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ABSTRACT

Single-cell analyses are important for providing new insights into cellular biology. Here we report an electrochemical reporter gene assay for single cells using a scanning electrochemical microscope (SECM)-microwell system. Each microwell trapped a single cell that synthesized a reporter protein, secreted alkaline phosphatase (SEAP). The SEAP catalyzed the hydrolysis of p-aminophenyl phosphate to p-aminophenol (PAP). A disk electrode in the SECM was positioned above the microwell and monitored the oxidation currents of PAP derived from SEAP. In addition, ring electrodes were prepared on the microwell device to induce redox cycling between the ring and disk electrodes, thus amplifying the electrochemical signals from the reporter protein. The redox cycling-based electrochemical reporter gene assay is useful for single-cell analyses.

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Keywords : Single-cell Analysis, SECM, Reporter Gene Assay, Redox Cycling

1. Introduction

A cell population is heterogeneous, and individual cells express a wide variety of genes at both the transcriptional and translational levels. Therefore, single-cell analyses are needed to provide new insights into cellular biology. Single-cell analyses are generally performed using either fluorescence-based or electrochemical-based assays. Scanning electrochemical microscopy (SECM) provides a non-invasive and sensitive method for measuring cell activity.1 We previously applied the SECM technique to a reporter gene assay.2,3 In this assay, HeLa cells were transfected with a vector encoding secreted alkaline phosphatase (SEAP) as a reporter protein, then seeded in microwells to prepare single-cell arrays. A solution containing p-aminophenyl phosphate (PAPP) as the enzymatic substrate was added to each microwell and the microwell was covered with the tip of a microelectrode in the SECM to accumulate the SEAP. Oxidation currents of the enzymatic product, p-aminophenol (PAP), were acquired to quantify the reaction. The designed device was characterized both by simulation and experiments. HeLa cells transfected with SEAP (SEAP-HeLa) were used as a model for the electrochemical reporter gene assay. The cells were trapped in the microwell device to provide single-cell arrays, and the electrochemical reporter gene assay was performed in redox-cycling and non-redox-cycling modes.

2. Experimental

2.1 Redox current simulation

Redox currents were calculated using COMSOL Multiphysics (ver. 5.2, COMSOL, Inc., USA) to characterize three types of microwell devices. Three-dimensional models comprising a probe and a microwell device were prepared. In all models, a probe (diameter: 130 µm) with a disk electrode (diameter: 50 µm) was placed 5 µm above a microwell. The first model comprised a microwell (diameter: 30 µm, height: 25 µm), the second model comprised a disk electrode (diameter: 30 µm) at the bottom of a microwell, and the third model comprised a ring electrode (inner diameter: 40 µm, outer diameter: 60 µm) at the top of a microwell surrounded by an outer microwell (diameter: 60 µm). Ferrocene-methanol (FcCH₂OH) was used as the redox compound. The electrochemical system was assumed to be a reversible one-electron reaction. The initial concentration of FcCH₂OH was 0.5 mM. The diffusion coefficients of FcCH₂OH and FcCH₂OH⁺ were set to 7 x 10⁻¹⁰ m²/s, indicating that the sum of FcCH₂OH and FcCH₂OH⁺ is equal to the initial concentration of FcCH₂OH during electrochemical detection. Amperometry in the redox-cycling mode was conducted by stepping the electrode in the probe (generator) to

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2.3 Cell culture and transfection
electrodes.
electrode (inside diameter: 40 µm, outside diameter: 60 µm) around 8 microwells (diameter: 60 µm, height: 5 µm) were then prepared on the microwells using lift-off lithography. The outer SU-8 Japan) and used for trapping single cells. Pt electrode arrays were fabricated on a microwell (diameter: 30 µm, height: 25 µm) were fabricated on a 0 V, respectively. was set to 0 and 0.5 mM on the surfaces of the electrodes at 0.5 and 0.30 V) and the QI is reduced back to PAP at the ring electrode produce PAP. The PAP is oxidized to QI at the disk electrode (0.00 V).

2.2 Fabrication of the microwell device
An outline of the device is shown in Fig. 1A. The inner SU-8 microwells (diameter: 30 µm, height: 25 µm) were fabricated on a glass slide using an SU-8 sheet (XP film TRIAL-25: MicroChem, Japan) and used for trapping single cells. Pt electrode arrays were prepared on the microwells with lift-off lithography. The outer SU-8 microwells (diameter: 60 µm, height: 5 µm) were then prepared on the electrodes using SU-8 3005 (MicroChem), providing a ring electrode (inside diameter: 40 µm, outside diameter: 60 µm) around each inner microwell. The ring electrodes were used as collector electrodes.

2.3 Cell culture and transfection
The culture and transfection of HeLa cells were described previously. Briefly, HeLa cells were transfected with the commercially available plasmid vector pSEAP2-control (Clontech Laboratories, Inc., USA) by lipofection. pSEAP2-control is a positive control vector expressing constantly SEAP under the control of the SV40 early promoter and the SV40 enhancer. Since SEAP is secreted from the cells, the reporter gene can be assayed from the culture medium without cell lysis. HeLa cells were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University).

2.4 Detection system
A Pt microdisk electrode (diameter: 50 µm) was used as a generator electrode for SECM measurements (Fig. 1A). The position of the probe was controlled using an XYZ stage (ANE-15: Chuo Seiki, Japan). The electrodes in the probe and device were connected to a multichannel potentiostat (HA-1512µM8: Hokuto Denko, Japan). An Ag/AgCl electrode was used as a reference/counter electrode. The tip of microelectrode approached the surface of the device to acquire an approach curve and allowed control of the distance between the tip of the probe and the device. The device was observed under a microscope.

2.5 Characterization of the SECM-microwell system
The device was treated by O2 plasma ashing, and the system was then characterized using FcCH2OH and PAP. A sample solution containing 0.50 mM FcCH2OH and 0.1 M KCl, or 0.50 mM PAP in HEPES (pH 9.5), was introduced into the device, and the probe was then vertically lowered to the top surface of the SU-8 microwell and the position of the tip was set to 0 µm from the top surface. In the redox-cycling mode, the tip potential was stepped from 0.00 to 0.50 V and from 0.00 to 0.30 V for FcCH2OH and PAP, respectively, while the potential of the ring electrode was kept at 0.00 V. In the non-redox-cycling mode, the potential of both electrodes was stepped from 0.00 to 0.50 V and from 0.00 to 0.30 V for FcCH2OH and PAP, respectively.

2.6 Electrochemical assay of a reporter gene at the single-cell level
Culture medium containing HeLa cells was placed in the microwells, left for 5 min to allow the cells to settle to the bottom of the microwell by gravity, and the culture medium was then aspirated to remove suspended cells from the surface of the device. HEPES buffer (pH 9.5) containing 4.7 mM PAPP (LKT Laboratories, Inc., USA) was introduced into the device, then the probe and Ag/AgCl electrodes were inserted into the solution. The tip of the microelectrode was positioned 0 µm above the surface of the microwell containing a single cell, and the cell was then incubated in the closed microwell for 1 min. In the redox-cycling mode, the tip potential was stepped from 0.00 to 0.30 V while the potential of the ring electrode was kept at 0.00 V, whereas in the non-redox-cycling mode, both potentials were stepped from 0.00 to 0.30 V.

3. Results and Discussion
3.1 Current simulation
The device configurations were designed based on current simulations in which only the disk electrode is placed on the microwell (Fig. 2A). Figure 2A shows that the oxidation current of FcCH2OH was rather low because FcCH2OH is consumed in the microwell. Most of the current signal at 5 s is derived from FcCH2OH diffusing from the bulk. In the next simulation, the disk electrode is placed on the bottom of the microwell (Fig. 2B). Redox cycling between the electrodes on the probe and on the device improves the electrochemical signal (Fig. 2B). However, the signal amplification (the ratio of the current from the disk electrode in redox-cycling mode to that in non-redox-cycling mode) is 2.0, indicating that the distance between the electrodes is too large. In addition, if a

Figure 1. (Color online) Scheme showing the detection system.
(A) Illustration of the probe (blue) with the microdisk electrode, and the microwell device with the ring electrode. The inner SU-8 device (B), or a disk electrode on the probe and a ring electrode on the microwell device (C). The chronoamperograms show oxidation currents at the probe.

Figure 2. (Color online) Redox current simulation of 0.5 mM FcCH2OH. The detection system consists of the disk electrode on the probe (A), a disk electrode on the probe and on the microwell device (B), or a disk electrode on the probe and a ring electrode on the microwell device (C). The chronoamperograms show oxidation currents at the probe.
spherical object such as a cell is placed in the microwell, the object disturbs the redox cycling. This problem was solved by designing a microwell device with a ring electrode (Fig. 2C). The oxidation currents with the ring electrode are higher compared to the other configurations because the gap between the electrodes is decreased, and the signal amplification is 7.2. In this configuration, redox cycling is not disturbed by a spherical object placed in the microwell. Thus, the device with a ring electrode is useful for signal amplification and was used for subsequent experiments.

3.2 Characterization of the SECM-microwell system

Figure 3 shows optical images of the detection system. Figure 3A shows that the diameters of the probe and disk electrodes were approximately 130 and 50 μm (RG: 2.6), respectively. Figure 3B shows the microwell device consisting of sensors and connector pads. Eight addressable ring electrodes in the sensor area were prepared.

Chronoamperometry was performed in redox-cycling and non-redox-cycling modes to evaluate signal amplification. Figure 4A shows that oxidation currents obtained using 0.50 mM FcCH2OH in redox cycling increased dramatically (by a factor of 11) compared to those in non-redox-cycling mode. The experimental results shown in Fig. 4A are roughly similar to the simulation results shown in Fig. 2C, indicating that the gap between the tip of the probe and the microwell device was approximately 5 μm, even though the position of the tip was set to 0 μm above the surface of the device. We therefore believe that the SU-8 layer was deformed when the tip was attached on the surface, thereby producing the 5 μm gap between the probe and the device.

The oxidation currents of PAP in the non-redox-cycling mode were twice as high as those obtained using FeC2H4OH (Fig. 4B) because the PAP/QI electrochemical system is a two-electron reaction and its diffusion coefficient is similar to that of FeC2H4OH. However, the signal amplification when detecting PAP was considerably lower than when detecting FeC2H4OH, suggesting fouling of the electrode due to redox cycling and indicating that the potentials might be insufficient for redox cycling. Even though a potential of 0.00 V might be insufficient to reduce QI back to PAP, we selected a potential of 0.00 V at the collector electrode for redox cycling to avoid consuming dissolved oxygen.

3.3 Electrochemical detection based on redox cycling for a reporter-gene assay at the single-cell level

HeLa cells transfected with a plasmid vector (pSEAP-2-control) were used as a model for the reporter gene assay. The optical images in Fig. 5A demonstrate that single-cell arrays were successfully fabricated on the device. Although the cells did not adhere chemically and physically on the bottom of the microwell, the cells were not flushed out by a small amount of flow, because the microwell blocked the flow. Thus, the microwell allowed the cells to stay in the microwells during the measurement. The current responses of the SEAP-HeLa cells were significantly larger than that of wild-type HeLa cells (Fig. 5B), indicating that SEAP was successfully measured. Wild-type HeLa cells possess endogenous alkaline phosphatases (ALP) and thus provided oxidation currents for PAP. The current signals in the redox-cycling mode were only approximately 1.4 times higher than those in the non-redox-cycling mode (Fig. 5B), but this was sufficient for successful application of the detection system. The signal amplification in the detection of the reporter gene assay was considerably lower than that of FeC2H4OH, and the reason is the same mentioned in the part of 3.2. The standard deviation in Fig. 5B are large, because of the variation among cells. However, the signal amplification is similar to that of FcCH2OH because the PAP/QI electrochemical system is a two-electron reaction and its diffusion coefficient is similar to that of FcCH2OH.

The future work is to monitor a signal transduction in cells by switching on/off the expression of the reporter gene under the control of promoter region.
The detection system can be applied to several kinds of single-cell analysis. For example, cell differentiation of embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, can be evaluated though detection of endogenous alkaline phosphatase (ALP). Thus, the system is applicable in tissue engineering and medical biotechnology.

4. Conclusions

We reported a redox-cycling-based electrochemical reporter gene assay for single cells using an SECM-microwell system. The system consists of a disk electrode in the SECM and a ring electrode in the microwell device. The detection system was characterized using simulation and experiments and indicated that redox cycling was successfully induced to amplify the electrochemical signal. In addition, a redox-cycling-based electrochemical reporter gene assay was successfully demonstrated. Thus, a model for an electrochemical reporter gene assay is proposed. We believe that this redox cycling-based electrochemical reporter gene assay is useful for single-cell analyses.

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