Sensors and Materials, Vol. 27, No. 9 (2015) 805–815
MYU Tokyo

S & M 1117

Carbon Nanotube-Enhanced Enzyme Sensor for Real-Time Monitoring of Cholesterol Levels in Free-Swimming Fish

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(Received January 29, 2015; accepted May 11, 2015)

Key words: sensor, fish, carbon nanotube, cholesterol, electrode

Temporal changes in the plasma total cholesterol concentration of fish are an indication of fish health, and a continuous *in vivo* monitoring method for total cholesterol is therefore desirable. An enzyme biosensor would be a good choice for continuous measurement. However, a biosensor as a foreign substance has a low sensitivity after a certain period of measurement owing to immune system attack. We introduced a highly sensitive carbon nanotube (CNT) to enhance sensor sensitivity for the real-time monitoring of total cholesterol in free-swimming fish (*Oreochromis niloticus*). The sensor was constructed using a Pt-Ir electrode that was dipped into a dispersed CNT solution and dried to adsorb the CNT onto the electrode. Cholesterol esterase, cholesterol oxidase, and a mediator were then immobilized on the sensor. The proposed sensor output current correlated well ($R = 0.9992$) with a cholestreyl oleate standard (10–300 mg dl^{-1}). The dynamic range matched the range of cholesterol concentrations in fish (50–300 mg dl^{-1}). A sensor with a mediator was not affected by changes in oxygen concentration. Changes in total cholesterol concentration could be continuously monitored in free-swimming fish using this sensor for 26 h.

1. Introduction

Fish have recently attracted attention as part of a healthy diet owing to their healthy nutrients. Both developed and developing countries have increased their demand for fish. In developed countries, the main reason for the increase is the consumer's interest in health. In developing countries, on the other hand, insufficient food availability caused by population growth is the main reason. To satisfy the increasing demand for fish, fish aquaculture practices have been promoted. However, fish farms often suffer from fish diseases[1–4] and parasites,[5,6] which lead to mass mortality. Studies have therefore been

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ISSN 0914-4935

performed to gain insight into fish health conditions. The total cholesterol concentration in fish blood plasma is a well-known barometer of health, especially infection.[7] One study showed a higher plasma total cholesterol concentration in fish that survived compared with those that died in an infection experiment.[8]

We developed a real-time biosensor that can measure total cholesterol concentrations in free-swimming fish.[9] Cholesterol esterase and cholesterol oxidase were immobilized on the sensor to recognize different types of cholesterol. To reduce the effects of changes in the dissolved oxygen concentration, the sensor was enhanced with a mediator (ferrocene). This sensor was implanted in the eye interstitial fluid (EISF) of the fish. EISF has fewer contaminants than blood, such as blood corpuscles. In addition, the total cholesterol concentration in EISF correlates well with the plasma total cholesterol concentration.[10] EISF, however, is subject to immune surveillance by the organism, and thus the sensor can be rejected as a foreign substance. When the sensor is attacked by the immune system, the sensitivity of the sensor decreases. To address this problem, we focused on carbon nanotubes (CNT), which have a high electrical conductivity.

CNTs are used in various fields, such as medicine, engineering, and chemistry.[11] Especially in the engineering field, CNTs are useful owing to their large surface area, high electrical conductivity, and mechanical properties. CNTs can also increase the sensitivity of electrodes because of their excellent electrical conductivity and large surface area.[13] Thus, in engineering, CNTs are often applied as an electrode auxiliary material[14–17] or an electrode.[18,19] Therefore, several studies have utilized CNTs to improve sensor sensitivity.[13–19] According to one study, CNTs were introduced into an immunosensor system, and the sensor dynamic range was amplified 100-fold compared with a sensor without CNTs.[14] Sensors with CNTs coating platinum working electrodes for detecting very small concentrations of cancer tumor markers had a higher sensitivity than those with bare working electrodes.[15]

Accordingly, the purpose of this study was to design a sensor enhanced by CNTs for measuring total cholesterol in free-swimming fish. We enhanced a cholesterol enzyme biosensor with CNTs and compared its performance with that of the original sensor, both in standard solution and EISF. We also tested the sensor performance under changing oxygen concentration. We applied the proposed sensor to test fish (*Oreochromis niloticus*) to monitor the total cholesterol concentrations in free-swimming fish.

2. Materials and Methods

2.1 *Reagents and measuring equipment*

Cholesterol esterase (from Pseudomonas fluorescens), cholesterol oxidase (from Streptomyces sp.), and nonaethylene glycol monododecyl ether were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). A 5% Nafion® dispersion solution (DE521 CS type), acetic acid, ferrocene carboxaldehyde, chitosan, sodium tetrahydroborate, 2-phenoxyethanol, heparin sodium, 25% glutaraldehyde solution, and Wako Cholesterol E test were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Cholestreyl oleate was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Disodium hydrogen phosphate 12-water, sodium dihydrogen phosphate dihydrate, and methanol were purchased from Kokusan Chemical Co. Ltd.

(Tokyo, Japan). CNTs were provided by Mr. Junzo Yana (Institute of Carbon Science and Technology, Shinshu University). All other reagents used for the experiments were of commercial or laboratory grade.

A wireless potentiostat for real-time monitoring was constructed in the laboratory. An optical fiber probe-type oxygen sensor was purchased from Ocean Optics, Inc. (Dunedin, FL, USA).

2.2 *Enzyme solution with mediator*

The enzyme solution comprised dissolved cholesterol esterase (7.0 µl, 99 unit ml^{-1}) and cholesterol oxidase (0.8 mg, 1.66 unit mg^{-1}) in phosphate buffer (100 µl, 0.1 M, pH 7.8). Ferrocene carboxaldehyde (215 mg) was dissolved in methanol (15 ml). Chitosan (161 mg) was dissolved in acetic acid (15 ml). These two solutions were then mixed. Tetrahydroborate (2 g) was added to the mixture to initiate a dehydration condensation reaction. This mixture (150 mg) was then placed into an Eppendorf tube and centrifuged in distilled water and methanol (500×*g*, 10 min). Subsequently, acetate buffer (0.05 M, pH 5.0) was added to the precipitate. Finally, 100 µl of enzyme solution was added. This solution is referred to as the enzyme solution with a mediator.

2.3 *Treatment of CNTs*

CNTs (20 mg) were placed in a mixture of sulfuric acid and nitric acid (3:1) with ultrasound treatment in a fume hood for 6 h. The CNT mixture was then diluted 20-fold in distilled water and filtered with a polycarbonate membrane (Merck KGaA, Darmstadt, Germany, TYPE HTTP 0.4 µm).

2.4 *Preparation of the sensor with CNTs and mediator*

We used a Teflon-coated Pt-Ir wire as the working electrode and Ag/AgCl paste as the reference electrode/counter electrode. The Pt-Ir wire was first cut into 1 cm pieces, and 2 mm of the coating was stripped from each end. We then connected one end to the lead wire (Teflon-coated copper wire), wrapped 1.5 cm of the copper wire (connected to the lead wire) around the Pt-Ir wire's Teflon coat, and applied Ag/AgCl paste to the copper wire. The Ag/AgCl paste was dried at 90 °C for 20 min and applied again. The lead wire's connections were covered with heat-shrink tubing. The other end of the Pt-Ir wire was considered the sensing surface. The treated CNTs were dispersed in Nafion® and adjusted to 30 mg ml^{-1}. The sensor was then dipped in the dispersion solution for 1 min and dried for 10 min. The sensor was dipped into the mediator-containing enzyme solution for 1 min and dried for 20 min. This process was performed two times, and the sensor was moved to a plate. Glutaraldehyde (0.05 ml) was added to immobilize the enzymes. Then the electrode was kept at 35 °C for 6 h. This sensor is referred to as the CNT/Med-type cholesterol sensor and was stored at 4 °C until use.

2.5 *Preparation of the sensor without CNTs*

We prepared a sensor without CNTs (mediator-type cholesterol sensor) to compare it with a CNT/Med-type cholesterol sensor. The preparation of the electrode was the same as that described in § 2.4. The sensor was dipped in Nafion® for 1 min and dried for 10 min. Thereafter, the manufacturing method was the same as that described in § 2.4.

2.6 *Preparation of the sensor without mediator*

We prepared a sensor without the mediator (CNT-type cholesterol sensor) to compare it with the CNT/Med-type cholesterol sensor. Electrode preparation and CNT immobilization were the same as those described in § 2.4. The sensor was then dipped in the enzyme solution (without the mediator) for 1 min and dried for 20 min, and the process was performed twice. Thereafter, the manufacture method was the same as that described in § 2.4.

2.7 *Amperometric total cholesterol measurement*

The sensor was dipped in the phosphate buffer (30 °C, 30 ml, 0.1 M, pH 6.5), and a voltage of +350 mV was applied according to our proposed research.[9] Once the output current stabilized, cholestreyl oleate standard solution was added to create a calibration curve in standard solution. We used a syringe (2.5 ml syringe, 20G injection needle) to collect interstitial fluid (300–500 µl) from fish eyes. The EISF was then transferred to an Eppendorf tube and the sensor was dipped in the EISF. We then added cholestreyl oleate standard solution to the EISF to create a calibration curve in the EISF. Because the EISF contains endogenous cholesterol, we measured the total cholesterol in the EISF using the Wako Cholesterol E test.

2.8 *Examination of the influence of oxygen concentration on the sensor*

A cholestreyl oleate standard (80 mg dl^{-1}, 30 °C) was prepared and the oxygen concentration was changed using nitrogen gas. An optical fiber probe-type oxygen sensor was used to measure the oxygen concentration in a beaker into which the sensor was placed.

2.9 *Blood collection and conventional cholesterol measurement*

For the anesthetic, we dissolved 2 ml of 2-phenoxyethanol in 5 L water. We rinsed the syringe (2.5 ml syringe, 23G injection needle) with heparin (3000 unit ml^{-1}). After anesthetizing the fish, we collected 200 µl of blood from the tail vein directly under the vertebra with the syringe, placed the blood in an Eppendorf tube, and stored it in a freezer (−28 °C). Conventional measurement of cholesterol was performed using the Wako Cholesterol E test.

2.10 *Wireless monitoring*

Fish (18 cm) were bred in an experimental aquarium without feeding for 48 h [Fig. 1(a)] to decrease the effect of feeding on cholesterol concentration and adapt the fish to the environment. To detect changes in plasma total cholesterol concentration, the fish were fed a high-nutrition fish food [Fig. 1(b)] and implanted with the CNT/Med-type cholesterol sensor [Fig. 1(c)]. During the monitoring, we collected blood 6 times and measured total cholesterol using the conventional blood sampling method. After monitoring, a one-point calibration method was used to calibrate the sensor output current to the EISF total cholesterol. We calibrated the sensor output current according to the total cholesterol measured from the first blood sample.

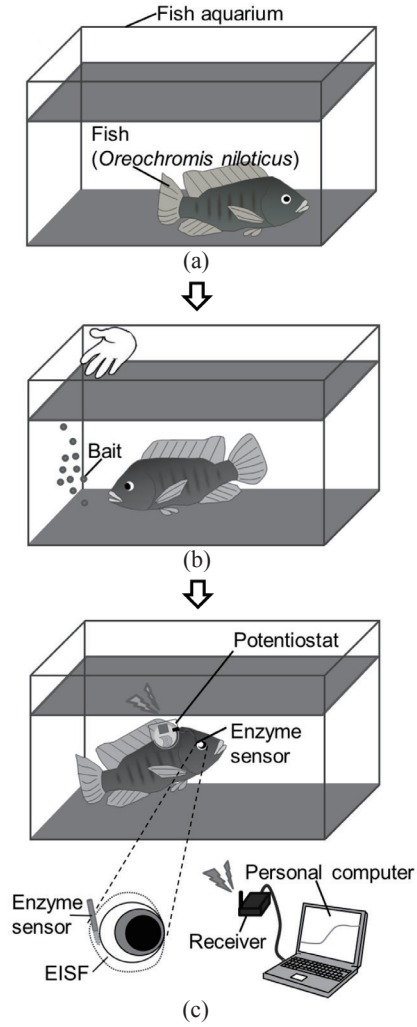

Fig. 1. Wireless monitoring. (a) Fish were bred in an experimental aquarium without feeding for 48 h. (b) The fish were fed a high-nutrition fish food. (c) After a 20 min break, the CNT/Med-type cholesterol sensor was implanted in the EISF and connected to the wireless potentiostat.

3. Results

3.1 *Calibration curve with standard solution*

The calibration curves for the CNT/Med-type cholesterol sensor and the mediator-type cholesterol sensor in standard solution are shown in Fig. 2. When the cholestreyl oleate standard concentration was in the range of 10 to 300 mg dl^{-1}, the CNT/Med/

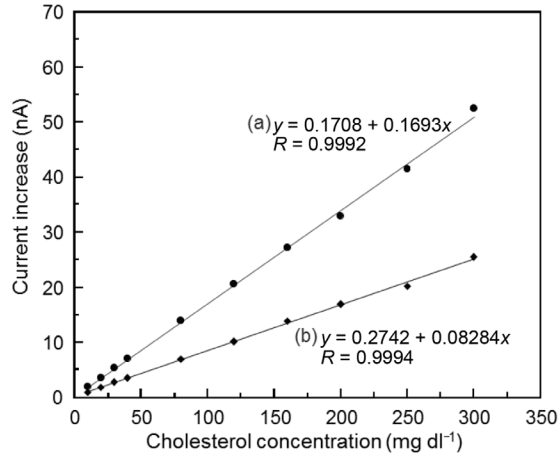

Fig. 2. Calibration curve in standard solution. (a) CNT/Med-type cholesterol sensor. (b) Mediator-type cholesterol sensor. The pH of the standard solution was 6.5 and the applied voltage was +350 mV.

cholesterol sensor output current increase strongly correlated with the cholestreyl oleate standard concentrations (R = 0.9992). When the cholestreyl oleate standard concentration was 300 mg dl^{-1}, the output current increase was 52.5 nA. Detection limits and quantification of biosensors are ultimately constrained by the noise-based precision. The detection limits of the sensor and quantification limits were 0.96 nA ($x_b + 3\delta$; x_b: average value of the base signal δ: standard deviation of the base signal) and 1.45 nA ($x_b + 10\delta$). The standard deviation (S.D.) and % relative standard deviation (R.S.D.) for 3 repetitive measurements (10 mg dl^{-1} cholesterol) were 0.135 and 0.541, respectively, validating good repeatability. The S.D. and R.S.D. for cholesterol detection (10 mg dl^{-1} cholesterol) using four different proposed sensors were 0.325 and 1.451, validating good reproducibility. The mediator-type cholesterol sensor output current increase and cholestreyl oleate standard concentration also strongly correlated in same range (R = 0.9994). When the cholestreyl oleate standard concentration was 300 mg dl^{-1}, however, the output current of the mediator-type cholesterol sensor was 25.5 nA, almost half that of the CNT/Med-type cholesterol sensor output current.

3.2 *Calibration curve with EISF*

The calibration curves for the CNT/Med-type cholesterol and mediator-type cholesterol sensors in the EISF are shown in Fig. 3. As with the standard solution, the CNT/Med-type cholesterol sensor output current strongly correlated with the cholestreyl oleate standard concentrations (R = 0.9690) when the cholestreyl oleate standard concentrations ranged from 65 to 319 mg dl^{-1}. The output current increased 12.9 nA when the cholestreyl oleate standard concentration was shifted from 104 to 309 mg dl^{-1}.

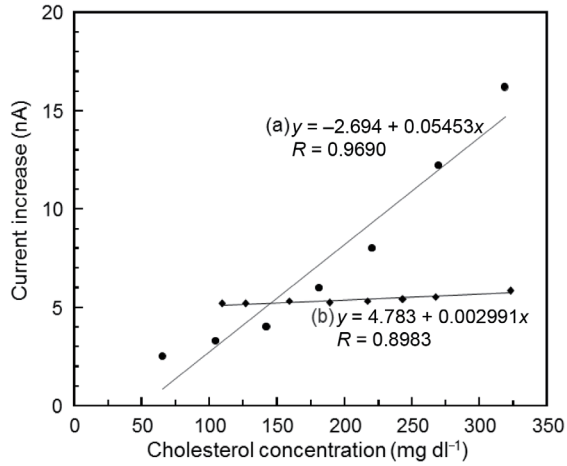

Fig. 3. Calibration curve in EISF. (a) CNT/Med-type cholesterol sensor. (b) Mediator-type cholesterol sensor. The applied voltage was +350 mV. The initial EISF total cholesterol concentrations were 6.5 mg ml^{-1} for (a) and 110 mg ml^{-1} for (b).

The mediator-type cholesterol sensor output current and cholestreyl oleate standard concentration were also strongly correlated (R = 0.8983). The output current, however, was extraordinarily low, only 0.7 nA, when the cholestreyl oleate standard concentration was shifted from 110 to 323 mg dl^{-1}.

3.3 *Influence of oxygen concentration on sensors*

The output current changed with changes in oxygen concentration (Fig. 4). We prepared a cholestreyl oleate standard (80 mg dl^{-1}) and measured it at various oxygen concentrations. The CNT/Med-type cholesterol and CNT-type cholesterol sensors were placed in these solutions. At an oxygen concentration of 8.5 ppm, the output current of the CNT/Med-type cholesterol sensor was defined as 100%. When the oxygen concentration was varied from 1.3 to 8.5 ppm, the output current ranged from 99.2 to 105.6%. Reducing the oxygen concentration did not lead to a decrease in output current. At an oxygen concentration of 8.4 ppm, the output current of the CNT-type cholesterol sensor was defined as 100%. In contrast to the CNT/Med-type cholesterol sensor output, however, the output current of the CNT-type cholesterol sensor decreased markedly when the oxygen concentration was reduced. The output current at an oxygen concentration of 2.0 ppm was 62.7%.

3.4 *Wireless monitoring of cholesterol concentrations in fish*

We used the CNT/Med-type cholesterol sensor to wirelessly monitor total cholesterol concentrations in free-swimming fish in an aquarium. Figure 5 shows the results of 26 h of wireless monitoring with the sensor. The *X*-axis indicates the time, and the *Y1*-axis

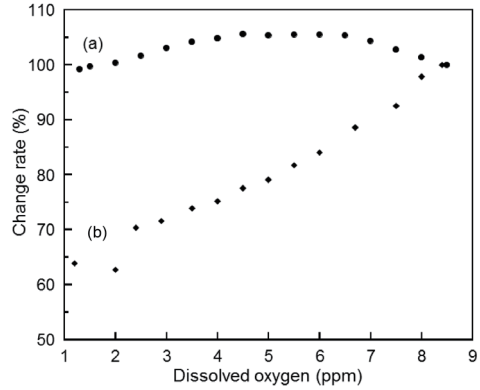

Fig. 4. Relationship between sensor output current and oxygen concentration. (a) CNT/Med-type cholesterol sensor and the applied voltage was +350 mV; (b) CNT-type cholesterol sensor and the applied voltage was +650 mV; the output current of the oxygen concentration of 8.5 ppm was defined as 100% in (a), and that of oxygen concentration of 8.4 ppm was defined as 100% in (b); the pH of the standard solution was 6.5; the cholesterol concentration was 80 mg ml^{-1}.

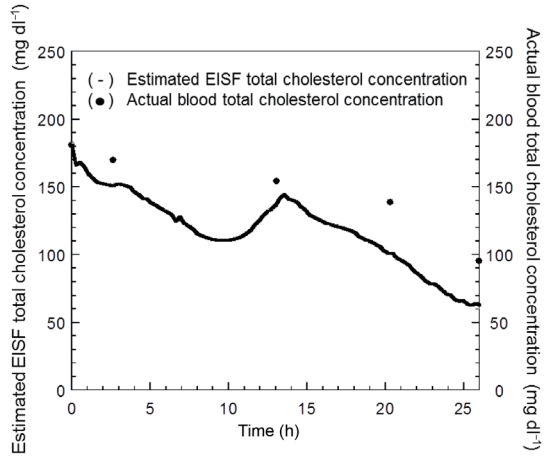

Fig. 5. Wireless monitoring using the proposed biosensor system. The one-point calibration method was used to calibrate the sensor output current to EISF total cholesterol.

shows the EISF cholesterol concentration, calculated from the sensor output current, and the *Y2*-axis shows the blood cholesterol concentration measured by the conventional blood sampling method. The EISF cholesterol concentration is indicated by a line and the blood cholesterol concentration by dots. The EISF total cholesterol concentrations correlated well with the blood total cholesterol concentrations, and the CNT/Med-type cholesterol sensor could be used to monitor total cholesterol *in vivo* for 26 h.

4. Discussion

A previous study revealed that EISF total cholesterol concentrations in a test fish (*Oreochromis niloticus*) range from 50 to 300 mg dl^{-1}.[10] The calibration curve for the proposed sensors was linear when the cholestreyl oleate standard concentration ranged from 10 to 300 mg dl^{-1}, indicating that the calibration curve of the CNT/Med-type cholesterol sensor can cover this range and is thus applicable for monitoring fish total cholesterol *in vivo*. The mediator-type cholesterol sensors covered the same range, but the output current was almost half that of the CNT/Med-type cholesterol sensor. A sensor with high sensitivity is necessary, because factors in the fish EISF reduce sensor sensitivity during monitoring. The CNT/Med-type cholesterol sensor is more suitable for *in vivo* monitoring than the sensor without CNTs. The excellent electrical conductivity of the CNTs increased the sensor's sensitivity. CNTs have a large surface area and thus immobilize more enzymes. Furthermore, no negative effects of the CNT were detected. There was a good correlation between the output current of the CNT/Med-type cholesterol sensor and the total cholesterol concentration, and the noise was as low as that of the sensor without CNTs.

In the standard solution, the CNT/Med-type cholesterol sensor output current strongly correlated with the cholestreyl oleate standard concentration. The linear portion of the calibration curve covered the total cholesterol concentration range of the test fish's EISF. The sensor output current was lower, however, than that measured in standard solution. Proteins in the EISF might adhere to the sensor surface and affect the measurement results. The mediator-type cholesterol sensor output current in the EISF was even lower than that of the CNT/Med-type cholesterol sensor. Thus, the CNT/Med-type cholesterol sensor was more sensitive than the sensor without CNTs in both the standard solution and the EISF.

We confirmed that the CNT/Med-type cholesterol sensor output was not affected by changes in oxygen concentration. Oxidizing enzymes, such as cholesterol oxidase, require electrons for the reaction. The electrons must come from either a mediator or oxygen. Without a mediator, therefore, sensor activity could be influenced by changes in oxygen concentration. The output current of the CNT/Med-type cholesterol sensor ranged from 99.2 to 105.6% when the oxygen concentration was varied from 1.3 to 8.5 ppm, indicating that the mediator CNT/Med-type cholesterol sensor was slightly affected by reduction in oxygen concentration. Without a mediator, however, the sensor output of the CNT-type cholesterol sensor markedly decreased as the oxygen concentration decreased. When the oxygen concentration was 2.0 ppm, the output current was only 62.7%. A mediator-type sensor, therefore, is required for *in vivo* wireless monitoring in an environment in which the oxygen concentration could change.

After using the CNT/Med-type cholesterol sensor for 26 h of wireless monitoring, we estimated the EISF total cholesterol concentration based on the sensor output current. The blood total cholesterol concentration was also measured using the conventional blood sampling method. The EISF total cholesterol concentration correlated well with the blood total cholesterol concentration. Therefore, the CNT/Med-type cholesterol sensor can monitor fish total cholesterol concentration *in vivo* for approximately 26 h

in real time. The estimated EISF cholesterol concentration was lower than the blood cholesterol concentration in the latter half of the measurement period, perhaps owing to a reduction in enzyme activity. The discrepancy between the EISF cholesterol concentration and the blood cholesterol concentration might increase with monitoring time, a problem that should be solved in future studies.

During monitoring, the fish exhibited no unusual behavior owing to the application of the sensor. The fish swam freely in the case without the sensor. Therefore, it is possible to monitor the total cholesterol concentration with the CNT/Med-type cholesterol sensor under free-swimming conditions in fish. After monitoring the fish, we examined the sensor activity by establishing a calibration curve in standard solution. The sensitivity was lower than before the *in vivo* monitoring, but there was still a good correlation between the sensor output current and the cholestreyl oleate standard concentration (data not shown). This indicates that the sensor can be used for at least 26 h. After 28 h, however, the sensor's output current increased suddenly and sharply. We consider that an immune reaction may have led to a loss of the enzyme from the sensor surface. Previous studies demonstrated that a cholesterol sensor without CNTs can effectively monitor the cholesterol concentrations for 48 h.[9,10] The CNT/Med-type cholesterol sensor had a CNT-dispersed Nafion® film under the enzyme film, so the films on the sensing surface were thicker than those on the sensors without CNTs. Perhaps a thicker film falls off more easily, thus decreasing the lifetime of the CNT/Med-type cholesterol sensor. The CNT/Med-type cholesterol sensor, however, requires some improvement. For longer monitoring, the enzyme activity reduction will lead to a discrepancy between the estimated EISF total cholesterol concentration and the blood cholesterol concentration. To address this problem, the monitoring data should be calibrated. Furthermore, the sensor life must be extended. Some studies have demonstrated that the application of a biocompatible membrane can extend the lifetime of sensors.[20] For longer monitoring, applying a biocompatible membrane to the sensor would be a good improvement.

5. Conclusions

The CNT/Med-type cholesterol sensor output current strongly correlated with the cholesteryl oleate standard concentration (R = 0.9992), and they were linear when the cholestreyl oleate standard concentration ranged from 10 to 300 mg dl^{-1}. The output current of the mediator-type cholesterol sensor and cholestreyl oleate standard concentrations were strongly correlated in the same range (R = 0.9994). The mediator-type cholesterol output current, however, was almost half that of the CNT/Med-type cholesterol sensor. The CNT/Med-type cholesterol sensor also had a higher sensitivity than the mediator-type cholesterol sensor in the EISF. The CNT/Med-type cholesterol sensor is a mediator type, so it is less affected by changes in oxygen concentration. The CNT/Med-type cholesterol sensor could monitor total cholesterol *in vivo* for 26 h, and the sensor's output current correlated well with the blood total cholesterol. Furthermore, the fish are not harmed by inserting the sensor. Therefore, this is a practical method suitable for monitoring the fish total cholesterol concentration under natural conditions. Our proposed sensor had a higher sensitivity than the original sensor as it could detect very small changes in total cholesterol in free-swimming fish.

Total cholesterol is an indicator of fish disease resistance, which decreases when fish are infected with a pathogenic microorganism.[8] Monitoring the total cholesterol change with the proposed sensor allows for observation of changes in fish health. The ability to easily monitor fish health will help fish aquaculture to improve the breeding environment of the fish and maintain high productivity.

Acknowledgements

This research was supported in part by a Grant-in-Aid for Scientific Research (B) from The Ministry of Education, Culture, Sports, Science, and Technology. We wish to thank Dr. Chiharu Ichioka (Ichioka Dental Office) and Mr. Junzo Yana (Institute of Carbon Science and Technology, Shinshu University) for their helpful discussion.

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