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Development of a label-free immunosensor system for detecting plasma cortisol levels in fish

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Abstract Fishes display a wide variation in their physiological responses to stress, which is clearly evident in the plasma corticosteroid changes, chiefly cortisol levels in fish. In the present study, we describe a novel label-free immunosensor for detecting plasma cortisol levels. The method is based on immunologic reactions and amperometric measurement using cyclic voltammetry. For the immobilization of the antibody on the surface of sensing electrode, we used a self-assembled monolayer of thiol-containing compounds. Using this electrode, we detect the CV signal change caused by the generation of antigen–antibody complex. The immunosensor showed a response to cortisol levels, and the anodic peak value linearly decreased with a correlation coefficient of 0.990 in diluted plasma. The specificity of the label-free immunosensor system was investigated using other steroid hormones, such as 17α, 20β-dihydroxy-4-pregnen-3-one, progesterone, estriol, estradiol, and testosterone. The specific detection of cortisol was suggested by a minimal change from −0.32 to 0.51 μA in the anodic peak value of the other steroid hormones. The sensor system was used to determine the plasma cortisol levels in Nile tilapia (*Oreochromis niloticus*), and the results were compared with those of the same samples determined using the conventional method (ELISA). A good correlation was obtained between values determined using both methods (correlation coefficient 0.993). These findings suggest that the proposed label-free immunosensor could be useful for rapid and convenient analysis of cortisol levels in fish plasma samples.

Keywords Cortisol · Biosensor · Immunoassay · Fish · Stress · Hormone

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Introduction

The physiologic responses to stressors generally serve an important survival function, and the pattern of the stress response is highly conserved in vertebrates. Repeated and chronic exposure to stressors has detrimental effects on many aspects of an organism's physiology, including changes in nervous system function, metabolism, growth, and development, reproductive function, and immune system function.

In the last couple of decades, several reviews have described the influence of stress on fish (Barton and Iwama 1991; Gamperl et al. 1994; Wendelaar Bonga 1997; Iwama et al. 1998; Barton et al. 2002; Ogawa et al. 2011), revealing that stressed fish have poor

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growth and impaired health, although the mechanisms involved in these detrimental changes remain unclear. A major focus of current research related to aquaculture is the identification of stress markers in fish that accurately reflect the stress status of the fish and measure fish health. Cortisol level is the well-studied marker of the stress response. A number of reviews have described plasma profiles of cortisol in response to various stressors and the physiologic consequences of elevated cortisol levels in fish (Barton and Iwama 1991; Gamperl et al. 1994; Wendelaar Bonga 1997; Mommsen et al. 1999; Nikaido et al. 2010). While the validations of cortisol were so fast, this steroid hormone is also considered to be the indicator of acute stress in fish instead of chronic stress (Barton et al. 2005; Fast et al. 2008; Douxfils et al. 2014). Thus, it allows us to identify the cortisol changes in fish in the early stage. The ability to accurately measure cortisol levels might be an effective method of monitoring fish health, leading to the development of husbandry practices to reduce or alleviate stress in aquaculture operations, and improved quality and production.

Various techniques for detecting steroid hormone levels have been reported, such as gas chromatography, mass spectrometry (Chang et al. 2005), liquid chromatography (Inoue et al. 2002), and fluorescence-based methods (Tschmelak et al. 2004). Chromatographic and fluorescence-based methods have the advantage of being highly sensitive and specific, but the time-consuming pre-treatment steps and high cost of the instrument make it difficult to use for on-site and rapid measurements. Radioimmunoassay (Kagawa et al. 1983) and enzyme-linked immunosorbent assay (ELISA) (Kobayashi et al. 2002) are also used to determine steroid hormone levels. The disadvantage on time and expense associated with maintaining a licensed radiation safety and disposal program in radioimmunoassay, and high levels of variability and time-consuming complicated steps in ELISA make these methods impractical for daily detection. In the present study, we focused on the development of an immunoassay system for steroid hormones (Muramatsu et al. 2011; Endo et al. 2011, 2012). In contrast to other immunoassay methods that require longer preparation times and multiple reaction steps, the label-free immunosensor technique (Thavarungkul et al. 2007; Piao et al. 2008; Qiu et al. 2010; Radi et al. 2009) is based on highly specific molecular recognition and the ability to directly detect extremely small amount of immune complexes formed on a modified electrode as a change in electrochemical activity in a relatively short time. This monitoring method is thus very attractive because of its simple assay procedure and high sensitivity.

The development of an electrochemical label-free immunosensor with features such as a single-step process and compact device size would allow for real-time monitoring of steroid hormones at fish farms. Here, we developed a basic analytical system as the foundation for real-time in vivo measurement of steroid hormones. The proposed method is based on immune reactions and electrochemical measurements using cyclic voltammetry (CV). CV is a type of potentiodynamic electrochemical measurement method. In CV experiment, the working electrode potential is scanned linearly versus time. The current at the working electrode is plotted versus the applied voltage (i.e., the working electrode's potential) to give the cyclic diagram trace. According to the trace, we can evaluate the electrochemical properties of an analyte in solution or monitoring the minor change on the electrode surface.

For biomolecular immobilization on the surface of the sensing electrode, we used self-assembled monolayers (SAM) of 3-mercaptopropionic acid (3-MPA) that form easily and remain stable (Choi et al. 2005; Li et al. 2005).

We aimed to develop a novel immunosensor system for steroid hormones. In this study, we introduce a novel method of detecting the levels of cortisol, an important steroid hormone in fish plasma. The proposed system is based on immune reactions and electrochemical measurements using CV. For biomolecular immobilization on the surface of the sensing electrode, we used SAM. The specificity of the immunosensor was evaluated. The proposed immunosensor system was applied to the measurement of cortisol levels in fish plasma samples, and the results were compared with those obtained using conventional ELISA.

Materials and methods

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). 3-MPA, N-hydroxysuccinimide (NHS), and bovine serum albumin (BSA, Fraction V, ~99 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and 2-morpholinoethanesulfonic acid monohydrate were purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). All other reagents used for the experiments were of commercial and laboratory grade.

Apparatus and electrode

The CV measurement system was constructed from an electrochemical analyzer (Model 802B, BAS, Tokyo, Japan). All experiments were performed with a conventional three-electrode system using a modified φ3.0-mm Au electrode (BAS) as a working electrode, a platinum wire (φ1-mm, 5 cm) as a counter electrode, and Ag/AgCl electrode saturated with 0.5 M KCl (BAS) as a reference electrode. For improved stability, the measurements were taken in a cell stand (CS-3, BAS).

Fabrication of the label-free immunosensor

The surface of the Au electrode (geometric surface area = 7.1 mm^2) was polished using diamond paste with φ1.0-μm particles and alumina slurries with φ0.05-μm particles. Polished electrodes were further cleaned by ultra-sonication in distilled water and ethanol absolute, 5 min in each solution. Cycling the electrode potential in a dilute sulfuric acid solution until a stable CV scan is achieved is a very common electrochemical cleaning technique (Fischer et al. 2009). The working electrode was cycled from 0 to 1500 mV (vs. Ag/AgCl) at a rate of 0.1 V s^{-1} in 0.5 M sulfuric acid for 50 cycled scans until the CV became stable. The electrode was then dried in flowing pure nitrogen gas.

The modification steps of the label-free immunosensor are shown in Fig. 1A–D. The thus-treated working electrode was immersed in 3-MPA solution in the dark at room temperature for 8 h. In this step, a SAM formed on the surface of the Au electrode (SAM/Au electrode; Fig. 1A). 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 (Fig. 1B). 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/SAM/Au electrode (Fig. 1C). Next, 1.0 mg ml^{-1} BSA was applied as a blocking agent to prevent non-specific binding (Fig. 1D).

After modification, the working electrode was immersed in 15 ml of 5 mM $K_3[Fe(CN)_6]$ solution containing 0.1 M KCl with a reference electrode and a counter electrode. The electric potential was swept from −0.2 to +0.6 V for 30 scans to determine the initial value of the anodic peak.

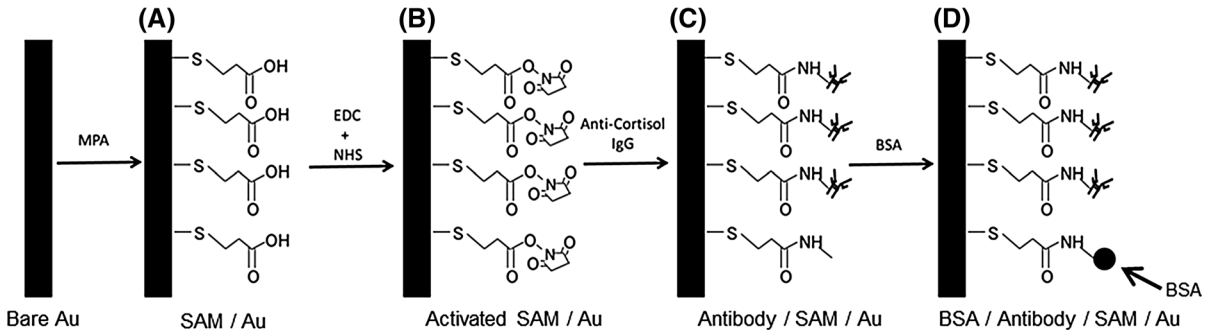

Fig. 1 Schematic diagram of surface modification of the proposed immunosensor. **A** Formation of the SAM, **B** activation of SAM, **C** antibody immobilization, **D** blocking with BSA

Specificity of the proposed immunosensor

The specificity of the label-free immunosensor system was investigated using steroid hormones that have structures similar to that cortisol, such as DHP, progesterone, estriol, estradiol, and testosterone. The immunosensor was incubated in 0.5 ml of each hormone solution with concentrations ranging from 6.6 to 422.3 pg ml^{-1} for 10 min. CV was then performed with the same parameters and solution for 30 cycles.

Calibration curve of cortisol concentration in plasma sample by using proposed system

A 1000 fold diluted actual plasma sample in which the concentration of cortisol was 10.3 pg ml^{-1} detected by conventional ELISA method was used in the measurements. We added cortisol standard solution into the diluted plasma sample to make the final cortisol concentrations of 10.3, 20.6, 41.2, 82.4, 164.8, 329.6, and 422.3 pg ml^{-1}. The electrochemical characteristics of the immunosensors were measured by CV. The electrode surface was dried and immersed in 0.5 ml of various concentrations of cortisol sample with the optimized analytical parameters. After immunoreaction, the working electrode was immersed in the same $K_3[Fe(CN)_6]$ solution and the change in the anodic peak current was evaluated.

Measurement of plasma cortisol concentration in fish

The proposed biosensor system was applied to measure cortisol levels in Nile tilapia (*Oreochromis niloticus*) plasma. The fish were cultured at the Tokyo University of Marine Science and Technology in a 500-L oxygenated tank with a controlled temperature of 26 °C and maintained under a natural photoperiod. Some fish were artificially altered by inducing physical stress. They were caught and exposed to air ($n = 4$) for 10 min or chasing them from behind by using a net (30 cm × 20 cm) for 2 min ($n = 8$). And another 5 fish were selected as control group without inducing artificial physical stress. After these protocols, all fish were netted from the preserve and anesthetized with 400 ppm 2-phenoxy ethanol by bath exposure. After the anesthetization, plasma samples were collected after stress induction and immediately centrifuged ($450 \times g$, 10 min) to separate the plasma. Plasma samples (100 µl) were transferred to clean test tubes and diluted with 900 µl phosphate-buffered saline (PBS, pH 6.5). The diluted plasma sample was further diluted 1/50 and frozen at −28 °C until use. Before the measurement, the samples were further doubling diluted. The cortisol contained in diluted plasma samples was reacted with the antibody on the surface of the sensor, and then the senor was immersed in a 15 ml of 5 mM $K_3[Fe(CN)_6]$ solution with the parameters described above to perform the measurements.

Analysis using ELISA as a conventional method

Cortisol levels of the same samples were also determined using the conventional ELISA method by using the kit purchased from Cayman Chemical (Ann Arbor, MI, USA). We used a 96-well strip plate pre-coated with goat anti-mouse IgG and prepared blank (Blk), non-specific binding (NSB), maximum binding (B_0), and sample wells. The absorbance of each well at 405 nm was measured using a Multiskan JX (Thermo Scientific, MA). Cortisol levels of blood plasma were calculated based on the calibration curve of absorbance, derived by the following equation.

$$\text{Abs.}\% = \frac{\text{ave. Sample} - \text{ave. Blk} - \text{ave. NSB}}{\text{ave. } B_0 - \text{ave. NSB}}$$

where ave.Sample: mean sample absorbance; ave.Blk: mean blank absorbance; ave.NSB: mean non-specific absorbance; and ave.B_0: mean maximum binding absorbance.

Results

Electrochemical characteristics of the modified electrode

The cyclic voltammograms of differently modified electrodes in 5 mM $K_3[Fe(CN)_6]$ with 0.1 M KCl are shown in Fig. 2A. The redox behavior of a bare Au electrode was observed with an anodic peak at 250 mV (Fig. 2A, curve a). The anodic peak current of the SAM/Au electrode (Fig. 2A, curve b) was smaller than that of the CV of the bare Au electrode. Moreover, in the CV of both the antibody/SAM/Au electrode and the BSA/antibody/SAM/Au electrode, the anodic peak currents continued to decrease

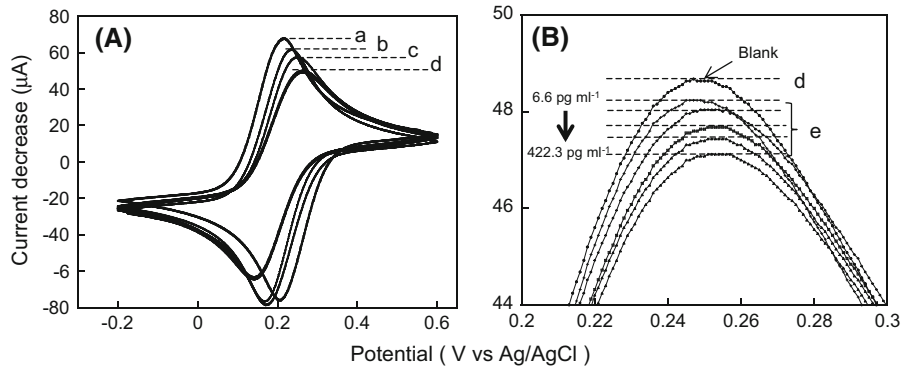

Fig. 2 Electrochemical behavior of the modified electrode. **A** Cyclic voltammograms of differently modified electrodes in 5 mM $K_3[Fe(CN)_6]$ with 0.1 M KCl at a scan rate of 100 mV s^{-1}. All potentials are given versus an Ag/AgCl reference electrode. The voltage ranged from −0.2 to 0.6 V. (*a*) Bare Au electrode, (*b*) SAM/Au electrode, (*c*) antibody/SAM/Au electrode, (*d*) BSA/antibody/SAM/Au electrode. **B** Typical response CV of the proposed label-free immunosensor. (*d*) Sensor output of BSA/antibody/SAM/Au electrode incubated in blank solution. (*e*) Sensor output of BSA/antibody/SAM/Au electrode incubated in different levels of cortisol standard solution

(Fig. 2A, curves c, d) to a final level of ∼31 % that of the bare electrode. A typical response of the proposed label-free immunosensor is shown in Fig. 2B. The CV anodic peak current of the proposed immunosensor (BSA/antibody/SAM/Au electrode) incubated in different concentrations of cortisol standard solution decreased compared to when it was incubated in blank solution (Fig. 2B, e → d).

Effects of analytical conditions on the output current

The effects of temperature, pH, and the immunologic incubation time on the CV anodic peak current are shown in Fig. 3. The decrease in the current gradually increased with an increase in the temperature or pH, reached a maximum, and then decreased. The maximum anodic peak current decrease was obtained at 30 °C and pH 6.5 (Fig. 3A, B). The decrease in current value gradually increased with an increase in the incubation time and then reached a plateau. The decrease in oxidation current value was essentially constant when the incubation time was greater than 10 min (Fig. 3C).

Specificity of the proposed sensor system

We examined the specificity of the proposed sensor system by measuring standard solutions of cortisol, progesterone, estriol, DHP, estradiol, and testosterone. The label-free immunosensor was incubated in each hormone solution, ranging from 6.6 to 422.3 pg ml^{-1}, for 10 min. Based on the relationship between the decrease in anodic peak current and cortisol concentration, the anodic peak current decrease value did not remarkably change (−0.32 to 0.51 μA) for any hormone examined other than cortisol (Fig. 4).

Calibration curve of cortisol concentration in plasma sample by using proposed system

A calibration curve of the label-free immunosensor is shown in Fig. 5. A good linear relationship between the anodic peak current decrease and the logarithm of cortisol concentrations in actual plasma sample was obtained in the range from 10.3 to 422.3 pg ml^{-1} ($y = -1.9313 + 2.6114 \log(x)$; $R = 0.990$).

Application of the proposed immunosensor system to actual fish plasma samples

To evaluate the potential application of the label-free immunosensor system for actual plasma samples, the proposed system was applied to determine the cortisol levels in plasma of Nile tilapia and the results were compared with the cortisol levels of the same samples determined by a conventional ELISA method. The plasma samples were diluted to 1/1000 for the label-free immunosensor system. The relationship between the data obtained with the proposed sensor system and data obtained with the ELISA method is shown in Fig. 6. There was a good correlation between values determined using the proposed sensor system and the

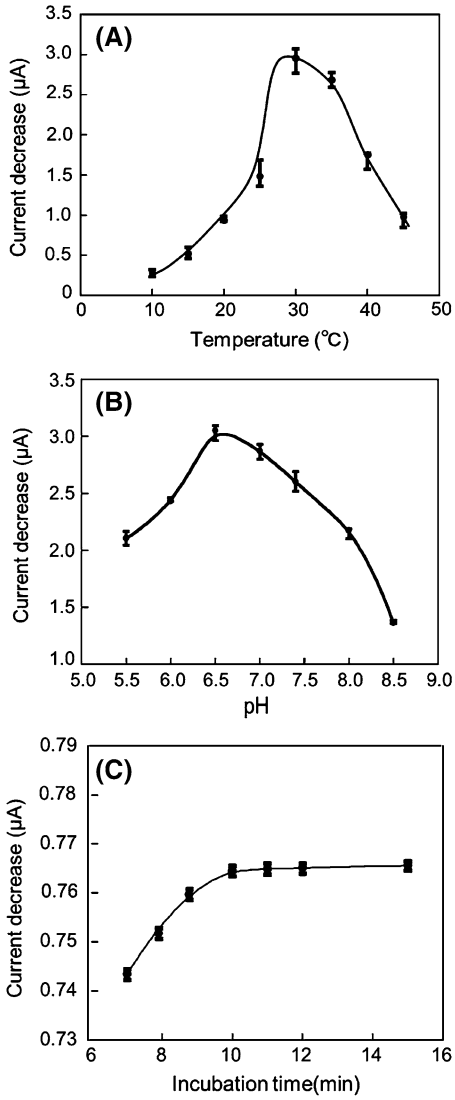

Fig. 3 Effects of assay conditions on the CV anodic peak current decrease. **A** Temperature (cortisol concentration 100 pg ml^{-1}), **B** pH (cortisol concentration 100 pg ml^{-1}), and **C** incubation time (cortisol concentration 6.6 pg ml^{-1})

ELISA in the range of 10.30–233.18 ng ml^{-1} (correlation coefficient 0.993).

Discussion

Electrochemical characteristics of the modified electrode

In the present study, the decrease in the anodic peak current of the modified electrode after incubation in

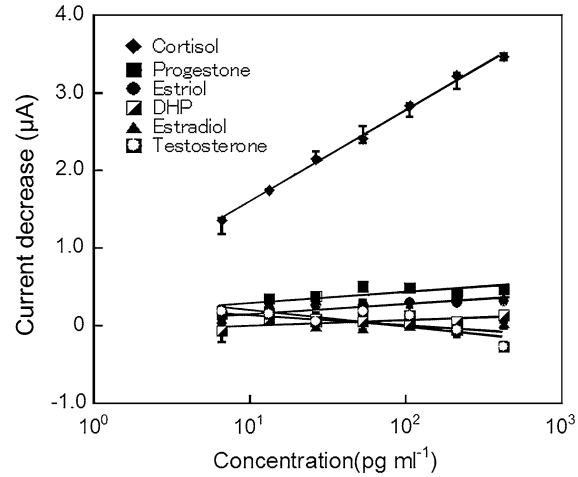

Fig. 4 Relationship between the anodic peak current decrease and each steroid hormone. The proposed sensor applied to cortisol, progesterone, estriol, DHP, estradiol, and testosterone. CV was performed in 5 mM $K_3[Fe(CN)_6]$ with 0.1 M KCl at a scan rate of 100 mV s^{-1}. The voltage ranged from −0.2 to +0.6 V. The assay conditions were as follows: temperature 30 °C, pH 6.5, and incubation time 10 min. The decrease in anodic peak current value was based on each CV result. Each sample was measured three times. Data points are the mean value, and the *error bars* indicate the maximum and minimum values

sample solution was used as the analytical response signal of the sensor system.

CV was often used to characterize the modifying process because CV provides useful information regarding the change in the electrode behavior after each modified step. In the present study, to preserve the decrease in the CV anodic peak current caused by the formation of each layer, 3-MPA with a short-chain alkanethiol was used to create the SAM on the Au electrode surface because of its orderly arrangement. Long-chain alkanethiol has a high electrical insulation and thus will have a strong influence on the measurement (the anodic peak disappears). We compared the cyclic voltammograms at different steps of the electrode modification. Compared with the CV of a bare Au electrode, the anodic peak current of the SAM/Au electrode was greatly decreased, indicating that a SAM of 3-MPA was formed on the surface of the Au and interfered with the electron transfer between the Au surface and $[Fe(CN)_6]^{3-/4-}$. Moreover, a further decrease in the anodic peak current was observed in the CV of the antibody/SAM/Au electrode, because the thickness

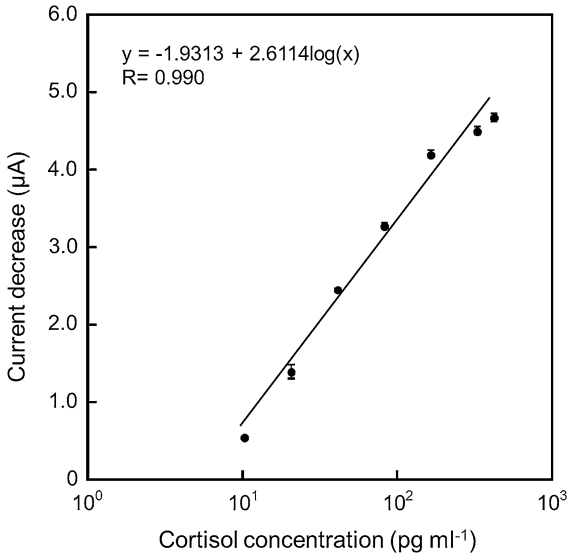

Fig. 5 Calibration curve of the proposed label-free immunosensor. Calibration curve of the proposed label-free immunosensor for diluted plasma sample. The voltage ranged from −0.2 to +0.6 V. Assay conditions were as follows: temperature 30 °C, pH 6.5, and incubation time 10 min. Each sample was measured three times. Data points are the mean value, and the *error bars* indicate the maximum and minimum values

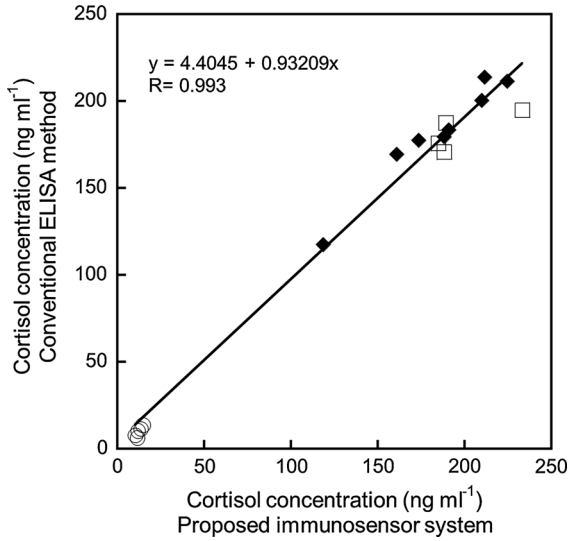

Fig. 6 Correlation between data obtained with the proposed label-free immunosensor system and with the ELISA method ($n = 17$). *Circle* control group; *square* stressed group (air exposure); *filled diamond* stressed group (net chasing)

of the insulating organic layer on the electrode surface was increased by the immobilization of anti-cortisol antibody. When BSA, which was used as a blocking solution, was immobilized on the antibody/SAM/Au electrode, the anodic peak current further decreased because it impedes electron transfer. These changes in anodic current support the notion that the modified layers shown in Fig. 1 had formed on the Au electrode.

Effects of analytical conditions on the output current

The anodic peak current of the CV signals produced by the antibody–antigen interactions was clearly influenced by the analytical conditions. Mason and Williams (1980) reported that the rate of dissociation is influenced by a variety of antibody and reaction conditions. We investigated the effects of temperature, pH, and incubation time. The amount of decrease in the anodic current increased from 10 to 30 °C (Fig. 3A) and suggested that antigen–antibody complexes were formed efficiently. On the other hand, the amount of decrease in current decreased when the temperature was over 30 °C. It is presumed that the antibodies were degraded at the higher temperature or that the antibody's affinity to the antigen decreased. We therefore determined the optimum temperature for the antigen–antibody reaction to be 30 °C. Next, we examined the effect of pH on the reaction. The amount of decrease in the anodic peak current increased between pH 5.5 to 6.5 and then decreased gradually when the pH was over 6.5 (Fig. 3B). We presumed that pH also has a great effect on the reaction, and we determined the optimum reaction pH to be 6.5. In studies of the effect of the incubation time, we used a standard solution of cortisol with a concentration of 6.6 pg ml^{-1}. The decrease in current stabilized after 10 min (Fig. 3C). Therefore, we determined that the optimum incubation time for the antigen solution was 10 min.

Specificity of the proposed sensor system

Fish plasma also contains other types of steroid hormones that have a structure similar to that of cortisol. Therefore, we investigated the specificity of the proposed sensor system for other steroid hormones. Compared with the results shown in Fig. 4, hormones other than cortisol did not induce a large change in the decrease in anodic peak current. These results indicate that the proposed label-free immunosensor system is sufficiently specific for cortisol measurement.

Calibration curve of cortisol concentration in plasma sample by using proposed system

The relationship between the CV anodic peak current decrease and the concentration of the cortisol in diluted actual plasma sample was investigated under the optimal parameters described above. The proposed sensor system showed a response to cortisol levels, and the anodic peak value linearly decreased with cortisol levels of 10.3–422.3 pg ml^{-1} contained in diluted plasma. The standard deviation (SD) and relative standard deviation (RSD) for 5 repetitive measurements for each standard sample were $\leq$0.031 and $\leq$0.431, respectively, validating good repeatability. The SD and RSD for cortisol measurement for each standard sample by using different proposed sensors were $\leq$0.365 and $\leq$0.673, validating good reproducibility. The range of the cortisol level in fish plasma is approximately dozens or hundreds ng ml^{-1} (Farrell 2011). Therefore, considering the calibration range of the proposed sensor system, the plasma sample would require a 1/1000 dilution.

Application of the proposed immunosensor system to actual fish plasma samples

We investigated the usefulness of the proposed sensor system for actual samples by measuring cortisol levels in fish 1000-fold diluted plasma. In the same sample, cortisol levels measured using the proposed sensor system were compared with those measured by the conventional ELISA method. The cortisol levels determined using the proposed sensor system were similar to those obtained using ELISA. And we compared our results with some studies' related reference (Delaney et al. 2005; Barreto and Volpato 2006). We found that our proposed biosensor system can cover the range shown in these studies. Thus, the proposed sensor system could be satisfactorily applied to monitor the changes in cortisol levels in actual fish plasma samples. Results obtained by this method may provide new knowledge in the field of fish stress, aquaculture, and fish physiology. The electrochemical principle of the proposed sensor can be a foundation of a portable device which can be used in field work. And it will be useful for rapid, reliable, and convenient analysis of fish health in a more complex living environment by combined with other indicators of fish health, such as blood glucose. On the basis of this knowledge, improvements to the aquatic environment and farming technology can be implemented to enhance fish-friendly fisheries.

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