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Flow immunosensor system with an electrode replacement unit for continuous cortisol monitoring for fish

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ABSTRACT

Blood cortisol concentration change in fish is widely used as an indicator of stress. In this study, we developed a flow system which equipped by an electrode replacement unit which can replace sensor conveniently. Multiple sensors sequentially disposed vertically in the holder, and the conductive portion of the sensor to be used was bonded with which set in the bottom of the holder. After measurement, next sensor was inserted into the holder and the replacement would be completed by sequentially moving. The flow system was constructed by replaceable working electrodes which sequence placed in the electrode replacement unit and count electrode into a reaction loop which operated by micro-pump. During the measurement, the potassium ferricyanide was transported into the flow loop and applied a voltage (+250 mV). Output current values were measured both before and after the immune-reaction and the change between them was calculated to determine the cortisol levels. As a result, replacement of the immunosensor performed in only several seconds and a good linear relationship was observed between the sensor response and cortisol in the range of 0–40 ng ml^{-1}. We also performed a measurement of cortisol levels in plasma of tilapia (*Oreochromis niloticus*) both by proposed biosensor system and ELISA which got a good correlation. We believe that the proposed system can provide a simple and rapid detection of cortisol levels for fish and its principle could be a foundation of a continuous in vivo monitoring of fish cortisol change in fish in the future.

1. Introduction

There are many studies on the effects of environmental factors on aquatic life. A general finding is that fish are acutely stressed by chemical changes in their living environment, such as pollution [1,2]. Besides, handing and man-made chemicals also cause unnecessary stress, which is induced by fish capture and transport, have negative effects on fish, including bad fish growth [3,4]. Especially, acute stress like handing disrupts reproductive function and may also cause morphological deformities [5]. To prevent or reduce these damaging effects, it is important to develop an analytical technique for measuring environmentally induced stress in fish. It is very difficult to determine the effect of each factor on fish ecology from the external change of fish because there are so many environmental factors that can induce stress in fish [6]. To address this problem, we have focused our efforts on understanding the effects of stress on fish health at an early stage from the inside of the fish from some important indicator of stress. Using this approach, we can determine the physiologic abnormalities in fish induced by stress early enough to prevent unnecessary mass death. Thus, the development of analytical techniques to test for indicators of stress in fish blood is expected to facilitate evaluation of the effects of stressors in the aquatic environment and to facilitate the construction of better fish culture environments, thereby improving fish welfare.

Among the stress indicators of fish, cortisol levels are most commonly measured and evaluated, especially in acute stress response of fish [7,8]. Cortisol, as a stress hormone, is a member of the glucocorticoid hormone family and is a very important metabolic regulator. When fish are under stress, cortisol is released from the internal gland, which is located in the head kidney, in response to adrenocorticotropic hormone. Cortisol activates the central nervous system and induces an increase in glucose levels to provide energy to combat the effects of the stressor [9]. Several studies have demonstrated that cortisol level fluctuations are related to stress following exposure to endocrine-disrupting compounds and heavy metals [10–12]. Increases in cortisol levels are also induced by other stress factors from changes in the water environment, such as acidification, hypoxia, and extreme temperature changes [13–16]. Thus, a rapid rise in the cortisol levels in fish indicates increased exposure to stressors. Moreover, because some studies indicate that excessive secretion of cortisol might cause immunosuppression in fish,

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simple and fast measurement of cortisol level changes in fish is important for comprehensive management of the aquatic environment.

Immunoassays based on the antigen–antibody-specific reactions are considered major analytical tools in clinical diagnoses, and in environmental and biochemical studies [17,18]. Various immunoassay protocols, such as surface plasmon resonance [19], quartz crystal microbalance [20], chemiluminescence [21], and electrochemical methods [22,23], have been extensively developed for detecting biomarkers like cortisol. Among these methods, electrochemistry with high sensitivity, low cost, low power requirements, and high compatibility is the preferred approach for clinical and environmental immunoassays. We developed some label-free immunosensor systems using cyclic voltammetry (CV) that allow for easy and rapid assay of various steroid hormones in fish [24,25], and we believe these systems will be as effective as the conventional detection methods used in fish farms while being simpler and more rapid to use. Though the sensors have a relatively high sensitivity, they require frequent electrode replacement and the measurement is performed by CV, which requires a large device and time-consuming cyclic scans. On the other hand, we also developed a biosensor for cortisol based on a flow injection analysis, which allows for rapid and convenient cortisol detection [26]. This system, however, requires a complex competitive immunologic reaction and immunomagnetic separation. Therefore, based on the knowledge gained from our previous studies, here we aimed to develop a cortisol measurement system in fish plasma based on flow analysis with easy electrode replacement via an electrode replacement unit and electrochemical measurement. We evaluated analytic parameters (pH and incubation temperature) and evaluated the sensitivity and specificity of this new sensor system. Finally, the proposed system was applied to the measurement of cortisol in fish plasma samples. The results were compared with those obtained using a conventional enzyme-linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Reagents

The cortisol enzyme immunoassay (EIA) standard, 17α,20β-dihydroxy-4-pregnen-3-one (DHP) EIA standard, progesterone EIA standard, estriol EIA standard, testosterone EIA standard, estradiol EIA standard, and cortisol EIA monoclonal antibody (anti-cortisol antibody) were purchased from Cayman Chemical (Ann Arbor, MI, USA). We purchased 3-mercaptopropionic acid, *N*-hydroxysuccinimide (NHS), and bovine serum albumin (Fraction V, ~99%) from Sigma-Aldrich (St. Louis, MO, USA), and *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC) and 2-morpholinoethanesulfonic acid monohydrate from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). All other reagents used for the experiments were of commercial and laboratory grade.

2.2. Preparation of anti-cortisol antibody-immobilized electrode and the electrode replacement unit

A 2-cm (φ0.1 mm) Au-wire purchased from Nilaco (Tokyo, Japan) was used as the basis of the electrode. The Au wire was polished using diamond paste with φ1.0-μm particles and alumina slurries with φ0.05-μm particles. Polished electrodes were further cleaned by ultrasonication in distilled water and ethanol absolute, 5 min in each solution. The Au wire was then cycled from 0 to 1500 mV (vs. Ag/AgCl) at a rate of 0.1 V/s in 0.5 M sulfuric acid with 50-cycle scans by CV until the CV curve became stable. The Au wire was then dried in flowing pure nitrogen gas and wrapped on a plastic electrode (4 × 15 mm) as the working electrode (Fig. 1A). One end of the Au wire was connected to a conductive port inside the electrode, and the other end adhered to the tip of the electrode. A cleat was used to maintain the position of the sensor in the electrode replacement unit (described below).

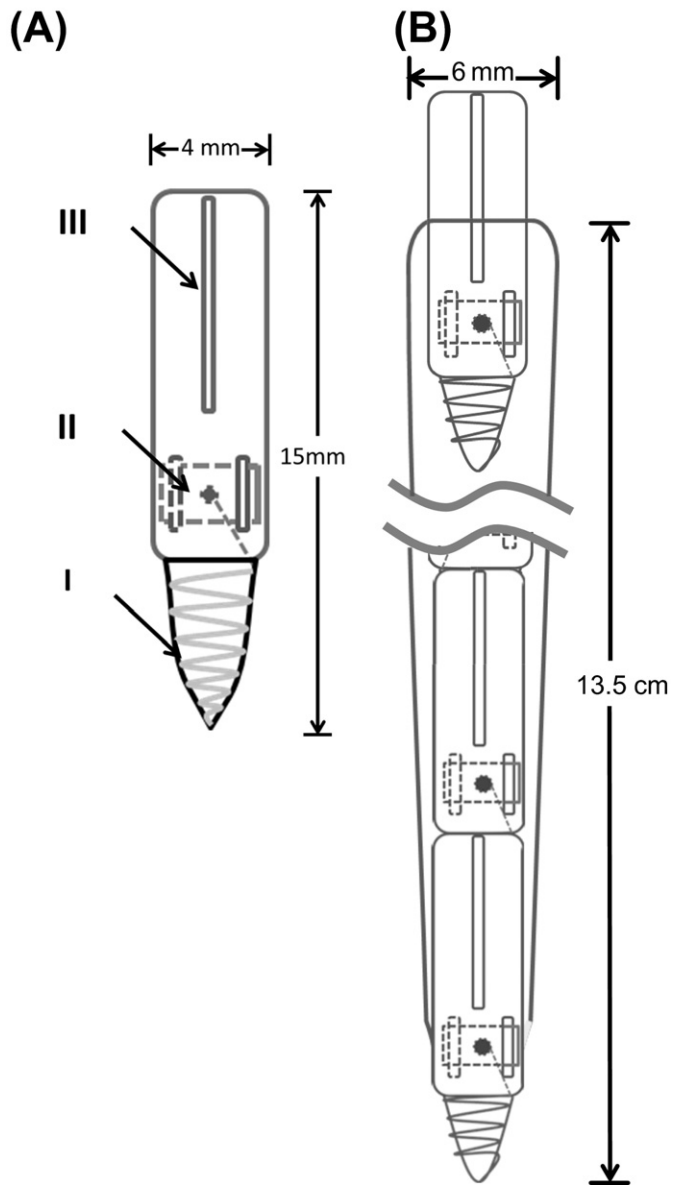

Fig. 1. Schematic view of the working electrode. (A) Au electrode. I: Au wire; II: conductive port; III: cleat; (B) electrode replacement unit. 12 electrodes can be arranged in the electrode replacement unit.

The thus-treated working electrode was immersed in the 3-mercaptopropionic acid solution in the dark at room temperature for 8 h. In this step, a self-assembled monolayer formed on the surface of the Au electrode. The electrode was then placed in EDC-NHS solution (EDC 100 mg ml^{-1}, NHS 100 mg ml^{-1}) at room temperature for 2 h to modify a carboxyl group to form an amine-reactive ester. The electrode was then rinsed with distilled water to remove non-specific, physically absorbed EDC and NHS, and dried under pure flowing nitrogen gas. Cortisol monoclonal antibody (0.5 mg ml^{-1}) in 0.1 M phosphate buffer (pH 7.4) was then placed on the modified surface of the working electrode overnight at 4 °C, allowing for the formation of the antibody/self-assembled monolayer/Au electrode. We then applied 1.0 mg ml^{-1} bovine serum albumin as a blocking agent to prevent non-specific binding. Finally, the proposed electrodes (12 electrodes) were sequentially arranged in a plastic holder whose top was wider than its bottom to make the replacement and fixation of electrodes easier and more effective (Fig. 1B).