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Effect of Valinomycin on the Respiration Activity of Zebrafish Embryos Using a Large-scale-integration-based Multiple Amperometric Biosensor

Kazuki Terao,[1] Masato Suzuki,[1,2*] Ryota Kunikata,[3] Atsushi Suda,[3] Kumi Y. Inoue,[4] Kosuke Ino,[5] Tomokazu Matsue,[6] and Tomoyuki Yasukawa[1,2**]

[1]Graduate School of Science, University of Hyogo, 3-2-1 Koto, Kamigori, Ako, Hyogo 678-1297, Japan
[2]Advanced Medical Engineering Research Institute, University of Hyogo, 3-264, Kamiya-cho, Himeji, Hyogo 670-0836, Japan
[3]Japan Aviation Electronics Industry, Ltd., 3-1-1 Musashino, Akishima, Tokyo 196-8555, Japan
[4]Faculty of Engineering, Graduate Faculty of Interdisciplinary Research, University of Yamanashi, 4-3-11 Takeda, Kofu, Yamanashi 400-8511, Japan
[5]Graduate School of Engineering, Tohoku University, 6-6-11 Aoba, Aramaki, Aoba, Sendai, Miyagi 980-8579, Japan
[6]Head Office of Enterprise Partnerships, Tohoku University, 468-1 Aoba, Aramaki, Aoba, Sendai, Miyagi 980-8545, Japan

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A large-scale-integration-based multiple amperometric biosensor (Bio-LSI) was used to obtain images of oxygen concentration around zebrafish embryos and monitor their respiration activity after valinomycin at different concentrations was injected. The regions with a low reduction current of oxygen coincided with the embryo positions. The reduction current decreased immediately after adding valinomycin owing to the increase in the respiration activity of the embryos. The decrement of the reduction current increased with valinomycin concentration. The estimation system is very simple because no alignment is required between the embryos and the electrodes arrayed at the bottom of the chamber. In general, the use of zebrafish embryos for toxicity tests has been expected as an alternative for animal tests. However, a relatively long time is required to estimate the mortality and morphological changes of the embryos caused by chemical substances by a standardized method. The use of the present system is expected to allow the rapid and simple sensing of the effects of chemical substances on the respiration activity of zebrafish embryos.

1. Introduction

New chemical substances such as medical supplies, agricultural chemicals, and cosmetics have been continuously developed to improve our quality of life. The effects of these substances on human health, ecological systems, and the environment should be estimated as a common challenge for the whole world. The Organisation for Economic Co-operation and Development

*Corresponding author: e-mail: suzuki@sci.u-hyogo.ac.jp
**Corresponding author: e-mail: yasu@sci.u-hyogo.ac.jp
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(OECD) has standardized estimation methods for these chemicals and products. There is the fish embryo toxicity OECD test guideline 236 (TG236) for estimating the effects of chemical substances by using zebrafish (*Danio rerio*) embryos.[1] The toxicity of chemical substances is estimated on the basis of the mortality and morphological changes of the embryos, when the embryos are cultured in sea water containing the chemical substances for 96 h. TG236 was developed as an alternative for the acute fish toxicity test (e.g., OECD TG203) to overcome ethical issues from the perspective of animal protection. In Europe, fish embryos at non-feeding developmental stages are categorized as non-animal by the European Union, Directive 2010/63/EU.[2]

The use of zebrafish embryos for toxicity tests has been expected as an alternative for animal tests[3–5] because the human genes related to diseases and drug metabolisms have 84% homology with zebrafish genes.[6,7] The toxicity tests of 31 chemical substances using zebrafish embryos showed that their developmental toxicities were in agreement with animal tests with accuracies higher than 80%.[8] Thus, the development of rapid and simple methods for estimating the effects of chemical substances will lead to an alternative for ecological and animal tests.

Techniques for the simultaneous estimation of different concentrations of chemical substances applied to zebrafish embryos using microfluidic devices have been reported.[9–12] However, the morphological changes observed by optical microscopy were used to estimate the effects of chemical substances on embryos. The quantitation of metabolic activity can be useful for the accurate estimation of the physiological effects of chemical substances on embryos. The oxygen consumption of embryos by respiration is one of the candidates for the estimation of physiological effects.[13] The oxygen consumption that depends on mitochondrial activity is a powerful indicator to discover cellular information such as metabolism and apoptosis. For example, the oxygen consumption of embryos was measured using a fluorescence molecule with oxygen sensitivity and immobilized on microwells with single embryos.[14] Furthermore, a commercially available extracellular flux analyzer was used to estimate the toxicity of chemicals to embryos at different developmental stages by indicating the oxygen consumption (mitochondrial activity).[15] The oxygen consumption of zebrafish embryos, as well as morphological changes, has been recognized as an important factor for estimating the effects of chemicals on embryos.

In this study, the oxygen consumption of zebrafish embryos in the presence of valinomycin at different concentrations was monitored using the reduction currents of oxygen obtained by a chip of large-scale-integration-based multiple amperometric biosensors (Bio-LSI).[16] The use of the chip with an array of 400 microelectrodes (20 × 20) in a 5 × 5 mm^2 region allows individual current responses to be obtained at each electrode, and thus the time courses of current images that reflect the distribution of the concentration of electrochemical species immediately above the electrode array can be obtained. Until today, Bio-LSI devices have been applied to the monitoring of the enzyme activities of embryoid bodies by amperometry[17] and potentiometry,[18] the imaging of dopamine release from the spheroids of PC12 cells,[19] multiple immunosensing,[20] the motion tracking of microorganisms,[21] the detection of multiple cell functions,[22] the fabrication of hydrogels at a localized area,[23] the detection of oxygen generated from spinach by photosynthesis,[24] and the estimation of endothelial permeability.[25] Our group reported the oxygen consumption of a single zebrafish enclosed in a small container by determining the

electrochemical reduction of oxygen.[26] In this work, the decrease in oxygen concentration caused by the respiration of embryos arranged on the electrode array was monitored with a Bio-LSI chip by adding valinomycin at different concentrations to determine its effect on the respiration activity of embryos. Valinomycin acts as an uncoupler to inhibit the synthesis of adenosine triphosphate (ATP) by oxidative phosphorylation. The continuous monitoring of oxygen concentration around embryos as images of reduction currents of oxygen allows for the estimation of respiration activity. In this system, the effect of valinomycin can be rapidly and simply estimated compared with the OECD tests because the respiration activity changes immediately after valinomycin is injected, and no difficult decision concerning the mortality and morphological changes of each embryo by the naked eyes is required. In addition, no precise control of the positions of the embryos on the electrode array is required, because the microelectrodes arranged under the embryos are used to obtain the reduction current of oxygen.

2. Materials and Methods

The Bio-LSI chip comprises 400 (20 × 20) platinum electrodes. The fabrication of the chip was detailed in a previous report.[16] The rows in the electrode array were named lines 1–20 from above, and the columns were named columns a–t from the left. Electrodes were covered by an insulating layer (5 mm thick) of negative photoresist (SU-8) to define the regions exposed to solutions (50 mm diameter). The distance between the centers of neighboring electrodes was 250 mm. A layer with an acrylic chamber (5 × 5 mm^2, 5 mm thick) and a layer with a type "H" fluidic channel were arranged on the electrode array [Fig. 1(A)]. The width of the type "H" channel and the lengths of the vertical and horizontal lines of the type "H" channel were set at 6, 30, and 10 mm, respectively. The chamber and channel were filled with artificial sea water (0.6 mL, SEALIFE, Nihonkaisui Co., Ltd., Tokyo). A platinum coil inserted at the bottom left of the type "H" channel and a Ag/AgCl electrode inserted at the top right were used as the counter and reference electrodes, respectively. Furthermore, two tubes were inserted at the top left and bottom right to inject and remove the artificial sea water containing valinomycin at different concentrations, respectively. The flow rate of the artificial sea water was 0.4 mL/min. Thus, the solution in the chamber and fluidic channel can be replaced with the solution containing valinomycin in 1.5 min.

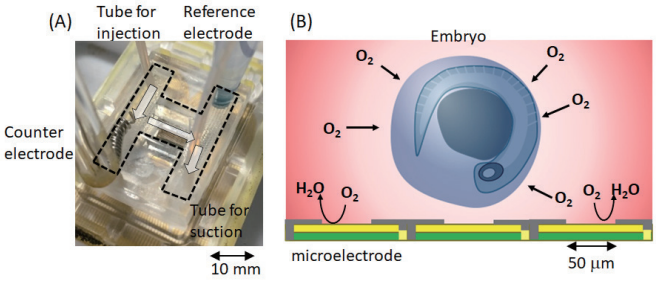

Fig. 1. (Color online) (A) Image of Bio-LSI chip with square chamber and type "H" fluidic channel. (B) Cross-sectional illustration of oxygen consumption of zebrafish embryos arranged on Bio-LSI chip.

The breeding facility of the zebrafish was illuminated for 14 h and exposed to darkness for 10 h in a day. The temperature of the artificial sea water for the zebrafish was maintained at 26 ℃. Fertilized embryos were cultured in the artificial sea water at 28.5 ℃ for 48 h. The embryos with a diameter of approximately 1 mm were put on the electrode arrays of chips.

A CMOS camera (VEX120, WRAYMER) was placed above the electrode array in the chamber to confirm the positions of the embryos. The potential of −0.5 V vs Ag/AgCl was applied to 400 electrodes at the same time for the same duration (36 min) to obtain the reduction currents of oxygen [Fig. 1(B)]. The reduction currents of oxygen obtained by the electrodes near the embryos decrease with the decrease in oxygen concentration owing to the consumption of oxygen by the embryos. The reduction currents of oxygen were used to investigate the effect of valinomycin on the respiration activity of the embryos.

3. Results and Discussion

3.1 Imaging of oxygen concentration around embryos

Three zebrafish embryos were arranged on the electrode array in the chamber filled with artificial sea water. The potential of −0.5 V was applied to 400 electrodes to obtain amperometric responses for reducing the oxygen dissolved in the artificial sea water. The artificial sea water containing 5.0 mmol/L valinomycin was then injected from the tube inserted at the top left of the type “H” channel, and at the same time, the artificial sea water in the chamber and type “H” channel was removed by the tube inserted at the bottom right of the type “H” channel at the same rate. Figures 2(A) and 2(D) show the image of three embryos on the electrode array and the image of reduction currents of oxygen, respectively. The centers of embryos a, b, and c were at the electrodes 3c, 3g, and 16l, respectively [Fig. 2(A)]. The reduction currents of oxygen obtained by the electrodes in the vicinity of the centers of the embryos were lower than those obtained by the other electrodes without an embryo [Fig. 2(D)]. The whitish regions in the current image,

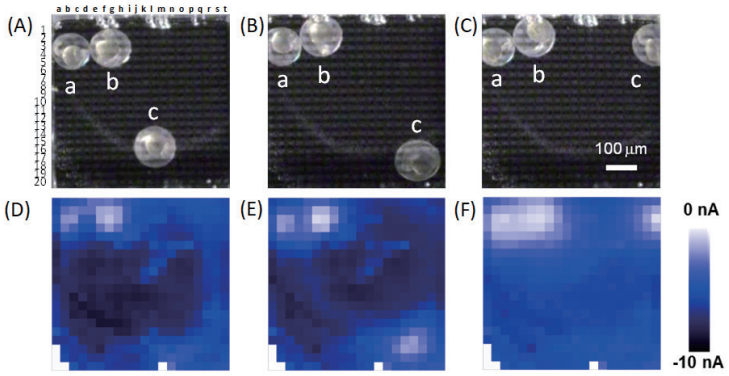

Fig. 2. (Color online) (A)–(C) Images of three embryos on electrode array and (D)–(F) images of reduction currents of oxygen (A, D) before the solution containing 5.0 μmol/L valinomycin was injected, and (B, E) 1.5 min and (C, F) 6.5 min after the injection of the solution containing 5.0 μmol/L valinomycin was stopped.

which indicate low currents, coincided with the positions of embryos a and b. The result suggests that the concentration of oxygen near the embryos decreased with increasing consumption of oxygen by the embryos. In addition, the decrease in reduction current may also be attributable to the inhibition of the oxygen diffusion by the embryos.

We injected the artificial sea water containing 5.0 mmol/L valinomycin within 1.5 min. Figures 2(B) and 2(C) show the optical images of the embryos and Figs. 2(E) and 2(F) show current images 1.5 and 6.5 min after the injection was stopped, respectively. In the current images, the whitish regions gradually spread in all directions and the whitish intensity was emphasized. The results indicate that the oxygen concentration around the embryos decreased with the addition of valinomycin owing to the increase in the respiration activity of the embryos. In the presence of valinomycin that cancels the membrane potential as an uncoupler to the oxidative phosphorylation, the embryos should consume more oxygen than in the absence of valinomycin to maintain ATP production. Therefore, the effect of valinomycin on the embryos can be qualitatively estimated from the images of the reduction currents of oxygen. Furthermore, no precise control of the positions of the embryos is required to estimate the respiration activity of the embryos. When the microelectrode was applied to the electrochemical estimation, the positional relationship between the electrode and the embryo should be regulated to obtain the electrochemical responses. In the present system, the introduction of the embryos to the chamber above the electrode array allows the arrangement of the embryos immediately above some of the 400 electrodes.

No current response was observed for embryo c in the current image [Fig. 2(D)], although embryo c was on electrode 16l. This is due to the transfer of embryo c on the electrode array. Embryos grow to be young fish 48 h after fertilization. The young fish in embryo c actively repeated the rotational motion and the swing of the tail, resulting in the random repeat of the movement and stillness on the electrode array. Embryo c moved to the right side of the image after passing electrode 16l and reached electrode 18q [Fig. 2(B)]. Then, embryo c again started to move toward the top of the image and reached electrode 2s [Fig. 2(C)]. When embryo c was on electrodes 18q and 2s without the spatial movement, decreases in the reduction currents of oxygen were observed [Figs. 2(E) and 2(F)]. However, the embryos that moved on the electrode array cannot be applied to this work because of the disturbance of the diffusion layer of oxygen formed on the electrodes by the movement of the embryos and the insufficient formation of the diffusion layer of oxygen around the embryos.

3.2 Investigation of the respiration activity of embryos in the presence of valinomycin

We investigated the amperometric responses of oxygen after injecting valinomycin at different concentrations as shown in Fig. 3(A). The potential of −0.5 V was applied to 400 electrodes at zero time, as shown in Fig. 3(A). The reduction currents reached almost the steady state 30 s after the potential was applied. The artificial sea water containing valinomycin at different concentrations was then injected 1.5 min after the potential was applied. The currents were plotted as averages of reduction currents obtained by four electrodes immediately below the arranged embryos and normalized with the average responses obtained immediately after the

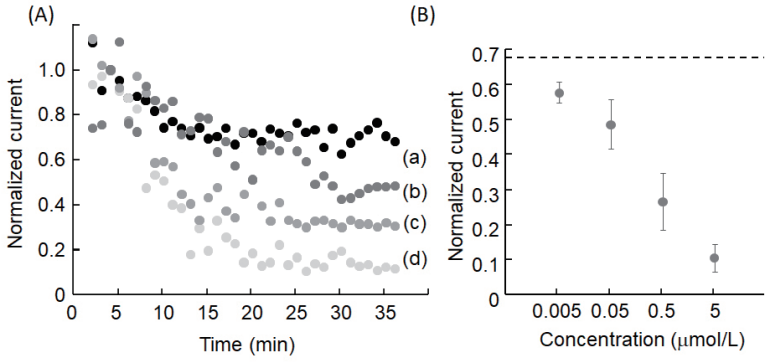

Fig. 3. (A) Normalized reduction currents of oxygen obtained by the electrodes immediately under the embryos upon injection of valinomycin at different concentrations. Concentrations of valinomycin: (a) 0, (b) 0.05, (c) 0.5, and (d) 5.0 μmol/L. (B) Normalized currents of oxygen as a function of the concentration of injected valinomycin. The number of trials for 0 and 5.0 μmol/L was six, and that for the other concentrations was three.

injection was stopped at 3 min. The embryos arranged around the center of the electrode array were used to remove the inhibition of the formation of the diffusion layer of oxygen around the arranged embryos by the walls of the chamber. The normalized currents obtained for the first 3 min were unstable owing to the disturbance of the diffusion layer generated by the reduction of oxygen at the 400 electrodes caused by the injection of the solution. When the artificial sea water without valinomycin was injected, the reduction currents showed a gradual decrease to reach an almost steady state in 10 min after stopping the injection. The decrease was attributed to the formation of the diffusion layer over the electrodes. When the solutions containing valinomycin were injected, the reduction currents also decreased and were lower than that obtained by injecting the solution without valinomycin. This is because of the decrease in the concentration of oxygen caused by the increase in the respiration activity of the embryos. The average currents between 30 and 35 min were used as responses for the calibration curve. Figure 3(B) shows these current responses as a function of the concentration of valinomycin. The results indicate that the responses decrease with increasing valinomycin concentration owing to the decrease in oxygen concentration caused by the increase in the respiration activity of the embryos. A good correlation between the valinomycin concentration and the current response was obtained in nano- and micromolar orders of valinomycin.

3.3 Investigation of the frequency of tail swing in the presence of valinomycin

We also investigated the frequency of the tail swing of young fish embryos after the injection of valinomycin. The frequency was calculated at minute intervals and normalized with the number at the first minute during the measurements of the reduction current of oxygen. The number at the first minute after the potential of −0.5 V was applied was found to be 10 times on average. The standard deviation for three embryos was 2.8 times. When the solution in the chamber was replaced with the solution containing 5.0 mmol/L valinomycin from 1.5 to 3.0 min, the frequency of the tail swing increased and became four times that observed before

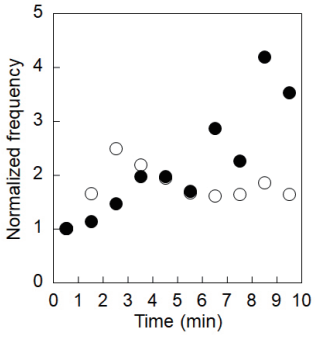

Fig. 4. Normalized frequency of the movement (tail swing) of young zebrafish embryos after the injection of valinomycin. Concentration of valinomycin: (white circle) 0 and (black circle) 5.0 μmol/L.

valinomycin was added (black circle in Fig. 4). The increment obtained by adding valinomycin was larger than that obtained by replacing the solution without valinomycin (white circle in Fig. 4). Therefore, this increase could be due to the stress applied to the embryos with the decrease in oxygen concentration caused by the enhancement of the respiration activity. However, a slight increase in swing frequency was observed by replacing the solution without valinomycin. This may be due to the decrease in oxygen concentration around the embryos caused by the consumption of oxygen by the electrochemical reaction with the 400 electrodes. The result shows the possibility of estimating the effects of chemical substances on the respiration activity of the embryos. In addition, there was no significant difference in the frequency of tail swing with and without a low valinomycin concentration.

4. Conclusions

The distribution of the oxygen concentration around the embryos was indicated as images of the reduction currents of oxygen based on the electrode array of Bio-LSI. The reduction currents of oxygen decreased immediately after adding valinomycin that acts as an uncoupler and reached a steady state within 10 min. The currents decreased with increasing concentration of valinomycin. The estimation system is very simple because no alignment is required between the embryos and the electrodes arrayed at the bottom of the chamber. The monitoring of the reduction currents allows for the rapid and simple estimation of the effect of valinomycin on the respiration activity of the embryos. Moreover, the frequency of the tail swing of young fish embryos also increased after adding valinomycin. Therefore, monitoring the reduction currents of oxygen and the frequencies of tail swing can be useful for estimating the effects of chemical substances on human health, ecological systems, and the environment. Oxygen was consumed not only by the embryos, but also by the 400 electrodes. In this system, the decrease in the reduction current of oxygen consumed by the embryos must be measured under low concentration conditions due to oxygen consumption at the 400 electrodes. Thus, the system should be improved to decrease the consumption of oxygen by electrodes.

Acknowledgments

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