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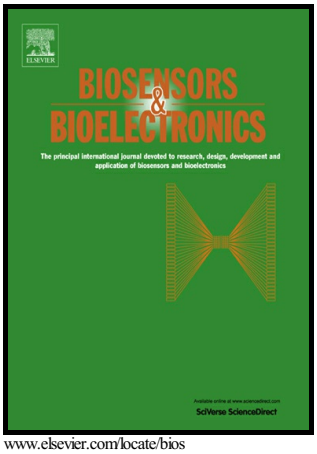

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New approach for monitoring fish stress: a novel enzyme-functionalized label-free immunosensor system for detecting cortisol levels in fish

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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. As a well-known indicator of fish stress, a simple and rapid method for detecting cortisol changes especially sudden increases is desired. In this study, we describe an enzyme-functionalized label-free immunosensor system for detecting fish cortisol levels. Detection of cortisol using amperometry was achieved by immobilizing both anti-cortisol antibody (selective detection of cortisol) and glucose oxidase (signal amplification and non-toxic measurement) on an Au electrode surface with a self-assembled monolayer. This system is based on the maximum glucose oxidation output current change induced by the generation of a non-conductive antigen-antibody complex, which depends on the levels of cortisol in the sample. The immunosensor responded to cortisol levels with a linear decrease in the current in the range of 1.25 to 200 ng ml^{-1} (R=0.964). Since the dynamic range of the sensor can cover the normal range of plasma cortisol in fish, the samples obtained from the fish did not need to be diluted. Further, electrochemical measurement of one sample required only ~30 min. The sensor system was applied to determine the cortisol levels in plasma sampled from Nile tilapia (Oreochromis niloticus), which were then compared with levels of the same samples determined using the conventional method (ELISA). Values determined using both methods were well correlated. These findings suggest that the proposed label-free immunosensor could be useful for rapid and convenient analysis of cortisol levels in fish without sample dilution. We also believe that the proposed system could be integrated in a miniaturized potentiostat for point-of-care cortisol detection and useful as a portable diagnostic in fish farms in the future.

Keywords: Cortisol; Fish stress; Enzyme-functionalized biosensor; Amperometry; SAM

1. Introduction

Physical, chemical, and biological stressors can evoke non-specific adaptive responses in fish, enabling fish to cope with the disturbance and maintain homeostasis. If the stressor is so severe or long lasting that the fish is not capable of regaining homeostasis, the responses themselves may become maladaptive and threaten fish health and well-being (Barton 2002). Physiologic responses to stress are classified as primary, which include endocrine changes such as measurable levels of corticosteroids, and secondary, which include changes in features related to metabolism, such as the production of glucose through gluconeogenesis. Tertiary or whole-animal changes in growth, disease resistance, and behavior can result from the primary and secondary responses, and possibly affect survival. Stress is a generalized response attributed to the fact that fish commonly have a complex of adaptive reactions to cope with stressors (Barton and Iwama 1991).

Biologists are familiar with the physiologic responses of fish to stress, but the mechanisms by which stress influences overall performance, especially the acute stress response, are not well understood and have not been thoroughly evaluated due to the lack of convenient, rapid, and accurate measurement methods for detecting stress indicators in fish plasma.

A major focus of current research related to aquaculture is identifying stress markers in fish that accurately reflect the stress status and measuring fish health (Schwaiger et al. 1997). Cortisol level is the best-studied marker of the stress response, and this steroid hormone is also considered to be the best indicator of acute stress in fish (Pickering and Pottinger 1989, Schreck and Lorz 2011, Pottinger and Carrick 1999). Cortisol activates a number of metabolic pathways that alter blood chemistry and hematology, such as carbohydrate metabolism by stimulating glucose production through gluconeogenesis, resulting in the elevation of plasma glucose (Barton et al. 2002). While fish farmers currently draw blood from breeding fish to monitor fish health, drawing blood requires not only special techniques and skills, but is also time-consuming. Moreover, fish blood cortisol levels are measured using clinical laboratory test kits designed for humans (Sink et al. 2008, Egan et al. 2009). To address this problem, we developed some enzyme biosensor systems to monitor stress, disease resistance, and fatigue indicators in fish (Endo et al. 2009, Yonemori et al. 2009, Wu et al. 2015a). Although glucose is still a very important indicator of fish stress,

it may also increase due to stress-independent factors, such as feeding and absorption of nutrients, sometimes cannot reflect the true stress variation of fish, especially acute stress. A number of studies describe the plasma cortisol profiles in response to various stressors and the physiologic consequences of elevated cortisol levels in fish (Rotllant and Tort 1997, Pankhurst and Van Der Kraak 2000, McCormick et al. 1998). Immunoassays based on antigen–antibody specific reactions are considered a major analytical tool for clinical diagnoses, and environmental and biochemical studies. Various immunoassay protocols, such as surface plasmon resonance (Li et al. 2007), quartz crystal microbalance (Uludag and Tothill 2012), chemiluminescence(Xu et al. 2011), and electrochemical methods(Chikkaveeraiah et al. 2012), have been extensively developed for detecting such as biomarkers or steroid hormones including cortisol. Among these methods, electrochemistry, which has 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 that allow for easy and rapid assay of various steroid hormones in fish, and believe these systems will be as effective as conventional detection methods used in fish farms, but are easier and faster to perform (Wu et al. 2016 , Wu et al. 2015b). Though the sensor (Wu et al. 2015b) we developed recently has a relatively high sensitivity (dynamic range: 156-10,000 pg ml^{-1}), the signal of the sensor was not sufficient to completely cover the normal cortisol variation range in fish (variation range: 10 - 200 ng ml^{-1}) (Barton and Iwama 1991, Pickering and Pottinger 1989, Schreck and Lorz 2011). If the signal can be further amplified, we believe the dynamic range of the sensor will be sufficient for monitoring cortisol levels in fish. Many promising strategies, such as enzymes, nanoparticles, liposomes, and DNA-based polymerase chain reaction, are used for signal amplification, but the development of new approaches to improve the simplicity and sensitivity of electrochemical immunoassays is challenging. In previous studies, bioactive enzymes were used to amplify electrochemical signals. Campas et al. (Campas et al. 2008) described an enzymatic recycling-based amperometric immunosensor for ultrasensitive detection of okadaic acid. The highlight of this method is its improved ability to label the secondary antibodies used for conventional enzyme-linked immunosorbent assay (ELISA), which uses double-enzyme labels in addition to binding of the substrate and product. Wilson (Wilson 2005) used enzyme-labeled electrochemical

immunoassays for the simultaneous detection of multiple tumor markers. The detection limit and sensitivity were greatly improved due to the bioelectrocatalytic reaction of the immobilized enzyme.

Here we describe a new approach and method using glucose oxidase (Gox) as an amplifier of signal (changes on the electrode surface due to the formation of the antigen-antibody complex) to enhance the immunosensor to get a wider range and reliable detection of plasma cortisol levels in fish. Formation of the antibody–antigen complex by a simple one-step immunoreaction between the immobilized anti-cortisol antibody and cortisol in the sample solution introduced a barrier against direct electrical transmission between the immobilized Gox and the electrode surface, and decreased the immobilized Gox toward the catalytic oxidation of glucose. In addition, the introduction of Gox eliminates the need for a mediator for the measurement. Thus, the measurement was achieved with amperometry instead of cyclic voltammetry, increasing the simplicity and rapidity of detection. Because amperometry can be easily performed, this system can also be a potential foundation for in vivo or point-of-care measurements. For biomolecular immobilization on the surface of the sensing electrode, we used self-assembled monolayers (SAM) of 3-mercaptopropionic acid that form easily and remain stable. We also applied the proposed immunosensor to measure actual fish plasma samples collected after exposing fish to various physical, biochemical, and behavioral physiologic stressors to evaluate the different physiologic states induced by each stressor. We believe the electrochemical principle of the proposed immunosensor can also be a foundation of a portable device that can be used in fieldwork.

2. Materials and methods

2.1. Reagents

The cortisol enzyme immunoassay (EIA) standard, 17α,20β-dihydroxy-4-pregnen-3-one (DHP) 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). Gox (from *Aspergillus niger*; E.C. 1.1.3.4, type VII-S; 158,900 unit g^{-1}) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St.

Louis, MO, USA). D(+)-Glucose and 2-phenoxy ethanol were purchased from Wako Pure Chemical Industries (Tokyo, Japan). D(+)-Glucose was dissolved in 0.1 M phosphate buffered saline (PBS) and allowed to mix by rotation for 24 h before use. All other reagents used for the experiments were commercial-grade or laboratory-grade.

2.2. Apparatus

The CV measurement system was constructed from an electrochemical analyzer (Model 802B, BAS). All experiments related with CVs measurements were performed with a conventional three-electrode system using a modified ø3.0 mm Au electrode (BAS) as a working electrode, a platinum wire (ø1 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 performed in a cell stand (CS-3, BAS).

The measurement system was constructed from a potentiostat (Model 3104, Pinnacle Technology, Lawrence, KS, USA). All experiments related with the amperometry method were performed with a dual-electrode system using a modified ø3.0-mm disc type Au electrode (BAS, Tokyo, Japan) as a working electrode, and a Ag/AgCl-pasted platinum wire (ø1-mm, 5 cm) as a counter electrode.

2.3. Fabrication of the label-free immunosensor

The surface of the Au electrode (surface area = 7.1 mm^2) was polished using diamond paste with ø3.0 μm and ø1.0 μm particles and alumina slurries with ø0.05 μm particles. Polished electrodes were further cleaned by ultra-sonication in distilled water and absolute ethanol, 5 min in each solution. The working electrode was then cycled from 0 to 1500 mV (vs. Ag/AgCl) at a rate of 10 mV s^{-1} in 0.5 M sulfuric acid for 50 cycle scan until the cyclic voltammetry signal 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–e. The treated working electrode (Fig. 1a) was immersed in a 10 mM 3-mercaptopropionic acid solution in the dark at room temperature for 8 h. In this step, SAM formed on the surface of

the Au electrode (SAM/Au electrode; Fig.1b). The working electrode was then placed in 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide solution (100 mg ml^{-1}) for 15 min at room temperature and 100 mg ml^{-1} N-hydroxysuccinimide was added at room temperature for another 90 min to modify a carboxyl group to form an amine-reactive ester (Fig. 1c). The electrode was then rinsed with ultrapure water to remove nonspecific, physically absorbed 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and N-hydroxysuccinimide, and dried under pure flowing nitrogen gas. Cortisol monoclonal antibody (0.5 mg ml^{-1}) and Gox (1598 units g^{-1}) in 0.1 M phosphate buffer (pH 7.5) was then placed on the modified surface of the working electrode overnight at 4 ℃ , allowing for the formation of the antibody/enzyme/SAM/Au electrode (Fig. 1d). At last, 1.0 mg ml^{-1} BSA was applied as a blocking agent to prevent non-specific binding (Fig. 1e). These electrochemical characteristics of the modified electrode were measured by cyclic voltammetry with a scan range from -0.2 to 0.8 V in a solution of 10 mM glucose. The fabricated immunosensors were stored at 4℃ when not in use. After modification, the working electrode was immersed in 15 ml of 500 mg dl^{-1} glucose solution (pH 7.5) with the counter electrode mentioned above. The initial output current value (blank) of the sensor was determined using a potentiostat after the output current became stable.

2.4. Measurement of cortisol standard solution

The optimal parameters of the immunoreaction were evaluated and determined by the rate of change of the output current compared with the blank. The electrode surface was then dried and immersed in 0.5 ml of various concentrations of cortisol standard solutions and fish plasma samples ranging up to 200 ng ml^{-1} (the common variable range for fish cortisol change) using the optimized parameters for cortisol measurement.

2.5. Specificity of the immunosensor

The specificity of the label-free immunosensor system was investigated using steroid hormones with structures and release time similar to that of cortisol, such as DHP, estriol, estradiol, and testosterone (Elskus 2004, Kobayashi et al. 1987, Nagahama 1997). The

immunosensor was incubated in 0.5 ml of each hormone solution with concentrations ranging from 1.25 to 32 ng ml^{-1} for 25 min. Amperometry was then performed with the same parameters and solutions as mentioned in 2.4.

2.6. Measurement of cortisol levels in fish plasma

The proposed biosensor system was applied to measure cortisol levels in plasma from Nile tilapia (*Oreochromis niloticus*). 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. Except the control group, fish were induced by exposure to different types of stressors (i.e., air, light stimulation, nitrite, and interaction between individuals) to artificially arise the cortisol levels in fish.

2.6.1 Cortisol levels changes aroused by interaction with other fish

Dominance hierarchies develop quickly in some species of fish, including Nile tilapia, and fish of lower status usually exhibit impaired feeding and poor growth as a result (Portz et al. 2006). Because stress (i.e., increased plasma cortisol levels) can reduce appetite, aggressive behavior may lead to cascading effects on fish growth and health. Aggressive social interactions may also inhibit foraging time and activity, thereby reducing food intake. In this experiment, two test fish, named Test fish α (smaller size, total length: 19.0 cm, weight: 183.77 g) and Test fish β (bigger size, total length: 24.5 cm, weight: 243.13 g) were used in the experiment. Test fish were reared in separate aquariums (normal breeding water) for at least 15 h. After acclimation, Test Fish β was transferred to an aquarium containing Test fish α for 30 min （blood was drew from Test A every 5 min, 6 times during the experiment）.

2.6.2 Cortisol levels changes aroused by air exposure

Stress in fish caused by physical disturbances encountered in aquaculture, such as handling and capture, evokes a variety of responses that may be adaptive or maladaptive

(Barton 2002). An aquarium with a water depth of 8 cm was prepared. Tilapia (Average length: 25.5 cm Average weight: 220.65 g, n=5) were used in the experiment after breeding more than rearing for at least 15 h in the aquarium (pH 6.8, water temperature 26°C in fresh water). To observe the extent to which air exposure caused by handling or capture would evoke fish stress, fish were scooped up in a net and exposed to air for 10 min after as a typical physical stressor in aquaculture.

2.6.3 Cortisol levels changes aroused by light stimulation

Fish are usually exposed to blue, green, or near infrared light, and fish have cone cells that enable them to discriminate colors (Volpato and Barreto 2001). Despite this, very few studies have evaluated the effects of light intensity and color on fish biology, especially how they affect the stress response. These few studies demonstrated both improvement and disruption of fish welfare due to environmental light, supporting the need for a better understanding of such effects, which may also be related to rearing conditions. In aquaculture, the environmental conditions, including light stimulation, should be monitored to guarantee fish welfare. In this experiment, an aquarium with a water depth of 8 cm was prepared. Tilapia (Average length: 22.3 cm, Average weight: 215.15 g, n=5) were used in the experiment after rearing for at least 15 h in the aquarium (pH 6.8, water temperature 26°C in fresh water). This aquarium was shielded with an opaque curtain to block the external light and darken the room. A compact stroboscope was placed at a distance of ~40 cm to the center of the experimental aquarium. When the experiment started, a white LED light flashed with a frequency of 10 Hz for 15 min.

2.6.4 Cortisol levels changes aroused by nitrite exposure

In this experiment, we monitored the fish stress response to nitrite exposure as an acute chemical stressor for fish. We tested the effects of 25 mg L^{-1} nitrites dissolved in water. Two aquariums (A, B) with a water depth of 8 cm were prepared and the conditions set (A: normal breeding water B: normal breeding water with 25 mg L^{-1} nitrites dissolved). Test fish (Average length: 23.3 cm, Average weight: 220.17 g, n=5) were used in the experiment after

rearing for at least 15 h in normal breeding water (ammonia: 0.25 mg L^{-1}, nitrite and nitrate: 0 mg L^{-1}; all tested by Tetra test kits, Germany). After acclimation, the fish were transferred from aquarium A to aquarium B, and exposure was continued for 20 min.

After stress induction, the test fish were anesthetized with 2-phenoxy ethanol, and blood samples were immediately collected and centrifuged (450×*g*, 10 min) to separate the plasma. The plasma samples were collected immediately and stored at below -28°C until use. For the experiment described in 2.6.4, plasma was collected from both test fish. Plasma sample measurements were performed in 15 ml of 500 mg dl^{-1} solution to obtain the maximum output current change rate with the optimal parameters described in 2.4.

3. Results and discussion

3.1. Electrochemical characteristics of the modified electrode by using CV

The cyclic voltammogram of the bare gold electrode in 10 mM glucose-PBS (pH 7.5) at 50 mV s^{-1} from a potential of -0.2 to 0.8 V is shown in Fig. 2A(a). No anodic and cathodic peaks were observed. The same phenomenon was observed after SAM immobilization [Fig. 2A(b)]. After the electrode was modified with anti-cortisol antibody and Gox, obvious anodic and cathodic peaks were observed [Fig. 2A(c)]. The anodic and cathodic peaks increased in the presence of glucose after Gox was immobilized to the electrode surface. When 10 mM glucose was added, an oxidation current increased around +0.65 V in the positive going sweeps and a sharp oxidation peak at +0.3 V in the negative going sweeps occurred for the Gox and anti-cortisol monoclonal antibody immobilized bio-electrode (Wu et al. 2015c). After that, the electrode surface was dried and immersed in 0.5 ml of 40 ng ml^{-1} cortisol standard solutions for 25 min and measured in the electrochemical cell. The anodic current decrease depended on the generation of the antigen-antibody complex, which is non-conductive and produces steric hindrance, depending on the cortisol level [Fig. 2A(d)]. For comparison, we also investigated the cyclic voltammogram of the immunosensor in glucose-free PBS (pH 7.5) [Fig. 2A(e)]. In contrast with Fig. 2A(c) and 2A(d), no peaks were observed for the same bio-electrode but without glucose addition, as shown in Fig. 2A(e). Thus, the reason for the

large current response might be due to amplification of the enzymatic bioelectrocatalysis signals. These results indicated that anti-cortisol antibody and cortisol was not involved directly in the oxidation of glucose but it produces the steric hindrance in the electron transfer, and that the oxidation of glucose was solely catalyzed by Gox. The reason for the large current response might be due to amplification of the enzymatic bioelectrocatalysis signals. While, typical responses of the proposed label-free immunosensor are shown in Fig. 2B. The CV current (+650 mV) of the proposed immunosensor [Fig.2B(a)] incubated in different concentrations of cortisol standard solution decreased compared to when it was incubated in blank solution (Fig 2B, b→f). It is obvious that there are significant differences in the values for different concentration of cortisol in sample. We think this phenomenon is caused by the increase of the resistance of the electrode or the space barrier between glucose and enzyme after the formation and increase of antigen-antibody complex.

3.2. Effects of analytical conditions on the output current of immunosensor

The effects of temperature, pH, and the antibody incubation time on the output current change rate are shown in Fig. 3. The rate of decrease of the current increased with an increase in the temperature or pH, reached a maximum, and then decreased. The maximum decreases in output current occurred at 30°C and pH 7.5 (Fig. 3A, B). For antibody incubation time, the rate of decrease in the output current increased gradually for 20 min, and then increased dramatically at 25 min, reaching a plateau at 30 min (Fig. 3C).

The output current decrease produced by the antibody-antigen interactions was clearly influenced by the analytical conditions. It is (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 20 to 30°C (Fig. 3A), suggesting that the antigen-antibody complexes were formed efficiently. On the other hand, the rate of decrease in the output current decreased when the temperature was greater than 30°C. It is presumed that the antibodies were degraded or that the antibody's affinity to the antigen decreased at the higher temperature. That is to say that the optimal temperature for this antigen-antibody reaction was 30 °C. But considering the actual use of the sensor system, a temperature

near the room temperature was more suitable for the measurement. We finally determined the operating temperature to 25℃ since the sensor also showed a good response. We then examined the effect of pH on the reaction. The rate of decrease of the output current increased from pH 6.5 to 7.5 and then gradually decreased when the pH was higher than 7.5 (Fig. 3B). We presumed that pH also had a great effect on the reaction, and determined the optimal reaction pH was 7.5. In studies of the effect of the antibody incubation time, we used a standard solution of 10 ng ml^{-1} cortisol. The decrease in current stabilized after 25 min and reaching a plateau at 30 min (Fig. 3C). But considering the efficiency of the reaction, we determined that the optimal antibody incubation time for the antigen solution was 25 min.

3.3. Calibration curve of immunosensor

A calibration curve of the proposed immunosensor in standard cortisol solution and fish plasma samples is shown in Fig. 4. A good linear relationship between the rate of decrease in the output current and the logarithm of cortisol concentration in standard cortisol solutions was obtained in the range from 1.25 to 200 ng ml^{-1} . The reproducibility of the immunosensor was evaluated by inter-assay relative standard deviation. The inter-assay relative standard deviation of 6 immunosensors was 4.2%, 3.8%, 6.9%, 7.7%, and 8.1% for cortisol concentrations of 1.25, 25, 50, 100, and 200 ng ml^{-1}, respectively. Thus, the reproducibility of the immunosensor was acceptable. We also examined the stability of the immunosensor. When the immunosensor was dried and stored at 4°C, it retained a mean of 93.2% (n=3) of its initial response after a storage period of 7 d. And the LOD of the sensor is 1.01 ng ml^{-1} calculated from the standard deviation and slope of the regression line. The range of the cortisol level in fish plasma ranged from dozens to hundreds of nanograms per milliliter. Therefore, given the calibration range of the sensor system, plasma samples would not need to be further diluted and could be directly measured in most common conditions.

3.4. Specificity of immunosensor

Fish plasma also contains other types of steroid hormones that have similar structures to that of cortisol. Therefore, we investigated the specificity of the sensor system for other

steroid hormones. We examined the specificity of the sensor system by measuring standard solutions of cortisol, estriol, DHP, estradiol, and testosterone (RSD ≤ 0.21). The sensor was immersed in 1 to 32 ng ml^{-1} of each hormone solution for 25 min. The rate of the output decrease did not remarkably change (~4.0 %) with changes in the concentration of each hormone examined other than cortisol (Fig. 5). These results indicate that the label-free immunosensor system was sufficiently specific for cortisol measurement and can be used for actual fish plasma measurements.

3.5. Measurement of cortisol levels in fish plasma

To evaluate the potential application of the immunosensor system for actual biologic samples, the sensor system was applied to determine the cortisol levels in plasma of Nile tilapia and compared with the cortisol levels of the same samples determined by the conventional ELISA method. Because the dynamic range of the sensor was up to 200 ng ml^{-1}, undiluted plasma samples could be used in the experiment. We performed an experiment on the stress response monitoring when smaller test fish α swim with bigger test fish β mentioned in 2.6.1. The cortisol levels of test fish α were measured by both proposed and conventional method and showed in a chronological order (Fig.6A). Each point showed the cortisol level of smaller fish immediately following a 5 min of contact with bigger individuals after a short duration of anesthesia and blood sampling. The rest of the periods were displayed some stress-independent operation in the experiment, such as the movement and resuscitation of anesthetized fish. It can be found that the cortisol levels of smaller fish varied and showed a very interesting change in the stress response of interaction with bigger one. Cortisol levels were temporarily increased at first, but the tendency changed into a subsequent gradually decrease. It can be considered that when the smaller fish swim with the bigger one, it will feel stress and the cortisol increased at the first stage. After that stage, cortisol levels turned into a tendency of decrease because the small fish was accept the reality then accustomed to the stress. In another study, we also performed a similar evaluation on the stress response of fish individual interaction. The stress responses were reflected from the temporal change of glucose which is another important fish stress indicator by using wireless biosensor system (Wu et al. 2015a.)

Compare our previous study, both of the results showed a similar trend when smaller fish swam with a bigger individual. But it should be noted that, cortisol changes faster than the glucose because cortisol was a kind of hormone secreted in the primary response of fish stress then promote gluconeogenesis.

Except the stress response of interaction between the individuals (△: interaction with other individual), both control (●:unstressed) and stressed fish's (○:air exposure, □:light stimulate, ◇ : nitrites addition) cortisol levels were measured individually by proposed and conventional method. The relationship between the data obtained with the sensor system and data obtained the ELISA method is shown in Fig. 6B. The correlation was very strong between values determined using the sensor system and the ELISA in the range of 17.30 to 183.93 ng ml^{-1} (correlation coefficient: 0.962). Fish will always express similar correspondence (swim rise to the surface for a fresh breath and rapid escaping, etc.) and hard to be found which stressor fish are suffering but their real stress will be reflect from their physiological changes indicator like cortisol levels. As Fig. 6B shows, by using our proposed biosensor system, it can be easily found an approximate order for fish acute stress (air exposure > light stimulate ≧ nitrites addition > unstressed) in a simpler and faster way. Conversely, from the approximate order, it may further help us to understand which stressor fish was suffering from the cortisol level changes (as a good indicator to acute stress). Since the measurement can be finished in 30 min, it can also help such as ichthyologists to observe fish stress response in a new way.

Overall, since the sensor showed a relatively wide dynamic range and well correlation with conventional ELISA method in real fish plasma sample, it indicated that the proposed sensor system could be satisfactorily applied to fast monitor changes in the cortisol levels in actual fish plasma samples under most stress-related conditions including stress-free condition (Delaney et al. 2005, Barreto and Volpato 2006).

4. Conclusion

This contribution describes a novel strategy for enhancing the electrochemical detection of cortisol in fish plasma by detecting the rate of current decrease produced by the catalytic

amplification of bioactive enzyme activity. Highlights of the proposed immunoassay system are its wide dynamic range; it is a direct, rapid, and simple process that does not require additional labeling. The findings of the present study confirmed that cortisol levels in fish plasma could be rapidly and sensitively measured using the described immunosensor system. Under optimal conditions, the linear range of the immunosensor using Gox as an enhancer was linear from 1.25 to 200 ng ml^{-1}. The formation of the antibody–antigen complex by a simple one step immunoreaction in sample solution introduced a barrier against direct electrical communication between the immobilized Gox and the base surface, and decreased the immobilized Gox toward the catalytic oxidation of glucose. In contrast to conventional methods, such as ELISA and labeled immunosensors, which require long incubation times and complicated steps, our sensor required only ~30 min per sample, including the immunoreaction time. This same mechanism may also be useful for detecting other kinds of important steroid hormones, such as testosterone and estriol.

In the present study, we used our immunosensor system to measure fish stress due to changes in the fish environment. The present findings revealed that cortisol levels in fish as an indicator of stress could be rapidly and conveniently monitored within 30 min using our biosensor. Acute stress in fish could be accurately monitored using the proposed immunosensor system. The 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 could be a foundation of portable devices for use in field work. Moreover, this immunosensor will be useful for rapid, reliable, and convenient analysis of fish health in combination with other indicators of fish health, such as blood glucose, which will guide improvements to the aquatic environment and farming technology that will enhance fish health in fisheries.

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Supplementary material caption

Supplementary material 1 Impedance analysis on differently modified electrodes. (a) bare Au electrode, (b) SAM/Au electrode, (c) BSA/antibody and enzyme /SAM/Au electrode (d) BSA/antibody and enzyme /SAM/Au electrode after a 10min immersion of 40 ng ml^{-1} standard cortisol solution. The Impedance analysis was performed at a Amplitude= 0.01V with a frequency range of 10000 Hz to 0.1 Hz in 5 mM potassium ferricyanide / potassium ferrocyanide solution (pH 7.5). The resistance increases with the formation of the molecular layer of each layer, especially after the immobilization of antibodies and enzymes. It can help us to prove the formation of the various molecular layers. In addition, once the antibody on the electrode surface combined with the antigen in the sample, the resistance is further increased. It can be proved that the antigen-antibody can be effectively combined and shows a certain effect on the electrochemical properties and resistance on the electrode surface.

Supplementary material 2 Amperometric curve on different levels of cortisol (a) 10 ng ml^{-1},(b) 40 ng ml^{-1}, (c) 200 ng ml^{-1}, (d) 150 ng ml^{-1}. The amperometric analysis was performed with the condition of temperature 30°C, pH 7.5, incubation time 25 min and applied voltage +650 mV. The curve was showed the changes of the current values before and after the immunoreaction. It is obvious that there is a significant decrease in the current values before and after the reaction. The change rates of the current of each sample were: 20.133% for 10 ng ml^{-1}, 33.010 for 40 ng ml^{-1}, 45.750 for 200 ng ml^{-1}, 43.298 for 150 ng ml^{-1}.

Supplementary material 3 Calibration curve of the label-free immunosensor for cortisol standard solutions. Assay conditions were as follows: temperature 30°C, pH 7.5, and incubation time 25 min. Each sample was measured three times. Data points are the mean value, and error bars indicate the maximum and minimum values. A good linear relationship between the rate of decrease in the output current and the logarithm of cortisol

concentration in standard cortisol solutions was obtained in the range from 0.0969 to 2.3010.

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Figure captions

Fig. 1 Stepwise fabrication process of the immunosensor.

Fig. 2 Electrochemical behavior of the modified electrode. (A)Cyclic voltammograms of differently modified electrodes (a) bare Au electrode, (b) SAM/Au electrode, (c) BSA/antibody and enzyme /SAM/Au electrode (d) BSA/antibody and enzyme /SAM/Au electrode after a 10min immersion of 40 ng ml^{-1} standard cortisol solution, (e) BSA/antibody and enzyme/SAM/Au in 10 mM glucose-free PBS (pH 7.5) after a 10min immersion of 40 ng ml^{-1} standard cortisol solution. (B) Typical response CV of the proposed label-free immunosensor. (a) Blank curve: 0 ng ml^{-1},(b) Response curve：10 ng ml^{-1}, (c) Response curve: 40 ng ml^{-1}, (d) Response curve：100 ng ml^{-1}, (e) Response curve：150 ng ml^{-1}, (f) Response curve：200 ng ml^{-1}. Each sample was used different levels of cortisol standard solution. All the cyclic voltammogram was performed at a scan rate of 50 mV s^{-1} with a potential change from -0.2 to 0.8 V in 10 mM glucose solution. (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. 3 Effects of assay conditions on the rate of output current decrease. (A) Temperature (cortisol concentration: 10 ng ml^{-1}, pH: 7.5, incubation time: 25 min), (B) pH (cortisol concentration 10 ng ml^{-1}, temperature: 25℃, incubation time: 25 min), and (C) Incubation time (cortisol concentration 10 ng ml^{-1}, temperature: 25℃, pH: 7.5)

Fig. 4 Calibration curve of the label-free immunosensor for cortisol standard solutions. Assay conditions were as follows: temperature 30°C, pH 7.5, and incubation time 25 min. Each sample was measured three times. Data points are the mean value, and error bars indicate the maximum and minimum values.

Fig. 5 Relationship between rate of output current decrease and concentration of various steroid hormones. The sensor was immersed in cortisol (●), estriol (△), DHP (□), estradiol (

◇), and testosterone (◪) solutions. Assay conditions were as follows: temperature 30°C, pH 7.5, and incubation time 25 min. Each sample was measured three times. Data points are the mean value, and error bars indicate the maximum and minimum values.

Fig. 6 Measurement of the actual sample (A) Monitoring of fish stress response on interaction between individuals. ●: proposed immunosensor system ○: conventional ELISA method (B) Correlation between data obtained with the immunosensor system and the ELISA method (n=26). ●: unstressed ○: air exposure, □: light stimulation, ◇: nitrite exposure, △: interaction with other individual.

Highlights

- We designed an amperometry immunosensor for simple and fast monitoring cortisol.
- Without blood-dilution procedures, fish stress monitoring become faster and easier.
- Signal amplification was achieved via both enzyme and antibody immobilized on gold electrode.

Fig.1

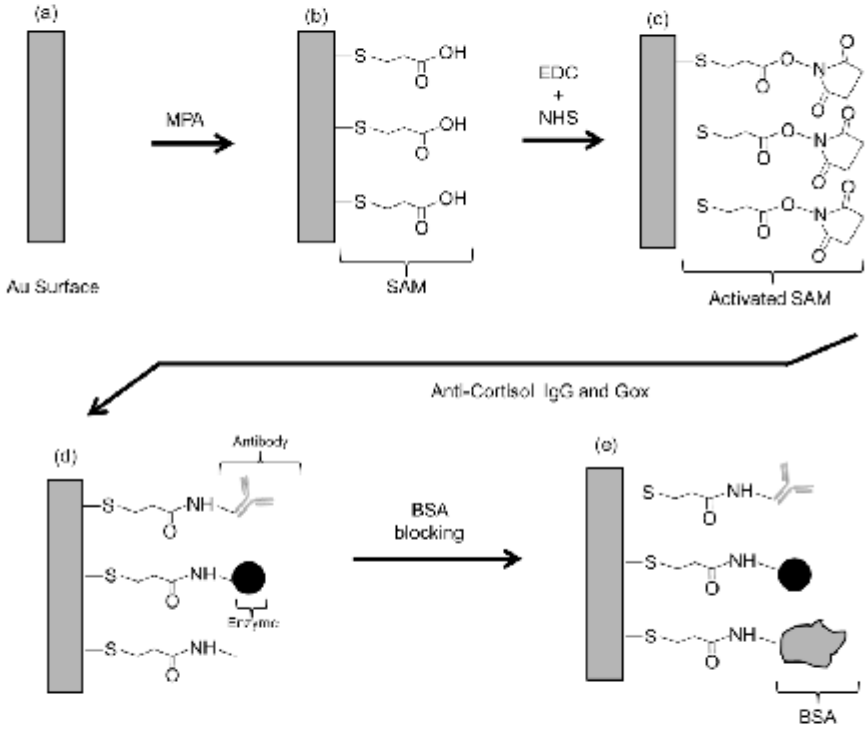

(a)
Au Surface
MPA
(b)
SAM
EDC
+
NHS
(c)
Activated SAM
Anti-Cortisol IgG and Gox
(d)
Antibody
BSA
blocking
(e)
BSA

Fig.2

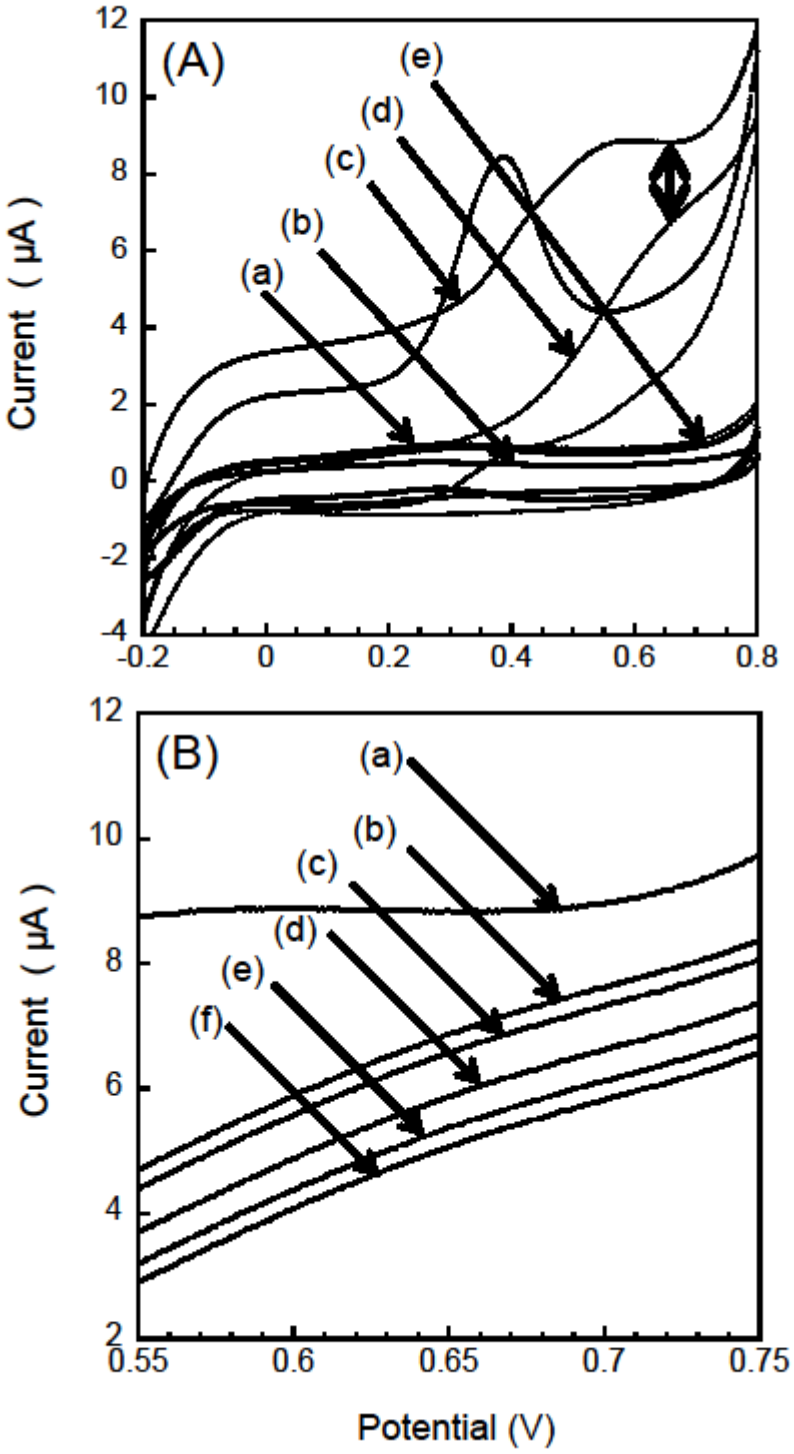

(A)
(a)
(b)
(c)
(d)
(e)
Current (μA)
12
10
8
6
4
2
0
-2
-4
-0.2
0
0.2
0.4
0.6
0.8
(B)
(a)
(b)
(c)
(d)
(e)
(f)
Current (μA)
12
10
8
6
4
2
0.55
0.6
0.65
0.7
0.75
Potential (V)

Fig.3

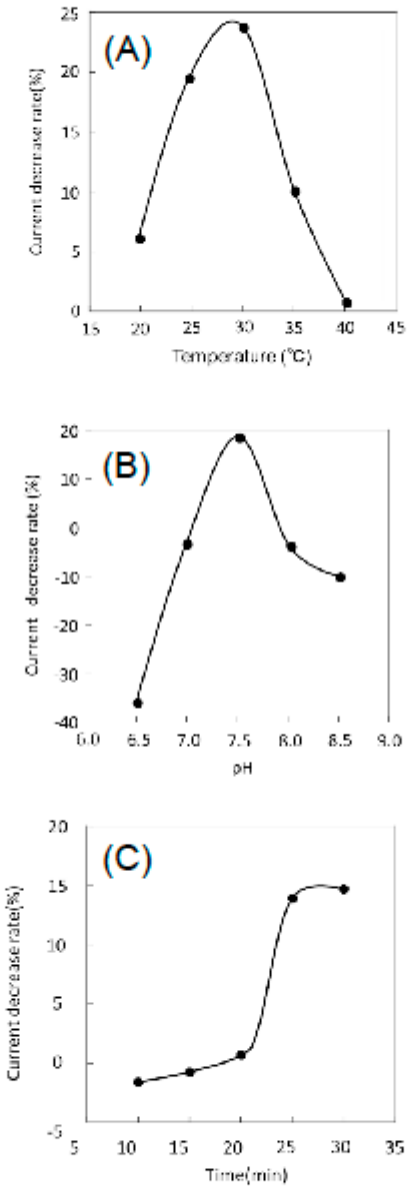

(A)
Current decrease rate(%)
Temperature (°C)
(B)
Current decrease rate (%)
pH
(C)
Current decrease rate(%)
Time(min)

Fig.4

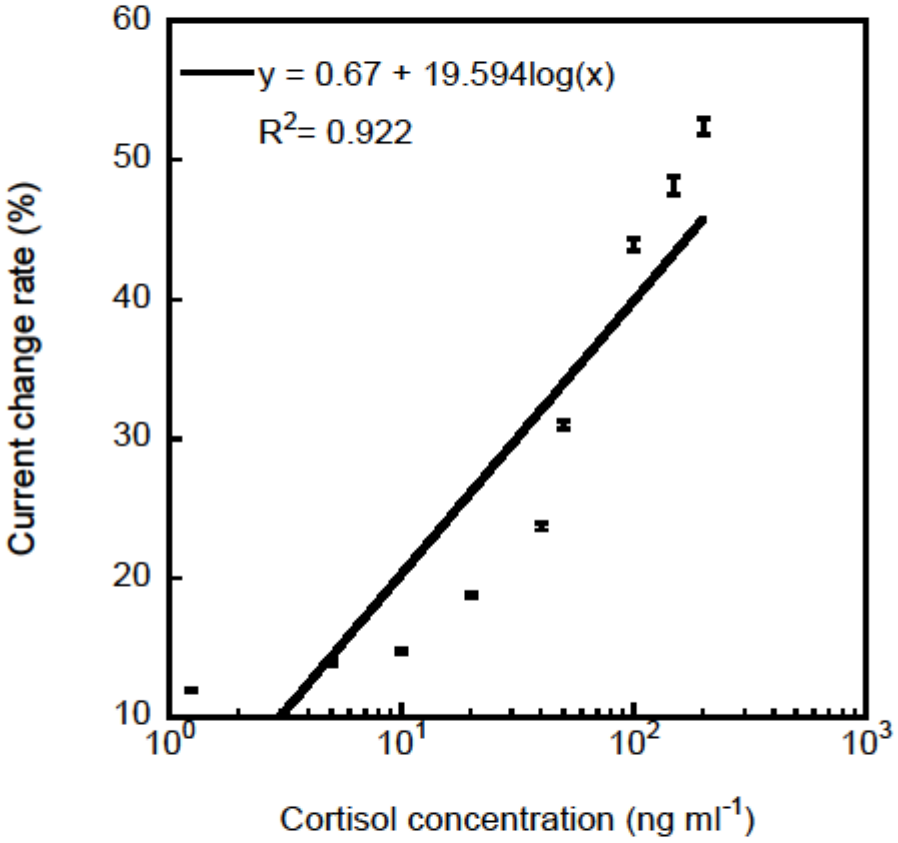

y = 0.67 + 19.594log(x)
R2= 0.922
Current change rate (%)
Cortisol concentration (ng ml-1)
60
50
40
30
20
10
100
101
102
103

Fig.5

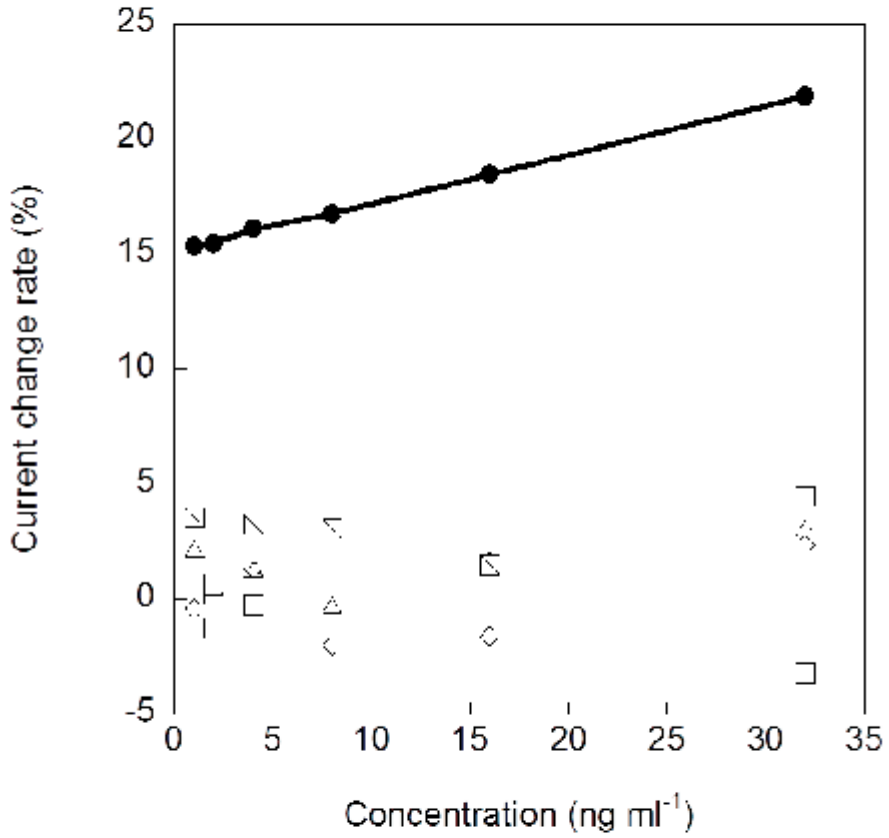
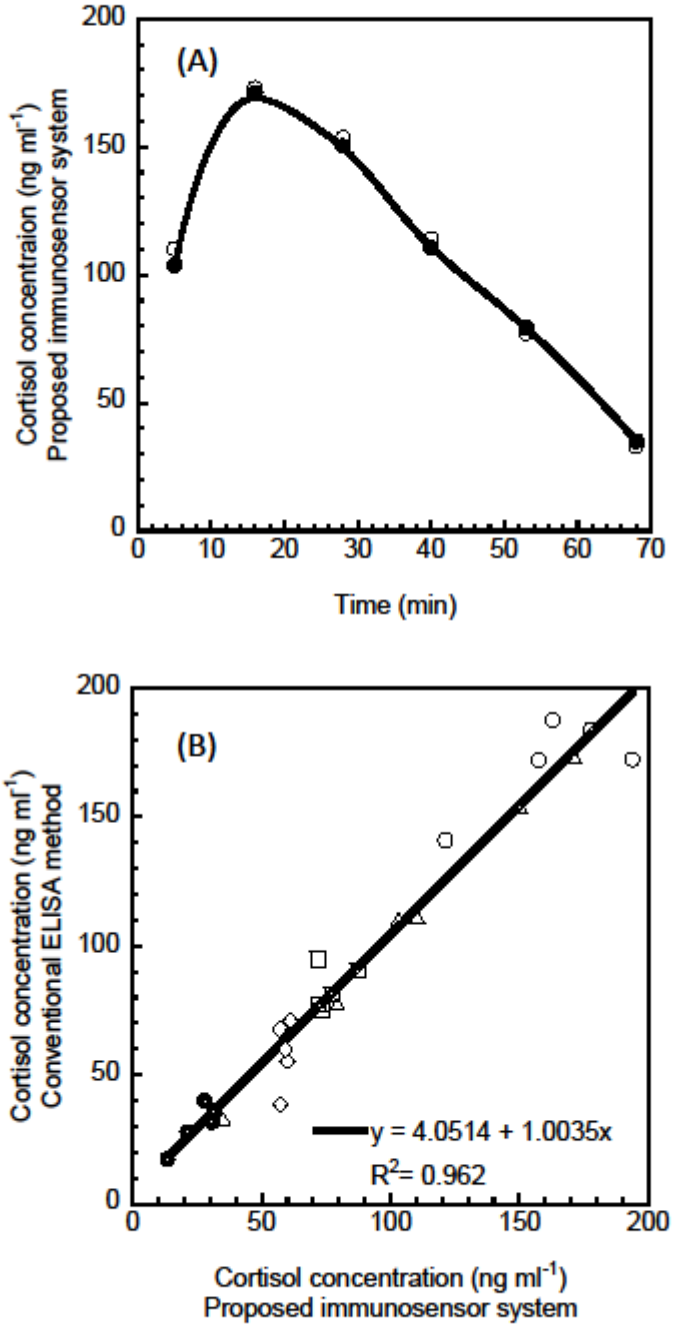

25
20
15
10
5
0
-5
0
5
10
15
20
25
30
35
Current change rate (%)
Concentration (ng ml-1)

Fig.6


(A)
Cortisol concentraion (ng ml-1)
Proposed immunosensor system
Time (min)
(B)
Cortisol concentration (ng ml-1)
Conventional ELISA method
y = 4.0514 + 1.0035x
R2= 0.962
Cortisol concentration (ng ml-1)
Proposed immunosensor system