Electrochemical Monitoring of Intracellular Enzyme Activity of Single Living Mammalian Cells by Using a Double-Mediator System

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Abstract

We evaluated the intracellular NAD(P)H:quinone oxidoreductase (NQO) activity of single HeLa cells by using the menadione–ferrocyanide double-mediator system combined with scanning electrochemical microscopy (SECM). The double-mediator system was used to amplify the current response from the intracellular NQO activity and to reduce menadione-induced cell damage. The electron shuttle between the electrode and menadione was mediated by the ferrocyanide/ferricyanide redox couple. Generation of ferrocyanide was observed immediately after the addition of a lower concentration (10 μM) of menadione. The ferrocyanide generation rate was constant for 120 min. At a higher menadione concentration
(100 μM), the ferrocyanide generation rate decreased within 30 min because of the cytotoxic
effect of menadione. We also investigated the relationship between intracellular reactive oxygen
species or glutathione levels and exposure to different menadione concentrations to determine
the optimal condition for SECM with minimal invasiveness. The present study clearly
demonstrates that SECM is useful for the analysis of intracellular enzymatic activities in single
cells with a double-mediator system.

Keywords
Scanning electrochemical microscopy; Menadione; Glutathione; Reactive oxygen species;
Ferricyanide

Abbreviations
RG, the ratio of the insulating glass sheath radius to the disk electrode radius

1. Introduction
The intracellular enzymatic activity of a single living cell is a beneficial indicator of its
metabolic vitality. Novel analytical tools permitting low-invasive, continuous, quantitative, and
single-cell level measurements to estimate the intracellular enzymatic activity are required.
Electrochemical methods have enabled the noninvasive quantitative analysis of redox enzyme
activity by using mediators that shuttle electrons between the electrode and enzymes. In
particular, hydrophobic redox mediators are known to undergo transmembrane diffusion
processes and can be utilized to investigate intracellular redox activity [1, 2]. In addition,
scanning electrochemical microscopy (SECM), which is a scanning probe microscopy technique
and uses a micro- or nano-electrode as a probe, is suitable to measure enzymatic activities with
high spatial resolution (less or equal to the size of a single cell) [3, 4]. Because SECM is useful to image and analyze the efflux or uptake of redox species from a sample surface, it has been used for single-cell measurements of stress-related chemicals such as neurotransmitters [5-7], nitric oxide [8], reactive oxygen species (ROS) [9, 10], and oxygen [11-13]. Intracellular and cell surface enzyme activities have also been detected with minimal invasiveness and characterized quantitatively [14-21].

Menadione is a quinone derivative with high cell membrane permeability and has been used to evaluate intracellular enzyme activities [14-16, 22, 23]. However, it is also known that menadione is cytotoxic. In the cell, menadione is detoxified by the NAD(P)H:quinone oxidoreductase (NQO) (EC 1.6.5.2) or eliminated by the conjugation reaction with glutathione (GSH). [24] NQO is a key enzyme that provides protection from quinone species. NQO protects against the deleterious effects of quinones by catalyzing their two-electron reduction without generating radical species. In general, quinones can readily undergo one-electron reduction reactions by other reductases and this process generates ROS, which are extremely cytotoxic because they induce lipid peroxidation, enzyme inactivation, and modification of DNA. This reaction also results in the generation of reactive semiquinone intermediates that can form adducts directly with cellular macromolecules, including DNA, thereby making them carcinogenic [25]. Another detoxification mechanism is the formation of a menadione conjugate with the reduced form of GSH. GSH is the major endogenous antioxidant, preventing damage of important cellular components from ROS attack. After the conjugation reaction of menadione with GSH, the complex is pumped out of the cell. Bard et al. detected the GSH-menadione complex in yeast cells [15] and Hep G2 cells (a human liver carcinoma cell line) [14] and calculated the efflux rate using on a constant-flux model.

The menadione-ferrocyanide double-mediator system has been used to amplify the
current response from intracellular NQO activities (Fig. 1) [22, 23, 26]. Ferricyanide helps to overcome the slow heterogeneous redox kinetics of menadione on Pt electrodes, which results in a current increase. The current was also amplified by redox cycling of the ferricyanide/ferrocyanide turnover between the detector electrode and the menadione/reduced form of menadione couple. Nagamine et al. previously reported evaluation of NQO activities of yeast cells by this system with SECM [16], and they optimized menadione concentration to maximize current responses. However, owing to menadione cytotoxicity, that condition is not suited for the time-dependent measurements. Conversely, there is no concentration limit on ferrocyanide because of its very low cell membrane permeability. If the ferricyanide concentration is much higher than that of menadione, it is not possible to detect menadione directly on the electrode because almost all menadione is reoxidized by ferricyanide near the cellular surface. If we assume that the concentrations of menadione and ferricyanide are in large excess over the value of the Michaelis constant of NQOs and that the microelectrode detects the diffusion-limited current, the rate-limiting step for the determination of the current response is the intracellular NQOs reaction [27].

In this study, the NQO activity of single living cells was monitored electrochemically with a double-mediator system and SECM at different concentrations of menadione as a membrane-permeable (hydrophobic) mediator. Ferricyanide, as a non-permeable mediator, was used for the highly sensitive detection of NQO activity. We optimized menadione concentration to minimize cytotoxicity and optimized ferricyanide concentration to obtain clear response. We also determined the amount of intracellular ROS and GSH at different menadione concentrations.
2. Materials and methods

2.1. Chemicals and materials

Potassium hexacyanoferrate (III), menadione, D-(+)-glucose, and 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid (HEPES) were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). All solutions were prepared using distilled and deionized water (Direct-Q, Millipore).

2.2. Cell Culture

HeLa cells were provided by the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). They were cultured on plastic petri dishes (Ø: 35 mm) in RPMI-1640 medium (Gibco Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified atmosphere containing 5% CO₂. For single-cell measurements, HeLa cells were seeded on a 35-mm dish at a low concentration (1 × 10³ cells per dish). After incubation for 1 day, these cells were used for SECM measurements.

2.3. SECM set-up

A disk-type Pt electrode with a diameter of 20 μm (RG = 2, RG, the ratio of the insulating glass sheath radius to the disk electrode radius) was used as a SECM microelectrode probe. The fabrication of the Pt microelectrode has been described previously [28]. In brief, a fine Pt wire was inserted into a glass capillary and shielded by thermal fusing of the glass. Finally, the tip of the capillary was carefully polished to give a disk-type microelectrode. SECM measurements were performed in a constant height mode. The electrode–substrate (petri dish) distance was set at 20 μm to avoid contact with the cells. The electrochemical current was
measured on the basis of a two-electrode configuration using an Ag/AgCl/KCl (sat.) electrode as reference electrode. The microelectrode was set at +0.5 V vs. Ag/AgCl/KCl (sat.) at room temperature (20°C) for the detection of ferrocyanide. The current was amplified using a current amplifier (428, Keithley). Movement of the microelectrode probe was performed using a motor-driven XYZ stage (K701-20RMS, Suruga Seiki) and a stage controller (D70, Suruga Seiki). The details of the SECM system have been reported elsewhere [17]. The measurements were conducted in a petri dish with HeLa cells in HEPES-based saline solution (10 mM HEPES, 150 mM NaCl, 4.2 mM KCl, 2.7 mM MgCl₂, 1.0 mM Na₂HPO₄, and 11.2 mM glucose; pH 7.4) containing 10 or 100 μM menadione and 200 μM ferricyanide. Before conducting the SECM measurements, the culture medium was replaced with HEPES-based saline solution.

2.4. Evaluation of the NQO activity

We evaluated the NQO activity as rate at which ferricyanide is generated using one-line scan SECM measurements. To monitor the NQO activity, one-line scan SECM measurements were carried out every several minutes. The baseline tilt of current response was subtracted by current response at start and last points of one-line scan where the microelectrode located away from the cell samples. Quantification of the mass transfer rate of the redox mediator (ferrocyanide) from a single HeLa cell was carried out using GC mode SECM imaging as described previously [29]. Briefly, when a spherically shaped cell is positioned on a flat substrate in a measurement solution, the mass transfer rate of the hemispherically diffusing redox mediator from the single HeLa cell \( F_{cell} \) in mol s⁻¹ can be expressed as

\[
F_{cell} = 2\pi r_s^2 \times \frac{\Delta C}{\Delta x}
\]  

(1)
where $D$ is the diffusion coefficient of a redox mediator (for ferricyanide, $D_{\text{ferro}} = 6.5 \times 10^{-6}$ cm$^2$ s$^{-1}$); $r_s$ is the radius of the single HeLa cell (in this study, $r_s = 10$ μm); and $\Delta C/\Delta x$ is the concentration gradient of the redox mediator at the cell surface (mol cm$^{-2}$). The concentration gradient of a redox mediator at the cell surface is calculated from the profiles of the redox current. For a conductive disk electrode, the redox current observed with the SECM system is expressed by

$$I = 4nFDCa$$

(2)

where $n$ is the number of electrons per molecule reduced; $F$ is the Faraday constant (96500 C mol$^{-1}$); $C$ is the local concentration of the redox mediator (mol cm$^{-3}$); and $a$ is the electrode radius (cm). The Faradaic current is also relatively independent of the RG. For a large RG, the experimental current should be close to the value expected from Eq. (2). However, at a RG < 10, a correction can be introduced by replacing the factor “4” in Eq. (2) with “4.43” (RG = 2) [30]. We assumed that the time scale in which the concentration gradient reaches a steady state is much faster than that in which the ferrocyanide generation rate varies. On the basis of this assumption, we also carried out real-time monitoring of the generation rate at different menadione concentrations. When we detect membrane-permeable mediators by the electrode near the cell surface, consumptions of redox mediators on the electrode induce local concentration gradients and cause a diffusion-controlled exchange of redox species across the lipid bilayer (called SECM-induced transfer mode) [31]. In addition, the closer an electrode is to the cell, the larger this effect becomes. However, in this case, the induced flux from inside the cell would be negligible because we used and detected hydrophilic ferricyanide. To investigate this effect, we also evaluated the ferrocyanide generation rate from a single cell at different electrode-substrate distances.
2.5. Viability assay, GSH assay and ROS assay

The cytotoxicity of menadione was examined using the Cell Counting Kit-8 (DojinDo, Kumamoto, Japan). Briefly, 100 μL of cell suspension (ca. $1 \times 10^5$ cells mL$^{-1}$) was added per well of 96-well microplates, followed by incubation at 37°C for 6 h under 5% CO$_2$ for cell attachment. Cells without menadione exposure (only HEPES-based saline solution) were used as control. To eliminate the influence of the medium, 100 μL of medium without sample was used as reference. After cultivation, the cells were exposed for 30 min to HEPES-based saline solution containing 0.1–1000 μM menadione and 200 μM ferricyanide, followed by washing and addition of medium containing 10 μL of Cell Counting Kit-8 solution. After incubation for two hours under the above-described condition, the absorbance at 450 nm was measured on a microplate reader. Cell viability was calculated using the following formula: $\frac{(A_s - A_b)}{(A_c - A_b)} \times 100$ (A$_s$: optical density at 450 nm [OD$_{450}$] of HeLa cells with menadione exposure, A$_c$: OD$_{450}$ of HeLa cells without menadione exposure, A$_b$: OD$_{450}$ of medium). These measurements were performed 8–12 times in each condition.

The intracellular ROS level was determined using the OxiSelect™ ROS Assay Kit (Cell Biolabs, CA). Cells were seeded on a 35-mm dish, followed by incubation at 37°C for 6 h under 5% CO$_2$ for cell attachment. DCFH-DA ($2',7'$-dichlorodihydrofluorescein diacetate) was added to the each dish and incubated for 1h under 5% CO$_2$. DCFH-DA is deacetylated by cellular esterases to non-fluorescent $2', 7'$-Dichlorodihydrofluorescein, which is rapidly oxidized to highly fluorescent $2', 7'$-Dichlorofluorescein (DCF) by ROS. The cells were exposed for 30 min to HEPES-based saline solution containing 0, 1 or 100 μM menadione and 200 μM ferricyanide. After suspending the cells, fluorescence measurements were performed with a flow cytometer (MoFlo™ XDR, Beckman Coulter, USA) at an excitation and emission
wavelength of 480 nm and 530 nm, respectively. Approximately $1 \times 10^4$ cells were analyzed in each condition.

The GSH-Glo Glutathione Assay (Promega, Madison, USA) was used to determine the intracellular GSH level. The assay is based on the conversion of a luciferin derivative into luciferin in the presence of glutathione. The cell samples in 96-well microplates were prepared under the same condition as described above. GSH standards (0–5 μM) were prepared from a 5-mM stock solution diluted in water. Five two-fold dilutions of the GSH stock were prepared and transferred to wells (50 μL). After cultivation, the cells were exposed for 30 min to HEPES-based saline solution containing 0, 4, 10, 40, or 100 μM menadione and 200 μM ferricyanide. Subsequently, the GSH-GloTM reagent, which was prepared according to the manufacturer’s instructions, was added to the experimental wells (50 μL per well) and the plate was incubated for 30 min at room temperature. Reconstituted luciferin detection reagent (50 μL) was added to each well and the plate was incubated for 15 min at room temperature. Luminescence was measured using an ICCD camera (PI-MAX 512RB; Princeton Instruments, USA). A standard curve was derived using the GSH standards (0–5 μM) and the GSH concentration in each sample was calculated from the equation of the regression line.
3. Results and discussion

3.1. Cytotoxic effect of menadione

Fig. 2 shows the viability of HeLa cells after exposure to various menadione concentrations for 30 min. The toxic effect was evident for menadione concentrations of more than 20 μM and could be due to the menadione-induced increase in ROS generation and decrease in GSH. This result is similar to those of other cytotoxicity studies performed with Hep-G2 cells [14]. Ferricyanide was not cytotoxic less than 20 mM for coexisting with the 10 μM menadione (Supporting Information (SI), Fig. S1).

The relationship between the menadione concentration and intracellular ROS and GSH levels is shown in Fig. 3A and 3B, respectively. Intracellular ROS levels were significantly increased at 100 μM menadione comparing with untreated controls (Fig. 3A). This is because menadione is reduced by one-electron reactions via other reductases and induces the generation of ROS. When the menadione concentration was 100 μM, high levels of ROS were generated and caused cell death. This result was consistent with that of the viability assay. ROS were also generated at a low menadione concentration (1 μM); however, cell viability was not affected (Fig. 2). Moreover, it was evident that the distribution of intracellular ROS levels became much broader. This is attributable to the differences in the detoxification ability of each cell suggesting a heterogeneous property of individual cells.

The intracellular GSH level significantly decreased when cells were exposed to more than 40 μM menadione, whereas it did not decrease at lower menadione concentrations (≤ 10 μM; Fig. 3B). Collectively, at a higher menadione concentration (100 μM), the elimination of menadione by formation of the GSH-conjugate becomes insufficient and the remaining menadione induces the generation of ROS, which leads to cell damage. At a menadione concentration of 10 μM, the cytotoxic effect is very small because of the GSH-mediated
detoxification of menadione even though ROS might also be generated. In the following SECM experiments, the menadione concentration was set at 10 μM to enable the measurement of electrochemical signals from the cells.

### 3.2. SECM imaging and flux analysis of a single cell

We measured the NQO activity of HeLa cells using a double-mediator system (Fig. 1). Menadione can readily diffuse into HeLa cells upon addition to the extracellular medium and can be reduced by intracellular NQO. Because of its hydrophobic nature, menadione can diffuse into the cell without the assistance of transport proteins or pumps. Outside of the cells, the reduced form of menadione immediately reacts with hydrophilic ferricyanide and ferrocyanide (Fe[CN]$_6^{4-}$) is then generated. The current signal of the ferrocyanide oxidation at the electrode indirectly reflects the intracellular NQO activity. The reduced form of menadione emitted from the cell is re-oxidized to menadione by ferricyanide and can react again with NQO again (redox cycling).

Fig. 4 shows SECM and optical microscope images of a single HeLa cell in HEPES buffer solution in the presence of 10 μM menadione and 200 μM, 2 mM or 20 mM ferricyanide. The current responses due to oxidation of ferrocyanide were successfully detected. At 200 μM ferricyanide current response was clear. However, at high ferricyanide concentration (2 mM and 20 mM), current response became unclear because of highly back ground current, not because of cytotoxicity (SI, Fig. S1). This observation indicates that SECM can be used to indirectly evaluate the NQO activity at a single-cell level by electrochemical detection of ferrocyanide. The area of higher current responses on the SECM image became slightly larger than the real area of the single cell on the optical image caused by diffusion of the mediators in buffer solution. At 100 μM menadione, a current response from the single cell could not be clearly
observed in most cases (SI, Fig. S2). This was due to cell death originating from the cytotoxicity of menadione.

The ferrocyanide generation rates of individual HeLa cells, $F_{\text{cell}}$, were calculated using Eq. (1) and were found to be $7.15 \pm 3.54 \times 10^{-17}$ mol s$^{-1}$ per cell ($N = 18$). This was larger than the generation rate detected from a single yeast cell previously reported by Nagamine et al. ($3.07 \times 10^{-19}$ mol s$^{-1}$ per cell) [16]. The main reason why the large generation rate for HeLa cell was observed was that the size of HeLa cell is larger than that of yeast cell. However, the concentrations of menadione and ferrocyanide in that study were different from those used in the present study. Furthermore, large variations in the current responses were observed (SI, Fig. S3), which is thought to be due to the heterogeneity in NQO activity of each HeLa cell.

Generation of ferrocyanide was observed immediately after replacing the culture medium with measurement solution containing menadione (Fig. 5). At 10 μM menadione, the generation rate was almost constant for 120 min. On the contrary, at 100 μM menadione, it decreased rapidly within 30 min after addition of menadione. This drastic decline could be attributed to cell death (Fig. 2) and was consistent with the difficulty to detect a current response at 100 μM menadione (SI, Fig. S2).

When the current response was 0.377 pA (one-line scan result at 60 min of Fig. 5A, SI, Fig. S4), with $F_{\text{tip}} = I_{\text{tip}}/nF$ [32], a ferrocyanide consumption rate of $3.91 \times 10^{-18}$ mol s$^{-1}$ was obtained from the SECM measurement. The consumption of ferrocyanide at the SECM electrode is 20-fold smaller than the generation of ferrocyanide by an individual living cell. However, it has been suggested that concentration changes of ferrocyanide near the cell due to the high reaction rate at the tip induces local concentration gradients and causes a diffusion-controlled exchange of redox species. Indeed, the ferrocyanide generation rate was slightly decreased when the electrode was placed closer to the cell (Fig. 6). It was not due to
cellular variability with a time-scale of several minutes. It is suspected that this slight decrease was caused by the hydrophilic property of ferrocyanide. Furthermore, in the constant-height mode (electrode-substrate distance is constant), the current responses could also include a feedback effect based on the electrode-cell surface distance, which could be affected by the height and shape of the cell. Although, the current profiles were in accordance with spherical diffusion theory when the electrode was not placed close to the cell as shown in SI, Fig. S5. To overcome those limitations, constant-distance mode (electrode-sample surface distance is constant) SECM techniques are inherently advantageous. In particular, combining SECM with scanning probe techniques, e.g., atomic force microscopy [33] and scanning ion conductance microscopy [7, 34, 35] as well as shear force [36-38] and impedance-based techniques [39], led to efficient strategies to control electrode-to-sample separation. In the future, the SECM system with distance control will be applied for more precise analyses of the NQO activity in single living cells. However, as indicated in this study, the flux from the tip electrode of the constant-distance mode SECM should be decreased by at least an order of magnitude smaller than the flux from the sample surface for precisely estimating the enzyme activity from the concentration gradient.
4. Conclusion

In summary, we successfully evaluated the intracellular NQO activity of single HeLa cells with a menadione-ferrocyanide double-mediator system combined with SECM. Oxidation currents of ferrocyanide were increased at the cell vicinity, and the ferrocyanide generation rates of single cells were found to be $7.15 \pm 3.54 \times 10^{-17}$ mol s$^{-1}$ per cell. Ferrocyanide generation was observed immediately after addition of 10 μM menadione. The ferrocyanide generation rate was constant for 120 min. At 100 μM menadione, the ferrocyanide generation rate decreased rapidly within 30 min because of the cytotoxic effect of menadione. We also investigated the relationship between intracellular ROS or GSH levels and the menadione concentration to which cells were exposed to determine the optimal menadione concentration for minimally invasive detection. We found that 10 μM menadione was optimal to detect NQO activity. The present study clearly demonstrated that SECM is useful for the analysis of intracellular enzymatic activities in single cells using a double-mediator system. In the future, the SECM system with distance control and a nano-electrode as probe will be applied for more precise analyses that include not only the estimation and monitoring of NQO activity or metabolites but also morphological changes in the cell surface.

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**Figures and captions**

Fig. 1 Principle of electrochemical detection of NAD(P)H:quinone oxidoreductase (NQO) activity in a HeLa cell. Fe(CN)$_6^{3-}$ and menadione were added in the measurement solution.

Fig. 2 Viability assay results for HeLa cells were evaluated by using Cell Counting Kit-8 (DojinDo, Kumamoto, Japan) normalized to the cells exposed with HEPES-based saline solution without menadione and ferricyanide. The absorbance at 450 nm was measured for HeLa cells after exposure for 30 min to different concentrations of menadione. The values represent several independent measurements for different menadione concentrations. Within each concentration, measurements for three or more replicates were performed.

Fig. 3 (A) Menadione increased intracellular ROS level. HeLa cells were pre-incubated with DCFH-DA for 60 min and then treated with 0, 1 or 100 μM menadione with 200 μM ferricyanide. The fluorescence of DCF was measured with a flow cytometer using excitation and emission wavelengths of 480 nm and 530 nm, respectively. (B) Menadione decreased intracellular GSH level. HeLa cells were pre-incubated with ruciferin derivative and then treated with 0, 4, 10, 40 or 100 μM menadione with 200 μM ferricyanide. The luminescence of luciferin was measured by ICCD camera and then GSH concentration was calculated from a standard curve.

Fig. 4 SECM images (left) and optical images (right) of a single HeLa cells. Measurements were performed in HEPES-based saline solution containing 10 μM menadione and ferricyanide (200 μM (A), 2 mM (B) or 20 mM (C)). The measurements were started at 30 min after addition of measurements solution and finished within 10 min. The electrode was positioned 20 μm.
above the substrate, and the scan rate was 50 μm s⁻¹. The scan range was 500 × 500 μm² and the step size was 10 μm. Scale bar: 100 μm. SECM images are displayed without correction of baseline shift of current response.

Fig. 5 Time course of the ferrocyanide generation rate from a single HeLa cell at 10 μM (A) or 100 μM (B) of menadione. Menadione was added at 30 min. The generation rates were calculated from modified current (SI, Fig. S4) of one-line scan SECM by using Eq. (1). The scan rate was 10 μm s⁻¹. The scan range was 500 μm.

Fig. 6 The ferrocyanide generation rates from a single HeLa cell at different electrode-substrate distances were calculated from modified current (SI, Fig. S5) of one-line scan SECM by using Eq. (1). Measurements at each distance were performed three times in the same sample. One measurement was completed within 1 minute, and all measurements (12 times) were completed within 60 minutes. The ferrocyanide generation rate at 20 μm was maintained virtually constant.
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Fig. 2: Relationship between Menadione concentration (µM) and normalized viability.
Fig. 3
Fig. 4
Fig. 5
NAD(P)H: quinone oxidoreductase (NQO) activity of single HeLa cells were evaluated by using the menadione-ferrocyanide double mediator system combined with scanning electrochemical microscopy (SECM).