*Sensors and Materials*, Vol. 33, No. 9 (2021) 3233–3244
MYU Tokyo



S & M 2684

# Development of Label-free Immunosensor System toward Gender Determination of Fish

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(Received July 2, 2021; accepted August 19, 2021)

***Keywords:*** 11-ketotestosterone, gender determination, fish, biosensor, immune reaction

It is imperative to discriminate between male and female fish during the juvenile stage and pre-adjust the required numbers of males and females to efficiently raise parent fish in aquaculture farms. One method to discriminate between males and females is to measure a fish-specific steroid hormone such as 11-ketotestosterone (11-KT) in fish plasma. However, conventional methods such as enzyme-linked immunosorbent assay (ELISA) are complicated to operate and require a long time for analysis. Here, we introduce a novel immunosensor system that combines immunological reaction and electrochemical measurement with the aim of achieving a rapid and straightforward measurement method in preparation for gender determination of fish. Using our system, we observed stepwise decreases in the oxidation peak current value with increasing concentration of 11-KT in the sample. Furthermore, a strong linear relationship was obtained between the standard solution concentrations of 0.21 and 3.30 pmol $ml^{-1}$ ($R$ = 0.9869) in only 20 min. Next, the optimum operating conditions of the sensor were evaluated. It was also confirmed that the sensor did not respond to other hormones. Moreover, a strong linear relationship was obtained between the values and those obtained by the conventional ELISA method ($R$ = 0.9927).

## 1. Introduction

The world's demand for seafood has been increasing yearly. However, the decline in the catch due to a decrease in the fish population has become a problem. In recent years, to overcome this problem, research on applying the complete aquaculture method has been conducted.(1) In this method, seedlings are artificially hatched from the eggs of parent fish then cultivated, without the need for natural seedlings existing in the wild.(2) In the artificial production of seedlings, the

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https://doi.org/10.18494/SAM.2021.3501

ISSN 0914-4935 

spawning of parent fish is directly linked to production efficiency, so adjusting the balance between males and females is essential. To achieve this, gender determination in the juvenile stage is essential.[3] Also, for fish species such as pufferfish and sturgeon, which have different commercial values depending on their gender, it is essential to distinguish between males and females to stably supply individuals with high commercial value.

However, some fish, including tuna, have few externally apparent gender differences, so it is challenging to distinguish their gender from their appearance, especially in the juvenile stage.[4] Moreover, discrimination between males and females by confirming the gonads by dissection is not suitable in the aquaculture industry because of the risk of injury and death. Therefore, the establishment of a new gender discrimination method has become an urgent issue.

The blood of fish contains various hormones.[5,6] Among them, 11-ketotestosterone (11-KT), which acts powerfully on biological phenomena (spermatogenesis, secondary sexual characteristics, and sexual behavior) that depend on male hormones, can be used to distinguish between males and females.[7] Moreover, 11-KT is a steroid hormone produced in the testes, and its concentration in the blood differs between males and females. Measurement of this hormone concentration is expected to help establish a new gender discrimination method independent of appearance and anatomy, enabling it to be used as an indicator of gender determination.

However, steroid hormones such as 11-KT are present only in trace amounts in biological samples or environmental water, meaning that a highly sensitive detection method is required for measurement. Therefore, a chromatographic mass spectrometer that combines liquid chromatography (LC) and gas chromatography (GC) is used to detect these substances.[8,9] Furthermore, by combining this analytical method with the above chromatography, measurements with high specificity and reproducibility are possible. However, the above methods require analyzers, which are large and expensive and have problems such as complicated pretreatment. Also, it is difficult to apply such methods to practical use in aquaculture farms because they require specialized knowledge such as selecting the optimal sample separation agent and searches for suitable measurement conditions for each sample. Enzyme-linked immunosorbent assay (ELISA) is a conventional detection method for 11-KT. ELISA uses a highly specific antigen–antibody reaction, and color measurements and luminescence based on the enzyme reaction are used as signals, enabling easy measurement.[10] However, ELISA has complicated operations such as washing, and time is required for the immune reaction. Therefore, in recent years, biosensors have attracted attention as a means of measuring specific substances in actual biological samples in a faster and easier way. Compared with the conventional method, biosensor technology has high sensitivity and specificity.[11] Some biosensor technology uses microfluidic technology,[12–14] is small enough to be portable,[15,16] and has shown potential for points of care.[17] Furthermore, some biosensors are also label-free, enabling the direct detection of target substances.[18,19] In this study, we developed a label-free immunosensor with the aim of achieving rapid and straightforward 11-KT measurement as a foundation for future point of care of fish (Fig. 1). Furthermore, as an application of the proposed sensor system to actual samples, we measured 11-KT contained in the plasma of Nile tilapia (*Oreochromis niloticus*) and compared the correlation with measured values obtained by the conventional method (ELISA).

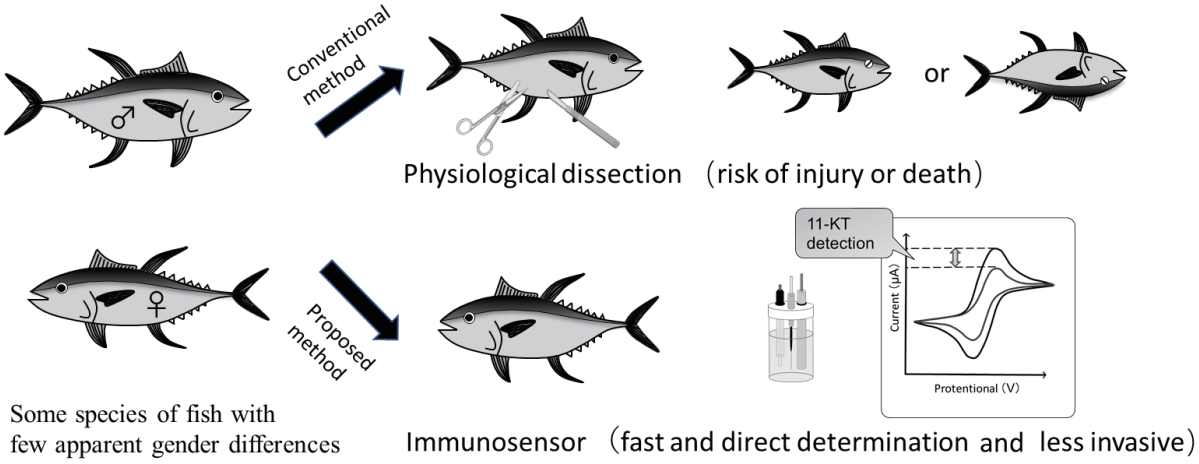



Fig. 1. Comparison of conventional and proposed methods.

## 2. Materials and Methods

### 2.1 Reagents

11-Ketotestosterone (11-ketotestosterone EIA Standard), 11-ketotestosterone antibody (11-ketotestosterone EIA Antiserum: 11-KT antiserum), 11-ketotestosterone acetylcholinesterase-labeled antigen (11-ketotestosterone AChE Tracer: 11-KT AChE-labeled antigen), and testosterone (Testosterone EIA Standard) were purchased from Cayman Chemical (Ann Arbor, MI, USA). 3-Mercaptopropionic acid (MPA), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethylenediaminetetraacetic acid, and tetrasodium salt hydrate were purchased from Sigma (St. Louis, MO, USA). 2-Phenoxyethanol and heparin sodium were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents used for the experiments were commercial or laboratory grade.

### 2.2 Test fish and actual sample preparation

Nile tilapia (*Oreochromis niloticus*) were used as the test fish, which were cultivated in Tokyo University of Marine Sciences and Technology and kept in a water tank (1200 × 600 × 450 $mm^3$, 324 L) containing around 25 fish at all times. The water used for breeding (breeding water) in the aquarium was neutralized tap water. The breeding water was maintained at a temperature of 23 ± 0.5 °C, constantly filtered by an upper filtration tank (physical filtration and biological filtration), aerated by an air pump, and illuminated for 9 h daily (9:00–18:00).

For actual sample preparation, a test fish was captured, and 2-phenoxyethanol solution was dissolved in 5 L of the breeding water to prepare an anesthetic solution of around 350 ppm. Anesthesia was performed by immersing the individual in the prepared anesthetic solution.

Next, after attaching a 23G syringe needle (0.60 × 25 $mm^2$) to a 2.5 ml Terumo syringe (Terumo, Tokyo), the syringe was filled with 3000 units $ml^{-1}$ heparin sodium solution. An anesthetized individual was laid on a wet towel moistened with the anesthetic solution, and another wet towel moistened with the anesthetic solution was placed on the head of the individual. After that, an injection needle was inserted from the base of the heel toward the vertebra, and blood was collected from the tail artery and vein. The collected blood was dispensed into microtubes and immediately centrifuged (450 × *g*, 3000 rpm, 10 min, 4 °C) to collect supernatant plasma. Plasma samples were stored at −20 °C until use.

### 2.3 Preparation of the immunosensor

Firstly, a φ 6.0 mm disk-type gold electrode (BAS, Tokyo) was swept 50 times in 0.1 M KOH solution (sweep range: −1.2 to −0.2 V) to remove thiol groups from the electrode surface. Next, the electrode surface was polished with φ 1.0 μm diamond and φ 0.05 μm alumina solution (BAS, Tokyo) and then ultrasonically cleaned in absolute ethanol followed by distilled water for 5 min each. In addition, the electrode was swept 50 times repeatedly in 0.5 M dilute sulfuric acid solution degassed with nitrogen gas for 15 min (sweep range: −0.2 to +1.6 V).

The pretreated gold electrode was then immersed in 10 mM MPA solution for 24 h at room temperature to form a self-assembled monolayer (SAM) on the detection surface. Next, the gold electrode on which the SAM was formed was immersed in 0.25 ml of 200 mg $ml^{-1}$ EDC solution (dissolved in 0.1 M MES solution, pH 5.5) for 15 min. After that, the solution was mixed with 0.25 ml of 200 mg $ml^{-1}$ NHS solution (dissolved in 0.1 M MES solution, pH 5.5) for 105 min, resulting in the formation of a carboxyl group with a highly active ester group. After that, it was immersed in 0.5 ml of 12 μg $ml^{-1}$ anti-rabbit IgG antibody solution at 25 °C for 2 h. Furthermore, the gold electrode was immersed in 0.5 ml of 1% BSA solution for 2 h (room temperature) for blocking. Finally, it was immersed in 0.25 ml of anti-11-KT antiserum at 4 °C for 12 h. Considering the activity of the antibody, we kept the sensor in the freezer for up to 7 days before testing.

### 2.4 Measurement

The immunosensor (working electrode, prepared in advance), a 5.0 cm platinum wire (counter electrode, φ 6.0 mm), and a silver/silver chloride (Ag/AgCl) electrode (reference electrode, manufactured by BAS, Tokyo, Japan) were used in the measurement. These electrodes were then immersed in 15 ml of 5.0 mM potassium ferrocyanide degassed with nitrogen gas for 30 min. After that, all the electrodes were connected to an electrochemical analyzer. To reduce noise, the measurement cell was stored in a cell stand, and cyclic voltammetry (CV) measurement was performed (sweep range: −0.2 to +0.6 V, sweep speed: 0.1 V $s^{-1}$, sweep cycle: 30 times). Before each subsequent measurement, we only needed to replace the prepared working electrode.

### 2.5 Effects of various conditions on output current of proposed sensor system

#### 2.5.1 Effect of immersion time in MPA solution on rate of current decrease of sensor

The immersion time of the pretreated gold electrode in 0.5 ml of MPA solution was set to 8 or 24 h. All the other steps for the fabrication of the sensor were performed as described above. After that, the prepared sensor was immersed in 5.0 mM potassium ferrocyanide solution, and CV measurement was performed before and after the antigen–antibody reaction.

#### 2.5.2 Effect of antigen–antibody reaction temperature on rate of current decrease of sensor

The prepared sensor was immersed in 0.5 ml of 11-KT standard solution (0.83 pmol $ml^{-1}$, pH 7.0) in a water bath at 20, 25, 30, and 35 °C and allowed to undergo the antigen–antibody reaction for 10 min. Then, CV measurement was performed in 5.0 mM potassium ferrocyanide solution.

#### 2.5.3 Effect of antigen–antibody reaction pH on rate of current decrease of sensor

A 0.83 pmol $ml^{-1}$ 11-KT standard sample was prepared using 0.1 M PB at pH 6.0, 6.5, 7.0, and 7.5. Next, the gold electrode was immersed in 0.5 ml of an 11-KT standard sample at each pH in a thermostatic chamber at 25 °C and allowed to undergo the antigen–antibody reaction for 10 min. Then, CV measurement was performed in 5.0 mM potassium ferrocyanide solution.

### 2.6 Calibration curve of sensor

The gold electrode was immersed in 0.5 ml of a prepared 11-KT standard sample (0.21–3.30 pmol $ml^{-1}$) in a water bath at 25 °C and allowed to undergo the antigen–antibody reaction for 10 min. Then, CV measurement was performed in 5.0 mM potassium ferrocyanide solution.

### 2.7 Effect of various steroid hormones on rate of current decrease of proposed sensor

Various steroid hormones (androstenedione, testosterone, and 11β-hydroxytestosterone) were prepared with concentrations of 0.21 to 3.30 pmol $ml^{-1}$ using PB (1.0 M, pH 7.4). A gold electrode was immersed in 0.5 ml of various steroid hormone solutions prepared in a water bath at 25 °C and allowed to undergo the antigen–antibody reaction for 10 min. Then, CV measurement was performed in 5.0 mM potassium ferrocyanide solution.

### 2.8 Measurement of 11-KT concentration in fish plasma using proposed sensor system

The plasma sample was collected and diluted fivefold with PB (1.0 M, pH 7.4). Next, the sensor was immersed in 0.5 ml of the diluted plasma sample and allowed to undergo the antigen–

antibody reaction for 10 min. Then, CV measurement was performed in 5.0 mM potassium ferrocyanide solution.

## 3. Results and Discussion

### 3.1 Response characteristics of label-free immunosensor

As described in Sect. 2, the preparation of the proposed sensor involved the formation of a SAM on the electrode surface by MPA, binding of anti-rabbit IgG antibody to the SAM by the EDC/NHS reaction, blocking by BSA, and reaction with the anti-11-KT antibody. These four steps of physicochemical modification were performed on the gold electrode. Therefore, the presence or absence of modification of each molecular layer on the electrode surface can be confirmed by electrochemically analyzing the fluctuation of the oxidation peak current value at each modification stage using CV. The results are shown in Fig. 2.

This figure confirmed that the oxidation peak current decreased with increasing number of substances modified on the electrode surface. This result is thought to be caused by the inhibition of electron transfer between the electrode surface and the potassium ferrocyanide solution due to the immobilization of some electrically inactive substances such as MPA and antibody. The above results confirmed that each molecular layer on the electrode surface was modified and that a label-free immunosensor could be easily fabricated.

Next, to confirm the response of the prepared immunosensor, CV measurement was performed when the electrode was immersed in a 0.83 pmol $ml^{-1}$ 11-KT standard sample for 10 min. The results are shown in Fig. 3.

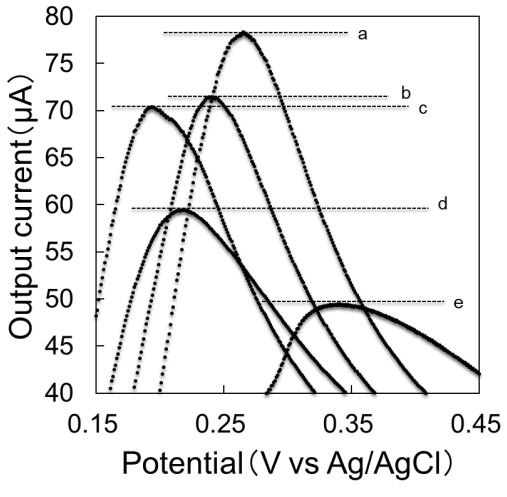



Fig. 2. Electrochemical evaluation of sensors. Measurement conditions (25 °C, pH 7.0, −0.2–+0.6 V, 0.1 V $s^{-1}$). (a) Au electrode; (b) SAM/Au electrode; (c) IgG/SAM/Au electrode; (d) BSA/IgG/SAM/Au electrode; and (e) Anti-11-KT antibody/BSA/IgG/SAM/Au electrode.

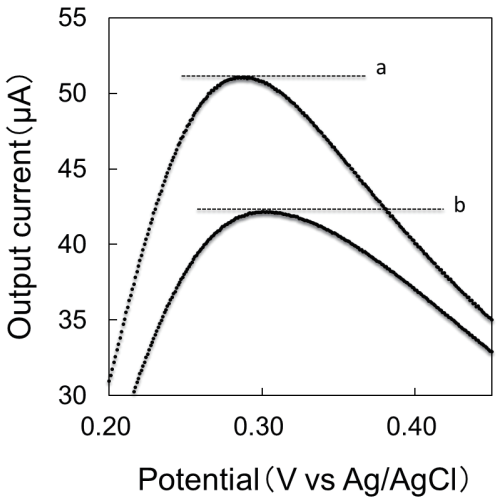



Fig. 3. Sensor response to 11-KT. Measurement conditions (25 °C, pH 7.0, −0.2–+0.6 V, 0.1 V $s^{-1}$). (a) Blank (anti-11-KT antibody/BSA/IgG/SAM/Au electrode) and (b) sensor response (11-KT/anti-11-KT antibody/BSA/IgG/SAM/Au electrode).

From this figure, a significant decrease in the oxidation peak current was confirmed between the CV curve before immersion in the 11-KT standard sample and the CV curve after immersion. The anti-11-KT antibody and 11-KT formed an electrically inactive antigen–antibody complex that inhibited electron transfer between the electrode surface and the potassium ferrocyanide solution. Therefore, the oxidation peak current before the antigen–antibody reaction was defined as a blank value. The value obtained by subtracting the oxidation peak current after the antigen–antibody reaction from this value was defined as the current decrease. The rate of current decrease was then calculated as current decrease/blank value × 100. We used this rate of current decrease as the response value of the sensor.

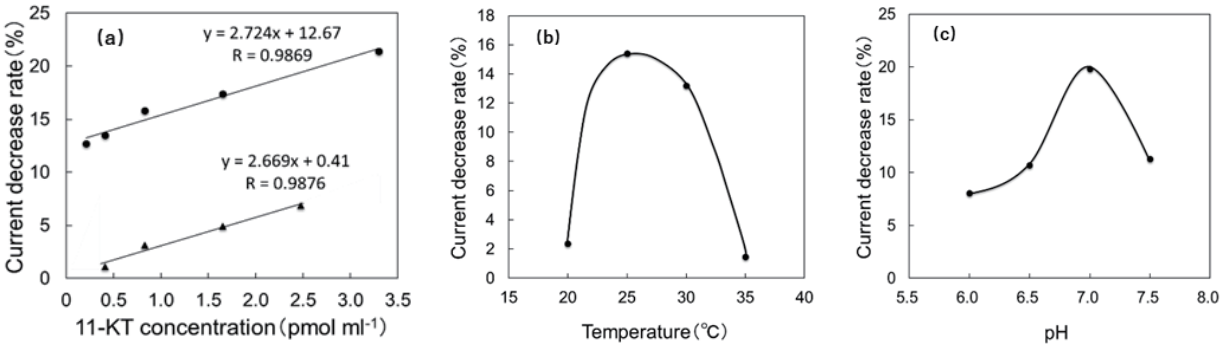
### 3.2 Effects of various conditions on output current of proposed sensor system

#### 3.2.1 Effect of immersion time in MPA solution on rate of current decrease of sensor

To investigate the effect of the immersion time in the MPA solution on the response value of this sensor, the sensor was fabricated with the immersion time set to 8 or 24 h, and the response value after the antigen–antibody reaction was measured. The result is shown in Fig. 4(a). A higher response value was observed when the immersion time was 24 h. The orientation of the SAM formed on the electrode surface increased with the duration of immersion in the MPA solution, and the amount of antibody that can bind increased, resulting in more antigen–antibody reaction with 11-KT.[20] As a result, electron transfer was inhibited.

#### 3.2.2 Effect of antigen–antibody reaction temperature on rate of current decrease of sensor

To investigate the effect of the antigen–antibody reaction temperature on the response value of this sensor, 11-KT standard solution (concentration: 0.83 pmol ml$^{-1}$) was used, and the reaction solution temperature was set to four values from 20 to 35 °C. The results of the




(a) (b) (c)

Fig. 4. Optimal operation condition of the sensor. (a) Effect of immersion time in MPA solution (pH 7.0, 25 °C, 10 min, −0.2 to +0.6 V, 0.1 V s$^{-1}$, 0.83 pmol ml$^{-1}$). ●: 24 h immersion ▲: 8 h immersion, (b) effect of antigen-antibody reaction temperature (pH 7.0, 10 min, −0.2–+0.6 V, 0.1 V s$^{-1}$, 0.83 pmol ml$^{-1}$), (c) effect of pH of antigen-antibody reaction (25 °C, 10 min, −0.2 to +0.6 V, 0.1 V s$^{-1}$, 0.83 pmol ml$^{-1}$).

measurement are shown in Fig. 4(b). From this figure, the response value increases up to a temperature of 25 °C. This is thought to be because up to 25 °C, the rate of molecular motion of the antigen increases with the reaction temperature, and the probability of a reaction between the antigen and antibody increases. On the other hand, the response value decreased from 30 °C. This is thought to be because the antibody immobilized on the electrode surface and the antigen in the reaction solution were denatured as the temperature increased, or the affinity between the antibody and the antigen decreased, and the amount of antigen–antibody complex on the electrode surface decreased. Therefore, the optimal reaction temperature during the antigen–antibody reaction was set at 25 °C.

#### 3.2.3 Effect of antigen–antibody reaction temperature on rate of current decrease of sensor

To investigate the effect of the antigen–antibody reaction pH on the response value of the sensor, 11-KT standard solution (concentration: 0.83 pmol $ml^{-1}$) was used, and the pH of the reaction solution was set to four values from 6.0 to 7.5. The results of the measurements are shown in Fig. 4(c). The highest response value was observed at pH 7.0. Proteins such as antibodies are ampholytes composed of amino acid polypeptide bonds, and the isoelectric point of IgG, such as the anti-11-KT antibody, is around pH 7.0.[7,21] When an antigen and an antibody react in a solution having a pH higher than the isoelectric point, the electric attraction between the antigen and antibody becomes stronger, and the formation of the antigen–antibody complex is promoted. This suggests that the response value increased until pH 7.0. On the other hand, a decrease in the response value was confirmed at pH 7.5. This is thought to be because the affinity of the antibody immobilized on the electrode to the antigen decreased as the pH of the antigen solution increased. Therefore, in this experiment, the optimum reaction pH during the antigen–antibody reaction was set to 7.0.

### 3.3 Calibration curve of 11-KT standard sample

The calibration curve of the proposed sensor was prepared using the 11-KT standard sample under the above optimal operation conditions. The results are shown in Fig. 5. From this figure, an excellent linear relationship ($R$ = 0.9869) was confirmed in the concentration range of 11-KT standard sample of 0.21 to 3.30 pmol $ml^{-1}$. From the measurement results of ELISA, the plasma 11-KT concentration of male tilapia is 1.79–9.90 pmol $ml^{-1}$,[22] so when measuring a plasma sample with a sensor, it should be diluted fivefold to ensure that the concentration is within the measurable range of the sensor.

### 3.4 Specificity of the proposed sensor

In addition to 11-KT, there is a wide variety of steroid hormones in fish plasma,[23] including testosterone,[24] which regulates spermatogenesis and secondary sexual development in the testes of fish, androstenedione,[25] a precursor of testosterone, and 11β-hydroxytestosterone,[26] a

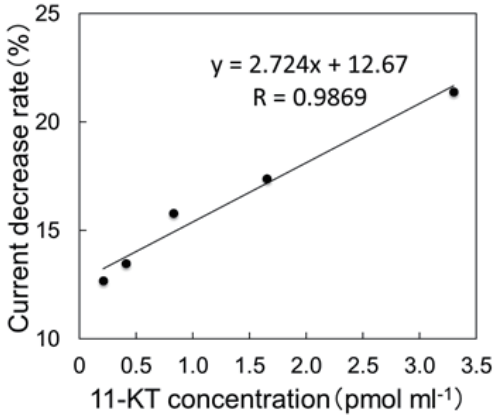



Fig. 5. Calibration curve of 11-KT (concentration of 11-KT: 0.21 to 3.30 pmol $ml^{-1}$, pH 7.0, 25 °C, 10 min, −0.2–+0.6 V, 0.1 V $s^{-1}$).

precursor of 11-KT. Therefore, in the actual measurement of 11-KT in the plasma of fish, it is necessary to examine the effect of the steroid hormones on the sensor response value. The results are shown in Fig. 6. From this figure, 11β-hydroxytestosterone showed a higher response value than testosterone and androstenedione. This may be because 11β-hydroxytestosterone is a precursor of 11-KT and is the closest structure to 11-KT among the steroid hormones. However, its response value is minimal compared with that of 11-KT, and the influence on the measurement is considered small. In addition, since testosterone is contained in fish plasma samples at a higher concentration than 11-KT, the effect on the sensor response value was examined using a standard testosterone solution with a concentration of 33.0 pmol $ml^{-1}$. The response value of the sensor was only 1.79% of that for 11-KT, suggesting its minimal effect on measurement results. Therefore, it was clarified that the proposed sensor system has high specificity for 11-KT and that its quantification is possible.

### 3.5 Application to actual fish samples

As an application to actual samples, we measured the plasma 11-KT concentration of fish (tilapia) using the proposed sensor and compared it with the measurement result of the conventional method (ELISA). The results are shown in Fig. 7. When the measured values of this system were compared with those of ELISA, a strong linear relationship was obtained ($R$ = 0.9927). The plasma 11-KT values for male tilapia ranged from 1.79 to 9.40 pmol $ml^{-1}$. On the other hand, the values for females were excluded from this figure because they were below the measurement limit of this system (0.21 pmol $ml^{-1}$). This is thought to be because the amount of 11-KT contained in the plasma of female tilapia is much less than that in the plasma of males. The value of 11-KT measured by ELISA was 0.035 pmol $ml^{-1}$.

On the other hand, the 11-KT value for plasma obtained with this system was slightly smaller than the ELISA measurement result. This is probably because the antigen–antibody reaction time of this system was very short compared with that of ELISA, and the amount of antigen–

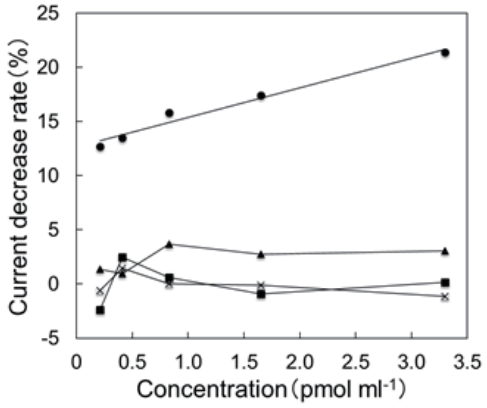



Fig. 6. Specificity of the sensor (concentration of hormones: 0.21 to 3.30 pmol $ml^{-1}$, pH 7.0, 25 °C, 10 min, −0.2–+0.6 V, 0.1 V $s^{-1}$), ●: 11-KT, ▲: 11β-hydroxytestosterone, ■: testosterone, ×: androstenedione.

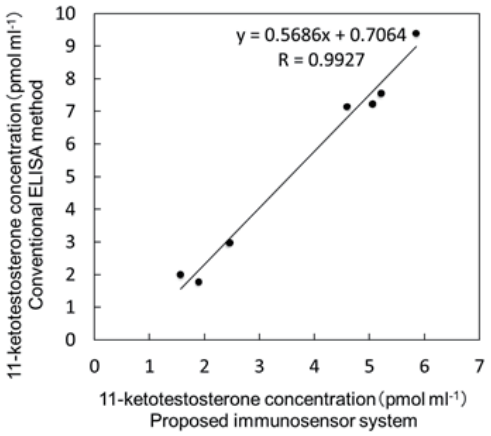



Fig. 7. Correlation between values measured by this sensor and ELISA ($n$ = 7).

antibody complex formed was small. However, this method can measure the 11-KT concentration in fish plasma more quickly and efficiently than the conventional method, suggesting the possibility of establishing a method for the rapid gender determination of fish.

## 4. Conclusions

A measurement method that can quickly and easily measure 11-KT, which is an indicator of the gender of fish, was developed. A SAM was used to immobilize an antibody on an electrode surface, and the detection of 11-KT was attempted by electrochemically measuring the inhibition of electron transfer by the antigen–antibody complex formed by the antigen–antibody reaction by CV. Firstly, the effects of various conditions (reaction temperature, pH, immersion time of SAM) on the antigen–antibody reaction of this sensor were examined. The optimal reaction temperature was 25 °C, the optimal pH was 7.0, and the optimal immersion time in the SAM was 24 h. Secondly, we attempted to find the quantification limit using an 11-KT standard sample. The dynamic range of the sensor for 11-KT was 0.21 to 3.30 pmol $ml^{-1}$ with a good correlation coefficient ($R$ = 0.9869). The specificity of this sensor was also examined using other steroid hormones (testosterone, androstenedione, 11β-hydroxytestosterone) that are present in fish plasma. There was almost no change in the response value even at relatively high concentrations of these hormones. Finally, as an application to actual samples, the proposed sensor was used to measure the 11-KT concentration in tilapia plasma and the measurement results were compared with those obtained by the conventional ELISA method, and a high correlation coefficient of 0.9927 was obtained. Possible future improvements include improved gender discrimination by expanding the dynamic range, simplifying the sensor fabrication process by directly immobilizing anti-11-KT on the SAM, and better response values by suppressing nonspecific adsorption.

## Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (B) (KAKENHI, Grant No. 21H02282) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We wish to thank Prof. Pierre Morizet-Mahoudeaux (University of Technology of Compiègne, France) and Kyoko Hibi (Kokugakuin Tochigi Junior College) for their helpful discussions.

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