Development of biosensor for measuring oxidative stress of fish

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Abstract

To elucidate the dynamics of oxidative stress in fish, it is necessary to know the concentration of superoxide anions as a precursor to various reactive oxygen species in the living body. Superoxides are unstable radicals, disappearing within a few seconds, which makes it challenging to measure them using conventional methods. We describe herein the development of a biosensor that can rapidly quantify superoxide concentrations in biological samples. The sensor was fabricated by immobilizing cytochrome c as an electron transfer element on the surface of a gold electrode as a self-assembled monolayer. The fabricated sensor was immersed in hypoxanthine solution as a working electrode with an Ag/AgCl counterelectrode. Xanthine oxidase standard solution at different concentrations was then added to generate superoxides. The sensor output correlated well with the superoxide concentration in the range of 0.58–9.33 μM . To confirm the specificity, various substances that may affect the sensor response were examined, revealing little effect on the sensor output. The sensor was applied to eyeball interstitial sclera fluid of Nile tilapia *Oreochromis niloticus* and exhibited acceptable performance.

Keywords Oxidative stress · Biosensor · Superoxide · Fish · Nile tilapia · *Oreochromis niloticus*

Introduction

Atmospheric oxygen is necessary for aerobic organisms to efficiently produce adenosine triphosphate (ATP) by respiration. During metabolism, some of the incorporated oxygen is converted to highly oxidative byproducts called reactive oxygen species (ROS). In addition, when foreign substances such as bacteria invade, ROS are actively produced by the

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system of neutrophils and macrophages immune cells, which play a central role in phagocytosis (Tan and Berridge 2000). Thus, ROS are constantly generated in vivo by physiologic factors, but they are usually regulated to maintain proper levels by decomposition and elimination via enzymatic and nonenzymatic antioxidant mechanisms. When excessive ROS are generated or when the antioxidant capacity is reduced, oxidative damage can occur to biologic components such as proteins, sugars, lipids, and nucleic acids. These phenomena are also caused by pathologic factors, ischemia–reperfusion injury, and external factors (e.g., chemical substances, ultraviolet rays, and radiation) (Kakuta and Sengoku 2009; Slegtenhorst et al. 2014; Gobi et al. 2018). An imbalance between the formation of ROS and their elimination or loss via antioxidant defense mechanisms is referred to as oxidative stress (Weseler and Bast 2010).

Research on oxidative stress has been actively conducted in humans and mice, mainly in fields such as pathophysiology, clinical medicine, and animal husbandry, but in recent years, this interest has spread to include the field of fisheries science; For example, in a study of coho salmon *Oncorhynchus kisutch*, excessive administration of oxytetracycline, which is commonly used as an antibiotic against bacterial

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diseases in farmed fish, could accelerate oxidization in fish and damage liver tissue in particular (Nakano et al. 2018). In addition, bisphenol A, which is used as a raw material for polycarbonate resins and epoxy resins worldwide, also induced oxidative stress and reduced the expression of growth-related factors and food intake in Nile tilapia *Oreochromis niloticus*, resulting in liver and kidney dysfunction (Tawwab and Hamed 2018). Besides, heat stress due to a sudden change in water temperature (Nakano et al. 2014) and ingestion of lipid peroxide contained in degraded feed also led to induction of oxidative stress, impaired growth, sickness, and hemolysis. Therefore, understanding the dynamics of oxidative stress in fish, mainly in the aquaculture industry, is important for sound breeding management, efficient production, and quality improvement.

ROS, a major cause of oxidative stress, is a general term for highly reactive substances containing oxygen in a broad sense, but more specifically superoxide anions (O_2^-), hydrogen peroxide, hydroxyl radicals, and singlet oxygen. Among these, O_2^- is a precursor to many other ROS, such as hydrogen peroxide, hydroxyl radicals, and peroxynitrite (Cai 2005). Therefore, accurately measuring the amount of O_2^- in the living body is extremely important to clarify the degree of oxidative stress. O_2^- is an unstable radical generated by one-electron reduction of normal oxygen molecules. In vivo, the mitochondrial respiratory chain electron transport system, xanthine oxidase produced in response to ischemia–reperfusion injury, and NADPH oxidase in phagocytes generate O_2^- . The physicochemical half-life of O_2^- in the physiologic aqueous space is approximately 5 s (Ewing and Janer 1995). Due to this short half-life, it is difficult to detect O_2^- directly in real samples.

The main conventional methods for measuring O_2^- include absorptiometry, chemiluminescence, and electron spin resonance (Tarpey and Fridovich 2001). The absorptiometry method is the simplest, because the measuring instruments and reagents are inexpensive, but it suffers from many problems related to selectivity and sensitivity. The chemiluminescence method can be applied to biological samples because it is inexpensive, simple, and more sensitive than the absorptiometry method, but its selectivity may be poor depending on the reagents and experimental conditions used. The electron spin resonance method has the highest specificity for monitoring O_2^- , but it is inconvenient because it requires a large measuring device and long-term measurements. In addition, spin-trapping agents used are expensive and toxic. Because these conventional methods involve complicated operations, tens of minutes are required for such measurements, making it difficult to quantify O_2^- accurately, due to its short half-life. In addition, because these methods are based on in vitro measurements, it is very difficult to measure changes in O_2^- in the living body over time.

In the present study, to establish an in vivo measurement system, we developed an electrochemical biosensor that

directly and rapidly detects O_2^- . This biosensor utilizes immobilized cytochrome c (Cyt c), which has high reactivity with O_2^- ; the electrons emitted from O_2^- are immediately captured by Cyt c to continuously generate O_2 . We further investigated the in vivo applicability of this sensor using various substances contained in biologic samples. Lastly, the sensor was applied to eyeball interstitial sclera fluid (EISF) of Nile tilapia *Oreochromis niloticus* to evaluate the performance of the sensor in vivo.

The measurement principle of the O_2^- biosensor is shown in Fig. 1. First, an electron transfer protein, Cyt c, is immobilized on the surface of a gold electrode using the self-assembled monolayer (SAM) method to fabricate a biosensor for O_2^- measurement. When this sensor is connected to a potentiostat and immersed in a sample solution, the oxidized Cyt c before the reaction changes to reduced Cyt c via one-electron reduction due to O_2^- in the sample. Furthermore, by applying a voltage of +150 mV to the electrode, the reacted Cyt c is returned to the oxidized form, generating one electron. Repeating this cycle while measuring the amount of electrons generated on the gold electrode enables continuous quantification of O_2^- .

Materials and methods

Reagent

Cytochrome c (Cyt c, from bovine heart), xanthine oxidase (XOD, 0.5 U mg^{-1} , from bovine milk), hypoxanthine (Hx), 3-mercaptopropionic acid (MPA), 3-mercapto-1-propanol (MPO), 11-mercaptoundecanoic acid (MUA), 11-mercapto-1-undecanol (MUD), *N*-hydroxysuccinimide (NHS), and bovine serum albumin (BSA) were purchased from Millipore

Fig. 1 Measurement principle of biosensor

Sigma (St. Louis, MO, USA). Superoxide dismutase (SOD, 4780 U mg⁻¹, from bovine erythrocytes) and β -nicotinamide adenine dinucleotide disodium (reduced form) were manufactured by Nacalai Tesque, Inc. (Kyoto, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 2-morpholinoethanesulfonic acid monohydrate (MES) were manufactured by Dojindo Laboratories (Kumamoto, Japan). Potassium hydroxide (KOH), sodium hydroxide (NaOH), potassium chloride (KCl), potassium dihydrogen phosphate (KH₂PO₄), disodium hydrogen phosphate dodecahydrate (Na₂HPO₄·12H₂O), D(+)-glucose (C₆H₁₂O₆), and citric acid monohydrate (C₆H₈O₇·H₂O) were special grade reagents manufactured by Kokusan Kagaku Co., Ltd. (Tokyo, Japan). The 95% sulfuric acid (H₂SO₄), sodium chloride (NaCl), 30% hydrogen peroxide (H₂O₂), uric acid (C₅H₄N₄O₃), ascorbic acid (C₆H₈O₆), and ultrapure water for liquid chromatography–mass spectrometry (LC–MS) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Biosensor fabrication

Electrode pretreatment

A Ø3.0-mm disk-shaped gold electrode (BAS, Tokyo, Japan) was polished using Ø3.0 μ m and Ø1.0 μ m diamond paste, and Ø0.05 μ m alumina solution (BAS). Next, ultrasonic cleaning was performed in both distilled water and absolute ethanol for 5 min each. The thiol group was then desorbed from the electrode surface by 50 sweeps (range -1.2 to -0.2 V, rate 0.1 V s⁻¹) in 0.1 M potassium hydroxide solution. Furthermore, electrochemical cleaning was performed to remove the oxide film on the electrode surface by repeating the 50 sweeps (range -0.2 to 1.5 V, rate 0.1 V s⁻¹) in 0.5 M dilute sulfuric acid.

Immobilization of Cyt c

The sensor fabrication procedure is shown in Fig. 2; refer to our previous research (Endo et al. 2012; Wu et al. 2020) for more details. The pretreated gold electrode was immersed in 0.5 ml SAM solution at room temperature for 24 h to form a highly dense and highly oriented thin film of alkanethiol on the electrode surface. Next, this electrode was immersed in 0.25 ml EDC solution (200 mg ml⁻¹) for 15 min, and after addition of 0.25 ml NHS solution (200 mg ml⁻¹), it was further immersed for 105 min. The terminal carboxyl group was replaced with a highly active ester group. It was then immersed in 0.5 ml Cyt c solution (200 μ M) prepared with PBS (pH 7.4) and stirred at 4 °C for 14 h to immobilize Cyt c. Finally, it was immersed in 0.5 ml BSA solution (1.0 mg ml⁻¹) for 2 h for blocking, resulting in the biosensor for O₂⁻ measurement.

Amperometric measurement

The Hx/XOD enzyme reaction system was used for the O₂⁻ generation system. This reaction is one of the oxidative reactions occurring in the body, where XOD in cytoplasm and blood rapidly transforms Hx (produced during ATP decomposition) to xanthine and uric acid. Two molecules are produced in each reaction step, resulting in a total of four O₂⁻. Therefore, in this study, the amount of O₂⁻ produced was controlled by changing the amount of XOD solution added to the sample solution.

For the measurement, a cell containing 10 ml Hx solution (300 μ M) was first placed on a waterproof stir plate in a constant-temperature (35 °C) bath, and the fabricated O₂⁻ biosensor (working electrode) and Ag/AgCl reference electrode (BAS, Tokyo, Japan) were immersed in the Hx solution. These electrodes were connected to a potentiostat,

Fig. 2 Sensor fabrication procedure. ● indicates Cyt c, and ■ indicates BSA

and after applying a voltage of +150 mV and waiting for the output current value to stabilize, the measurement was started.

Comparison of SAM solution combinations used in sensor fabrication

In this study, Cyt c was immobilized on the surface of a gold electrode by SAM. In general, SAM has a three-part structure consisting of a “bonding group” that binds to the surface of the substrate, a “functional head group” that has a functional group such as carboxyl or amino, and an “alkyl chain” that connects them. SAMs with various surface properties are formed depending on the alkyl chain length and type of head group. In this experiment, we prepared four types of SAM (MPA, MPO, MUA, and MUD) solutions with different alkyl chain lengths and head groups, and examined the optimum SAM solution to prepare the sensor.

The fabricated sensor and Ag/AgCl electrode were immersed in Hx solution (300 μM , 10 ml, 35 $^{\circ}\text{C}$), each was connected to a potentiostat, and a voltage of +150 mV was applied. The characteristics of each sensor were then compared by measuring the output current value at each XOD concentration by adding XOD solution dropwise so that the XOD concentration in the Hx solution ranged from 10 to 80 mU ml^{-1} , generating O_2^- .

Confirming the sensor performance

A Cyt c-immobilized sensor was prepared using the SAM solution determined in the optimal condition. The fabricated sensor was immersed in Hx solution (300 μM , 10 ml, 35 $^{\circ}\text{C}$), connected to a potentiostat, and a voltage of +150 mV was applied. Then, XOD solution was added dropwise so that the XOD concentration in the Hx solution ranged from 1.25 to 80 mU ml^{-1} , generating O_2^- . The output current value at each XOD concentration was measured. The output current value before adding XOD solution was used as the blank value, and the value obtained by subtracting the blank value from the values measured at 7 s after addition were used as the measured values to plot a calibration curve.

Additional items related to sensor specificity and measurement value correction

The fabricated sensor was immersed in Hx solution (300 μM , 10 ml, 35 $^{\circ}\text{C}$) and connected to a potentiostat, and a voltage of +150 mV was applied. XOD solution was added dropwise so that the XOD concentration in the Hx solution was 20 mU ml^{-1} , generating O_2^- . After 1 min, various solutions considered to be interfering substances were added at the concentrations presented in Table 1.

Table 1 Concentration of each substance in Hx solution

	Concentration in Hx solution (μM)	Dropping amount (μl)
H_2O_2	57.00	5.84
Glucose	2,775.00	101.30
NADH (red)	1.41	14.16
Uric acid	416.39	435.73
Citric acid	123.73	125.64
Ascorbic acid	5.86	5.70

After 1 min, 40 μl SOD solution (4,780 U ml^{-1}) prepared with PBS (pH 7.4) was added dropwise, and the fluctuation of the output current was measured. SOD is an enzyme that specifically eliminates O_2^- , catalyzing the disproportionation reaction of O_2^- in Eq. 1.

Measurement using actual samples

Test fish

The test fish used was Nile tilapia, cultivated at Tokyo University of Marine Science and Technology. An upper filtration tank (RF-120; Iwaki Co., Ltd., Tokyo, Japan) was installed on a water tank (1200 mm $\times$ 600 mm $\times$ 450 mm; Kotobuki Co., Ltd., Nara, Japan) with continuous aeration using an air pump. The tank density was approximately 20 fish. The room temperature was set to 25 $^{\circ}\text{C}$, with 9 h photoperiod (09:00–18:00). This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of Tokyo University of Marine Science and Technology.

EISF collection

After attaching a Terumo injection needle to a Terumo syringe, an appropriate amount of heparin sodium solution was withdrawn into the syringe. EISF (0.2 ml) was collected by inserting the needle toward the outer membrane of the eyeball of the fish. The collected EISF was dispensed into an Eppendorf tube and stored in a refrigerator (4 $^{\circ}\text{C}$).

Measurement of actual samples

The fabricated sensor immersed in 10 ml PBS was connected to a potentiostat, and a voltage of +150 mV was applied. EISF solution (0.2 ml, 35 $^{\circ}\text{C}$, samples a and b from individual A and B with size of 30.3 and 28.4 cm, respectively) was then added. After 1 min, 40 μl SOD solution (10

U ml⁻¹) prepared with PBS (pH 7.4) was added dropwise, and the output current was monitored.

Results

SAM solutions used for sensor fabrication

The results are shown in Fig. 3. We confirmed that sensors a and b using MPA and MPO with short alkyl chain length showed a gradual increase in the output current as the XOD concentration was increased. On the other hand, for sensors c and d using MUA and MUD with relatively long alkyl chain length, the output current decreased as the XOD concentration was increased from 60 to 80 mU ml⁻¹, and the overall output current was also five or six times lower than measured using sensor a or b.

Sensor performance

Sensors were prepared using MPA:MPO mixed SAM solution (1:1), as judged to be optimal above, and the amount of XOD added was varied so that the XOD concentration in the

Hx solution ranged from 1.25 to 80 mU ml⁻¹. A calibration curve was plotted based on the output current of the sensor. In addition, the theoretically estimated O₂⁻ concentration at each stage of XOD concentration was calculated using Eq. 2, and a calibration curve versus the output current value was also plotted.

$$[\text{O}_2^-] = [\text{XOD}] \times t \times 4, \quad (2)$$

where [O₂⁻] indicates the estimated O₂⁻ concentration and [XOD] indicates the XOD concentration. Besides, *t* indicates the reaction time (min) between Hx and XOD. In this experiment, the output current value at 7 s after dropping XOD was used, so *t* = 0.11667 min.

Figure 4a shows the correlation between the XOD concentration and output current, and Fig. 4b shows the correlation between the O₂⁻ concentration calculated from Eq. 2 using the XOD concentration and the output current. The results reveal a gradual increase in the output current as the XOD concentration was increased in the concentration range of 1.25 to 20 mU ml⁻¹ (O₂⁻ concentration 0.58–9.33 μM). The correlation between the XOD concentration and current output was very strong (*R* = 0.9920).

Fig. 3 Response of sensors fabricated with different SAM solutions to XOD solution addition: **a** sensor A using MPA solution, **b** sensor B using MPA:MPO mixed solution (1:1), **c** sensor C using MUA solution, and **d** sensor D using MUA:MUD mixed solution (1:1)

Fig. 4 Calibration curve of XOD or estimated O₂⁻ concentration. Hx: 300 μM, 10 ml, 35 °C; **a** XOD (1.25–80 mU mL⁻¹); **b** estimated O₂⁻ concentration (0.58–33.73 μM)

Effect of substances on sensor specificity

These results are shown in Fig. 5. Adding hydrogen peroxide, glucose, or NADH (red) had minimal effects on the sensor output (Fig. 5a–c). Adding uric acid or citric acid decreased the output current value (Fig. 5d, e). When adding ascorbic acid solution, the output current value increased and then gradually decreased. With addition of SOD, the output current value declined to nearly the initial blank value but was slightly higher than the level before adding XOD (Fig. 5f).

Measurement of actual samples

These results are shown in Fig. 6. After adding EISF, the output current increased greatly. We then added SOD to remove any O₂⁻ remaining in the sample, which cannot be detected by the sensor. The blank value was subtracted from the measured value to give the response value (signal). The signal obtained from the sensor was 0.13 nA (sample A) and

0.15 nA (sample B), corresponding to O₂⁻ concentrations of 0.17 μM (sample A) and 0.20 μM (sample B).

Discussion

Regarding the selection of the SAM, it is likely that the current value decreased because the distance between Cyt c and the electrode was longer due to the longer alkyl chain length, resulting in decreased electron transfer efficiency (Marcus and Sutin 1985; Feng et al. 1995; Reddy and Gobi 2012). The electron transfer rate between the electrode and Cyt c decreases with increasing alkyl chain length of the SAM, which is consistent with the results in the present work. Also, the overall output current of sensor b was approximately 1.5 times higher than that of sensor a. In other words, a higher output current value was obtained at all the XOD concentration levels when using the mixed SAM comprising MPA and MPO than when using MPA alone. In this regard, although the detailed mechanism has not yet been clarified, results similar to those in the present experiment are often reported in studies of alkanethiols that have a hydroxyl group (Ge and Lisdat 2002; Puig et al. 2009). Gobi et al. prepared a sensor using MPA for immobilization of Cyt c on a gold electrode and a sensor using a mixed SAM comprising MPA and MPO, and measured the electron transfer between the gold electrode and Cyt c by cyclic voltammetry (Gobi and Mizutani 2000). The electron transfer rates in each sensor were measured, and determined to be 12 s⁻¹ and 2800 s⁻¹, respectively. The magnitude of the response current to O₂⁻ was also higher in the sensor using the MPA:MPO mixed SAM. These findings suggest that MPO acts to facilitate electron transfer between the electrode and the immobilized probe molecule. When considering the correction formula depending on the SAM for the sensor sensitivity, we found that the sensors obtained using MPA and MPO with short alkyl chain length (sensors a and b) showed similar linear or logarithmic correlations (around 0.9500). Meanwhile, the sensors obtained using MUA and MUD with long alkyl chain length (sensors c and d) showed similar but poor linear or logarithmic correlations (around 0.7500). That is to say, the sensor's response to O₂⁻ showed a good correlation for MPA or MUA but was not strongly correlated for MPO or MUD. Also, MPA exhibited better performance in terms of providing a more uniform and directional method of immobilization of Cyt c compared with MUA. On this basis, the SAM solution prepared by mixing MPA and MPO solutions at 1:1 ratio was considered to be the most suitable for preparing the sensor.

On the other hand, for the calibration curve plotted using the optimal SAM formula, good correlation was observed with the sensor output current in the O₂⁻ concentration range from 0.58 to 9.33 μM ($R = 0.9920$). In addition, when

Fig. 5 Effect of each substance on sensor response. Hx: 300 μ M, 10 ml, 35 $^{\circ}$ C; **a** H_2O_2 ; **b** glucose; **c** NADH (red); **d** uric acid; **e** citric acid; **f** ascorbic acid

the concentration of O_2^- exceeded 9.33 μ M, a good sensor response was still obtained. However, these response values are hard to be believe at this time because of the unstable enzyme reaction due to the shortage of hypoxanthine as a substrate. At this time, the response for the relatively high concentrations of O_2^- in Fig. 4b can only be used as a reference.

For application of this sensor to in vivo measurements, it is necessary to confirm that the sensor does not exhibit

a positive response to substances other than O_2^- present in biologic samples. In particular, Cyt c may be affected by antioxidants and reducing substances in the body (Ukeda 2004). Substances that may affect the measurement by this sensor include hydrogen peroxide and glucose, which are widely present in animal body fluids. In particular, hydrogen peroxide is electrolyzed at around +650 mV to generate electrons, so in this system where a voltage of +150 mV is applied to the electrode part of the sensor, a measurement

Fig. 6 Measurement of actual samples. 0.2 ml EISF, 35 °C **a** sample A; **b** sample B

error could occur. NADH, which functions as an electron carrier in blood and cytosol, is a reducing substance and may participate in the reduction reaction of oxidized Cyt c. Furthermore, uric acid, citric acid, and ascorbic acid, which are typical water-soluble antioxidants, capture and eliminate active oxygen species generated in the aqueous phase of the body and act as a defense mechanism against oxidative stress. However, because these substances are easily oxidized, they may cause an oxidation current when they reach the electrode. Therefore, in this experiment, the specificity of the sensor was examined using six substances: hydrogen peroxide, glucose, NADH, uric acid, citric acid, and ascorbic acid, with reference to previous study (Wilson et al. 2011).

For uric acid and citric acid, it is considered that the output response decreased because some of the O_2^- in the sample was eradicated by the antioxidant effect of these substances. When the sensor is applied to a living body, however, the measurement target is the amount of O_2^- , which is apparently in a steady state after subtracting the amount eradicated by antioxidant effects. The effect of the antioxidants on the output response is considered to be extremely

small. On the other hand, when ascorbic acid solution was added, the output current increased and then slowly decreased. With addition of SOD, the output current value declined to nearly the initial blank value but was slightly higher than the level before adding XOD. It is likely that the ascorbic acid was oxidized and decomposed when applying a voltage of +150 mV, and the oxidation current was also detected, so that the output response increased. Therefore, in the future, to reduce the measurement error and improve the selectivity of the sensor, we will modify the sensor surface with a selective permeation film to suppress the permeation of large-molecular-weight substances such as ascorbic acid.

Finally, we applied the proposed sensor to measure the concentration of O_2^- in EISF. Although the mean response was only around 0.185 μ M, this result was obtained from a sample that had been diluted 50 times, so the original concentration of O_2^- would be approximately 9.25 μ M. Besides, adding SOD seemed to have almost no effect on the current of the sensor, indicating that the sensor reacted with all the O_2^- and there is no O_2^- remaining in the sample. This shows that the proposed sensor can detect the presence of O_2^- in vitro.

Because EISF is an interstitial environment of living cells in multicellular organisms, it may be a good site for monitoring oxidative stress. In the future, to better understand the dynamics of oxidative damage in fish, we will obtain real-time data by implanting the sensor into the EISF.

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