Carbon-Nanotube-Enhanced Label-Free Immunosensor for Highly Sensitive Detection of Plasma Cortisol Level in Fish

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Fish display a wide variation in their physiological responses to stress, which is clearly evident in plasma corticosteroid changes, chiefly the cortisol level, in fish. Cortisol is a well-known indicator of fish stress; thus, a simple and speedy approach to detecting plasma cortisol should be developed. Here we described a novel disposable carbon-nanotube-enhanced label-free immunosensor for detecting plasma cortisol level. The principle of the sensor system is based on differences in the electrochemical change in oxidation peak current induced by an immunoreaction that depends on the cortisol level of the sample. We used a single-walled carbon nanotube to improve the sensitivity and conductivity of the system. The proposed sensor system showed a linear correlation \((R = 0.992)\) between cortisol level and oxidation peak current in the range from 156 to 10000 pg ml\(^{-1}\). The specificity of the label-free immunosensor system was investigated using other steroid hormones; the specificity of cortisol was suggested by a minimal change in the oxidation peak current of the other steroid hormones. The sensor system was used to determine the plasma cortisol level of Nile tilapia \((Oreochromis niloticus)\) and the results were compared with those of the same samples determined using a conventional method (ELISA). A very good correlation was obtained between results determined using both methods (correlation coefficient, 0.999).

1. Introduction

Over the past few decades, extensive studies have focused on the metabolic and physiologic effects of stress in fish, and several recent reviews have summarized the findings.\(^{(1-4)}\) Cortisol level is the most common indicator of stress in fish.\(^{(5-7)}\)

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majority of the above studies tend to correlate the physiologic changes associated during stress with the circulating level of cortisol. Cortisol is a multifaceted steroid hormone, especially physiologically and metabolically. Plasma cortisol level can be accurately measured using commercially available radioimmunoassay (8) or enzyme-linked immunosorbent assay (ELISA) kits. (9) Radioimmunoassay, however, requires the use of radioactive compounds, and ELISA produces highly variable results and is time-consuming. Thus, these methods are impractical for the rapid and safe detection of cortisol. In contrast to other immunoassay methods, the label-free immunosensor technique is based on highly specific molecular recognition and the ability to directly detect antigen-antibody complexes formed on a modified electrode as a change in electrochemical activity. This monitoring method is very attractive because it is simple and does not require a complicated labeling process. (10,11) The label-free immunosensor detects the target on the basis of a change (amplitude, conductive, piezoelectricity, etc.) due to the binding of the target compound to the immobilized antibody on the sensor bioreceptor region. (12–14) Thus, a method that can ensure orientation of immobilized antibody and sufficient high density on the surface of the device is desired. In the present study, we developed a label-free immunosensor using self-assembled monolayers (SAMs) to immobilize the antibody. As previously reported, SAMs maintain a good orientation of high-density monolayers formed by the spontaneous chemical adsorption of molecules. Tlili et al. (15) reported the electrical characterization of a thiol SAM on gold and demonstrated very stable and efficient effects.

Moreover, to accommodate the range of cortisol levels observed in fish, the dynamic range of the sensor must be amplified. Accordingly, we focused on carbon nanotubes (CNTs) with a high electrical conductivity. CNTs have attracted attention as high-capacity nanomaterials with various physical characteristics that are now applied to the development of highly sensitive immunosensors. (16–19) Moreover, CNTs have excellent chemical and mechanical stability. (20) CNTs are divided into two classes on the basis of the number of graphene sheets comprising the cylindrical structures. In this study, we used single-walled carbon nanotubes (SWCNTs), which have various stereostructures and changeable electronic properties (e.g., metals or semiconductors). (21)

We aimed to develop an immunosensor system that is highly sensitive and has a wide dynamic range for detecting cortisol. The proposed method is based on immunologic reactions and electrochemical changes caused by generating an antigen-antibody complex. We measured electrical current, which changed according to the progression of immuno-reactions on a Au electrode (working electrode) using cyclic voltammetry (CV). SWCNTs were selected to amplify the dynamic range of the sensor. Because electron transfer between the electrode surface and the redox-active substance was inhibited by the antigen-antibody complex of cortisol and the antibody, oxidation peak current decreased as cortisol level increased. The immunoreaction parameters and specificity of the immunosensor were evaluated. The proposed immunosensor system was used to measure the cortisol level of fish plasma samples and the results were compared with those obtained using conventional 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). 3-Mercaptopropionic acid, N-hydroxysuccinimide (NHS), and bovine serum albumin (BSA, Fraction V, ~99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). (3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) and 2-morpholinoethanesulfonic acid monohydrate were purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). 5% Nafion® dispersion solution (DE521 CS type) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). SWCNTs were provided by Mr. Junzo Yana (Institute of Carbon Science and Technology, Shinshu University). All the other reagents used for the experiments were of commercial or laboratory grade.

2.2 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 the working electrode, a platinum wire (ϕ1 mm, 5 cm) as the counter electrode, and a Ag/AgCl electrode saturated with 0.5 M KCl (BAS) as the reference electrode. For improved stability, the measurements were performed in a cell stand (CS-3, BAS).

2.3 Fabrication of the carbon nanotube enhanced label-free immunosensor

The surface of the Au electrode (geometric surface area = 7.1 mm²) was polished using diamond paste with ϕ1.0 μm particles and alumina slurries with ϕ0.05 μm particles. Polished electrodes were further cleaned by successive ultra-sonication in distilled water and absolute ethanol for 5 min each. Cycling the electrode potential in a dilute sulfuric acid solution until a stable CV curve is achieved is a very common electrochemical cleaning technique. The working electrode was cycled from 0 to 1500 mV (vs Ag/AgCl) at a rate of 0.1 V/s in 0.5 M sulfuric acid for 50 cycles until the CV curve became stable. The electrode was then dried in flowing pure nitrogen gas.

The modification steps of the label-free immunosensor are shown in Fig. 1. The treated working electrode was immersed in 3-mercaptopropionic acid 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. 1(a)]. 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 convert carboxyl groups into amine-reactive esters [Fig. 1(b)]. The electrode was rinsed with distilled water to remove non-specific, physically absorbed EDC and NHS, and dried under pure flowing nitrogen gas. A 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 immunoglobulin G (IgG)/SAM/Au electrode [Fig. 1(c)]. We then applied 1.0 mg ml⁻¹ BSA as a blocking agent to prevent nonspecific binding [Fig. 1(d)]. Finally, a solution containing 25 mg ml⁻¹ SWCNT dispersed in 5% Nafion® was applied to the modified surface of the electrode and dried under pure flowing nitrogen gas [Fig. 1(e)].

After modification, the working electrode was immersed in 15 ml of 5 mM K₃[Fe(CN)₆] solution containing 0.1 M KCl with the reference electrode and counter electrode. The electric potential was cycled 15 times from −0.2 to +0.6 V to determine the initial oxidation peak current.

2.4 Evaluation of optimal immunoreaction parameters

In order to find optimal immunoreaction parameters, sensors were immersed in standard solutions at different pH or temperature. The reaction times of the immunoreaction were also evaluated by varying the incubation time. After the immunoreaction, the working electrode was washed with distilled water and dried with nitrogen gas (abbreviated as washing procedure), then immersed in the same K₃[Fe(CN)₆] solution. The CV optimal immunoreaction parameters were evaluated by the rate of change of the oxidation peak current.
2.5 Calibration curve of cortisol concentration in plasma sample using proposed system

Electrodes were immersed separately in 0.5 ml of cortisol standard solution at various concentrations (156 to 10000 pg ml\(^{-1}\)) using optimized immunoreaction parameters. After the procedure, electrodes were washed and dried, then immersed in K\(_3\)[Fe(CN)\(_6\)] solution to make measurements for creating a calibration curve for cortisol concentration.

2.6 Specificity of the proposed immunosensor

The specificity of the label-free immunosensor system was investigated using steroid hormones with structures similar to that of cortisol, such as DHP, progesterone, estriol, estradiol, and testosterone. The immunosensor was incubated in 0.5 ml of each hormone solution at concentrations ranging from 156 to 10000 pg ml\(^{-1}\) for 10 min. CV was then performed with the same parameters and solution as cortisol for 15 cycles after the washing procedure.

2.7 Measurement of plasma cortisol concentration in fish

The proposed biosensor system was used to measure cortisol levels in Nile tilapia (Oreochromis niloticus) plasma. The fish were cultured at Tokyo University of Marine Science and Technology in a 300 L oxygenated tank with a controlled temperature of 26 °C and maintained under a natural photoperiod. The cortisol levels of some fish were manipulated by inducing physical stress. Target fish were caught and exposed to air for 10 min. Plasma samples were then collected and stored below −28 °C until use. The blood samples were immediately centrifuged (450×g, 10 min) to separate the plasma. The plasma (100 μl) was transferred into a clean test tube and diluted with 900 μl of phosphate-buffered saline (pH 7.0). The diluted plasma sample was further diluted to 1/10 and frozen at −20 °C until use. The measurement of the diluted plasma samples was performed in 15 ml of 5 mM K\(_3\)[Fe(CN)\(_6\)] solution with the parameters described previously.

2.8 Analysis using conventional ELISA

The cortisol levels of the same samples were also determined using a conventional ELISA method. We used an ELISA kit purchased from Cayman Chemical (Ann Arbor, MI, USA) including a 96-well strip plate pre-coated with goat anti-mouse IgG and prepared blank (Blk), nonspecific 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). Blood plasma cortisol level was calculated using the calibration curve of absorbance, derived using the following equation:

\[
\text{Abs.} \% = \frac{\text{ave.} \text{Sample} - \text{ave.} \text{Blk} - \text{ave.} \text{NSB}}{\text{ave.} \text{B}_0 - \text{ave.} \text{NSB}},
\]

where \(\text{ave.} \text{Sample}\) is the mean sample absorbance, \(\text{ave.} \text{Blk}\) is the mean blank absorbance, \(\text{ave.} \text{NSB}\) is the mean nonspecific absorbance, and \(\text{ave.} \text{B}_0\) is the mean maximum binding absorbance.
3. Results

3.1 Electrochemical characteristics of the modified electrode

The cyclic voltammograms at each stage of the modified electrode in 5 mM K₃[Fe(CN)₆] with 0.1 M KCl are shown in Fig. 2. The redox behavior of a bare Au electrode was observed using oxidation peaks [Fig. 2(a)]. The oxidation peak current of the Au electrode modified with SAM, IgG, and BSA [Fig. 2(b)] decreased compared with that of the bare electrode [Fig. 2(a)]. Moreover, the oxidation peak current increased markedly after SWCNTs were applied to the surface of the electrode [Fig. 2(c)]. After immunoaction, the oxidation peak current decreased according to the concentration of cortisol in the sample [Fig. 2(d)].

3.2 Evaluation of optimal immunoreaction parameters

The effects of temperature, pH, and immunologic incubation time on CV oxidation peak current are shown in Fig. 3. The amount of decrease in the current gradually increased with increasing temperature or pH, reached maximum, and then decreased. The maximum oxidation peak current decrease was obtained at 30 °C and pH 7.0 [Figs. 3(a) and 3(b)]. The amount of decrease in current gradually increased with an increase in incubation time and then saturated. The decrease in oxidation current was essentially constant at incubation times longer than 10 min [Fig. 3(c)].

Fig. 2. Electrochemical behavior on the modified electrode. Cyclic voltammograms at each stage of the modified electrode were recorded in 5 mM K₃[Fe(CN)₆] with 0.1 M KCl at a scan rate 100 mV s⁻¹. All potentials are given versus the Ag/AgCl reference electrode. The voltage ranged from −0.2 to 0.6 V. (a) Bare Au electrode, (b) BSA/antibody/SAM/Au, (c) SWCNT/BSA/antibody/SAM/Au, and (d) antigen (cortisol)/SWCNT/BSA/antibody/SAM/Au.
3.3 **Cortisol calibration curve of the proposed system**

A calibration curve of the label-free immunosensor using a cortisol standard solution is shown in Fig. 4. A linear relationship between the amount of current decrease of the oxidation peak and the logarithm of cortisol level was obtained in the range from 156 to 10000 pg ml\(^{-1}\) \[ y = 41.669 + 9.0167\log(x), \quad R = 0.992 \].

3.4 **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 in concentration from 156 to 10000 pg ml\(^{-1}\), for 10 min. From the relationship between the decrease in oxidation peak current and cortisol concentration shown in Fig. 4, the amount of oxidation peak current decrease did not markedly change for any hormone examined other than cortisol (Fig. 5).

3.5 **Application of the proposed immunosensor system to actual fish plasma samples**

To evaluate the potential application of the label-free immunosensor system to actual plasma samples, the proposed system was used to determine the cortisol level of Nile tilapia plasma and to compare it with the cortisol levels of the same samples determined by conventional ELISA. The plasma samples were diluted to 1/100 for the label-free immunosensor system. The relationship between the data obtained with the proposed sensor system and data obtained using ELISA is shown in Fig. 6. The correlation between the results determined using the proposed sensor system and ELISA in the range of 25–169 ng ml\(^{-1}\) was very high (correlation coefficient, 0.999).
In the present study, the decrease in the oxidation peak current of a modified electrode after incubation in sample solution was used as the analytical response signal of the sensor system. CV is often used to characterize a modified process because CV provides useful information regarding the change in electrode behavior after a

Fig. 4 (left). Calibration curve for the proposed label-free immunosensor for cortisol standard solutions. The voltage ranged from −0.2 to +0.6 V. Assay conditions were as follows: temperature 30 °C, pH 7, and incubation time 10 min. Each sample was measured three times. Error bars indicate the maximum and minimum values.

Fig. 5 (right). Relationship between the anodic peak current decrease and each steroid hormone. The proposed sensor was used to measure cortisol, DHP, progesterone, estradiol, estriol, and testosterone levels. 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 7, and incubation time 10 min. The decrease in oxidation peak current was determined by each CV result.

Fig. 6. Correlation between data obtained with the proposed label-free immunosensor system and the ELISA method (\( n = 8 \)). Results were calculated from data according the dilution factor.

4. Discussion

In the present study, the decrease in the oxidation peak current of a modified electrode after incubation in sample solution was used as the analytical response signal of the sensor system. CV is often used to characterize a modified process because CV provides useful information regarding the change in electrode behavior after a
modification step. To confirm the molecular layer construction, we compared the CV results after each modification of the electrode surface. The oxidation peak currents of the electrodes modified with SAM, IgG, and BSA decreased sharply compared with that of the bare electrode. We presumed that the electron transfer between the electrode surface and the redox-active substance was inhibited because conductivity was affected by 3-mercaptopropionic acid, IgG, BSA and antigen, which are electrically inactive. Accordingly, the findings indicate that each molecular layer was formed on the surface of the electrode. On the other hand, the oxidation peak current increased markedly after the immobilization of the SWCNTs, demonstrating that the electron exchange on the electrode surface was amplified by the deposition of the SWCNTs due to its high conductivity. This procedure allows sensor dynamic range (oxidation peak current variable range) to be expanded and the sensor signal amplified, thus making the sensor more sensitive. Moreover, in the present study, the oxidation peak current before the immune-reaction was defined as the blank, and the deviation between the oxidation current and the blank was used as the response of the sensor.

The proposed sensor system is based on the principle that the antigen is detected using CV by measuring the inhibition of electron transfer caused by the formation of the antigen-antibody complex. The oxidation peak current of the CV signals produced by antibody-antigen interactions was clearly influenced by a variety of antibody and reaction conditions. We investigated the effects of temperature, pH, and incubation time. The amplitude of the decrease in the oxidation current increased from 10 to 30 °C [Fig. 3(a)], suggesting that antigen-antibody complexes were formed efficiently. On the other hand, the amplitude of the decrease in the current decreased when the temperature was over 30 °C. It is presumed that the antibodies were denatured at a higher temperature or that the antibody’s affinity to the antigen decreased. We therefore determined the optimum temperature for the immunoreaction to be 30 °C. We then examined the effect of pH on the reaction. The amplitude of the decrease in the oxidation peak current increased between pH 5.5 to 7.0 and then decreased gradually when the pH was over 7 [Fig. 3(b)]. It is presumed that the change in pH influences the binding speed and changes the affinity of the antibody for the antigen. This indicates that pH also has a marked effect on the reaction, and we determined the optimum reaction pH to be 7. In studies of the effect of incubation time, we used a standard cortisol solution with a concentration of 156 pg ml\(^{-1}\). The decrease in current stabilized after 10 min [Fig. 3(c)]. Therefore, we determined that the optimum incubation time for the antigen solution was 10 min.

On the basis of these findings, we determined the optimum conditions to be 30 °C, pH 7, and 10 min for incubation. The relationship between the CV oxidation peak current decrease and the concentration of the cortisol standard solution was investigated under these optimal parameters. The calibration curve for cortisol using the proposed sensor system was linear from 156 to 10000 pg ml\(^{-1}\). Moreover, because procedures such as plate washing and the addition of reagents required in the conventional method are not necessary in our proposed method, the assay could be completed within 15 min.

Fish plasma also contains other types of steroid hormones with similar structures to cortisol, such as estriol, an estrogen secreted from granulosa cells of ovarian follicles involved in the specialization or development of the genital gland. Another hormone,
DHP, is closely related to fish ovulation.\(^{(26,27)}\) Therefore, we investigated the specificity of the proposed sensor system for other steroid hormones. Hormones other than cortisol did not induce a large decrease in anodic peak current. These results indicate that the proposed label-free immunosensor system was sufficiently specific for cortisol measurement.

We demonstrated that the proposed sensor can be used to monitor the cortisol level of Nile tilapia samples and compared these to the cortisol levels of the same samples determined by a conventional ELISA method. Plasma samples were diluted to 1/100 for the label-free immunosensor system.\(^{(28)}\) The cortisol levels determined using the proposed sensor system were similar to those determined using ELISA. Thus, the proposed sensor system could be satisfactorily used to monitor changes in the cortisol level of actual fish plasma samples.

5. Conclusions

In the present study, we investigated the electrochemical characteristics of a modified electrode with improved antibody immobilization and amplification of electron exchange on the electrode surface caused by SWCNT immobilization. We determined the optimal reaction conditions for the sensor, and the calibration curve for cortisol was linear from 156 to 10000 pg ml\(^{-1}\). We next used our proposed sensor system to measure actual fish plasma samples and found a good correlation with the conventional method.

The present findings reveal that cortisol level in fish as an indicator of stress can be rapidly and conveniently monitored using our proposed biosensor. Results obtained by this method may provide new knowledge in the field of fish stress, aquaculture, and fish physiology. The proposed biosensor system will be useful for rapid, reliable, and convenient analysis of ‘actual stress’ in combination with other indicators of fish health, such as blood glucose. On the basis of this knowledge, improvements in the aquatic environment and farming technology can be implemented to enhance fish-friendly fisheries.

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References

1. B. A. Barton: Annu Rev. Fish Dis. 1 (1991) 3.
2. L. Tort: Dev. Comp. Immunol. 35 (2011) 1366.
3. R. M. G. Wells and N. W. Pankhurst: J. World Aquacult. Soc. 30 (1999) 276.
4. M. W. Davis: Fish Fish. 11 (2010) 1.
5. T. P. Mommsen, M. M. Vijayan and T. W. Moon: Rev. Fish Biol. Fisher. 9 (1999) 211.
6. B. A. Barton and G. K. Iwama: Annu. Rev. Fish Dis. 1 (1991) 3.
7. A. K. Gamperl, M. M. Vijaya and R. G. Boutiler: Rev. Fish Biol. Fisher. 4 (1994) 215.
8. T. Ellis, J. D. James, C. Stewart and A. P. Scott: J. Fish Biol. 65 (2004) 1233.
9 J. S. F. van der Vyver, H. Kaiser, W. M. Potts and N. James: J. Appl. Ichthyol. 29 (2013) 1275.
10 M. Piao, H. B. Noh, M. A. Rahman, M. S. Won and Y. B. Shim: Electroanal. 20 (2008) 30.
11 L. Qiu, C. Wang, P. Hu, Z. Wu, G. Shen and R. Yu: Talanta 83 (2010) 42.
12 D. Brondani, J. V. Piovesan, E. Westphal, H. Gallardo, R. A. Fireman Dutra, A. Spinelli and I. C. Vieira: Analyst 139 (2014) 5200.
13 J. D. Qiu, R. P. Liang, R. Wang, L. X. Fan, Y. W. Chen and X. H. Xia: Biosens. Bioelectron. 25 (2009) 852.
14 N. Kim, I. Park and D. Kim: Sens. Actuators, B 100 (2004) 432.
15 A. Tlili, A. Abdelghani, S. Hleli and M. A. Maaref: Sensors 4 (2004) 105.
16 G. A. Rivas, M. D. Rubianes, M. C. Rodriguez, N. F, Ferreyra, G. L. Luque, M. L. Pedano, S. A. Miscoria and C. Parrado: Taranta 74 (2007) 291.
17 Q. Gao, Y. Guo, W. Zhang, H. Qi and C. Zhang: Sens. Actuators, B 153 (2011) 219.
18 H. Endo, M. Igarashi, A. Banba, H. Ohnuki, U. Hishio, T. Hayashi, H. Ren and G. Yoshizaki: Int. J. Environ. Anal. Chem. 91 (2011) 174.
19 H. Endo, T. Muramatsu, G. Yoshizaki, H. Ren and H. Ohnuki: Fish. Sci. 78 (2012) 391.
20 J. Wang: Electroanalysis 17 (2005) 7.
21 Z. Yao, H. W. C. Postma, L. Balents and C. Dekker: Nature 402 (1999) 273.
22 M. G. Sullivan, B. Schnyder, M. Bärtsch, D. Alliata, C. Barbero, R. Imhof and R. Kötz: J. Electrochem. Soc. 147 (2000) 2636.
23 F. Malem and D. Mandler: Anal. Chem. 65 (1993) 37.
24 D. W. Mason and A. F. Williams: Biochem. J. 187 (1980) 1.
25 A. A. Elskus: Mar. Environ. Res. 58 (2004) 463.
26 M. Kobayashi, K. Aida and I. Hanyu: Gen. Comp. Endocrinol. 67 (1987) 24.
27 Y. Nagahama: Steroids 62 (1997) 190.
28 B. A. Barton: Integr. Comp. Biol. 42 (2002) 517.