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TECHNICAL NOTE

Carbon nanotube enhanced mediator-type biosensor for real-time monitoring of glucose concentrations in fish

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Abstract We have developed a mediator-type biosensor to rapidly monitor blood glucose concentrations in fish, which are an indicator of stress. Glucose oxidase was used to detect glucose concentrations and ferrocene was used to limit the effect of oxygen. We also improved the sensitivity and durability of the sensor for better performance. Single-walled carbon nanotubes were used to enhance sensor sensitivity. Affixing the carbon nanotubes (30 mg ml^{-1}) to the working electrode increased the sensor sensitivity to 61.9 mM nA^{-1} mm^{-2}, twice the value for the sensor without single-walled carbon nanotubes. A fabricated mediator-type biosensor sensor was used to perform real-time in vivo measurements. The sensor was implanted into the interstitial fluid of a fish eyeball, and detection was transmitted to a personal computer by a wireless potentiostat. Continuous measurement of the glucose concentration was possible for 78 hours. Stress was artificially applied to the fish during the measurement, and the change of blood glucose concentrations were observed. Our proposed sensor is applicable for effectively monitoring stress in free-swimming fish.

Keywords Mediator-type biosensor · Single-walled carbon nanotube · Continuous in vivo measurement · Poly(ethylene glycol) · Fish

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Introduction

In the aquaculture industry, a rapid and easy method to monitor the health of fish is desired. Maita et al. [1] proposed that the health of fish can be monitored on the basis of hemodiagnosis. We developed a wireless glucose biosensor system which allows easy and rapid assay of blood substances in free-swimming fish [2]. However, there were some technical problems with the initially developed biosensors. First, a change in the dissolved oxygen (DO) concentration affected the sensor output current since an oxidizing enzyme was used. Second, the glucose concentration is measured by the enzyme layer formed on top of the detection site. This layer comes into direct contact with in vivo substances, which might result in early deactivation of the enzyme.

To limit the effect of oxygen concentration, we focused on ferrocene, a mediator which substitutes for the oxidation–reduction reaction of oxygen and catalyzes the reaction between the electrons and the enzyme. Chitosan is often used to fix ferrocene to electrodes. Chitosan has amino groups which covalently bond substances to itself, enhancing the functionality of a membrane [3, 4]. This characteristic allows ferrocenecarboxylic acid to attach well to chitosan. With use of the combination of ferrocene and chitosan, it is possible to firmly fix a mediator to the sensor detection site.

To improve the sensor characteristics, we used carbon nanotubes (CNTs) and poly(ethylene glycol) (PEG) to fabricate our sensor. CNTs are popular in the field of electronic devices [5]. A mediator-type glucose biosensor using single-walled CNTs (SWCNTs) has been reported to enhance the oxidation–reduction reaction of the mediator [6, 7]. Therefore, fixing SWCNTs along with enzymes to the detection site will result in the construction of a sensor with better sensitivity. Several substances are used to fix SWCNTs to

the electrode surface. Nafion® is especially popular since SWCNTs are known to disperse especially well in that solution [8]. In addition, PEG was studied to limit the environmental factors affecting the steric structure or the enzyme. It is also known to prevent detachment of enzymes from electrode surfaces [9] and to stabilize enzyme activity [10, 11], which are characteristics essential for long-term in vivo monitoring.

In this study, we developed a mediator-type biosensor system using SWCNTs and PEG to construct a glucose biosensor that will be less affected by in vivo substances during monitoring. We then inserted the constructed sensor into a fish to perform real-time monitoring of the glucose concentration in fish.

Materials and methods

Reagents

Glucose oxidase (197 U mg^{-1}, EC 1.1.3.4, from *Aspergillus niger*) was purchased from Sigma-Aldrich (Tokyo, Japan). Nafion® (5 %), chitosan 5, ferrocenecarbaldehyde, and sodium tetrahydroborate were purchased form Wako Pure Chemical Industries (Tokyo, Japan). The SWCNTs were provided by Junzo Yana (Institute of Carbon Science and Technology, Shinshu University). PEG (SUNBRIGHT series) was purchased from Nippon Oil Factory (Tokyo, Japan). All other reagents used for the experiments were commercial or laboratory grade.

Fabrication of the mediator-type enzyme sensor

A needle-type sensor was constructed using the same method as Yonemori et al. [2]. SWCNTs (1–12 mg) were dissolved in 5 % Nafion® solution (0.5–5 wt%). One microliter of the SWCNTs dissolved in Nafion® was applied to an exposed Pt–Ir wire to fix the SWCNTs. Ferrocenecarbaldehyde (215 mg) was dissolved in 15 ml methanol, 161 mg chitosan was mixed with 15 ml acetic acid, and the two mixtures were combined. Sodium tetrahydroborate (2 g) was added to produce chitosan modified with ferrocene (Chit-Fc). Chit-Fc was rinsed with distilled water and methanol by centrifugation and dissolved in an acetic acid buffer solution (pH 5.5). Glucose oxidase (2 mg) and PEG (5 mg) were dissolved in 200 μl phosphate buffer (0.1 M, pH 7.0). This mixture was stirred for 6 hours using a MicroMixer E-36 (Titec, Tokyo, Japan); resulted in attachment of glucose oxidase and PEG. After modification, ultrafiltration was performed by centrifuging (11,000*g*, 10 min) the solution with an Ultra YM-30 (Millipore, Japan). A 100-μl aliquot of the condensed solution and 100 μl phosphate buffer (0.1 M, pH 7.0) were mixed with 300 μl Chit-Fc to make the enzyme solution. The enzyme solution (1 μl) was applied to the working electrode with the fixed SWCNTs and dried for 12 hours at 35 °C.

Apparatus

A potentiostat (model 3140, Pinnacle Technology, Lawrence, KS, USA) was used for amperometric measurements. A handmade wireless potentiostat and receiver were used for wireless real-time monitoring. The wireless potentiostat was constructed from a PIC MicroChip (ADC 12 F675, Japan) and an iTx 315S FM-type wireless module (ITEC, Tokyo Japan).

In vivo measurement

A Nile tilapia fish was used for this experiment. The glucose concentration of its eyeball interstitial sclera fluid (EISF) is known to correlate well with the blood glucose concentration [2]. Therefore, EISF was used for real-time monitoring of the glucose concentration.

The wireless potentiostat in this system transmits 512-MHz radio waves to the receiver and data are displayed on a computer screen. Measurement began directly after the first blood draw. Nitrogen gas was applied to the water during the measurement to decrease the oxygen concentration, thereby inducing a form of stress in fish. In vivo measurement differs from in vitro measurement in that external factors which may affect the measurement reside in fish. Therefore, it is imprecise to use a calibration curve obtained in vitro from a standard solution for in vivo measurements. One-point calibration method [12–14] was used in this experiment for sensor calibration.

Results and discussion

Sensor characteristics

The effects of the SWCNT concentration on the sensor output current were analyzed (Fig. 1a). Nafion® solutions with different SWCNT concentrations were used to construct the sensors. We found an SWCNT concentration of 30 mg dl^{-1} was optimal for construction of the sensor. The reasons are as follows. The sensor sensitivity increased as the SWCNT concentration increased in the range from 5 to 30 mg ml^{-1}, but the desired sensitivity was not achieved at SWCNT concentrations of 40 and 60 mg ml^{-1} (data not shown). SWCNT concentrations of 40 and 60 mg dl^{-1} might cause excess fixation of SWCNTs at the detection site, thereby disturbing electron

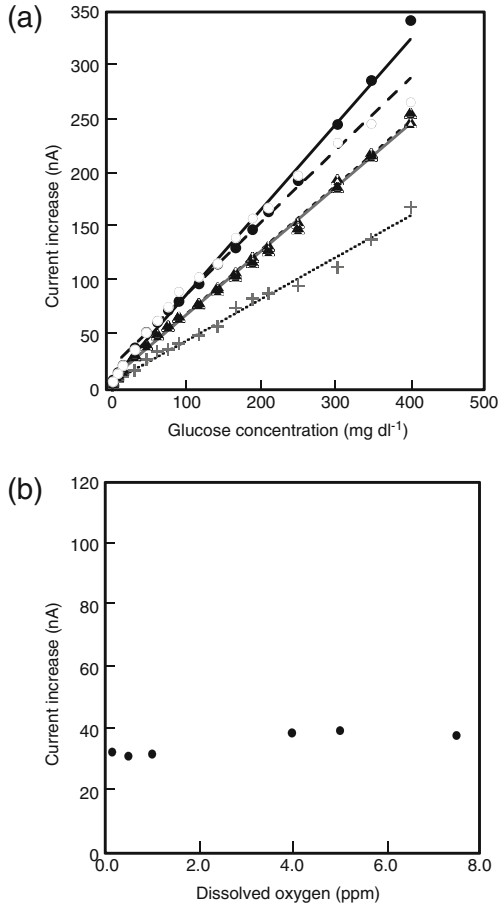

Fig. 1 Analysis of sensor characteristics. **a** Calibration of the sensor with different SWCNT concentrations. For this experiment, we used 0.1 M phosphate buffer, pH 7.0. A voltage of +350 mV was applied and the temperature was set to 30 °C. *Filled circles* 30 mg dl^{-1}, *open circles* 20 mg dl^{-1}, *filled triangles* 10 mg dl^{-1}, *open triangles* 5 mg dl^{-1}, *crosses* 0 mg dl^{-1}. **b** Effect of dissolved oxygen on sensor output current. For this experiment, we used 0.1 M phosphate buffer, pH 7.0. A voltage of +350 mV was applied and the temperature was set to 30 °C.The dissolved oxygen concentration was altered in the range from 0 to 8 ppm

transfer. The sensitivity of the sensor with SWCNTs at a concentration of 30 mg dl^{-1} was 61.9 mM nA^{-1} mm^{-2}, which was twice as high as that of the sensor without SWCNTs, 30.3 mM nA^{-1} mm^{-2}. Therefore, this sensor was used for further experiments.

The effect of the DO concentration on the sensor output current was analyzed by changing the DO concentration of a glucose standard sample (pH 7.0, 100 mg dl^{-1}). The result is shown in Fig. 1b. The sensor output current remained stable in the range from 0 to 8 ppm and was unaffected by the DO concentration. The DO concentration in fish is known to change with the DO concentration of the surrounding water [15], which in nature changes readily. This suggests the rapid change in the DO concentration inside the body of fish. In this situation, if a sensor without a mediator is used, error resulting from a change in the DO concentration may affect the in vivo measurement.

There are several factors that induce stress in fish. A change in the DO concentration is one of those. The goal of our wireless monitoring system is to detect stress in fish; however, if the sensor output current is affected by the change in the DO concentration, accurate monitoring of stress may not be achieved. Therefore, in terms of the effect of the DO concentration, our proposed sensor will be suitable for further measurements.

Real-time in vivo measurement

The results of real-time in vivo measurements are shown in Fig. 2. The fabricated sensor was inserted into the EISF of a living fish for approximately 15 hours prior to the measurement. By this procedure, we continuously monitored the blood glucose concentration for 78 hours. The calibrated blood glucose concentration showed changes similar to those of the actual blood glucose concentration. The sensor output current decreased in the first 20 hour, suggesting that insertion of a sensor into the EISF potentially causes stress, which then decreases over time. At the 25th and 53 rd hour, nitrogen was applied to the water to decrease the oxygen concentration: nitrogen induces stress by suffocating fish. The oxygen concentration at the 25th hour changed from 7.64 to 2.51 ppm and that at the 53 rd hour changed from 6.52 to 2.76 ppm. The calibrated blood glucose concentration increased with the decrease in oxygen concentration, demonstrating that the wireless monitoring successfully reflected the stress level. Real-time in vivo measurement was succesfully performed for 78 hours.

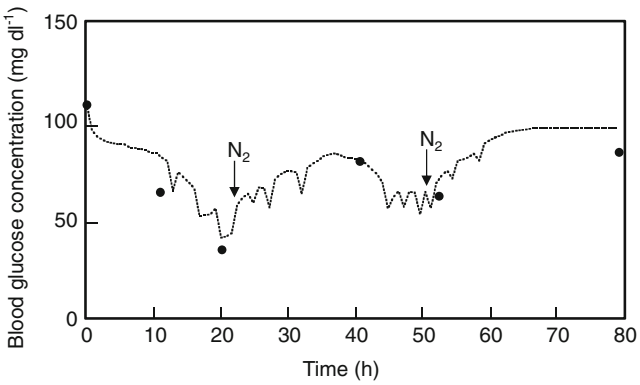

Fig. 2 Real-time monitoring of blood glucose concentration. For this experiment, we used 0.1 M, phosphate buffer, pH 7.0. A voltage of +350 mV was applied and the temperature was set to 30 °C. *Circles* blood glucose, *dotted line* estimated glucose concentration

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