Simulation and Experiment for Electrode Coverage Evaluation by Electrochemical Impedance Spectroscopy using Parallel Facing Electrodes

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Abstract

Parallel facing electrodes (PFE) for adherent cell monitoring by electrochemical impedance spectroscopy (EIS) was developed, and its characteristics were investigated by both computer simulation and experiment. The PFE consists of two facing gold electrode strips separated by 40 μm, and the area of its intersection is 500 μm × 500 μm. Computer simulation of EIS with adherent cells showed distinct difference in solution resistance for different cell coverage, which was confirmed by experimental results using latex beads suspension. A well-defined relationship between solution resistance and cell coverage in our PFE is promising for quantitative evaluation of cell density, morphology and fatality.

**Keywords:** Electrochemical impedance spectroscopy, Electrochemical sensor, Finite element method simulation.
Introduction

Cell is the basic unit of living organism, and medical research and drug development by cell analysis have widely been conducted.\(^1\)\(^2\) Cell analysis provides information such as physiological parameters of cell and the effect of drugs.\(^3\) Microscopy using fluorescent stains and flow cytometry are widely accepted as a principal cell analysis method.\(^4\) However, fluorescent stains may inhibit inherent cell functions, and thus it is desirable to conduct the analysis by a label-free method.\(^5\) Meanwhile, a flow cytometry analyzes individual cell in a flow by dispersing the cell in solution. It is a powerful tool for evaluation and sorting of the single floating cell, but it is not suitable for the long-term monitoring of adherent cell.\(^5\) Electrochemical impedance spectroscopy (EIS) is one of the promising analysis method which enables long-term monitoring of the adherent cell without any damaging or labelling.\(^6\) The EIS can measure the time course of identical cells cultured on the sensor,\(^7\) and it offers the various information on metabolism,\(^8\) morphology and changes in cell membrane and cytoplasm.\(^9\) As the EIS does not require any optical measurement system, it is easy to downsize the whole measurement system by an electrode array with CMOS technology.\(^10\)\(^-\)\(^12\)

Co-planar electrodes are accepted as an electrochemical sensor by many researchers due to its ease of fabrication and handling.\(^13\) There are some reports on the EIS measurement of antibody-antigen reaction,\(^14\)\(^15\) cell cycle,\(^16\)\(^17\) barrier function,\(^18\) apoptosis\(^19\) and differentiation of stem cell\(^20\) using co-planar electrodes such as interdigitated electrodes. In case of EIS using co-planar electrode, an electric field concentrates along the edges of the electrodes, and a non-uniform electric field is applied to the cells.\(^21\) Dispersion of cell positions also occurs on the sensor substrates.\(^22\) These aspects complicate formulation of impedance between two electrodes, thus it is difficult to quantitatively evaluate the cell density and morphological information.\(^23\) In contrast, parallel facing electrodes (PFE) can apply a uniform electric field to cells and measure
only the cells existing between the electrodes. Thanks to the uniform electric field, impedance is described by a relatively simple and well-defined formula showing unambiguous dependence on cell coverage of the sensor electrode. As the cell coverage is proportional to cell density, cell size or existence/absence of the cells, EIS using the PFE will give a quantitative information of living cells. Based on this idea, we have verified it previously with an experimental approach.24

In this work, we demonstrate that the impedance analysis using the PFE can be used to determine cell coverage of the sensor electrode surface in a quantitative way. The influence of cell coverage on impedance was examined by simulation in addition to experiments. The simulation results confirmed that the cell density can be quantitatively evaluated by EIS using PFE and equivalent circuit analysis. In fact, the results of latex beads suspension measurement showed the linear relationship between the impedance and the inverse of the area uncovered by latex beads. These results indicate that our PFE can be used to determine the cell coverage ratio, which reflects long-term changes in cell density, size or fatality.

This paper is organized as follows: Section 2 shows the theory of equivalent circuit model used in the EIS analysis of cell. Section 3 explains the simulation and experimental methods. The results and discussion are described in Sect. 4. Section 5 gives the conclusion.

Theory

The equivalent circuit model shown in Fig. 1 is used for the cell analysis by EIS measurement.25 Electrical double layer capacitance ($C_{dl}$), solution resistance ($R_{sol}$) and solution capacitance ($C_{sol}$) can be considered regardless of existence/absence of cells. For solution not containing redox species, charge transfer resistance ($R_{ct}$) can be ignored. Cells are considered with intercellular solution resistance ($R_{gap}$), cell membrane capacitance ($C_{mem}$) and cytoplasm resistance ($R_{cp}$) inserted in series with $R_{sol}$. The $R_{gap}$ is simply written as
\[ R_{\text{gap}} = \frac{1}{\sigma_{\text{sol}}} \cdot \frac{h}{A - A_{\text{cell}}} = \frac{h}{\sigma_{\text{sol}} A} \cdot \beta, \]  

where \( \sigma_{\text{sol}} \) is the conductivity of solution, \( h \) is the height of cell, \( A \) is the total area of electrode and \( A_{\text{cell}} \) is the electrode area covered by the cells. Defining the cell coverage ratio \( \alpha \) as \( \alpha = A_{\text{cell}} / A \), \( R_{\text{gap}} \) can be rewritten in terms of \( \beta = 1 / (1 - \alpha) \), as shown in Eq. (1). In case of PFE, cells on the electrode are measured regardless of their positions. This contributes to the linear dependence of \( R_{\text{gap}} \) on \( \beta \) for fixed \( h \) and \( \sigma_{\text{sol}} \).

Figure 2 shows a schematic Bode plot drawn from the equivalent circuit model shown in Fig. 1. The \( f_{c1} \) is the corner frequency depending on \( C_{\text{dl}} / 2 \) and \( R_{\text{sol}} + R_{\text{gap}} \), while \( f_{c2} \) is the corner frequency depending on \( R_{\text{sol}} + R_{\text{gap}} \) and \( C_{\text{mem}} / 2 \). The \( f_{c1} \) and \( f_{c2} \) generally appear at several hundred kHz and several MHz, respectively.\(^{23}\) Measurement of \( R_{\text{gap}} \) is thus hindered by \( C_{\text{dl}} \) below \( f_{c1} \). Impedance of \( C_{\text{dl}} \) decreases as the frequency increases, and \( R_{\text{gap}} \) becomes noticeable when frequency becomes higher than \( f_{c1} \). When frequency exceeds \( f_{c2} \), current flows through the cell membrane. The \( R_{\text{gap}} \) representing the cell density and morphology can therefore be observed in the frequency range between \( f_{c1} \) and \( f_{c2} \).

**Simulation and Experimental Methods**

*Finite element simulation of impedance dependence on adherent cell density*

The simulation was performed using COMSOL Multiphysics 5.3a, where differential forms of Maxwell’s equations are solved by finite element method. Table 1 shows the parameters used for the simulation, where almost all of the values were based on the literature of Sun et al.\(^{26}\) The double layer capacitance per unit area was a calculated value.\(^{22}\) This study
focuses on cell density and thus the cytoplasm conductivity was set to be same as that of the solution. The simulations were performed with various electrode coverage of cells existing between PFE. The simulation model is shown in Fig. 3. Spherical cell and adhesive cell modeled as cylinder or hemisphere\textsuperscript{27-29} in the rectangular cuboid model were assumed. The distance between the cell and the electrode was set to 100 nm.\textsuperscript{27} The spherical cell diameter was 10 $\mu$m and adhesive cell diameter and height were 10 $\mu$m and 5 $\mu$m, respectively.\textsuperscript{22} The cell nucleus within the cell was ignored because it does not affect the $R_{gap}$ depending on the extracellular ion conduction. The top and bottom faces of the model domain were defined as working electrode (WE) and counter electrode (CE), respectively. The mirror boundary condition was imposed at the side faces to represent a space infinitely repeated. Therefore, even if only one cell was set on the model, the simulation results were equivalent to that of multiple cell groups. Zero DC bias and an AC voltage with an amplitude of 5.0 mV was applied to WE, while CE was kept grounded. The distance between WE and CE was 40 $\mu$m which is the same as the proposed PFE used in experiments. The $\alpha$ value was changed from 0% to 70% by changing the electrode width $W$ while fixing the cell diameter. The $\beta$ and $W$ corresponding to each $\alpha$ are shown in Table 2. Since the magnitude of impedance is different for different electrode area, the simulation results are multiplied by the electrode area $W^2 [\mu m^2]$ and converted to the impedance per 1 $\mu$m$^2$ $|Z_0| [\Omega \cdot \mu m^2]$. To properly reproduce the infinite repetition of the model domain, the simulation model considered here is not a cylinder but a rectangular cuboid. The electrode coverage of 80% or more cannot therefore be reproduced by the rectangular cuboid model. However, it will not be an issue for simulating the situation of an actual experiment because the adherent cell cultures are generally passaged when they grow to about 70% - 80% confluent. The simulation was conducted in the frequency range of 1 Hz to 100 MHz.
PFE sensor fabrication

Figure 4 shows the electrode structures used in the experiment.\(^4\) A cover glass (Matsunami Glass CG 00024) was used as an electrode substrate with spacers that had a width of 1.0 mm and a height of 20 \(\mu\)m along the long edge. Titanium and gold were deposited on the glass substrate in this order with a thickness of 50 nm for titanium and 100 nm for gold, respectively. The titanium acts as an adhesive layer between the gold and the substrates, and the gold acts as a sensor electrode. Let the two electrodes be substrate 1 (S1) and substrates 2 (S2). One of them functions as a WE and the other functions as a CE. As shown in Fig. 4(b), S1 and S2 are overlaid crosswise so as to cross each other to form a parallel facing structure. The width of narrow part of electrode is 500 \(\mu\)m, and the area of the electrode intersection which is the sensing area is 0.250 \(\text{mm}^2\). The electrode distance is 40 \(\mu\)m which is the total of two spacers on the surface of the glass substrate. The electrodes were fixed with the 3D printed jig shown in Fig. S1(a) (Supporting Information) and rubber band. Firstly, S2 was placed on the jig. Subsequently the target solution was dropped onto the center of S2, then S1 was placed crosswise above S2. Lastly the cover was placed above S1. The S1 was detached from S2 for each measurement to exchange the sample solution. The actual photograph of the proposed PFE is shown in Fig. S1(b) (Supporting Information).\(^4\) After fixing the electrodes with the jig and rubber band, the electrodes were connected to a measurement equipment via clip to improve the electrical connection.

Reagents

The latex beads suspension with diameter of 10 \(\mu\)m (Sigma 55463) was prepared. The latex beads are made of polystyrene and its surface has no coating. Latex beads were used as substitutes for cells to evaluate the performance of our sensor. Latex beads are different from real cells in that they have no membrane structure, but they are used as simulated cells to
evaluate impedance sensors.\textsuperscript{4,30,31} The experiment was performed in the range of $\alpha = 0\% - 30\%$ since $\alpha$ of the undiluted suspension was 30%. The latex beads suspension was diluted with 10 mM phosphate buffered saline (Wako 164-18541) so that the electrode coverage $\alpha$ became 0%, 10%, 20% and 30% when all the beads were precipitated on the electrode surface. The supernatant of the diluted latex beads suspension was used as 0% suspension. A micrograph of $\alpha = 20\%$ suspension dropped on the electrode is shown in Fig. S2 (Supporting Information). Negligible aggregation that might affect the measurement has occurred, and the latex beads has mostly diffused to a monolayer. The conductivity of the prepared suspension was measured using a conductivity meter (Horiba B-771).

\textbf{EIS measurements}

The EIS measurement was performed using an electrochemical analyzer (ALS/CH Instrument Model 610DR). To modify the electrode surface as hydrophilic,\textsuperscript{32} the electrodes were coated with 4 $\mu$L of albumin from bovine serum (BSA; Sigma A6003) diluted to 1% w/w with a buffer solution for 40 min at the beginning. The electrodes were then rinsed with ultrapure water (UPW; Wako 214-01301). Before the measurement of each latex beads suspension, the electrodes were also rinsed with 0% suspension. The 2 $\mu$L of each target solution was dropped on the electrode, then EIS measurement was conducted in the range of 1 Hz to 1 MHz. In this experiment, the impedance of the suspension containing latex beads dispersed in solution is measured. However, the position of latex bead is independent in Eq. (1), and the position does not influence the result in principle. Although the proportionality constant of the calibration curve might change, it should not affect the linearity. Thus the impedance of the suspension was measured without considering the effect of adsorption to the electrodes. The impedance of each suspension was measured five times, and the suspension was changed after every measurement. To avoid unintentional increase of the electrode coverage of the latex beads,
the electrodes were rinsed with UPW and 0% suspension after every replacement of the suspension. The measurements were performed at room temperature.

Results and Discussion

Impedance dependence with electrode coverage of cell

Figure 5(a) shows the Bode plot of the simulated impedance using the spherical model shown in Fig. 3(a). The Bode plot of the cylindrical and the hemispherical model are shown in Fig. S3. The low frequency range \( f < 10 \text{ kHz} \) is the region where the \( C_{dl} \) is dominant. The impedance change due to the difference of the electrode coverage of cell appears between 10 kHz and 1 MHz due to an increase of \( R_{gap} \). The impedance due to \( C_{mem} \) and \( R_{cp} \) is seen above 1 MHz, and finally \( C_{sol} \) becomes dominant near 100 MHz. The shape of the bode plot was almost the same as the schematic shown in Fig. 2.

The \( R_{gap} \), representing the cell density, was observed where the phase difference is closest to 0°. Figure 5(b) shows the impedance magnitude of each \( \alpha \) where the phase difference is closest to 0° plotted against \( \beta \). The figure shows the linear relationship between the impedance and \( \beta \) for all spherical, cylindrical and hemispherical cell (\( R^2 \) are 0.9907, 0.9956 and 0.9837 respectively) as mentioned at Sect. 2. Thus, a linear relationship between impedance and \( \beta \) can be obtained for any practical cell shape. It suggests that the cell coverage change can be estimated backward from the impedance change if a calibration curve is drawn for a certain cell. This is because a uniform electric field is applied to the cell by using the PFE. However, it should be noted that the slope of the calibration curve depends on the cell shape. In Eq. (1), only cell height \( (h) \) and cell coverage \( (\alpha) \) were considered as parameters regardless of cell shape. Hemispherical cell is smaller in volume than cylindrical cell with same height and area of base. The cell modeled as hemisphere do not limit the current path as much as the cylinder and thus
the impedance magnitude was smaller than the cylindrical cell. For the same reason, the slope of the calibration curve for the spherical cell is almost the same as the cylindrical one even though the cell height is double of the other cell models. This indicates a universal calibration curve that can be used for all cells cannot be drawn from the equivalent circuit model considering only cell height and coverage.

Our simulation thus demonstrated the possibility that the cell coverage could be quantitatively evaluated by EIS using PFE. However, it is not possible to draw a universal theoretical line that perfectly matches the simulation result using the equivalent circuit model.

**EIS with latex beads suspension**

Figure 6(a) shows the experimental results of the latex beads suspension. Error bars represent the maximum, the median and the minimum values at each measurement point. The parasitic resistance of the electrodes was fluctuated due to displacement of the connection position of the clip, the change of position and angle where the electrode was overlaid. To eliminate such variation due to the human handling, the median value was adopted.

When the electrodes onto which the suspension was dropped were set on the jig, the suspension spreads to the fringe around the electrode intersection, leading to an undesired fringe impedance between two electrode strips outside the intersection. The $C_{dl}$ impedance of the intersection, which has a small electrode area, is higher than the fringe impedance at the low frequency range, and therefore the fringe impedance is dominant below 10 kHz. Since the $C_{dl}$ impedance of the intersection becomes lower at the high frequency range, the current concentrates only at the intersection and the impedance at the intersection becomes dominant. The shape of the impedance spectrum is similar to that of Fig. 2, but note that the resistance between 100 Hz to 10 kHz is not the $R_{gap}$ derived from the intercellular solution resistance but the solution resistance at the fringe of the intersection. The $f_{c1}$ and $f_{c2}$ appear above 10 kHz at
which the $C_{ai}$ impedance of the intersection appears.

The phase difference is closest to $0^\circ$ at 1 MHz and $R_{gap}$ notably appeared at that frequency. As latex beads are homogeneous insulating spheres, they can be represented by replacing cell membrane capacitance and cytoplasm resistance in the equivalent circuit with merely a capacitance of latex beads. The $R_{gap}$ increases because the higher electrode coverage of the latex beads become, the more current pathway between the electrodes is limited by latex beads.

Figure 6(b) shows the measured impedance at 1 MHz plotted against $\beta$, along with the calibration curve. As shown in Fig. 6(b), the impedance increases linearly with respect to $\beta$. The possibility of quantitative evaluation of cell coverage by EIS was thus confirmed experimentally as well as the simulation.

Thus, we showed that it is possible to quantitatively evaluate the latex beads concentration up to 30% from $R_{gap}$ by EIS measurement using our proposed PFE. Our results suggest the possibility of quantitative evaluation of cell density and cell morphology. Reducing the variations due to the handling during the measurement contributes to more accurate measurements. The simplification of the assembling and disassembling process of the electrodes must be done.

Conclusions

We demonstrated the simulation and experimental aspects to examine the possibility of quantitative evaluation of cell density by EIS using PFE. The simulation results have shown that $R_{gap}$ was proportional to the inverse of the electrode area uncovered by cell, thus cell density and morphological information such as cell size should be quantitatively evaluated. The relationship between $R_{gap}$ and electrode coverage of the latex beads was also confirmed experimentally. Our study suggests that PFE enables the quantitative and continuous evaluation of cell density, cell
size and cell death.

Supporting Information: A 3D CAD image of the electrode jig, picture of the actual electrode, micrograph of latex beads suspension and simulated impedance spectra using the cylindrical and hemispherical cell model. These materials are available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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Table 1  Parameters used in the simulation.

| Parameter                                           | Value     |
|-----------------------------------------------------|-----------|
| Double layer capacitance per unit area [F/m²]        | 0.89      |
| Solution relative permittivity                       | 80        |
| Solution conductivity [S/m]                          | 1.6       |
| Cell membrane thickness [nm]                         | 5.0       |
| Cell membrane relative permittivity                  | 5.0       |
| Cell membrane conductivity [S/m]                     | 1.0 × 10⁻⁸|
| Cytoplasm relative permittivity                      | 60        |
| Cytoplasm conductivity [S/m]                         | 1.6       |

Table 2  Electrode coverage of cells or latex beads $\alpha, \beta = 1 / (1 - \alpha)$ and electrode width $W$.

| $\alpha$ [%] | $\beta$  | $W$ [μm] |
|--------------|----------|----------|
| 70           | 3.333    | 10.6     |
| 60           | 2.500    | 11.4     |
| 50           | 2.000    | 12.5     |
| 40           | 1.667    | 14.0     |
| 30           | 1.429    | 16.2     |
| 20           | 1.250    | 19.8     |
| 10           | 1.111    | 28.0     |
| 0            | 1.000    | 28.0     |
Figure Captions

Fig. 1  The equivalent circuit model for EIS measurement of cell. The $C_{\text{mem}}$ and $R_{\text{cp}}$ correspond to cell membrane capacitance and cytoplasm resistance. These components represent the cell.

Fig. 2  Bode plot of the equivalent circuit model. Impedance between $f_{c1}$ and $f_{c2}$ reflects $R_{\text{gap}}$. The symbol $(R_{\text{gap}}||R_{\text{cp}})$ indicates the combined resistance of $R_{\text{gap}}$ and $R_{\text{cp}}$ in parallel.

Fig. 3  Simulation model for impedance dependence on density of (a) spherical, (b) cylindrical and (c) hemispherical adherent cell between PFE. The top and bottom surfaces were defined as WE and CE. The remaining surfaces were mirrored. Cell-electrode gap height was 100 nm.

Fig. 4  Schematics of the PFE used in our experiments. (a) Dimensions of gold electrode deposited on the glass substrates. (b) Assembly of two electrode substrates (S1 and S2). The intersection of two gold electrodes forms the PFE structure. (c) Cross-sectional view of the sensing area. Reproduced from Ref. 24.

Fig. 5  Simulated impedance spectra using the spherical cell model. (b) Simulated impedance magnitude of each $\alpha$ where the phase difference is closest to 0° versus $\beta = 1 / (1 - \alpha)$ with calibration curve.

Fig. 6  (a) Impedance spectra of latex beads suspension. Reproduced from Ref. 24. (b) Impedance magnitude of each $\alpha$ at 1 MHz versus $\beta = 1 / (1 - \alpha)$ with calibration curve.
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