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Development of Self-powered Biosensor for Fish Health Monitoring

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Meeting the global demand for marine resources requires high efficiency in the aquaculture industry. This makes it necessary to monitor the stress level of cultured fish and control their health and breeding environment. Blood glucose level has been reported to increase with increasing stress level in cultured fish, and wearable glucose sensors have been used for fish, which can continuously monitor their blood glucose level. Here, we demonstrate the construction of a self-powered biosensor without a power supply. This biosensor operates through the generation of electricity during the glucose oxidation reaction at the anode and the simultaneous O_2 reduction reaction at the cathode, with the connection of the anode and cathode in the same circuit. The prepared anode exhibits a response that depends on the glucose concentration, and the cathode detects O_2 reduction reaction. The developed biosensor detected current responses when 3 mM glucose was added to black sea bream. Because the self-powered biosensor does not require a power supply, the overall system is smaller than the conventional biosensor system. The developed biosensor is expected to serve as a continuous and wearable monitoring device for cultured fish.

1. Introduction

Recently, the global demand for marine products has led to overfishing.[1,2] Consequently, fishery resources have started to diminish.[3–7] To circumvent this problem, it is necessary to focus on aquaculture fisheries. To achieve sustainable aquaculture production, efforts are

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required to ensure that aquaculture production is suitable for the aquaculture fishing environment (no overcrowding), fish are fed an appropriate amount of food (i.e., no overfeeding), and the fishing environment is protected from fouling.[8–12] However, improper aquaculture is carried out at some sites, where overcrowding and overfeeding are used to increase production efficiency, resulting in the mass mortality of cultured fish owing to infectious diseases and environmental degradation. One indicator of cultured fish health is stress level. Changes in the stress level of cultured fish have been reported to be accompanied by changes in the blood composition, the endocrine system, and other parameters.[13,14] By monitoring the stress level of cultured fish and managing their health and environment, the problems associated with aquaculture fisheries may be reduced.

Cortisol and glucose levels in the blood of cultured fish have been reported as indicators of stress.[15–17] Cortisol is a steroid hormone secreted into the blood during a stress response. When fish are stressed, the concentration of cortisol in the blood temporarily increases by an amount depending on the intensity of the stressor. Additionally, glucose production by the glycogenesis pathway is promoted owing to cortisol secretion. Therefore, glucose is a secondary response substance to stress.[18,19]

Liquid chromatography-mass spectrometry (LC-MS) and enzyme-linked immunosorbent assay (ELISA) are generally used to measure steroid hormones such as cortisol.[20–22] LC-MS provides high detection sensitivity, selectivity, and reproducibility; however, the analytical equipment is large, expensive, and requires complex sample pretreatment and expertise, such as the selection of measurement conditions. In contrast, ELISA is a simple measurement technique that does not require special equipment or institutions; however, complex sample treatment and long reaction times are required. Therefore, these methods are not suitable for rapid and continuous monitoring.

In the measurement of glucose, colorimetric and fluorescence assays with enzyme reactions are applied. However, these methods require absorbance analysis while enzymatically reacting one sample at a time, and it is difficult to rapidly and continuously measure the blood glucose concentration in cultured fish. In contrast to colorimetric and fluorescence assays, the enzyme electrode method has the advantages of easy device miniaturization and rapid and easy measurements. Therefore, the focus of this study is the application of the enzyme electrode method to biosensor technology. Glucose biosensors use glucose as a substrate and have immobilized enzymes on the electrode, which catalyze oxidation. Glucose oxidase (GOx) and glucose dehydrogenase (GDH) are examples of these enzymes.[23–27] The electrons obtained via the oxidation of glucose by these enzymes are detected as an electrical signal. Glucose biosensors can indirectly measure the glucose concentrations because the response current increases with increasing glucose concentration. Moreover, they are easy to operate, provide rapid onsite analysis, and do not require complex pretreatment, such as sample extraction and isolation. The most common electrochemical biosensor is the amperometric biosensor, which applies a constant voltage to the working and reference electrodes and measures the current between the working and counter electrodes. This type of biosensor is widely used because it requires simple electronic equipment and is highly sensitive. There is a report that wearable glucose sensors for fish, which continuously monitor their blood glucose level, was developed.

[28] However, amperometry and other electrochemical methods are unsuitable for long-term monitoring, because they require an external power supply for the potentiostat or galvanostat.

In this study, we aimed to develop a self-powered biosensor, which combines an anode that oxidizes glucose to gluconolactone and a cathode that reduces O_2 to H_2O (Fig. 1). We hypothesize that this biosensor can continuously measure the glucose concentration without an external power supply because the sensor signal is a flow of electrons between the electrodes, which is produced by the glucose oxidation reaction. It is important to construct a biosensor that can simultaneously generate and detect electricity and does not require an external power supply.

GOx, which is employed in many biosensor studies as an anode enzyme, uses O_2 as an electron acceptor; therefore, the concentration of O_2 in the body can easily cause a reduction in output. Therefore, in this study, pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) from microorganisms [29–31] is used as the anode, which does not require O_2 as an electron acceptor, and the progress of the oxidation reaction is independent of the O_2 concentration in the blood. PQQ-GDH does not use O_2 as an electron acceptor, but instead catalyzes the dehydrogenation of glucose. As the cathode enzymes, bilirubin oxidase (BOD) from *Myrothecium* sp. [32–34] is used, which catalyzes the oxygen reduction reaction.

2. Materials and Methods

2.1 Chemicals and reagents

PQQ-GDH was purchased from Toyobo (Osaka, Japan); 1-[3-(Succinimidyloxycarbonyl) propoxy]-5-ethylphenazinium triflate (amine-reactive PES; AR-PES) was obtained from Dojindo Laboratories (Kumamoto, Japan); 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) was purchased from Nacalai Tesque (Kyoto, Japan); multiwalled carbon nanotubes (MWCNTs), BOD, and Nafion were purchased from Sigma-Aldrich (Tokyo, Japan), and D (+)-glucose, L (+)-ascorbic acid (AA), urea (UA), and sodium hydroxide (NaOH) were purchased from FUJIFILM Wako Chemicals (Osaka, Japan). O_2 and N_2 gases were obtained from Uno Sanso (Fukui, Japan). Black sea bream blood was provided by Kindai University. The blood was collected using a syringe with heparin coated on the interior wall. After blood collection, erythrocytes were killed by freezing at –20 °C for 1 h. All reagents were of first grade and used without further purification. All solutions were prepared using Milli-Q water. A glassy carbon electrode (GCE) was obtained from BAS (Tokyo, Japan). Its outer diameter was 6.0 mm and its inner (conductive part) diameter was 3.0 mm, giving an electrode area of 28.28 μm^2.

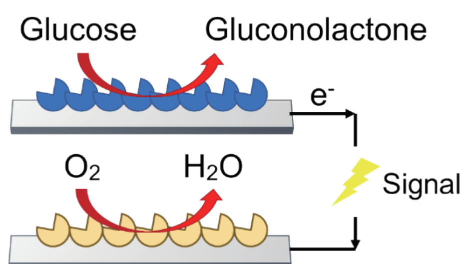

Fig. 1. (Color online) Schematic image of the self-powered biosensor.

2.2 Preparation of PQQ-GDH/PES-modified anode electrode

The PQQ-GDH enzyme was used as the anode electrode. Firstly, to improve conductivity, 10 µL of 0.5 mg/mL MWCNT dispersion was added dropwise onto a GCE and dried under ambient conditions. Subsequently, 10 µL of 1 mg/mL PQQ-GDH solution was added to the electrode dropwise and dried under ambient conditions. Next, 10 µL of 5 mM AR-PES solution was added to the electrode dropwise and dried under ambient conditions. Finally, 10 µL of 0.1 wt% Nafion solution was added to the electrode dropwise to immobilize and prevent the withdrawal of PQQ-GDH and AR-PES, followed by drying under ambient conditions [Fig. 2(a)]. The obtained electrode is referred to as the PQQ-GDH/PES-modified electrode.

2.3 Preparation of BOD-modified electrode

The BOD enzyme was used as the cathode. Firstly, 10 µL of 0.5 mg/mL MWCNT dispersion was added dropwise onto a GCE and dried under ambient conditions. Subsequently, 10 µL of 1 mg/mL BOD solution was added to the electrode dropwise, and dried under ambient conditions. Finally, 10 µL of 0.1 wt% Nafion solution was added dropwise and dried under ambient conditions [Fig. 2(b)]. The obtained electrode is referred to as the BOD-modified electrode.

2.4 Evaluation of prepared electrode

The PQQ-GDH/PES-modified electrode was evaluated by cyclic voltammetry (CV). This was performed in a three-electrode system using the PQQ-GDH/PES-modified electrode as the working electrode, a Pt wire as the counter electrode, and a Ag/AgCl electrode as the reference electrode. The CV was performed at a sweep rate of 100 mV/s in 50 mM HEPES-NaOH buffer (pH = 7.0) with 0 or 5 mM glucose.

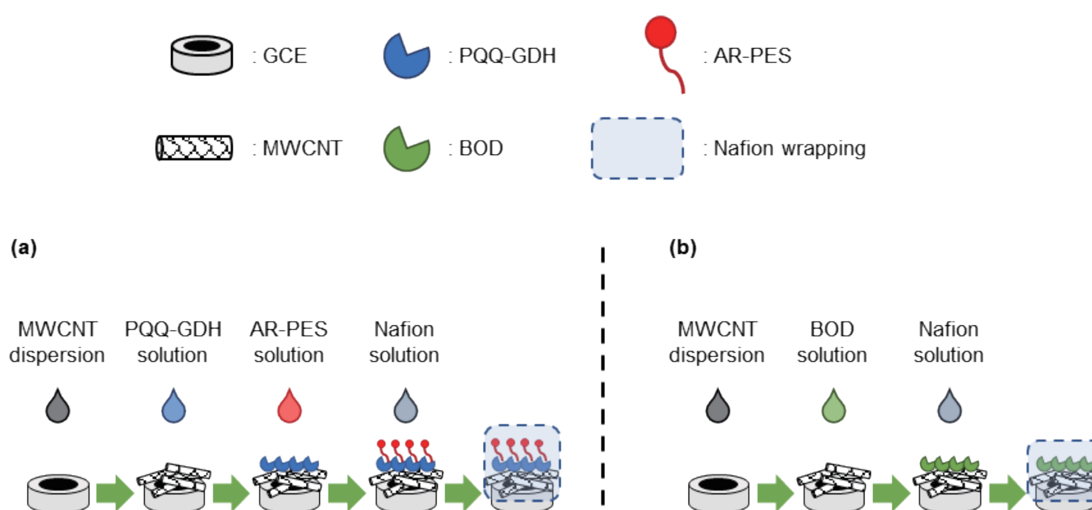

Fig. 2. (Color online) Preparation process of (a) PQQ-GDH/PES-modified electrode and (b) BOD-modified electrode.

The BOD-modified electrode was evaluated by chronoamperometry (CA). This was performed in a three-electrode system using the BOD-modified electrode as the working electrode, a Pt wire as the counter electrode, and a Ag/AgCl electrode as the reference electrode. The CA was performed in 50 mM HEPES-NaOH buffer (pH = 7.0) while degassing using N_2 gas, and O_2 gas was bubbled through the electrolyte while applying a voltage of 0.01 V.

2.5 Evaluation of assembled self-powered biosensor

A self-powered biosensor was assembled by incorporating the fabricated PQQ-GDH/PES-modified electrode and BOD-modified electrode into the same circuit. The assembled self-powered biosensor used a nonresistive ammeter to measure the current produced without an external power supply for the electrochemical reactions. The distance between the PQQ-GDH/PES-modified and BOD-modified electrodes was 5 mm. During the measurement, a magnetic stirrer was used to gently stir the mixture (Fig. 3).

3. Results

3.1. Characterization of PQQ-GDH/PES-modified anode electrode

Figure 4 shows the CV curves of the PQQ-GDH/PES-modified electrode at different glucose concentrations. Here, the oxidation current was analyzed. In the absence of glucose (0 mM), a PES-derived oxidation wave was observed at approximately −0.08 V. This suggests that the AR-PES was modified with the PQQ-GDH. At a glucose concentration of 5 mM, the oxidation current at −0.08 V increased by 1.2 μA compared with that at the 0 mM glucose concentration. These results confirm that enzyme-catalyzed reactions involving glucose occur at the anode.

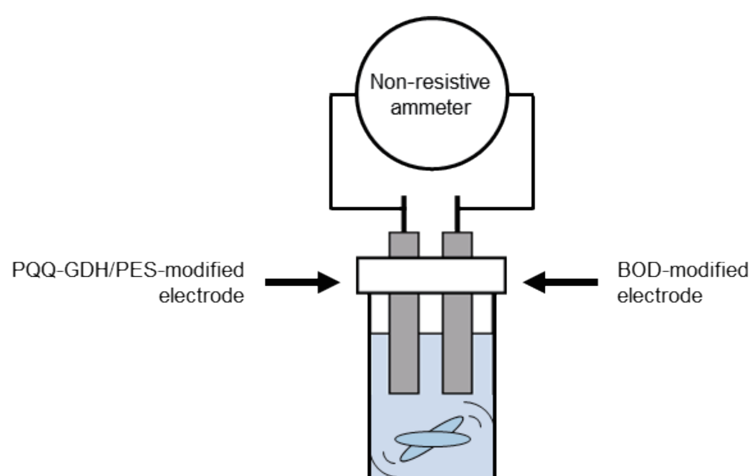

Fig. 3. (Color online) Schematic image of experimental system for evaluating self-powered biosensor.

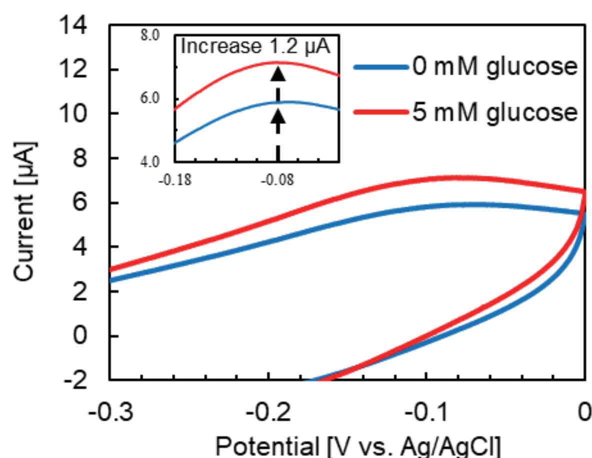

Fig. 4. (Color online) CV curves of glucose oxidation reaction using PQQ-GDH/PES-modified electrode with 0 and 5 mM glucose in 50 mM HEPES-NaOH buffer (pH = 7.0) at a sweep rate of 100 mV/s. Inset: Magnified CV curves, where arrows indicate the peak potential.

3.2 Characterization of BOD-modified cathode electrode

CA measurements were performed on the BOD-modified electrode. The electrolyte was degassed using N_2 gas, and O_2 gas was bubbled through the electrolyte while applying a voltage of 0.01 V. Figure 5 shows the change in current when the BOD enzyme (O_2) used as a substrate was continuously added for approximately 1800 s. During this period the current was stable. A reduction in the current was measured immediately after O_2 induction, thus confirming that the O_2 reduction reaction was catalyzed by the BOD enzyme.

3.3 Evaluation of self-powered biosensor assembled using the PQQ-GDH/PES-modified anode and BOD-modified cathode

A self-powered biosensor was assembled using the PQQ-GDH/PES-modified electrode as the anode and the BOD-modified electrode as the cathode. The selectivity of the assembled self-powered biosensor for common biological species was tested. Figure 6 shows the current response when 1 mM UA, AA, and glucose were added dropwise. As shown in Fig. 6, no clear change in current was observed when UA and AA were added. However, when glucose was added, a change in current was observed. This result indicates that the assembled self-powered biosensor exhibited excellent selectivity to glucose.

The current response of the assembled self-powered biosensor was evaluated at different glucose concentrations. Figure 7 shows the current responses to 1, 2, and 3 mM glucose solutions added dropwise. An increase in current was observed with increasing glucose concentration, suggesting that glucose was oxidized at the anode. As shown in Fig. 7, the relationship between the glucose concentration and current showed good linearity.

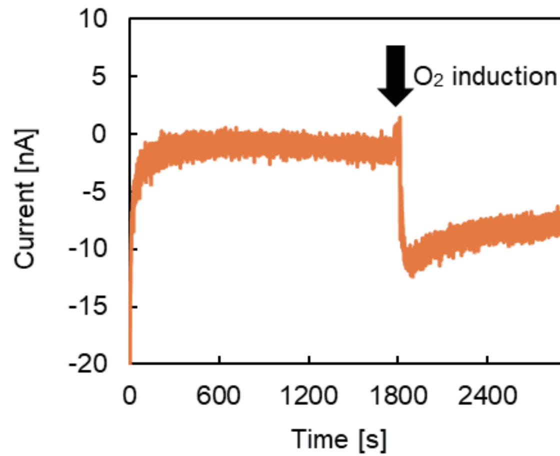

Fig. 5. (Color online) CA curve of O_2 reduction reaction using BOD-modified electrode in N_2-saturated 50 mM HEPES-NaOH buffer (pH = 7.0) at 10 mV (vs. Ag/AgCl) with O_2 induction at 1800 s.

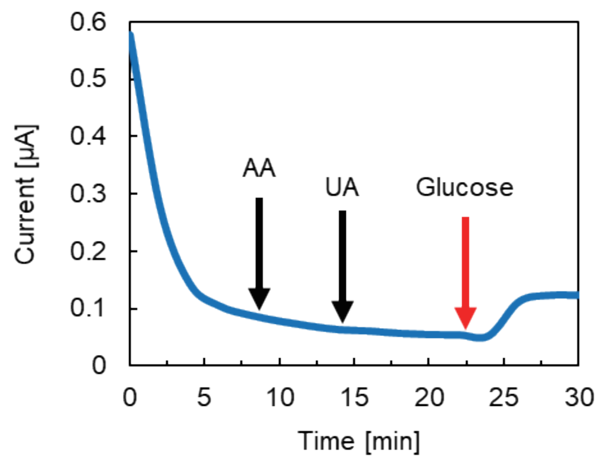

Fig. 6. (Color online) Amperometric response of self-powered biosensor in 50 mM HEPES-NaOH buffer (pH = 7.0) to AA, UA, and glucose.

Under these conditions, the nonresistive ammeter measuring the current does not use an external power supply but is driven by the electromotive force of the assembled self-powered biosensor. Therefore, the biosensor demonstrated glucose responsivity and selectivity as well as self-power generation.

3.4 Evaluation of glucose response of self-powered biosensor using black sea bream blood

Figure 8 shows the current response when 3 mM glucose was added dropwise to 5 mL of black sea bream blood 4 min after starting measurement at room temperature. The current increase was confirmed immediately after the glucose addition. This indicates that the biosensor

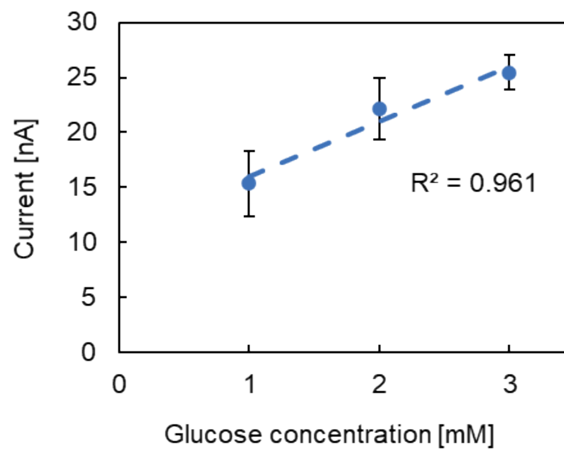

Fig. 7. (Color online) Linear dependence of response current on glucose concentration (n = 3) in a 50 mM HEPES-NaOH buffer (pH = 7.0).

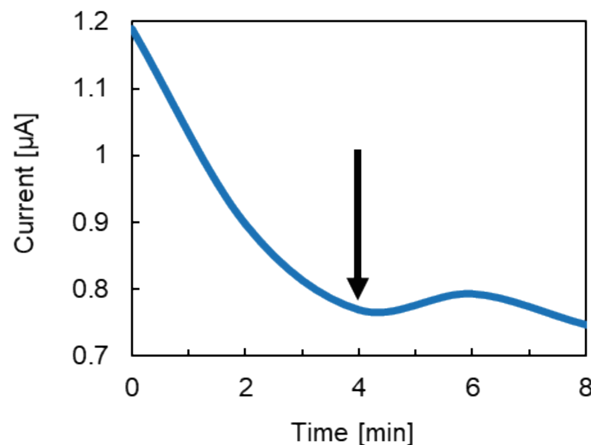

Fig. 8. (Color online) Amperometric response of glucose oxidation reaction when using self-powered biosensor in 50 mM HEPES-NaOH buffer (pH = 7.0) to analyze black sea bream blood; 3 mM glucose was added at 4 min (arrow).

is not inhibited by other substances in the black sea bream blood and selectively responds to glucose, enabling the detection of variations in the glucose concentration when cultured fish are stressed.

Note that the nonresistive ammeter measuring the current did not use an external power supply; it was driven by the electromotive force of the assembled self-powered biosensor. This biosensor does not require an external power supply, and it demonstrate responsiveness to glucose in black sea bream blood while generating its own power.

4. Conclusion

In this study, we developed a self-powered and glucose-responsive biosensor, composed of an anode that oxidizes glucose and a cathode that reduces O_2, to detect stress in cultured fish based

on the fact that the blood glucose concentration increases when fish are stressed. PQQ-GDH/PES-modified and BOD-modified electrodes were prepared as the anode and cathode, respectively. Notably, in this study, the nonresistive ammeter was not connected to an external power supply, and the glucose oxidation reaction at the anode and O_2 reduction reaction at the cathode occurred simultaneously, thus realizing a self-powered biosensor with good glucose responsivity and selectivity. This biosensor can be miniaturized because it does not require an external power supply. In the future, we aim to develop a biosensor that can continuously monitor stress levels in cultured fish.

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