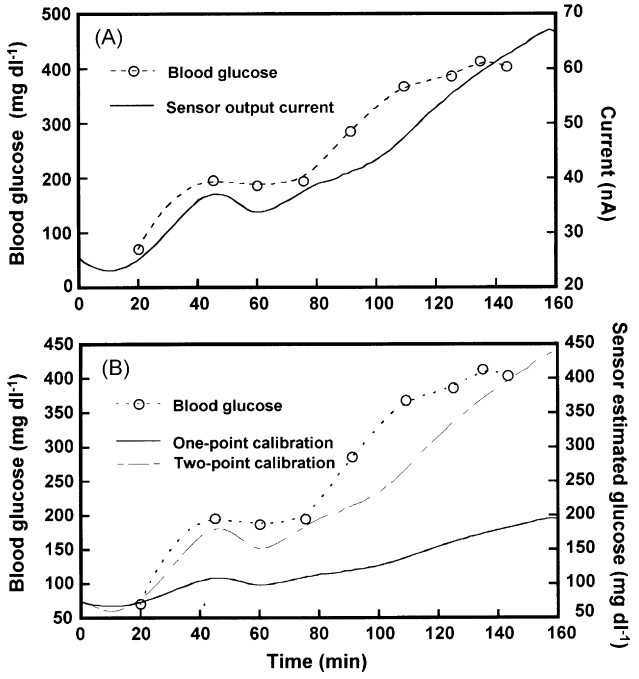

Fig. 5. Continuous glucose measurement for short-term under light anesthesia (A) Comparison between blood glucose change and sensor output current. (B) Comparison between blood glucose change and sensor-estimated glucose using the two calibration methods.

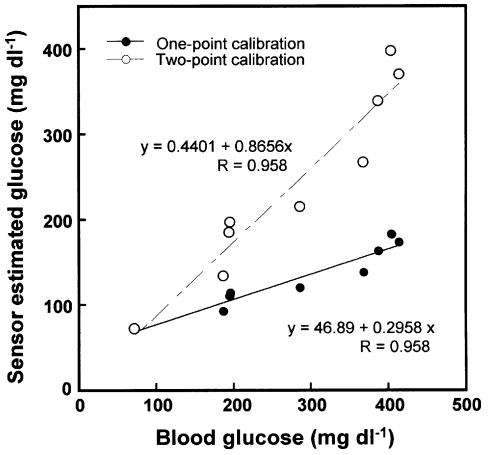

Fig. 6. Correlation between blood glucose and sensor-estimated glucose calculated by the one-point and two-point calibration methods.

calibration method; $y = 46.89 + 0.2958x$, $R = 0.958$; two-point calibration method; $y = 0.4401 + 0.8656x$, $R = 0.958$). The slope of the correlation curve obtained for the one-point calibration method was lower than that of the two-point calibration method. This difference is probably reflects the fact that the background output current (I_0) was not given enough time to stabilize. If the sensor output current is not stabilized after sensor insertion, the calibration values (G_1, I_1) will be different from the true sensor output current. As a result, the slope of the sensitivity (S) becomes too steep, and the sensor-estimated glucose level determined by one-point calibration is not accurate. In fact, at least 2 h is needed for the background current to stabilize [37].

We used the two-point calibration method to determine sensor sensitivity (S) and background current (I_0) for two different current responses (I_1, I_2) to glucose concentrations (G_1, G_2) obtained at two time points. The theoretical background current calculated using the two-point calibration method is important for determining accurate sensor sensitivity (S) for short-term in vivo glucose monitoring. For example, if one-point calibration is performed under a stable background current, the sensor-estimated glucose based on one-point calibration method might be as accurate as that calculated using the two-point calibration method. It is unlikely, however, that there will be enough time to obtain a stable background current because the test fish are lightly anesthetized. Moreover, the interstitial glucose concentration is dependent on the balance between the local production rate and the clearance by the local blood flow. Under anesthesia, glucose metabolism in fish may be lower in both capillary blood vessels and the EISF pool might decrease due to a decrease in stroke volume. If long-term measurements are performed under these conditions, it might be difficult to obtain a high correlation between blood and sensor-estimated glucose concentrations.

Here, we confirmed the correlation between EISF and blood glucose concentrations in fish, which led us to develop a needle-type glucose sensor for short-term continuous glucose monitoring in fish. Our sensor system allows for the estimation of blood glucose concentrations using EISF as a reference in water creatures under regulated experimental conditions. Our future research plans are to develop a telemetry system for continuous glucose monitoring. The original purpose of our study was to develop a method for continuous glucose monitoring in fish to monitor health conditions. In the present study, a needle-type glucose sensor was connected to a multi-channel potentiostat. The sensor system was thus too large to allow the fish to swim freely. This fixed-line sensor system cannot measure blood glucose under non-stressful conditions, as indicated by the increase in blood glucose concentrations induced by anesthesia and physical stress. Therefore, the fixed-line biosensor system is not appropriate for swimming fish in an actual field, such as a fish farm. Thus, it is necessary to develop a telemetry biosensor system for long-term monitoring. The development of such a sensor system will facilitate and simplify the management of cultured fish health.

4. Conclusion

In the present study, we developed a system to continuously monitor glucose concentrations in fish. The fish species Nile tilapia was selected as the fish model. We used the interstitial fluid from the eyeball sclera as the ISF sampling site. The relationship between EISF glucose concentrations and blood glucose concentrations was investigated. The EISF glucose concentration was closely correlated with that in blood ($R = 0.960$, $n = 112$). We prepared a needle-type enzyme sensor for subcutaneous glucose monitoring. The sensor provided a rapid response with good linearity (51–460 mg dL^{-1}), with a correlation coefficient of 0.995 calculated using linear regression analysis. Continuous glucose monitoring was performed by implanting the sensor into the eye. The results of the study confirmed that both glucose concentration and sensor output current increased over time, and the blood glucose change was reflected by EISF glucose concentrations, which could be measured continuously. In this study, the glucose concentration was estimated on the basis of both a one-point and two-point calibration method. The two-point calibration method allowed for accurate glucose monitoring (blood glucose range of 70–420 mg dL^{-1}) over 160 min. A good correlation was observed between the sensor-estimated glucose and whole blood glucose concentrations ($y = 0.4401 + 0.8656x$, $R = 0.958$). Although the measurement was short-term and under regulated conditions, we consider that our novel measurement method will be useful for continuous estimation of blood glucose levels in fish.

Acknowledgments

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