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Development of mediator-type biosensor to wirelessly monitor whole cholesterol concentration in fish

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Abstract We developed a wireless monitoring system to monitor fish condition by tracking the change in whole cholesterol concentration. The whole cholesterol concentration of fish is a source of steroid hormones or indicator of immunity level, which makes its detection important for tracking physiological condition of fish. Wireless monitoring system comprises of mediator-type biosensor and wireless transmission device. Biosensor is implantable to fish body, and transmission device is so light, in that fish is allowed to swim freely during monitoring. Cholesterol esterase and oxidase were fixated on to the detection site of biosensor and used to detect the whole cholesterol concentration. However, cholesterol oxidase incorporates oxidation–reduction reaction of oxygen for detection, which concentration fluctuates easily due to change in environmental condition. Meanwhile, mediator-type biosensor enables monitoring of whole cholesterol concentration by using mediator to substitute that oxidation–reduction reaction of oxygen. Characteristic of fabricated mediator-type biosensor was tested. The sensor output current of mediator-type biosensor remained stable compared to output current of non-mediator-type biosensor under fluctuating oxygen concentration of 0–8 ppm, which implied that this sensor is less affected by change in dissolved oxygen concentration. That biosensor was then implanted into fish for wireless monitoring. As a result, approximately 48 h of real-time monitoring was successful.

Keywords Fish · Whole cholesterol concentration · Blood · Mediator-type biosensor · Wireless monitoring · In vivo monitoring

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Introduction

Many of researches related to fish physiology are based on hemodiagnosis, analysis of component concentration in blood plasma, which enables chemical diagnosis of fish condition (Koedprang et al. 2002). For example, changes in plasma whole cholesterol concentration are used to analyze immunity

(Davis et al. 1999), effect of toxics (John 2007; Gül et al. 2012; Singh et al. 2012; Skolness et al. 2012; Seel-audom et al. 2013), metabolism (Pavlidis et al. 1999; Rehulka and Minarík 2012), and sperm functionality (Lahnsteiner et al. 2009) of target fishes. Tracking of whole cholesterol concentration is important to analyze and study the state, metabolism, and reproduction of fish; deeper studies based on whole cholesterol concentration might lead to improvement in aquaculture methods.

Whole blood is first collected from Cuvierian duct or arteriovenous blood vessel of fish and centrifuged to obtain plasma, where there are less proteins and fibrinogens. However, there are only two major ways currently applied to obtain blood samples: capturing and anesthetizing or killing. Capturing fish is reported to be not only stressful for fish but also as one of the reasons of altering plasma component concentration (Biswas et al. 2006; Di Marco et al. 2011). In addition, killing fish is attributive method since investigation of that specific fish can no longer be conducted.

In 2009, Yonemori et al. developed a wireless monitoring system of fish physiological condition (Endo et al. 2009; Yonemori et al. 2009). This system enables stress-free and non-killing analysis of plasma component concentration, such as whole cholesterol concentration, and will eliminate risk of monitoring error by handling and blood drawing. Wireless monitoring system comprises amperometric biosensor, transmitter, and receiver. The amperometric biosensor detects component concentration and sends the signal to the transmitter. Transmitter reads the electrical data and converts the information to radio waves, which is then sent to the receiver. The biosensor and transmitter are connected and attached to fish body, while receiver is connected to personal computer which is located afar from fish tank.

This biosensor is implanted into fish eyeball interstitial sclera fluid (EISF), which component concentration correlates well with that of blood component concentration. For example, EISF whole cholesterol concentration correlates with blood whole cholesterol concentration in a correlation coefficient of $R = 0.8818$ (Yoneyama et al. 2009). The size of biosensor is small, and weight of transmitter is light in that monitoring of target component concentration of free-swimming fish is possible. By using this monitoring system, wireless and real-time monitoring of EISF whole cholesterol concentration was successfully monitored for about 40 h (Yoneyama et al. 2009).

Previous amperometric biosensor, however, uses oxidizing enzymes, which activity is easily affected by changes in oxygen concentration, to monitor the EISF whole cholesterol concentration. The reaction for detection of the whole cholesterol concentration using oxidizing enzymes is as follows.

$$\text{Cholesterol ester} \xrightarrow{\text{(cholesterol esterase)}} \text{cholesterol} + \text{fatty acid} \quad (1)$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{(cholesterol oxidase)}} \text{cholestenone} + H_2O_2 \quad (2)$$

Electrons released by the oxidation–reduction reaction of oxygen (2) are detected by biosensor. However, oxygen concentration in water changes rapidly due to environmental factors such as red tides (Dai et al. 2011). In addition, oxygen concentration in fish body is affected by change in dissolved oxygen concentration in breeding water. The decrease in dissolved oxygen concentration is likely to decrease the output of biosensor, which will malfunction the monitoring. Therefore, the development of a biosensor that allows for monitoring without being influenced by oxygen level is desired.

A mediator-type biosensor (Dicks et al. 1993) is a type of biosensor which is least unaffected by fluctuation of oxygen levels. Oxidizing enzymes use the oxidation–reduction reaction of oxygen to detect the target molecules, while mediator-type biosensor substitutes that reaction with a mediator (Van Staveren and Mezler-Nolte 2004). For example, the reaction of cholesterol oxidase incorporating mediator is shown below.

$$\text{Cholesterol} + \text{Mediator}_{ox} \xrightarrow{\text{(cholesterol oxidase)}} \text{cholestenone} + \text{Mediator}_{red}$$

Dissolved oxygen concentration fluctuates easily by factors such as sunrise, sunset, water depth, and breeding density. By constructing mediator-type biosensor, wireless monitoring of EISF whole cholesterol concentration will become possible under various conditions.

In the present study, we aimed to develop a mediator-type biosensor to wirelessly monitor EISF whole cholesterol concentration without the influence of dissolved oxygen concentration. The characteristics of developed mediator-type biosensor were first

analyzed, and this sensor was implanted into fish to actually conduct wireless in vivo monitoring of EISF whole cholesterol concentration.

Materials and methods

Reagents

Cholesterol oxidase (1.80 U mg^{-1}, E.C.1.1.3.6, from *Streptomyces* sp.) and cholesterol esterase (1.66 U mg^{-1}, E.C.3.1.1.13, from *Pseudomonas* sp.) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and chitosan 5, methanol, ferrocenecarbaldehyde, Cholesterol E-Test Wako, heparin sodium, 5 % Nafion® dispersion solution, sodium tetrahydroborate, and 2-phenoxyethanol were purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). All other reagents used for the experiments were of commercial and laboratory grade.

Measurement apparatus

A Model 8102 potentiostat and its data analysis software, Pinnacle Acquisition Laboratory, purchased from Pinnacle Technology, Inc. (Lawrence, KS, USA) were used for in vitro amperometric measurements. A transmitter and its receiver, both made in-house, were used for in vivo amperometric monitoring. The wireless potentiostat sends data to a receiver (model iRX315A, iTEC Corp, Japan) using radio waves at a frequency of 312 MHz.

Construction of needle-type electrode

First, 15-mm-long Teflon coated Pt–Ir wire (Φ 0.178 mm, Nilaco Co., Tokyo, Japan) was cut and soldered to a lead wire (Φ 0.1 mm, Unique Medical Co., Tokyo, Japan). A 15-mm-long copper wire and another fresh lead wire were cut, and one end of the copper wire was soldered to the lead wire. The copper wire was wrapped around the Teflon coating of Pt–Ir wire. The wrapped wire was coated with Ag/AgCl paste and dried at 90 °C for 20 min. This drying process was repeated twice. To complete electrode construction, solder joint was covered using heat-shrinkable tubing (Sumitomo Electric Industries, Ltd., Tokyo, Japan). The Pt–Ir wire became working electrode, while Ag/AgCl became counter electrode.

Fabrication of the mediator-type biosensor

Cholesterol oxidase (COx) was first dissolved in phosphate buffer solution (0.1 M, pH 6.5) to prepare a 168 U ml^{-1} COx solution. Then, 3 µl phosphate buffer solution was added to 2 µl COx solution. Next, 0.4 mg cholesterol esterase (CEst) was dissolved in 5 µl phosphate buffer solution and mixed with the COx solution.

The mediator-type biosensor is a type of biosensor that uses "mediator" to enhance the oxidation–reduction instead of oxygen and is therefore unaffected by change in oxygen concentration. Ferrocene is a substance that sandwiches ferric oxide with cyclopentadienyl magnesium. The oxidation–reduction reaction of ferrocene is inducted by applying a voltage of +350 mV. This voltage is lower than that of original oxidation–reduction reaction voltage of COx and oxygen, which is +650 mV. Substituting this reaction with ferrocene and lowering the application voltage to +350 mV will eliminate the effect of change in dissolved oxygen (DO) concentration.

The mediator can be fixed to the detection site of the biosensor in many ways. In this experiment, we focused on physical encapsulation method and selected chitosan as fixator (Yang et al. 2007). The amino base of chitosan reacts with ferrocenecarbaldehyde by dehydrogenation reaction and chemically binds to ferrocene. In addition, chitosan adsorbs to metallic surfaces and can be fixed to the detection site by dipping the metallic surface into chitosan solution.

Two hundred and fifteen milligram ferrocenecarbaldehyde was dissolved in 15 ml methanol, and 161 mg chitosan 5 was dissolved in 15 ml acetate. Both solutions were mixed together, and 2 g sodium tetrahydroborate was added to conjugate ferrocenecarbaldehye to the chitosan 5 (Chit-Fc). One hundred and fifty milligram Chit-Fc was washed by centrifugation at 500×*g* for 10 min. Distilled water was used for the first wash and methanol for the second wash. Rinsed Chit-Fc was dissolved in 150 µl acetic buffer (pH 5.0) and 100 µl phosphate buffer. The previously prepared solution with dissolved enzymes was added to the Chit-Fc solution and mixed well to make the enzyme solution. Detection site of sensor was first immersed in 5 % Nafion® solution for 1 min and dried for 10 min to prevent impurities from effecting the monitoring. Then, it was immersed into enzyme solution for 1 min and dried for 10 min. This process

was repeated twice. Finally, the sensor was dried at 35 °C for 6 h. From this process, Nafion® membrane and enzyme layer were formed on top of Pt–Ir wire, respectively. The Nafion® membrane was formed directly on Pt–Ir wire to prevent enzymes from coming in direct contact with the Pt–Ir wire, where electrochemical detection takes place. Furthermore, the enzymes were fixated on to the detection site using biocompatible chitosan. Therefore, the chitosan layer was fabricated last in order to protect the detection site from fouling. The mediator-type biosensor, the Nafion/Chit-Fc/CEst/COx sensor, was then fully fabricated (Fig. 1).

Wireless monitoring of EISF whole cholesterol concentration

First, the transmitter was waterproofed. A small bag of polyethylene vinyl was prepared, and a hole was cut in one of the corners. A 1 cm length of polyethylene microtube (diameter 10 mm) was cut and fixed into the hole of polyethylene vinyl using Araldite adhesive. The lead wires of the mediator-type biosensor were inserted through the microtube and connected to the transmitter using a pitch connector. The side of the polyethylene vinyl was sealed firmly with a heat sealer to encapsulate the transmitter. When enclosing the potentiostat, the antenna of the device was coiled up to fit in the bag. At the end, the remaining hole of the microtube was filled with Alardite adhesive to prevent water from leaking in.

Nile tilapia (*Oreochromis niloticus*) were used in this experiment. The fish were cultivated in an aquarium (80 × 60 × 50 cm, 240 l) under controlled conditions until use. The fish were not fed for 2 days prior to the experiment to prevent changes in the whole cholesterol concentration due to foraging and metabolism. The fish were anesthetized by placing them in 400 ppm of 2-phenoxyethanol solution for 10–15 min until their gills stopped moving. Then, the fish were placed on a wet paper towel, and the water around the eyeball was carefully wiped away. Avoiding direct contact with the fish with the bare hand, pressure was gently applied to the side of eyeball closest to the snout to express the EISF. A needle was used to puncture a hole for the sensor implantation near the eyeball. The surface membrane enclosing the EISF is extremely delicate and subject to tearing. Therefore, the hole was placed not directly into the EISF but from a site near the eyeball. A larger needle was used to widen the hole, and the mediator-type whole cholesterol biosensor was carefully inserted into EISF. The biosensor implanted in the EISF was connected to the wireless potentiostat. This wireless potentiostat sends out radio waves every second. The device operates by a 3-V battery, and as long as the battery lasts, it continuously sends out radio waves. The EISF whole cholesterol concentration was monitored by converting current information detected by the mediator-type whole cholesterol biosensor into a 312-MHz signal by the wireless potentiostat. The signal is transmitted to a receiver that is directly connected to a personal computer. In addition, the wireless potentiostat used in this experiment is designed to attach to fish. It is 25 mm in diameter and weighs 6 g. The weight of fish used for this experiment is around 300 g; therefore, attachment of the monitoring device is assumed to be stress free. This characteristic allows for real-time monitoring of the EISF component concentration away from the fish tank.

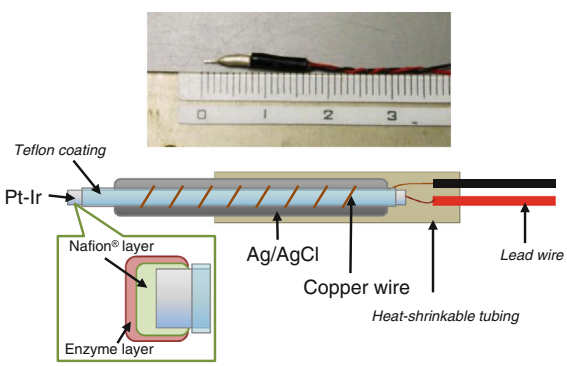

Fig. 1 Picture and diagram of a mediator-type biosensor

Results

In vitro testing of basic Nafion/Chit-Fc/CEst/COx sensor characteristics

The mediator-type biosensor receives electrons from oxidation–reduction reaction of mediator and emits sensor output current at the applied voltage of +350 mV. The sensor output current of newly fabricated Nafion/Chit-Fc/CEest/COx sensor and standard whole cholesterol correlated well in the range of 9.52–166.67 mg dl^{-1} with coefficient of $R = 0.9990$

(Fig. 2a). Temperature dependence of this sensor was tested by altering the temperature of the standard cholesterol solution (Fig. 3a). A standard solution with 80 mg dl^{-1} of cholesterol was prepared, and the temperature was altered in the range of 10–65 °C. As a result, sensor output current increased up to 60 °C and decreased thereafter. pH dependence was tested by preparing a 80 mg dl^{-1} standard cholesterol solution with pH varying from 5.5 to 8.0 (Fig. 3b). The sensor output current increased up to pH 6.5 and decreased afterward. The reproducibility of Nafion/Chit-Fc/CEst/COx sensor was then tested. Three Nafion/Chit-Fc/CEst/COx sensors were constructed to test the reproducibility of the sensor. The standard deviation produced by each sensor was 1.68, 0.79, 0.53 (mean ± SD, 1.00 ± 0.60).

Correlation of sensor output current with EISF whole cholesterol concentration was evaluated in vitro. The wireless monitoring of EISF whole cholesterol concentration is conducted by directly implanting the Nafion/Chit-Fc/CEst/COx sensor into fish EISF, which contain biological substances that may affect the sensor output current. The sampled EISF whole cholesterol concentration was 83.39 mg dl^{-1} (determined using a Wako Cholesterol E-Test), and standard cholesterol solution was added to EISF to increase the cholesterol concentration. The sensor output current of the Nafion/Chit-Fc/CEst/COx sensor correlated well with the EISF whole cholesterol concentration in the range from 83.39 to 163.39 mg dl^{-1} (Fig. 2b).

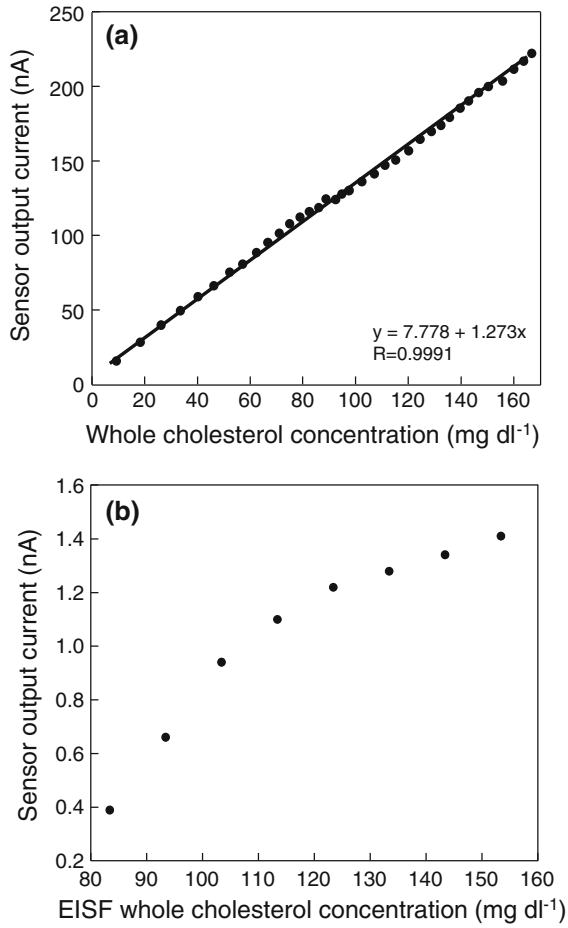

Fig. 2 Correlation of whole cholesterol concentration with sensor output current **a** in standard solution and **b** in EISF pH of standard solution was 6.5, and original EISF whole cholesterol concentration was 83.39 mg dl^{-1} (determined from Wako Cholesterol E-Test). Sodium azide was added to EISF samples as preservative. Applied voltage was +350 mV for both experiments

Effect of dissolved oxygen concentration on Nafion/Chit-Fc/CESt/COx sensor

To assess the efficiency of fixated ferrocene, effect of DO concentration on sensor output current was tested (Fig. 4). DO concentrations in standard solution was altered using nitrogen gas, and the fluctuation of sensor output current was recorded. An optical fiber probe sensor from Ocean Optics Co. (FOXY-R probe) was used to monitor the change in oxygen concentration of the standard solution. We also fabricated a cholesterol biosensor without using the mediator (Nafion/CEst/COx sensor) and performed the same experiment. Sensor output current at the highest oxygen concentration was set as 100 %, and rest of the sensor outputs were converted into percentage based on that output. The values of the Nafion/Chit-Fc/CEst/COx sensor were stable in the oxygen concentration range from 0.27 to 7.14 ppm. On the other hand, those for Nafion/CEst/COx sensor increased with an increase in the DO concentration.

Wireless monitoring of EISF whole cholesterol concentration

Two types of calibration methods, one-point calibration method and two-point calibration method, are focused for in vivo monitoring (Choleau et al. 2002; Csoreg et al. 1994). Equation for one-point (A) and two-point (B) calibration method is shown below.

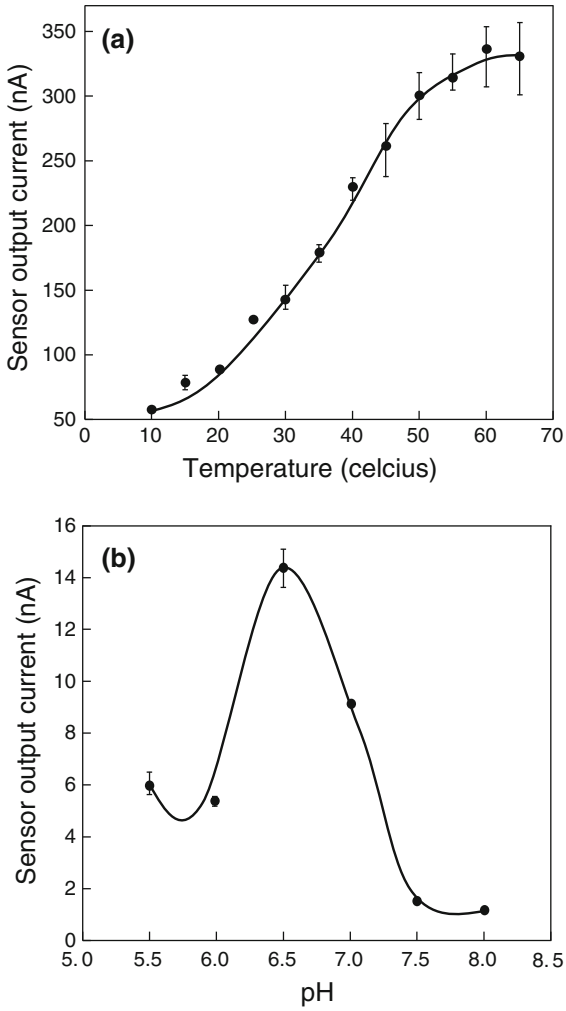

Fig. 3 **a** Effect of temperature to sensor output current. **b** Effect of standard solution pH to sensor output current. pH of standard solution was 6.5 for (**a**), temperature of the solution was 25 °C for (**b**), and applied voltage and concentration of standard whole cholesterol were +350 mV and 80 mg dl^{-1} for both experiments

$$\begin{aligned} &\text{Cali} - \text{w.cholesterol}\ (t) = I\,(t)/S \\ &\qquad S = I_1/\text{Act} - \text{w.cholesterol}_1 \end{aligned} \tag{A}$$

$$\begin{aligned} &\text{Cali} - \text{w.cholesterol}\ (t) = \left(I_{(\text{t})} - I_{(0)}\right)/S \\ &\quad S = (I_2 - I_1)/(\text{Act} - \text{w.cholesterol}_2 - \text{Act} \\ &\qquad - \text{w.cholesterol}_1) \\ &\quad I_0 = I_1 - (S \times \text{Act} - \text{w.cholesterol}_1) \end{aligned} \tag{B}$$

The sensor sensitivity (S), actual whole cholesterol concentration (Act-w.cholesterol), and sensor output current (I) were used for both types of calibration to

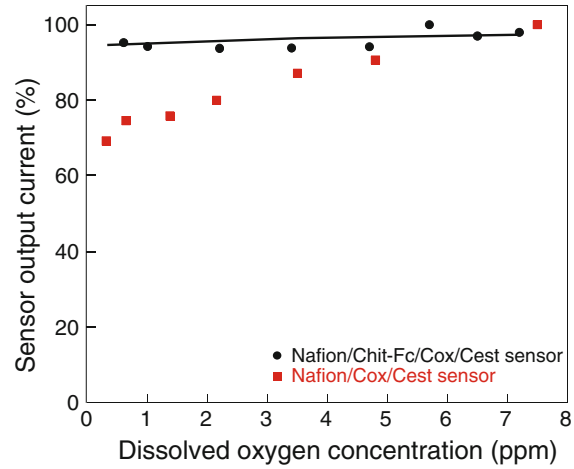

Fig. 4 Effect of dissolved oxygen concentration on sensor output current. *Square* is the output of Nafion/CEst/COx sensor, and *circle* is the output of Nafion/Chit-Fc/CEst/COx sensor. pH of standard solution was 6.5, applied voltage was +350 mV, and concentration of standard whole cholesterol was 80 mg dl^{-1}

calculate calibrated whole cholesterol concentration (Cali-w.cholesterol). One-point calibration method uses actual whole cholesterol concentration from the first blood draw and sensor output current at the time of blood draw, whereas two-point calibration method uses actual whole cholesterol from first and from subsequent blood draws during monitoring to calculate calibrated whole cholesterol. To select calibration method for upcoming wireless monitoring of EISF whole cholesterol concentration, short-term monitoring was conducted (Fig. 5a). The monitoring was operated for 6 h, and actual whole cholesterol concentration and sensor output current showed similar fluctuations. Five blood samples were collected during the experiment, and its actual whole cholesterol and sensor output current at the time of blood draw were used for calibration. Calibrated whole cholesterol for one- and two-point calibration and actual whole cholesterol is shown in Fig. 5b. Two-point calibration using actual whole cholesterol at every blood draw was conducted (data not shown), and two-point calibration using first and fourth blood draw was selected since it showed similar fluctuation with that of actual whole cholesterol and one-point calibrated whole cholesterol. All three cholesterol concentrations showed similar fluctuations. Then, calibrated whole cholesterol from both calibration methods was compared with actual whole cholesterol (Fig. 5c). Those from one-point and two-point calibration showed

similar correlation: Correlation coefficient for both calibration methods was $R = 0.78101$.

Finally, in vivo monitoring of EISF whole cholesterol concentration was performed (Fig. 7). The picture of implanted Nafion/Chit-Fc/CEst/COx sensor is shown in Fig. 6. The whole cholesterol concentration was continuously monitored for approximately 48 h, and sensor output current and actual blood whole cholesterol concentration, determined by Test Wako, showed similar fluctuations. The light was emitted to fish at the 16th hour of monitoring and turned off around 30th hour. EISF whole cholesterol concentration increased with light emission and decreased after it was turned off. The changes in calibrated whole cholesterol concentration were similar to the sensor output current and actual blood whole cholesterol concentration.

Discussion

The characteristics of newly fabricated Nafion/Chit-Fc/CEst/COx sensor were tested in vitro prior to wireless monitoring.

First, the correlation of sensor output current with standard whole cholesterol solution showed good quality of Nafion/Chit-Fc/CEst/COx sensor. The measureable standard whole cholesterol range, 9.52–166.67 mg dl^{-1}, covered the average EISF whole cholesterol concentration of Nile tilapia, 50–150 mg dl^{-1} (Yoneyama et al. 2009). Second, Nafion/Chit-Fc/CEst/COx sensor is enzymatic biosensor which output current is affected by factors such as temperature and pH, which alters enzyme activity. The optimum temperature (Fig. 3a) is higher than water temperature which Nile tilapia inhabit in. On the other hand, calibration curve of Fig. 2a was drawn under optimum breeding temperature of Nile tilapia (25 °C) and good correlation was seen. The optimum pH was lower than pH of EISF. The actual pH of EISF is around 7.8–8.0 (Yonemori et al. 2009), and the point at which a decrease in the sensor output current was observed. To study the function of the sensor under this condition, the correlation between sensor output current and standard cholesterol solution concentration was tested using standard solution at pH of 8.0. As a result, the sensor output was strongly correlated ($R = 0.999$) with standard cholesterol concentrations ranging from 9.52 to 142.86 mg dl^{-1} (data not shown).

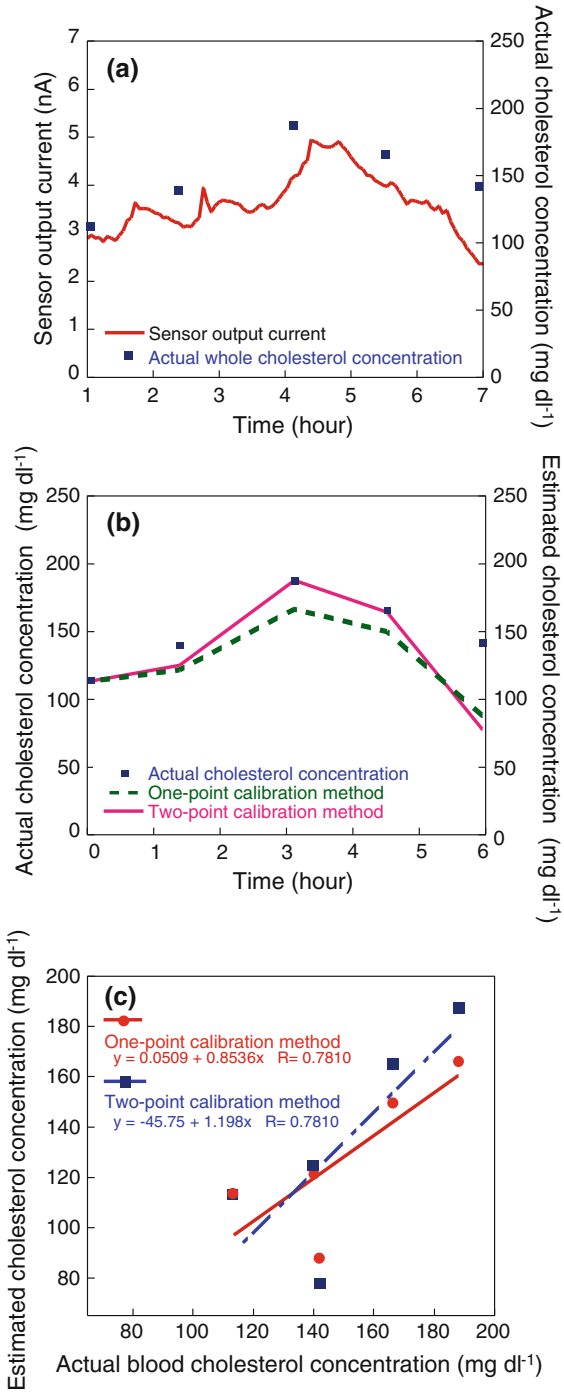

Fig. 5 Actual whole cholesterol concentration and calibrated whole cholesterol concentration. **a** Short-term monitoring of EISF whole cholesterol concentration. **b** Fluctuation of actual whole cholesterol concentration, one-point calibration cholesterol concentration, and two-point calibrated cholesterol concentration. **c** Correlation among actual cholesterol concentration and calibrated cholesterol concentration

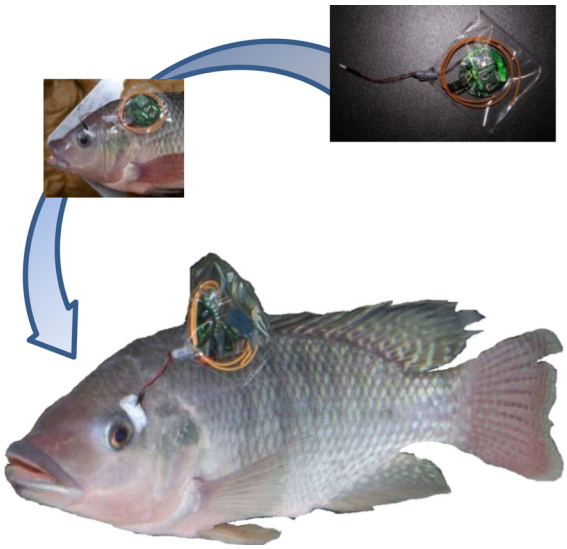

Fig. 6 Picture of Nile tilapia (*O. niloticus*) with wireless monitoring system

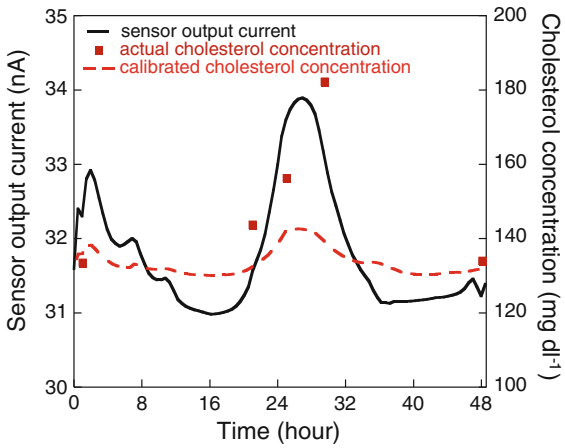

Fig. 7 Result of wireless in vivo monitoring of whole cholesterol concentration in fish EISF. Fish were not fed for 2 days prior to the experiment. One-point calibration method was used to calibrate the sensor output current

Biological sample such as EISF contains impurities that may affect sensor output current. Testing of biosensor characteristics in actual samples is required prior to in vivo monitoring. Nafion/Chit-Fc/CEst/COx sensor was immersed in EISF, and correlation of sensor output current with EISF cholesterol concentration was analyzed. Good correlation was seen in between the range of 83.39–163.39 mg dl^{-1} (Fig. 2b); however, the change in sensor output current decreased slightly around 120 mg dl^{-1}. In addition, while the color of EISF was clear at the beginning of experiment, it changed to yellow-green after the addition of the standard solution to make the total concentration 50 mg dl^{-1}. This color change might have occurred due to EISF deterioration, thereby causing a change in the absolute increase in sensor output current. After this experiment, sensor was washed and immersed again in the standard whole cholesterol solution. The correlation between sensor output current and standard concentration was checked, and a good correlation was seen (data not shown). Therefore, the decrease in sensor output current was likely due to impurities in the EISF. Moreover, the impurities in the EISF may have adhered to the sensor detection site, decreasing sensor precision. The Nafion/Chit-Fc/CEst/COx sensor, however, was able to measure the cholesterol concentration in the required range.

The sensor output current of Nafion/Chit-Fc/CEst/COx sensor was more stable in standard solution with various DO concentrations. This result suggests that at the lower DO concentrations, the Nafion/CEst/COx sensor did not have enough oxygen available to detect target concentration, while the Nafion/Chit-Fc/CEst/COx sensor detected the whole cholesterol. However, values of the Nafion/Chit-Fc/CEst/COx sensor decreased slightly with a decrease in DO concentration. This was hypothesized to have occurred from electrons transferred to oxygen. For Nafion/Chit-Fc/CEst/COx sensor, electrons emitted from cholesterol oxidase are likely to be transferred to ferrocene; on the other hand, there are possibilities of electrons delivered to oxygen, malfunctioning the monitoring. However, as of fabricated Nafion/Chit-Fc/CEst/COx sensor, decrease in sensor output was small compared to Nafion/CEst/COx sensor, avoiding the effects of variations in DO concentration.

From the in vitro experiments, from the result of temperature, pH, and DO, this Nafion/Chit-Fc/CEst/COx sensor is plausibly operable when implanted into fish EISF. In addition, a good characteristic was also seen in EISF. Therefore, this sensor is operable in breeding factors and in the body of Nile tilapia.

One of the important characteristics of biosensor constructed for in vivo implantation is reproducibility. Biosensors dedicated for in vivo application require reproducibility of less than 5 % (Treviño et al. 2009). Result shows that mean reproducibility was 1.00 ± 0.60 %, and this biosensor is applicable for

in vivo use. The difference in sensor output current is assumed to be due to difference in the surface area of electrodes and the amount of attached enzymes. Therefore, the rather large difference between sensors requires further study to provide more precise measurements.

Biosensors need to be calibrated in order to function with precision. One-point calibration and two-point calibration method are two of the major methods used to calibrate implantable biosensors for in vivo monitoring. To select calibration methods applicable for wireless monitoring system, results from one-point calibration and two-point calibration methods were compared. From the results, no major difference was seen in between one-point and two-point calibration methods. The major goal of developing this wireless monitoring system of EISF whole cholesterol concentration is to provide method to monitor change in whole cholesterol concentration under stress-free condition. If two-point calibration method is selected to calibrate, fish needs to be recaptured after being released. Therefore, one-point calibration method, which enables calibration of Nafion/Chit-Fc/CEst/COx sensor without excessively stressing fish, was selected for further experiments.

Finally, the wireless monitoring of EISF whole cholesterol concentration was successfully performed for about 48 h. Blood whole cholesterol concentration from the first blood draw was used for the one-point calibration method. The changes in calibrated whole cholesterol concentration were similar to the sensor output current and actual blood whole cholesterol concentration. During the monitoring, the light was emitted to fish to artificially fluctuate EISF whole cholesterol concentration: fish was kept in dark before exposing to light. Relationship between whole cholesterol concentration and light remains unclear; however, fish metabolism is affected by sunlight. To further enlighten this phenomenon, research in fish physiology field is required.

The values of the calibrated whole cholesterol concentration were lower than that of the actual blood whole cholesterol concentration. This difference may be due to the plateau in the sensor output current increase at higher concentrations of cholesterol (Fig. 2b). The impurities found in EISF may have affected the sensor output current, keeping the calibrated whole cholesterol concentration low even when the actual blood whole cholesterol concentration increased. Further research and development of the implanted biosensor are required to reduce the effect of in vivo impurities. However, because the fluctuations in the calibrated whole cholesterol concentration were similar to the actual whole cholesterol concentration and sensor output current, this monitoring was successful. This wireless monitoring system could contribute to prognosis of fish condition under conditions with dissolved oxygen concentration. Therefore, it can be used to monitor fish condition under oxygen-decreasing circumstances, such as red tides. In addition, it could be applied to studies of fish physiology and to deepen the knowledge of fish cultivation, leading to improvements in cultivation method for aquaculture.

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