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Wireless biosensor system for real-time cholesterol monitoring in fish "Nile tilapia"

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ABSTRACT

The rapidly increasing demand for cultured fish as a food resource requires simple, effective methods for controlling fish health in culture conditions. Plasma total cholesterol levels are significantly related to fish mortality following bacterial challenge, and are thus a good indicator of the general health of fish. We developed a wireless biosensor system to continuously monitor the total cholesterol concentration in fish (Nile tilapia, *Oreochromis niloticus*). The biosensor was constructed with Pt–Ir wire (φ0.178 mm) as the working electrode and Ag/AgCl paste as the reference electrode. Cholesterol oxidase and cholesterol esterase were immobilized on the working electrode using glutaraldehyde. The sensor output was linear and strongly correlated with the cholesterol level ($R = 0.9970$) in the range of 2.65–403 mg dl^{-1}. This range covers the range of total cholesterol levels in fish. To avoid blood coagulation and proteins coalescing on the sensor, we implanted the sensor in the fluid under the scleral surface of the eyeball (EISF). The EISF is presumed to reflect the levels of most blood components and does not include the substances contained in blood that inhibit sensor measurement. Total cholesterol concentrations in blood and EISF were strongly correlated ($R = 0.8818$, $n = 72$) in the blood total cholesterol range of 74–480 mg dl^{-1}. Therefore, we used EISF as an alternative to blood and performed continuous *in vivo*-monitoring of the total cholesterol concentration in fish. We also investigated the application of the calibration method and wireless monitoring system. These applications enabled us to securely monitor total cholesterol levels in free-swimming fish in an aquarium for over 40 h. Thus, our newly developed sensor provided a rapid and convenient method for real-time monitoring of total cholesterol concentrations in free-swimming fish.

1. Introduction

There is growing concern that many resources, such as food and energy, are in limited supply and may eventually run out. Fisheries are also at a crisis point because of increased worldwide consumption, over-fishing, and regulatory demands. The demand for fishery products will eventually exceed supply. The development of aquaculture is expected to resolve this problem, providing society with a sustainable food supply. Aquaculture, however, must still overcome many issues before it can be regarded as a mainstream method of fish production. In particular, aquafarms are plagued by fish disease. Due to overcrowded farming conditions, the diseases quickly spread through the water to other fish. Dead or diseased fish have no value as food and aquafarmers thus sustain considerable financial hardship. Although antibiotics are effective, their use affects the risk of exposing the consumer to residual drug. Therefore, a method to maintain fish health without using drugs is in high demand. Several blood tests have been developed for monitoring fish health [1]. For example, plasma total cholesterol concentration is significantly related to fish mortality following bacterial and viral challenge, and unhealthy fish have below normal plasma total cholesterol concentrations [2]. Therefore, plasma total cholesterol concentration is a good indicator of the general health of fish. Currently, in aquaculture, plasma total cholesterol concentration is measured using a colorimetric determination method. This method, however, is complicated, costly, and time-consuming. Therefore, a simple, cost-effective, and convenient system for monitoring fish health is needed.

Electrochemical technology, especially biosensor technology, is potentially useful for solving this problem. Many types of

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biosensors for measuring blood components, such as glucose [3], cholesterol [4,5], and lactic acid [6] have been developed. In 1982, Karube et al. [7] developed a sensor system comprising an immobilized enzyme and an oxygen electrode. Since then, various sensors have been developed based on the same principle and that implemented innovations such as the use of a hydrogen peroxide electrode [8], quartz crystal [9], silicate sol–gel [10], and Langmuir–Blodgett films [11]. Sensors for measuring total cholesterol concentration in the blood have also been developed [12,13], but these sensor systems do not provide continuous measurement.

We have developed enzyme sensor systems for measuring total cholesterol and glucose concentration in fish blood. These sensor systems are based on flow injection analysis [14], which allows for rapid and convenient measurement of total cholesterol and glucose, but separate procedures are required for collecting the blood samples for testing. Moreover, the flow injection analysis system is large and complicated compared with a batch-type measurement system. To miniaturize and simplify the sensor system for measuring blood components in fish, we developed a needle-type enzyme sensor comprising a needle-type hollow container, an immobilized enzyme membrane, and a fiber optic probe coated with ruthenium complexes [15]. In this system, direct insertion of the sensor into the caudal vein allows for rapid and convenient measurements of blood glucose concentrations, but continuous long-term measuring is difficult because the sensor output is decreased by blood coagulation and coalescing protein (e.g., albumin, γ-globulin) deposits on the sensor. Methods for continuous *in vivo*-monitoring of glucose concentrations in human and other mammals have been studied, focusing on measurements of glucose in interstitial fluid (ISF) [16,17]. ISF is the biologic fluid present between the blood vessels and cells. The reason for selecting ISF for the sampling site is that ISF is presumed to reflect the concentration of most blood components and does not include the substances in blood that hamper sensor measurement. We focused on the fluid under the scleral surface of the eyeball (EISF) in fish as a site for collecting ISF and developed a needle-type enzyme sensor to wirelessly monitor fish glucose levels [18]. The sensor system was constructed using a platinum–iridium (Pt–Ir) Ag/AgCl electrode, controlled potentiostat, electric wave sensor, and personal computer. The sensor was inserted into the external layer of the fish (Nile tilapia, *Oreochromis niloticus*) eyeball. Real-time glucose monitoring was performed using the sensor system. Glucose concentrations reflecting those in fish blood could be monitored in free-swimming fish.

The present paper describes the development of a rapid and convenient method for real-time monitoring of cholesterol concentrations in fish, as follows; (1) investigating the relationship between EISF and blood total cholesterol levels in fish, (2) determining the characteristics of the needle-type cholesterol biosensor, (3) developing a wireless system for monitoring total cholesterol levels under free-swimming conditions in an aquarium.

2. Experimental

2.1. Reagents

Cholesterol esterase (EC 232-808-6, 2.8 U mg^{-1}), cholesterol oxidase (EC 232-842-1, 2.4 U mg^{-1}), and nonaethylene glycol monododecyl ether were purchased from Sigma–Aldrich (St. Louis, MO). Bovine serum albumin, cholesterol oleate, 2-phenoxy ethanol, and 5% nafion dispersion solution were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Standard solutions of cholesterol ester were prepared by first dissolving cholesterol oleate in nonaethylene glycol monododecyl ether and then in 0.1 M phosphate buffered saline (PBS). Approximately 3 g of nonaethylene glycol monododecyl ether was added to 100 mg cholesterol oleate. The solution was then stirred until it became clear and colorless at 60 °C and 600 rpm. PBS (pH 8.0) was added to bring the solution to the desired volume, and the solution was stirred again until it became clear and colorless. A fresh standard solution was prepared for each experiment.

2.2. Total cholesterol analysis for evaluating the relationship between total cholesterol concentration in blood and EISF

2.2.1. Collection and analysis of the sample

We used Nile tilapia (*O. niloticus*) as the test fish, cultured at Tokyo University of Marine Science & Technology. Total cholesterol concentrations between blood and EISF were measured. Each fish (body weight; ca. 100–300 g, body length; ca. 15–25 cm) was netted from the preserve and anesthetized with 400 ppm 2-phenoxy ethanol by bath exposure. Blood samples were collected from the caudal vein along the backbone by inserting a heparinized syringe fitted with a 23G needle (0.65 mm × 25 mm) into the basal anal fin. The blood samples (100–200 μl) were then centrifuged (550 × *g*) for 10 min to separate the plasma. EISF samples were collected by inserting a syringe fitted with a 27G needle (0.40 mm × 19 mm) into the sclera of the eyeball. To minimize measurement error, the collection procedure never lasted more than 10 min per fish. Each collected sample was transferred to a test tube and stored at −80 °C until analysis.

Total cholesterol concentrations were determined by an enzymatic colorimetric method (E-test, Wako Pure Chemical Industries, Tokyo, Japan). Each sample (20 μl) was mixed with 3 ml buffer solution (pH 6.1) containing enzymes (cholesterol esterase, cholesterol oxidase, horseradish peroxidase) and a color-producing reagent. Total cholesterol was hydrolyzed and then oxidized to produce 4-cholesten-3-one and hydrogen peroxide. The hydrogen peroxide reacted with 4-aminoantipyrine and D-amino acid oxidases to produce a blue color (600 nm). Total cholesterol concentrations were measured using a UV/Vis spectrophotometer (JASCO Co., Tokyo, Japan).

2.2.2. Temporal change of total cholesterol concentration between blood and EISF in individual fish over days

We evaluated whether the temporal changes in total cholesterol concentrations in the blood were reflected by changes in the total cholesterol concentration in EISF in individual fish. The testing system comprised a 45-L plastic tank (50 cm × 30 cm × 30 cm). During the test, the water temperature was maintained at 30 °C with continuous aeration and recirculation through a biologic filter. The photoperiod, provided by a fluorescent light, was set to be on from 10:00 to 20:00. Blood and EISF samples were collected at 12:00 daily, as described above. The testing period spanned 30 days and the fish were fed on days 7 and 15.

2.2.3. Correlation of total cholesterol concentration between blood and EISF in randomly selected fish

We evaluated whether there were differences in the relationship between total cholesterol concentration in the blood and EISF in individual fish. Our stock population consisted of 72 adults Nile tilapia (ca. 100–300 g). The fish were randomly selected, netted from the tank, and blood and EISF were collected by the methods described above.

2.3. Needle-type total cholesterol sensor

2.3.1. Preparation of hydrogen peroxide electrode

The sensor was constructed using a 15-mm Teflon-coated platinum/iridium (Pt–Ir) wire (φ0.178 mm), 10-mm copper wire (φ0.1 mm), Ag/AgCl coating paste (BAS, Tokyo, Japan), and two copper lead wires (Fig. 1). The sensor was composed by two electrodes (working electrode and reference electrode/counter

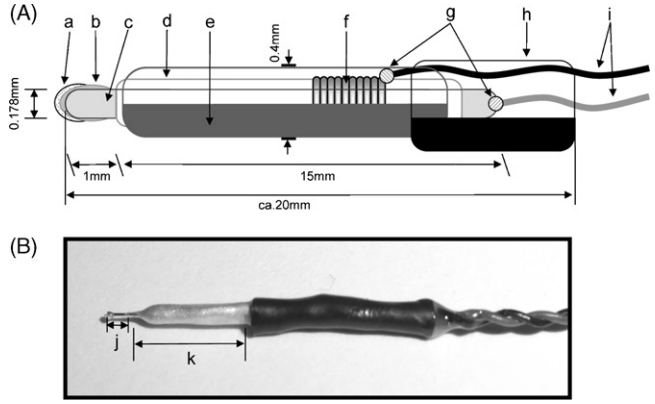

Fig. 1. Diagram (A) and photograph (B) of needle-type total cholesterol sensor. (A): (a) enzyme membrane, (b) nafion, (c) platinum/iridium, (d) Teflon-coat, (e) Ag/AgCl paste, (f) copper wire, (g) connection, (h) heat-shrink tubing, (i) lead wire, (B): (j) working electrode (Pt–Ir) and (k) reference electrode/counter electrode (Ag/AgCl).

electrode) because of less invasive for biological body. Thus, a material electric potential of which is relatively stable was used as reference electrode/counter electrode. The Teflon-coated Pt–Ir wire was stripped of 1.0-mm Teflon to expose the Pt–Ir wire as a sensing cavity (working electrode). The copper wire was wound around the Teflon-coated surface as extended line of lead wire. Then, the copper wire was coated with the Ag/AgCl paste and used as a reference electrode/counter electrode. After soldering each lead wire to either the Pt–Ir or the copper wire, each connection was covered with heat-shrink tubing and epoxy adhesive.

2.3.2. Enzyme immobilization on the working electrode

First, a 5% nafion dispersion solution (10 μl) was applied to the working electrode to prevent noise induced by oxidized substances in the EISF. Then, the enzyme membrane was immobilized on the tip of the working electrode. An enzyme solution containing cholesterol oxidase (8.4×10^{-4} units), cholesterol esterase (3.36×10^{-4} units), and 20 mg bovine serum albumin dissolved in 1.0 ml of 0.1 M PBS (pH 7.8) was applied to the tip of the working electrode and the enzymes were immobilized by a cross-linking reaction using 25% glutaraldehyde (20 μl) for 6 h. This immobilization method was repeated 2 times per electrode.

2.4. Amperometric total cholesterol measurement

The enzyme sensor was connected to a multichannel 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. Then, a 650-mV potential (vs. Ag/AgCl) was applied to the Pt–Ir working electrode using the potentiostat. Amperometric total cholesterol measurement was then performed based on the following 3 reactions that occurred on the surface of the working electrode.

$$\text{Cholesterolesters---(cholesterolesterase)} \rightarrow \text{cholesterol} + \text{fattyacid} \quad (1)$$

$$\text{Cholesterol} + O_2\text{--(cholesteroloxidase)} \rightarrow \text{4-cholesten-3-one} + H_2O_2 \quad (2)$$

$$H_2O_2\text{--(electrocatalyticoxidation)} \rightarrow O_2 + 2H^+ + 2e^- \quad (3)$$

Reaction (3) produced an electric output current that was recorded via the computer-controlled data acquisition software (Pinnacle Acquisition Laboratory).The sensor was placed in 50 ml of 0.1 M PBS (pH 8.0). After the output current stabilized, a 1.0-ml aliquot of the 1000 mg dl^{-1} cholesterol oleate standard solution was added to the 0.1 M PBS. After the output current again stabilized, another 1.0-ml aliquot of the cholesterol standard solution was added. This procedure was repeated until 20 aliquots had been added. The sensor output current was measured after each addition of cholesterol and the background current was subtracted from the response current. Because cholesterol generally exists as a cholesterol ester, cholesterol oleate was used for the cholesterol standard.

2.5. Total cholesterol monitoring in fish

2.5.1. Wired sensor system for continuous total cholesterol monitoring

Sensor implantation and fixation were performed after anaesthetizing fish with 400 ppm 2-phenoxy ethanol. A small hole was created in the sclera using a 20G catheter (φ1.10 mm) consisting of an outer Teflon layer and inner puncture needle (22G, 23 mm, Serflow™ Terumo, Tokyo, Japan). The inner puncture needle was then removed, and the excess exposed catheter on the skin was trimmed off. The sensor was then inserted into the scleral ISF, immersing the working and reference electrodes of the sensor in the EISF. The sensor was then fixed in place using a biomedical adhesive (Aronalpha-A Sankyo, Toagosei, Tokyo, Japan). The adhesive was applied around the insertion point to fill the hole and the sensor was stapled to the fish's body. After implantation and fixation, the sensor was connected to a multichannel potentiostat. Monitoring of total cholesterol was performed after the background current of the sensor became stable. During monitoring, EISF from the contralateral eyeball was collected periodically using a syringe fitted with a 27G needle (0.40 mm × 19 mm) to compare the obtained sensor response to the actual total cholesterol concentration. To prevent the fish from moving while collecting the EISF sample, the fish was transferred to a 7.5-L water tank containing 200 ppm of 2-phenoxy ethanol as a light-anesthetic agent. The fish was held in place using a string to simplify the EISF sample collection process.

2.5.2. In vivo calibration

Continuous estimation of total cholesterol in EISF was performed using one-point or two-point *in vivo* calibration methods [19–21]. The one-point calibration method is represented by the following formula:

$$\text{TC}(t) = \frac{I(t)}{S}$$

The sensor sensitivity (S), expressed in nA/(mg dl^{-1}), was determined from a single total cholesterol measurement in EISF as the ratio between the concomitant sensor output current (I; expressed in nA) and the total cholesterol (TC) concentration. Subsequently, the TC 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. An EISF sample was obtained from the other sclera using a syringe fitted with a 27G needle. TC concentration in EISF was determined by an enzymatic colorimetric method. These parameters (I, TC) were used as the calibration reference.

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

$$\text{TC}(t) = \frac{I(t) - I_0}{S}.$$

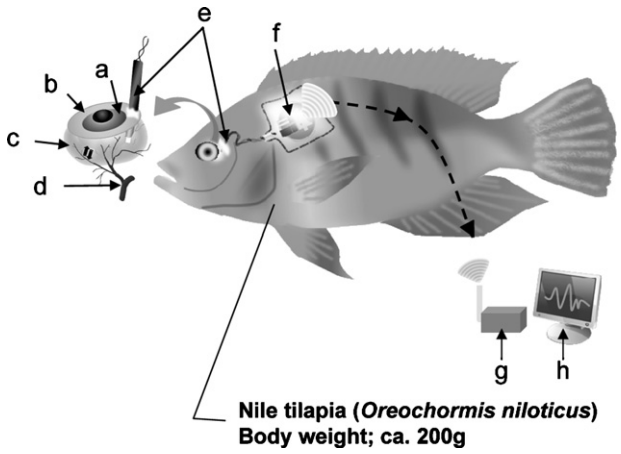

Fig. 2. Schematic diagram of the wireless monitoring system for fish (Nile tilapia). (a) Eyeball, (b) sclera, (c) EISF, (d) blood vessel, (e) needle-type total cholesterol sensor, (f) wireless potentiostat, (g) receiver and (h) personal computer.

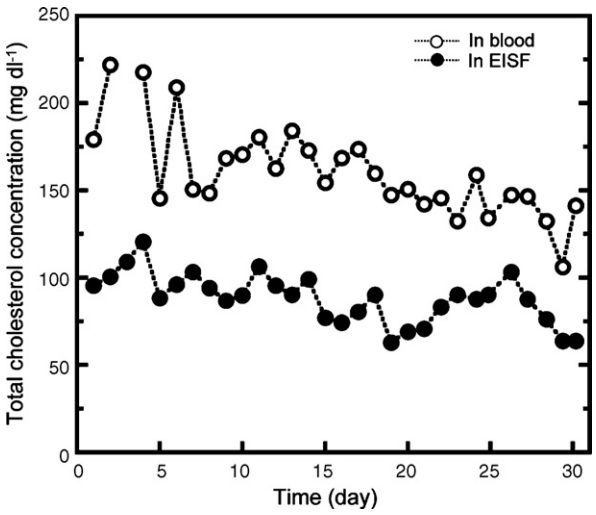

Fig. 3. Representative plot of total cholesterol concentration in the blood and EISF in an individual fish over 30 days. Total cholesterol concentration in blood was plotted as white circles and that in EISF was plotted as a black circles.

The formula consists of the sensor sensitivity (S) and the intercept I_0 of the sensor output. Parameters (S) and (I_0) were calculated from the change of total cholesterol concentration in EISF from TC_1 to TC_2, the change in the sensor current I from I_1 to I_2: $S = (I_2 - I_1)/(TC_2 - TC_1)$, followed by calculation of I_0 using the following formula: $I_0 = I_1 - (S \times TC_1)$.

2.5.3. *Wireless sensor system for total cholesterol monitoring under free-swimming conditions*

A schematic diagram of the wireless monitoring system is shown in Fig. 2. Sensor implantation and fixation were performed according to the same method used for the wired monitoring. The sensor was connected to a wireless potentiostat (3102BP, Pinnacle Technology, Inc) and covered with a waterproof polypropylene sheet. The waterproofed wireless potentiostat was attached to the dorsal and pectoral fins of the fish using nylon thread. The signal of the sensor output current signal was sent to a receiver (3100RX, Pinnacle Technology Inc) using a radio wavelength of 916.5 MHz. The fish were then transferred to a water tank (40 cm × 24 cm × 50 cm). During the monitoring, EISF samples from the contralateral eyeball were collected periodically from fish anesthetized with 400 ppm 2-phenoxy ethanol to prevent noise signals due to fish movement. After collecting the EISF, the water dissolved 2-phenoxy ethanol in tank was changed with new one. During the experiment, the water was maintained at 30 °C, aerated, and filtered. Continuous monitoring of total cholesterol was performed after the sensor output current became stable.

3. Results and discussion

3.1. *Comparison of total cholesterol between blood and EISF*

It is difficult to perform continuous monitoring of blood components using a biosensor because the sensor output decreases over time due to blood coagulation and protein coalescing on the sensor. We investigated the correlation between total cholesterol concentrations in blood and EISF. Changes in cholesterol levels in individual fish over time were measured to assess whether fluctuations of cholesterol concentrations in the blood were reflected by changes in cholesterol concentrations in the EISF. We examined the correlation between total cholesterol concentration in blood and in EISF over 30 days in a fish. The temporal changes in total cholesterol concentration in a single representative fish are shown in Fig. 3. The fluctuations of the total cholesterol concentrations in the blood and EISF over 30 days were significantly correlated. Additionally, two specific properties of EISF were discovered by comparing the two temporal cholesterol concentration profiles. First, total cholesterol concentration in EISF was approximately half that in blood. Second, the kinetics of the changes in EISF cholesterol concentrations were dependent on the valence of the change; increases in the blood concentration were slowly reflected by increases in the EISF concentration, whereas decreases in blood concentration were rapidly reflected by decreases in the EISF concentration. This difference in kinetics likely reflects the fact that the EISF cholesterol concentration is a product of cholesterol moving into the EISF along a concentration gradient and cholesterol being removed from the EISF by the cells, which depends on the requirements of the cells for cholesterol. An increase in cholesterol availability can lead to a rapid increase in cell demand. In addition, the amount demanded by the cell has an established minimum. Thus, total cholesterol concentration in EISF was reflected by blood concentrations, especially when the total cholesterol concentration in the blood decreased.

We next examined interindividual variations in the relationship between blood and EISF cholesterol concentrations at a single time point. The tilapia were captured randomly from the water tank ($n = 72$), and both blood and EISF samples were obtained. Total cholesterol concentration in EISF was strongly correlated ($R = 0.8818$) with blood total cholesterol in the range of 74–480 mg dl^{-1} (Fig. 4), thus indicating that the EISF is a useful alternative to blood collection for measuring total cholesterol. In general, in the aquafarm setting, blood tests are performed once a day. Although there is a small time-lag between total cholesterol concentration in the blood and that in the EISF, total cholesterol concentration in the EISF generally reflects the total cholesterol concentration in the blood. Thus, sampling the EISF to determine total cholesterol levels should be a good indicator of fish health.

3.2. *Characteristics of the needle-type total cholesterol biosensor*

Fig. 5 shows a relationship between output current and cholesterol oleate concentration. Cholesterol oleate, which is a kind of esterified cholesterol, was prepared as standard sample of total cholesterol. Good linearity was observed in the concentration range of 2.65–403 mg dl^{-1} (correlation coefficient: 0.9970). The mean total cholesterol concentration in the blood of Nile tilapia is 140.35 mg dl^{-1} [22] and the concentration in the EISF is usually lower than that in blood (Fig. 3). Thus, the physiologic cholesterol

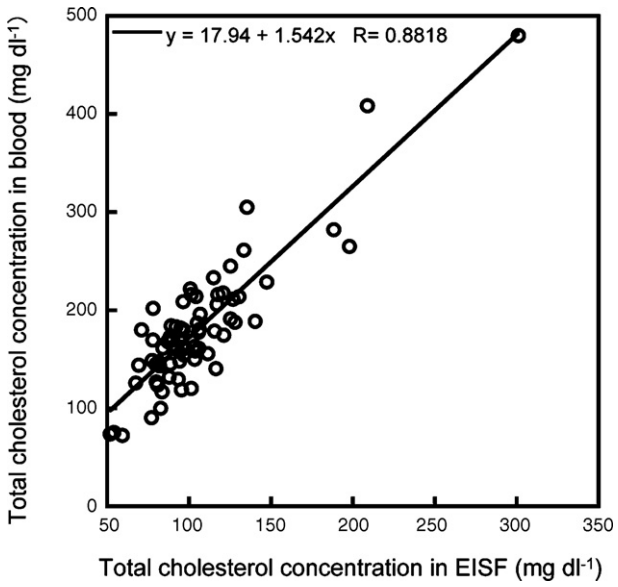

Fig. 4. Correlation of total cholesterol concentration in blood and EISF in randomly selected fish ($n = 72$, 74–480 mg dl^{-1}).

concentrations in EISF are well within the linear range of the sensor.

As the sensor comprises a biologic catalyst (enzyme), the response can vary depending on the pH and temperature of the solutions in which the immobilized enzyme is placed. The effects of pH and temperature on the sensor induced current for a 100 mg dl^{-1} standard solution are shown in Fig. 6. The sensor current gradually increased in the range of pH 5.5–6.5 and rapidly decreased at pH greater than 6.5 (Fig. 6 A). The EISF pH in Nile tilapia is 8.0 [23]. Although pH affects enzyme activity, even at pH 8.0 the sensor induced enough current to detect changes in the total cholesterol concentration. Temperature also has important effects on enzyme activity. For temperatures ranging from 10 to 40 °C, the sensor current increased rapidly up to 30 °C and then became nearly constant (Fig. 6 B). Above 40 °C, the current

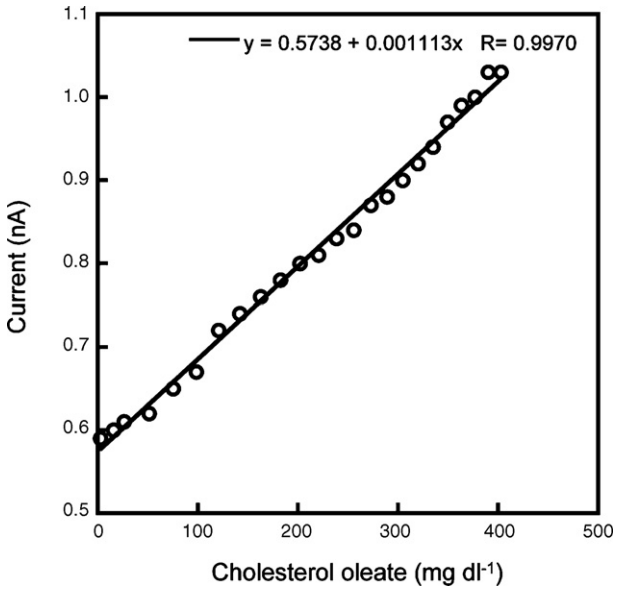

Fig. 5. Relationship between sensor output current and cholesterol oleate concentration (range of cholesterol oleate concentration 2.65–403 mg dl^{-1}). The measurement was performed by sequential addition of 100 mg dl^{-1} cholesterol oleate standard solution in pH 8.0, 30 °C PBS under a +650 mV potential (vs. Ag/AgCl).

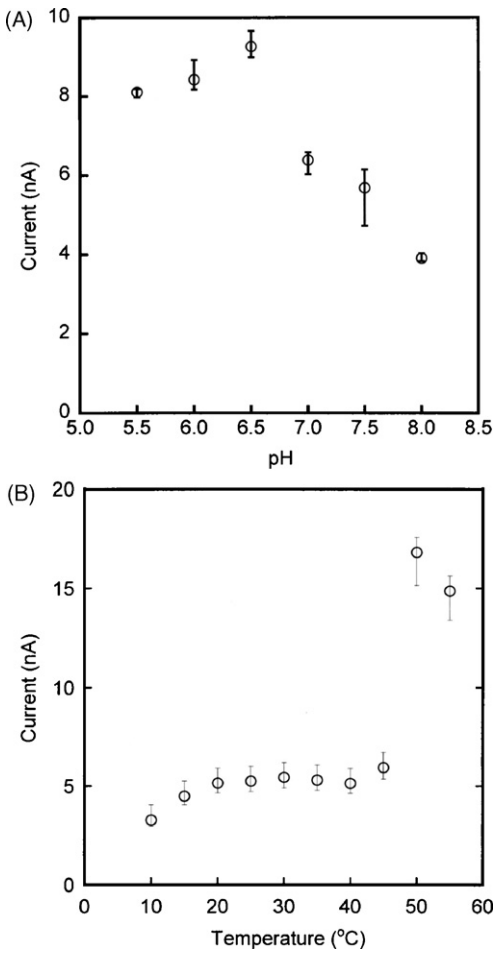

Fig. 6. Typical effects of pH (A) and temperature (B) on the total cholesterol sensor response in PBS. In (A) the temperature was 30 °C, in (B) the pH was 8.0. The measurements were performed using 100 mg dl^{-1} cholesterol oleate standard solution under a +650 mV potential (vs. Ag/AgCl).

increased rapidly again and reached the maximum output. At temperatures above 40 °C, however, the signal was very noisy and the output current was not stable. EISF is assumed to be at the same temperature as the environmental water temperature since the internal body fluid temperature of fish is influenced by its environment. The water temperature used for rearing Nile tilapia is in the range of 15–30 °C. Thus, the sensor induces sufficient output current under typical EISF temperature conditions and can be applied to *in vivo*-measurements.

The reproducibility of sensor measurements is shown in Table 1. The reproducibility of the measurements was demonstrated by immersing a single sensor in 100 mg dl^{-1} standard solution (30 °C).

Table 1
Reproducibility of total cholesterol measurements using the cholesterol biosensor ($n = 20$).

Electrode no.	Relative standard deviation (RSD %)
1	4.02
2	2.40
3	2.68
4	9.37
5	4.45
Mean ± SD	4.58 ± 2.52

The measurement was performed using 100 mg dl^{-1} cholesterol oleate in PBS, pH 8.0, 30 °C under a +650 mV potential (vs. Ag/AgCl).
Absolute response value for five sensors: 2.97–6.49 nA.

Each of five enzyme sensors was tested 20 times. The relative standard deviation (RSD %) was used to evaluate the reproducibility because of lot-dependent variability in the sensor output current. The RSD was 4.58 ± 2.52% in the absolute response values for five sensors was from 2.97 to 6.49 nA. The low RSD values indicated that the sensor can be used to determine total cholesterol concentrations with satisfactory precision. Therefore, the sensors have excellent reproducibility for measuring total cholesterol, and can be applied to continuous measurement of total cholesterol concentration *in vivo* with stable and accurate results.

3.3. Continuous cholesterol monitoring using the wired enzyme sensor system

We performed continuous total cholesterol monitoring in Nile tilapia using a wired sensor system. The fish were immobilized in the water tank to minimize experimental error while collecting the EISF samples. After sensor implantation in the EISF, the fish were not touched until the output current became stable (approximately 240 min). The total cholesterol concentration was then continuously monitored for 230 min. The time course of the total cholesterol concentration in EISF and the sensor output current were similar. In fact, the correlation coefficient between the actual total cholesterol concentration and sensor output current was 0.8961 ($n = 6$), confirming the corresponding relationship between them.

Sensor-calibrated cholesterol concentrations were estimated using either a one-point or two-point calibration method. The one-point calibration method used the calibration value (TC_1, I_1) in which TC_1 was the total cholesterol concentration in EISF (165 mg dl^{-1}) and I_1 was the subsequently obtained sensor output current (5.05 nA). The two-point calibration method used the first parameter (I_1; 5.05 nA, TC_1; 165 mg dl^{-1}) and the total cholesterol concentration in EISF and sensor output current obtained 90 min after sensor implantation (I_2; 4.53 nA, TC_2; 157 mg dl^{-1}). The relationship between the actual concentration and calibrated curve of the sensor is shown in Fig. 7. The two-point calibration method appeared to be more accurate than the one-point calibration method for determining the actual cholesterol concentration.

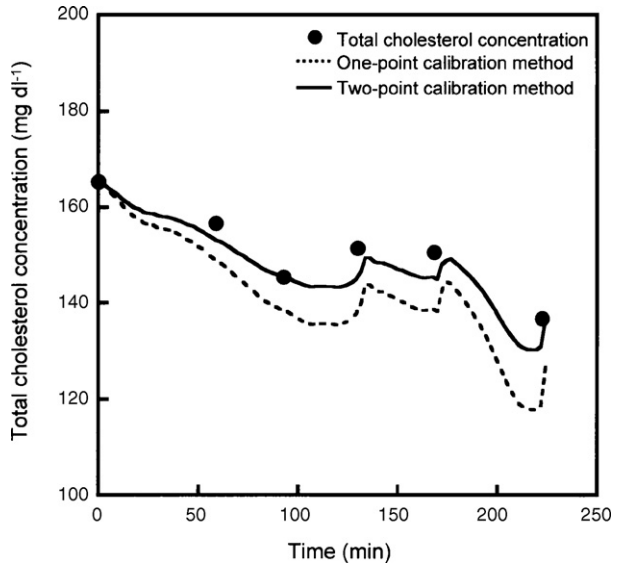

Fig. 7. The relationship between total cholesterol concentration in EISF and the calibrated sensor response. Total cholesterol concentrations were plotted as black circles, the calibrated response using the one-point calibration was plotted as the broken line, and the calibrated response using the two-point calibration was plotted as the solid line.

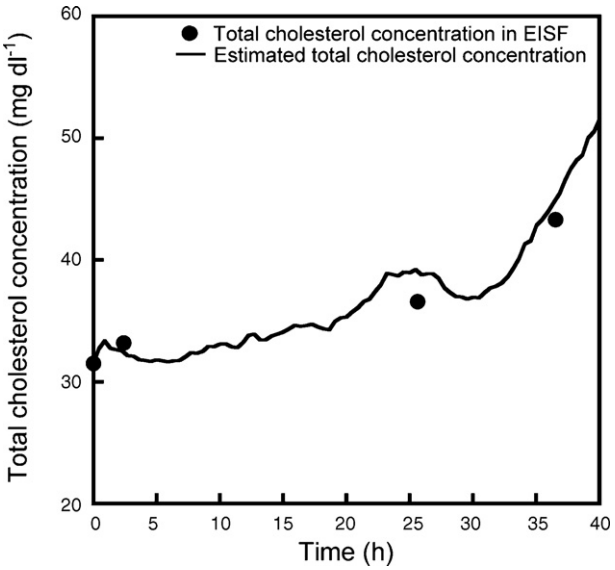

Fig. 8. The change in estimated total cholesterol concentrations in the wireless monitoring system. Total cholesterol concentrations in EISF at 0 and 90 (min), plotted as black circles, were used in the two-point calibration method.

To judge this relationship objectively, we compared the range of error between the actual and estimated cholesterol concentration values. The range of error was calculated as the mean value of the difference between the estimated value and the actual value for all 6 time points. The results indicated that the error for the two-point calibration method was lower (−1.18 mg dl^{-1}) than that for the one-point calibration method (−13.11 mg dl^{-1}). Therefore, the two-point calibration method should be used to measure total cholesterol concentration in fish. These findings confirm that the needle-type sensor monitoring system can be used for real-time measuring of total cholesterol concentration in fish.

3.4. Total cholesterol monitoring using the wireless enzyme sensor system under free-swimming conditions

We applied the sensor system to wirelessly monitor total cholesterol concentrations in free-swimming fish in an aquarium. Nile tilapia was anesthetized with 400 ppm 2-phenoxy ethanol, and then the wireless sensor system was attached. The fish were transferred to a 50-L fish water tank containing no anesthetic. The time courses of the output current of the sensor and sensor-calibrated total cholesterol levels are shown in Fig. 8. The two-point calibration method was adopted using the first measurement (I_1; 3.65 nA, TC_1; 31.5 mg dl^{-1}) and a second measurement obtained 90 min later (I_2; 3.79 nA, TC_2; 3.32 mg dl^{-1}). Wireless monitoring was continued for over 40 h and the results indicated that this system can be used for short-term measurement of total cholesterol in free-swimming fish. Actually, the calibration curve showed actual concentration of total cholesterol in EISF well. The estimated total cholesterol levels that were monitored for 40 h were within the range of standard values for Nile tilapia. The point of notice is that periodical calibration will be essential for practical chronic monitoring, which range for several weeks or a month, of total cholesterol levels. The results of wireless monitoring showed potential of the sensor as ubiquitous tool for aqua farm.

4. Conclusion

The present findings confirm that a needle-type enzyme biosensor-based monitoring system can be used to continuously

measure total cholesterol concentrations in fish. We used EISF as an alternative to blood and investigated the relationship between total cholesterol concentration in the blood and EISF. The total cholesterol concentration in EISF strongly correlated with that in blood. We prepared a needle-type enzyme sensor for measuring total cholesterol in fish. The sensor size was ca. 0.4 mm width × 20 mm length and provided a rapid response, good linearity, and good reproducibility. Continuous *in vivo*-monitoring was performed when the sensor was implanted under the scleral surface of the fish eyeball. The changes in the cholesterol concentrations in EISF and those in the sensor output current were similar. We investigated two steps for practical application; (1) establishing an accurate calibration method and (2) application of a wireless monitoring system. The two-point calibration method was more effective than the one-point calibration method for calculating the actual cholesterol concentration. Additionally, the wireless monitoring system enabled us to securely monitor total cholesterol levels in free-swimming fish in an aquarium. The wireless monitoring system detected changes in total cholesterol concentration and thus the ability of the fish to fight disease. Several issues, however, remain. One is determining how to protect the sensor output response from changes in the *in-vivo* environment of the fish, such as those due to changes in temperature and dissolved oxygen in EISF. Another is that the monitoring space must be enlarged so that the free-swimming fish can be placed in larger tanks that are typically used for aquaculture. Future improvements should address these issues. This system will contribute to monitoring fish health in aquaculture.

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