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such as metabolism, respiration, and viability of bacteria. In addition, it was found that the response obtained in a dead bacterium was 3 times larger than that obtained in a living cell. We concluded that the difference in the electrochemical response is dependent on the biological functions such as metabolism, respiration, and viability of bacteria.

ABSTRACT

Fluorescent dye is a useful tool in qualitative analysis. Acridine orange (AO) is one such dye, which fluoresces when bound to biomolecules. In this study, we investigated whether the electrochemical activity of AO can be used for bacterial quantification. We observed that the oxidation peak current of AO depended on the amount of AO-labeled bacteria present on the electrode. The electrochemical response was estimated as $8.4 \times 10^{-5}$ A for a single cell. In addition, it was found that the response obtained in a dead bacterium was 3 times larger than that obtained in a living cell. This difference is attributed to the differences in the electrochemical response depending on the biological functions such as metabolism, respiration, and viability of bacteria.

Keywords: Bacterial Detection, Acridine Orange, Differential Pulse Voltammetry

1. Introduction

Pathogenic bacteria are a major public health concern worldwide as they cause hospital-acquired infections and food poisoning. Simple detection methods for pathogenic bacteria in food, clinical, and environmental samples are required to prevent and diagnose these diseases.1–5 On the other hand, bacteria play various kinds of essential roles in the ecological system. For example, degrading bacteria that convert organic materials to their inorganic forms, which provide nutrition to plants, is essential for soil fertility.6,7 Recently, the amount of bacteria in the soil has reduced because of soil pollution and climate changes due to human activity. This has caused considerable economic damage to the agricultural industry. Therefore, it is necessary to determine the amount of bacteria in the soil for the maintenance of soil fertility.6,7 Besides, bacteria that benefit humans have attracted research interest. Some types of bacteria are expected to be useful as an alternative for chemical fertilizers.8–10 Bacteria that can reduce metal ions are used for the remediation of heavy metal pollution.11,12 Moreover, photosynthetic bacteria are applied in the manufacture of microbial fuel cells.13,14 In order to utilize these bacteria efficiently, it is essential to calculate the bacterial amount, and evaluate their viability and vital activity following cultivation.

Acridine orange (AO) shows unique properties and has a wide range of applications. It is used as a pH indicator, as a photosensitizer, and in bacterial staining.15–17 AO exists in two forms in the aqueous medium: neutral and cationic states (AOH$^+$) (Fig. 1).18,19 Neutral AO penetrates the cell membrane, and then transforms into AOH$^+$ by accepting a proton inside the cell.20 AOH$^+$ cannot move out of the cell; it intercalates into the double helical structure of DNA and fluoresces green, and binds to RNA, which results in red fluorescence.21 The fluorescence of AO is useful in the qualitative analysis of biomolecules. However, it is difficult to quantify biomolecules using AO because the molar extinction coefficient and quantum yield of AO strongly depend on its binding state.15,22,23

In this study, we investigated the electrochemical activity of AO and the differences between the electrochemical response in the buffer solution and the bacterial cell. For bacterial quantification using AO as an electrochemical label, we quantitated the electrochemical response of AO in a single bacterial cell. Further, we investigated the effect of bacterial biological functions such as metabolism, respiration, and viability on the electrochemical response.

2. Experimental

2.1 Reagents and apparatus

Ultrapure water (>18 MΩ cm) sterilized by UV light was used for all experiments. 3,6-Bis-(dimethylamino)acridine hydrochloride (acridine orange, AO) was purchased from Dojindo, Japan. Nutrient broth was obtained from Eiken Chemicals (Japan). Bacterial sample of Pseudomonas aeruginosa was acquired from National Institute of Technology and Evaluation Biological Resource Center (NBRC, Japan). Bacterial viability was checked by LIVE/DEAD Baclight L7007 (Molecular Probes). Image of scanning electron microscopy (SEM) was obtained using TM-3030 Miniscope (Hitachi, Japan). Fluorescence spectra were obtained using FP-6300 spectrofluorometer (JASCO, Japan).

2.2 Bacterial cultivation

All bacterial experiments were carried out in a biosafety level 2 (BSL-2) laboratory, developed and managed in accordance with safety regulations. A strain of P. aeruginosa was cultured in an agar growth medium at 30°C for 48 hours. Colonies were suspended in liquid growth medium (30 mL) and cultured at 30°C for 18 hours. This suspension was centrifuged at 6,500 rpm for 15 min. The precipitate was redispersed in 30 mL of ultrapure water, and the suspension was centrifuged again under the same conditions described above. These procedures were repeated three times. We confirmed over 95% of bacterial viability in the suspension.

Figure 1. Equilibrium equation of neutral and cationic AO.
Scan rate and pulse amplitude of DPV was 10 mVs⁻¹ respectively, throughout this study. Scan rate of CV was 20 mV.

2.4 Electrochemical measurement

All microbial experiments were performed under strictly sterile conditions. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed with a potentiostat (ALS 842B, BAS, Japan) in 3.0 mL of phosphate buffer (90 mM, pH 7). An Ag/AgCl|saturated KCl electrode and a platinum mesh electrode (10 × 10 mm) were used as reference and counter electrodes, respectively, throughout this study. Scan rate of CV was 20 mV. Scan rate and pulse amplitude of DPV was 10 mVs⁻¹ and 50 mV, respectively.

2.5 Sterilization of the bacteria

The bacterial suspension (0.30 mL) was added to 2-propanol (0.70 mL), and the mixture was stirred for 1 hour at room temperature for sterilization. We checked for almost 0% of bacterial viability. The suspension was centrifuged at 6,500 rpm for 15 min to remove extra AO. The precipitate was redispersed in 1.0 mL of ultrapure water, and the suspension was centrifuged again under the same conditions described above. These procedures were repeated three times. The resulting AO-labeled bacterial suspension (5.0 µL) was poured over a glass carbon (GC) disc electrode (diameter: 1.6 mm, BAS, Japan) and then left to dry in atmospheric air, as shown in Fig. 2.

2.3 Preparation of an electrode deposited with AO-labeled bacteria

AO aqueous solution (3.3 mM, 0.50 µL) was added to the bacterial suspension of P. aeruginosa (8.1 × 10⁶ cells/mL), and then the mixture was left for 15 minutes in a dark place at room temperature in order to label the bacteria. This suspension was centrifuged at 6,500 rpm for 15 min to remove extra AO. The precipitate was redispersed in 1.0 mL of ultrapure water, and the suspension was centrifuged again under the same conditions described above. These procedures were repeated three times. The resulting AO-labeled bacterial suspension (5.0 µL) was poured over a glass carbon (GC) disc electrode (diameter: 1.6 mm, BAS, Japan) and then left to dry in atmospheric air, as shown in Fig. 2.

To electrochemically quantify the bacteria, DPV was performed at 10 mVs⁻¹ scan rate and 50 mV pulse amplitude in phosphate buffer, as shown in Fig. 3A. A 5.0 µL bacterial suspension was poured over a GC disc electrode of 0.020 cm² surface area, and was allowed to stand at room atmosphere for 1 h. The bacteria-deposited electrode thus obtained, was used as a working electrode. An oxidation peak was observed at 0.80 V, which increased with increase in the amount of bacteria. Figure 5B indicates a linear relationship (R² = 0.9606) between the magnitude of current at 0.80 V and the amount of the bacteria in a range of 2.0 × 10⁵–4.1 × 10⁶ cells. We obtained the electrochemical response of 8.4 × 10⁻¹⁵ A per a single cell.

The area of P. aeruginosa that was projected and had a rod-shape with 0.60 width and 1.7 µm length was calculated to be about structural changes, as shown in Fig. 4. The oxidation peak at 0.72 V was attributed to the oxidation of neutral AO to the radical cation form (AO⁺) in Fig. 4a. The AO⁺ immediately transforms into AOH⁺ owing to its low structural stability. Subsequently, the oxidation peak at 0.88 V was attributed to the oxidation of AOH⁺ to the radical dication form (AOH⁺⁺) in Fig. 4b. From the above, it can be seen that the oxidation peak observed for the AO-labeled bacteria-deposited electrode is attributable to the oxidation of AOH⁺. Because most of the AO is present as AOH⁺ in the bacterial cell, it is possible to determine the amount of bacteria by focusing on the electrochemical response of AOH⁺.

3. Results and Discussion

3.1 Electrochemical property of AO

CV was performed using a GC electrode deposited with AO-labeled bacteria as a working electrode in phosphate buffer. An oxidation peak was observed at 0.90 V vs. Ag/AgCl (Fig. 3a). On the other hand, there were no peaks observed when bacteria without an AO label had been deposited on a GC electrode (Fig. 3b). This means the oxidation peak current is derived from AO molecule. To investigate further, we performed CV using a bare GC electrode in phosphate buffer with AO. Two oxidation peaks were observed at 0.72 and 0.88 V, while there was no reduction peak in the potential range (Fig. 3c). Because the reduction of AO has been observed in more negative potential range around −1.0 V vs. SCE in the previous work. It is well-known that acridine and similar structures give a characteristic electrochemical response depending on their interactions with the electrode surface.
1.0 × 10⁻⁸ cm². We can estimate that 2.0 × 10⁶ cells of bacteria are required to infill the electrode surface. SEM image of the electrode deposited with bacteria (2.0 × 10⁶ cells) is shown in Fig. 6. The bacteria observed as dark rods markedly differed from the electrode surface (bright area) owing to an insulating property of bacteria. From the SEM observation, we estimated that the dark portions, caused by the aggregation of bacteria, occupied about 41% of the electrode surface. This indicates that the current response depends strongly on the bacterial coverage over the electrode.

3.3 Electrochemical response of a single AO-labeled bacterium

AO aqueous solution (1.7 nmol in 2.0 mL) indicated a typical fluorescence spectrum which has an emission intensity (I₀) at 527 nm (excitation at 500 nm), as shown in Fig. 7. AO aqueous solution (1.7 nmol) was added to 2.0 mL of bacterial suspension (4.5 × 10⁸ cells), and then the mixture was centrifuged at 6,500 rpm for 15 min. The supernatant indicated a decrease in fluorescence intensity. The fluorescence intensity (I) depends on the concentration of AO (c).

\[ I = \varepsilon \phi c \quad (1) \]

where is determined by specific values such as molar extinction coefficient (ε), quantum yield (φ), and the intensity of the excitation (I₀). The difference observed in the intensity (ΔI) depends on the amount of AO molecules staining the bacteria (n_bacteria).

\[ \Delta I/I_0 = (c_0 - c)/c_0 \quad (2) \]

\[ = (n_0 - n)/n_0 = n_{bacteria}/n_0 \quad (3) \]

where the n is the mole number of AO. From the above, we calculated 8.1 × 10⁻¹⁰ mol as the n_bacteria and estimated 1.8 × 10⁻¹⁵ mol of AO per single bacterium (1.8 × 10⁻¹⁵ mol cell⁻¹).

Therefore, it was confirmed that 3.6 × 10⁻¹³–7.4 × 10⁻¹² mol of AO on the electrode was modified by bacteria in the range of 2.0 × 10⁵–4.1 × 10⁶ cells in Fig. 5B. On the other hand, we obtained the electrochemical response of 8.4 × 10⁻¹⁵ A per a single cell in Fig. 5B. Therefore, the current per mole of labeling AO can be determined as 4.7 × 10³ A mol⁻¹.

3.4 Dependence of the electrochemical response on living and dead bacteria

DPVs were obtained using GC electrodes deposited with living (a) and dead bacteria (b), as shown in Fig. 8. Although the oxidation peak was observed at 0.80 V in both voltammograms, the peak current obtained in the dead bacteria was larger than that of living bacteria. It is well known that bacteria have uptake and efflux pumps in their membranes that play a key role in the ejection of antimicrobial compounds and exogenous materials from the cells. Living bacteria eject AO as an exogenous material coming into the cell by penetrating through the membrane. On the other hand, dead bacteria keep all of AO molecules coming into the cell through penetration of the cell membrane. The amount of AO molecules in a single dead bacterium was estimated to be 5.2 × 10⁻¹⁸ mol from the peak current and the current per mole of labeling AO (4.7 × 10³ A mol⁻¹). The AO mole number obtained in a dead bacterium was 3 times greater than that obtained in a living cell (1.8 × 10⁻¹⁵ mol).
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electrochemical response is independent of the binding state of AO

larger than that obtained in a living cell. We conclude that the

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of AO in a single dead bacterium (5.2 \times 10^5 mol) was 3 times

more than that obtained in a living cell. We conclude that the
electrochemical response is independent of the binding state of AO

in the cell.

We gratefully acknowledge the financial support provided by the
Ministry of Agriculture, Forestry, and Fisheries through a science
and technology research promotion program for agriculture, forestry,
fisheries, and food industry. We also acknowledge financial support
from the Japan Society for the Promotion of Science (JSPS) through
a Grant-in-Aid for Scientific Research (B) (KAKENHI 25288039,
25288069) and Grant-in-Aid for Challenging Exploratory Research
(KAKENHI 26620072, 26620123).

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