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A simple mathematical model for electric cell-substrate impedance sensing with extended applications

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ABSTRACT

This paper presents a simple mathematical model to predict the impedance data acquired by electric cell-substrate impedance sensing (ECIS) at frequencies between 25 Hz and 60 kHz. With this model, the equivalent resistance (R) and capacitance (C) of biological samples adhered on gold surfaces could be more precisely measured at 4 kHz. ECIS applications were extended for real-time monitoring of living bacteria cultivated in Luria Bertani (LB) culture medium by two different approaches. In the former, we used a ferri/ferrocyanide redox couple in LB medium as an indicator for bacterial multiplication. Because the redox couple was toxic to some bacteria, we developed a second approach, in which L-cysteine self-assembled monolayers (SAM) on gold electrodes were used to detect living bacteria. The L-cysteine SAM could also be detected by ECIS. Unlike traditional impedance microbiological methods which need special culture media with low ions, our procedures significantly enhanced signal/noise ratios so bacteria could be detected in general purpose culture media. It was easy and convenient to obtain bacterial doubling times and evaluate the resistance of bacteria to antibiotics from ECIS spectra.

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1. Introduction

Cell spreading, morphology, and micromotion, three important parameters of cell behavior, have been quantified using an electrical method referred to as electric cell-substrate impedance sensing (ECIS) (Giaever and Keese, 1984; Mitra et al., 1991). Upon inoculation, cells drift downward through the culture medium and settle on the bottom of tissue culture microwells, each containing one or ten circular microfabricated gold electrodes. The cells then begin to attach and spread on the electrode surface, precoated with a binding protein, known as extracellular matrix (ECM). The confluent cell layer is eventually formed and affects the current flow since adhered cells will act as insulating particles due to their plasma membrane. The measured impedance then changes drastically after the cell attachment and spreading, due to an interference with the free space above the electrode. The changing impedance can be continuously monitored and interpreted to reveal information about cell spreading and mobility (Keese et al., 1998; Kowolenko et al., 1990). This method has been used as a general tool for probing cell spreading and motility as well as an alternative to animal testing for toxicology studies. To date, mammalian cells have been used extensively with ECIS to probe cell

behavior including cytotoxicity (Ko et al., 1998; Lo et al., 1994; Luong et al., 2001; Male et al., 2008a; Smith et al., 1994; Xiao and Luong, 2003; Xiao et al., 2002a,b). Recently, ECIS was used to probe inhibitory effects of *Antrodia camphorata* isolates (Male et al., 2008b) and destruxins (Male et al., 2009) on insect cells. A slight change in chemical structures of such compounds results in significant effects on inhibition which can be probed by cell-based impedance spectroscopy.

The principle of ECIS is based on Ohm's law and a biological sample (adhered cell) can be treated as an equivalent RC circuit (a resistor with resistance R and capacitor with capacitance C connected in series). However, interpretation of the resulting impedance data is very complicated as the values of R and C are dependent upon the operating frequency. Even if adhered and spreading cells are assumed to have a regular disk shape, the mathematical model (Giaever and Keese, 1991) to fit impedance data is extremely complex. In brief, the impedance measurement is related to the cellular capacitance, cell-to-cell junction, and the distance between the electrode's surface and the ventral side of the cell. Based on experimental data, it was observed (Xiao and Luong, 2003) that the ECIS instrument reports very different R and C values at different frequencies for solid electric RC circuits with known resistance and capacitance. Even at a fixed frequency and with a fixed capacitor in the test RC circuit, the ECIS instrument would report very different capacitance of the RC circuit if R is changed. It was also reported (Xiao et al., 2002b) that for a 125-cm

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cable, connecting an ECIS chip to the lock-in phase amplifier would contribute a parasitic impedance Z_p with $R_p = 1 \text{ k}\Omega$ and $C_p = 0.2 \text{ nF}$. Owing to the contribution of Z_p , any biological sample impedance modulus reported by ECIS would be close to $1 \text{ k}\Omega$ at very high frequencies.

This paper describes a simple mathematical model for predicting ECIS measurements at frequency ranging from 25 Hz to 60 kHz. Together with this model, ECIS was capable of precisely measuring resistance and capacitance of any ECIS sample at 4 kHz. Extended ECIS applications included the detection of L-cysteine self-assembled monolayers on gold electrodes, and the monitoring of living bacteria in real time and bacterial resistance to antibiotics.

2. Materials and methods

2.1. A simple mathematical model for ECIS and experimental confirmation

The electric circuit of a commercial ECIS system and an ECIS sensing chip (Applied Biophysics, Troy, NY) is shown in Fig. 1. An 8W1E sensing chip consists of eight detecting electrodes deposited on the bottom of eight separate mini-culture wells, whereas an 8W10E chip has 10 detecting electrodes in each mini-culture well. The diameter of each detecting electrode on the two chips is $250 \mu\text{m}$. A common counter electrode ($7 \text{ mm} \times 46 \text{ mm}$) is shared by the detecting electrodes on the chip with an exposed area of $2 \text{ mm} \times 9 \text{ mm}$ in each well. Each detecting electrode and the counter electrode are linked with a square pad at one end of the chip (Fig. 1). These electrodes and pads are thin gold films (50 nm) sputtered on a polycarbonate substrate (Keese et al., 1998). A ribbon cable (length of 125 cm) connects a chip in the CO_2 incubator to a control box which was connected to a lock-in phase amplifier (model SR830, Stanford Research Systems, Sunnyvale, CA). The sine wave frequency (f) and amplitude (V_o) can be set by ECIS software. Usually V_o was set at 1 V ac , and f could be set at a frequency between 25 Hz and 60 kHz. Two chips (chip A and chip B) were connected to the control box with the arrangement of wells on each chip shown in Fig. 1. The wells on the two chips are named $A_1, A_2, \dots, A_8$, and $B_1, B_2, \dots, B_8$. By default, the well A_1 is connected to the lock-in phase amplifier. Although the impedance of an electrolyte/gold interface is very complex, an equivalent RC circuit (Warburg, 1899) could be used to simulate its electric property. The

equivalent RC circuit of a well filled with culture medium is shown in Fig. 1. In brief, R_1C_1 and R_2C_2 are the equivalent circuits for the electrolyte/gold interfaces on the counter electrode and detecting electrode, respectively, while R_3 ($\sim 800 \Omega$) represents the resistance of the culture medium between the two electrolyte/gold interfaces. The well with a culture medium ($\geq 200 \mu\text{L}$) could be treated as an equivalent RC circuit with a resistance $R_s = R_1 + R_2 + R_3$ and a capacitance $C_s = 1/(1/C_1 + 1/C_2)$. At a given frequency f , the impedance of the well should be $Z_s = R_s + 1/(j\omega C_s)$ where $j = \sqrt{-1}$ and $\omega = 2\pi f$. The capacitance of the electrolyte/gold interface resulting from an electrical double layer is proportional to the electrode area. In an ECIS chip, C_1/C_2 is >300 because the counter electrode area is 300 times higher compared to the detecting electrode. The capacitance of the detecting electrode interface can be considered as the capacitance of the well filled with the culture medium. The well resistance is inversely proportional to the interface area, therefore, the detecting electrode resistance dominates the well resistance. With an AC signal (sine wave) $V = V_o e^{j\omega t}$ applied to the two electrodes through a $1 \text{ M}\Omega$ resistor (R_o), the potential between the detecting and counter electrodes becomes:

$$u_1 = \frac{Z_s}{R_o + Z_s} V_o e^{j\omega t} \quad (1)$$

If $|Z_s| < 0.01 R_o$, i.e. $|Z_s| < 10 \text{ k}\Omega$, the above formula is simplified as

$$u_1 = \frac{Z_s}{R_o} V_o e^{j\omega t} \quad (2)$$

Consequently, the amplitude of the system current is $1 \mu\text{A}$, whereas the amplitude of the voltage between the two electrodes is in the mV range. The amplitude (u_o) and the phase (φ) of the voltage between the detecting and counter electrodes can also be directly measured by a lock-in phase amplifier:

$$u_2 = u_o e^{j(\omega t + \varphi)} \quad (3)$$

With the condition $|Z_s| < 0.01 R_o$, the voltage predicted by Ohm's law (2) and the voltage directly measured by the lock-in phase amplifier (3) should be equal

$$\frac{Z_s}{R_o} V_o e^{j\omega t} = u_o e^{j(\omega t + \varphi)} \quad (4)$$

With u_o and φ known from the lock-in phase amplifier, and the values of V_o and R_o are also known from the ECIS circuit, the sample impedance Z_s directly reported by an ECIS instrument is defined as

Fig. 1. The circuit of an electric cell-substrate impedance sensor (ECIS) and the scheme of an ECIS sensing chip (8W1E or 8W10E) with eight mini wells ($\sim 0.7 \text{ mL}$). An equivalent circuit of the two electrolyte/gold interfaces (R_1C_1, R_2C_2) and the culture medium (R_3) in a mini well was also shown.

Z_{ECIS}

$$Z_{ECIS} = R_o \frac{u_2}{V} = R_o \frac{u_o}{V_o} e^{j\varphi} \quad (5)$$

At lower frequencies, the sample impedance modulus $|Z_s|$ might be bigger than $1 \text{ M}\Omega$ (R_o). When the condition $|Z_s| < 0.01 R_o$ is not satisfied, (5) is no longer valid and the following equation (6) should be used to relate Z_{ECIS} to the sample impedance Z_s by substitution of u_2 in (5) by u_1 in Eq. (1):

$$Z_{ECIS} = R_o \frac{u_2}{V} = R_o \frac{u_1}{V} = R_o \frac{(Z_s/(R_o + Z_s))V_o e^{j\omega t}}{V_o e^{j\omega t}} = \frac{R_o Z_s}{R_o + Z_s}$$

or

$$\frac{1}{Z_{ECIS}} = \frac{1}{R_o} + \frac{1}{Z_s} \quad (6)$$

Since Z_s is always connected in parallel with the parasitic impedance Z_p contributed by the connecting leads, the impedance reported by ECIS for a sample Z_s at any frequency becomes:

$$\frac{1}{Z_{ECIS}} = \frac{1}{R_o} + \frac{1}{Z_s} + \frac{1}{Z_p} \quad (7)$$

Therefore, the impedance reported from ECIS is not equal to the sample impedance. At frequency $< 1 \text{ kHz}$ or above 10 kHz , the difference between Z_{ECIS} and Z_s could be very significant. Previous work (Xiao et al., 2002b) described a simple method to precisely extract Z_s from Z_{ECIS} at frequencies between 1 kHz to 10 kHz . Briefly, a balance RC circuit with known impedance Z_b was used to connect with Z_s in parallel. In the beginning ($\sim 5 \text{ min}$) of an experiment, the impedance of each empty well on chips was measured. Because the Z_s of an empty well was infinite, impedance indicated by ECIS for the well with a balance RC circuit would be: $(1/Z_\infty) = (1/R_o) + (1/Z_p) + (1/Z_b) + (1/\infty)$; then a biological sample was inoculated into the well, the impedance of the well monitored by ECIS would be: $(1/Z_{ECIS}) = (1/R_o) + (1/Z_p) + (1/Z_b) + (1/Z_s)$. Without knowing the parameters of the ECIS instrument, it is possible to extract the sample impedance by (8). The sample equivalent resistance and equivalent capacitance are obtained from $R_s = \text{Re}(Z_s)$ and $C_s = -(1/(2\pi f \text{Im}(Z_s)))$. However, at lower and higher frequencies, Z_s can no longer be treated as an equivalent $R_s C_s$ circuit with a constant resistance and a constant capacitance

$$Z_s = \left(\frac{1}{Z_{ECIS}} - \frac{1}{Z_\infty} \right)^{-1} \quad (8)$$

From (8), ECIS could be used as an Ohm meter to precisely measure resistance and capacitance of solid elements in electric circuits. Fig. 2 shows the results of ECIS measurements for three RC circuits: $15 \text{ k}\Omega/10.0 \text{ nF}$, $4.7 \text{ k}\Omega/4.7 \text{ nF}$ and $1.5 \text{ k}\Omega/4.0 \text{ nF}$. From 25 Hz to 60 kHz , the impedance moduli $|Z_{ECIS}|$ of the three RC circuits measured by the ECIS are shown by dots. The solid curves in the figure are simulation results by applying Eq. (7) to the three RC circuits. A MathCAD® program was written for the numerical simulations. In the program, the frequency f changed from 25 Hz to 60 kHz according ECIS measurements; R_o was set as a constant ($1 \text{ M}\Omega$) which was decided by the hardware of the ECIS instrument; R_p and C_p were set to $1 \text{ k}\Omega$ and 0.2 nF , respectively. The original $|Z_{ECIS}|$ data of ECIS measurements and numerical simulations were shown in Table 1. For these RC circuits' impedance moduli, numerical simulation data agreed very well with the ECIS data. The impedance difference between the measurement and the model value becomes noticeable only at frequencies below 60 Hz . At very low frequencies, the sample impedance could be over $1 \text{ M}\Omega$, and the lock-in phase amplifier impedance could not be treated as infinite. The inset of the figure shows a comparison between (R_{ECIS}, C_{ECIS}) and (R_s, C_s) of five RC circuits at 4 kHz . The five solid dots in the figure were results of ECIS measurements of the five RC circuits: $1.5 \text{ k}\Omega/4.0 \text{ nF}$, $2.7 \text{ k}\Omega/4.0 \text{ nF}$, $4.7 \text{ k}\Omega/4.7 \text{ nF}$, $8.2 \text{ k}\Omega/4.7 \text{ nF}$, and $15 \text{ k}\Omega/10.0 \text{ nF}$; the

Fig. 2. Verification of the ECIS mathematical model. Solid dots came from ECIS measurements of three RC circuits at frequencies between 25 Hz and 60 kHz . The solid lines were numerical simulation results from the ECIS model (Eq. (7) with $R_o = 1 \text{ M}\Omega$, $R_p = 1 \text{ k}\Omega$ and $C_p = 0.2 \text{ nF}$). The $|Z|$ data of the figure are shown in Table 1. In the inset, the solid dots and open dots showed the comparison between (R_{ECIS}, C_{ECIS}) and (R_s, C_s) at 4 kHz for five solid RC circuits: $1.5 \text{ k}\Omega/4.0 \text{ nF}$, $2.7 \text{ k}\Omega/4.0 \text{ nF}$, $4.7 \text{ k}\Omega/4.7 \text{ nF}$, $8.2 \text{ k}\Omega/4.7 \text{ nF}$, and $15 \text{ k}\Omega/10.0 \text{ nF}$.

five open dots were generated from Eq. (8). It was obvious that (R_s, C_s) was more accurate than (R_{ECIS}, C_{ECIS}) as shown in this figure.

2.2. Luria Bertani (LB) medium

12.5 g of LB broth media powder (Fisher Scientific, Hampton, NH) dissolved in 500 mL distilled water was adjusted to $\text{pH } 7.0$ with 5 M NaOH . The culture medium was autoclaved for 30 min prior to storage. The LB medium (400 mL) was mixed with 6.0 g of granulated agar (Fisher Scientific, Hampton, NH) and stored at room temperature after being autoclaved for 30 min . The agar jelly was melted by microwave heating to prepare LB agar plates in Petri dishes (Fisher Scientific, $100 \times 15 \text{ mm}$ Slide Dish).

2.3. Bacterial culture and titration measurement

Bacterial strains were purchased from American Type Culture Collection (ATCC, Manassas, VA). After reconstitution of the lyophilized powders, glycerol stock tubes were stored at -80°C . Master plates were prepared by scratching the surface of these glycerol stocks with a plastic inoculation loop and drawing zigzag lines on a LB agar plate. The inverted plates were incubated at 37°C overnight and stored at 4°C for the next 3 weeks. Weekly stock solutions were prepared from the master plate with a plastic inoculation loop by transferring 3–5 colonies to 4 mL of LB medium in a 13-mL tube. The tube was incubated in a shaker at 37°C for 6 h and stored at 4°C for 1-week use. Bacterial titration was measured by colony forming unit (CFU) counting.

2.4. Doubling time (τ) measured by CFU counting

The LB medium (4.5 mL) was added to six 13-mL snap tubes marked E8, E7, E6, E5, E4 and E3. These tubes and the tube with the weekly stock *E. coli* were preheated for 30 min at 37°C . The seven tubes were removed from the incubator and the time was set to zero ($t = 0$). The weekly stock *E. coli* solution (0.5 mL) was mixed with the contents of E8; then 0.5 mL E8 liquid was injected into E7 and this process was repeated until E3. After $11.1 \mu\text{L}$ aliquots of E7, E5 and E3 were taken for titration, these 10-fold series dilution tubes were quickly placed back in the incubator. At 40 min intervals over the course of 8 h , $11.1 \mu\text{L}$ aliquots from the tubes marked E7, E5 and E3 were taken for titration $N(t)$. In the exponential or log phase, a

Table 1

[Z] Comparisons between ECIS measurements and numerical simulations.

f (Hz)	$R_s C_s = 1.5 \text{ k}\Omega 4.0 \text{ nF}$		$R_s C_s = 4.7 \text{ k}\Omega 4.7 \text{ nF}$		$R_s C_s = 15 \text{ k}\Omega 10.0 \text{ nF}$	
	$ Z_{\text{ECIS}} $ (Ω)	$ Z_{\text{simulate}} $ (Ω)	$ Z_{\text{ECIS}} $ (Ω)	$ Z_{\text{simulate}} $ (Ω)	$ Z_{\text{ECIS}} $ (Ω)	$ Z_{\text{simulate}} $ (Ω)
25	1,400,000	834,367	1,200,000	791,178	610,000	520,000
50	720,000	603,491	610,000	543,119	310,000	290,000
75	480,000	450,474	410,000	395,986	210,000	200,000
100	360,000	353,932	310,000	307,744	150,000	150,000
200	180,000	185,919	160,000	159,691	78,000	78,000
500	74,000	75,482	65,000	64,701	34,000	34,000
750	50,000	50,413	43,000	43,313	25,000	25,000
1000	37,000	37,842	33,000	32,633	21,000	21,000
2000	19,000	18,971	17,000	16,778	16,000	16,000
3000	13,000	12,693	12,000	11,674	15,000	15,000
4000	9600	9567	9300	9244	15,000	15,000
5000	7700	7701	7900	7869	15,000	15,000
6000	6500	6465	7000	7009	15,000	15,000
7000	5600	5590	6400	6434	15,000	15,000
8000	5000	4940	6000	6032	14,000	14,000
9000	4500	4439	5700	5739	14,000	14,000
10,000	4100	4043	5500	5520	14,000	14,000
15,000	2900	2896	4900	4955	14,000	14,000
20,000	2300	2366	4700	4729	14,000	13,000
30,000	1900	1898	4500	4525	13,000	12,000
40,000	1600	1702	4300	4405	12,000	11,000
50,000	1500	1600	4200	4299	11,000	10,000
60,000	1500	1540	4100	4194	10,000	9200

linear relationship between $\log[N(t)]$ and time t is expected. The bacterial doubling time is $\tau = \log(2)/k$, where k is the slope of the straight line of the $\log[N(t)]$ vs. t plot.

2.5. Ferri/ferrocyanide solution

A 200 mM stock solution of ferri/ferrocyanide was prepared from an equal volume of 400 mM potassium ferricyanide $\text{K}_3\text{Fe}(\text{CN})_6$ and 400 mM tetrapotassium ferrocyanide $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ (American Chemicals). The stock solution was filtered using a 0.22 μm filter. Just before the ECIS experiment, 2.5 mM ferri/ferrocyanide was prepared by mixing the LB medium with the stock solution.

2.6. L-Cysteine and ampicillin solutions

L-Cysteine, ampicillin and sodium chloride were products of Sigma-Aldrich (St. Louis, MO). L-Cysteine powder was dissolved in 0.15 M NaCl to 100 mM and filtered by a 0.22 μm filter as the stock. The ampicillin powder was dissolved in doubly distilled water to 100 mg/mL.

2.7. Bacterial resistance to antibiotics monitored by L-cysteine coated ECIS chips

Each well of the two 8W1E chips was initially filled with 400 μL of 0.15 M NaCl, followed by dropwise addition of 10 μL L-cysteine (100 mM). After overnight incubation, the wells were emptied and washed by the LB medium. Finally, the wells were filled by 400 μL LB medium containing different ampicillin concentrations. The ampicillin stock (10 μL) was mixed with 90 μL of the LB medium. The diluted ampicillin (40 μL) was mixed with 3600 μL LB, resulting in 100 $\mu\text{g/mL}$ ampicillin. The 100 $\mu\text{g/mL}$ ampicillin solution was doubly diluted by LB medium. The final ampicillin concentration in well 1, 2, 3, 4, 5 and 6 on the two chips was 100, 50, 25, 12.5, 6.25, and 3.125 $\mu\text{g/mL}$, respectively. There was no ampicillin in wells 7 and 8 of the two chips, which served as the control. Two subtypes of *S. typhimurium*: DT104A and DT104E were used because of their resistance to ampicillin. The titrations of these two bacteria in the stationary phase were the same: 2×10^9 CFU/mL.

3. Results and discussion

3.1. Using ECIS to monitor L-cysteine binding to gold surface

Many life phenomena occur on solid/liquid interface, some techniques such as surface plasmon resonance (Liedberg et al., 1983), piezoelectric crystal microbalance (Caruso et al., 1997) and electrochemical impedance spectroscopy (K'Owino and Sadik, 2005) have been developed to detect biological molecules bond to gold surfaces. With a thiol group ($-\text{SH}$) in its side chain, L-cysteine can covalently bind to gold atoms on gold electrodes (Feyter and Schryver, 2003), known as self-assembled monolayer (SAM). The experimental data revealed that ECIS was able to detect L-cysteine bound to the gold surface in real time. Fig. 3A and B shows that resistance R_s and capacitance C_s of 8W1E wells (each with 600 μL saline) was $\sim 1.8 \text{ k}\Omega$ and 2.6 nF, respectively at 4 kHz. At $t = 0.6$ h and $t = 1.8$ h, diluted L-cysteine solution (6 μL) was added in each well, so that the final cysteine concentration in these wells was 1.0, 0.5 and 0.05 mM, respectively. To the well with 1.0 mM L-cysteine, the well capacitance quickly increased to ~ 5.3 nF and the resistance increased to $\sim 2 \text{ k}\Omega$. To the two wells with 0.5 and 0.05 mM L-cysteine, the capacitance was somewhat smaller, 4.8 and 4.2 nF, respectively. For the control well (saline without cysteine), the capacitance and resistance were unchanged as expected. The resistance changes of the three wells with L-cysteine were less than 10%, but the capacitance changes of the three wells with 0.05, 0.5 and 1.0 mM cysteine were 61%, 85% and 104%, respectively. At $t = 3.4$ h, all the four wells were washed and refilled by the saline, and only a small part of L-cysteine was washed away.

Fig. 3C shows $|Z_s|$ of four samples from 2 to 9 kHz. One of the samples was a solid RC circuit ($2.2 \text{ k}\Omega/4.7 \text{ nF}$) which was used as reference, whereas the other three samples were a well with 0.85% NaCl, a well with 50 mM L-cysteine in saline and a well with the Dulbecco's Modified Eagle medium plus 10% fetal bovine serum. The R_s value of the three wells was $\sim 2 \text{ k}\Omega$. The C_s of the saline well was ~ 2.5 nF, but the C_s of the other wells were ~ 5 nF. At any frequency, the $|Z_s|$ value of the saline well was the highest because its C_s was the lowest. The $|Z_s|$ vs. f curves of the three wells were almost parallel to the solid RC circuit $|Z_s|$ - f curve. Such a result implied that the wells could be treated as an equivalent RC circuit

Fig. 3. Application of ECIS to detect L-cysteine SAM. (A) and (B) Resistance R_s and capacitance C_s change with time at 4 kHz after diluted L-cysteine (6 μ L) was injected into 8W1E wells with 0.6 mL 0.85% NaCl saline. The final L-cysteine concentrations in the wells were 1.00, 0.50 and 0.05 mM, and the control was a well just with saline. (C) Impedance moduli of four samples between 2 and 9 kHz. L-Cysteine or protein adsorbed on ECIS electrodes could significantly decrease $|Z_s|$ because C_s was significantly increased. The dash line with “x” symbols was for a control to show that a biological sample on an ECIS detecting electrode could be treated as an equivalent RC circuit.

with a constant R and C at frequencies from 2 to 9 kHz. From C_s or $|Z_s|$ change, it is easy to monitor biological molecules binding to the gold surface. In the last four decades, many impedance spectroscopy methods were developed for biological sample detection on gold surfaces (Pejicic and Marco, 2006). The ECIS spectra provided an alternative method in this area.

3.2. Using ferri/ferrocyanide for ECIS to detect living bacteria

Due to bacterial metabolism, mostly uncharged and/or weakly charged nutrients in culture medium are converted into highly charged and smaller molecules (Stewart, 1899). For example, the metabolism of electrically inert carbohydrates into lactates and carbonates would increase the culture media's electrical conductivity. However, the conductivity change caused by bacterial metabolism is so small that special culture media with low ion levels (high resistance) must be used in traditional impedance instruments for detection of living bacteria. If no such media were available, indirect impedance methods had to be used for bacteria detection (Dèzenclos et al., 1994). Any fluctuation in temperature (>0.1 °C) and/or water evaporation will overwhelm impedance signals produced by bacterial metabolism (Silleby and Forsythe, 1996). To our knowledge, ECIS has not been attempted for detection of living bacteria because of their much smaller sizes compared to mammalian cells. The ferri/ferrocyanide redox system is one of the most extensively studied redox couples in electrochemistry (Beriet and Pletcher, 1993). The experimental data revealed that ferri/ferrocyanide could be used as an indicator for ECIS to detect living bacteria. Without the introduction of the redox couple, the ECIS signal response effected by bacteria was insignificant compared to the signal background. Fig. 4A and B shows R_s and C_s changed with time when *E. coli* K91 was cultured in three wells on an 8W10E chip. The vertical arrows indicated the time when the diluted bacterial stock was injected into the 8W10E wells with 0.4 mL of the LB medium. The curves marked by symbols ①, ② and ③ indicated the initial bacterial titration in the three wells was 5×10^7 , 5×10^4 and 5×10^1 CFU/mL, respectively. The dash lines from a well without bacteria served as a control. There were changes in R_s and C_s for the three wells with *E. coli* K91, but they were in the same levels of the control. For this reason ECIS could not be directly used for living bacteria detection.

Fig. 4C and D shows R_s and C_s spectra during the *E. coli* K91 culture in three wells of the same 8W10E chip. Each of four wells was filled with 0.4 mL LB medium containing 2.5 mM ferri/ferrocyanide.

The dash lines came from controls (without bacteria). After bacteria inoculation in the three wells, their R_s and C_s spectra were different from the controls, but differences of C_s spectra were more significant. Before *E. coli* K91 inoculation, the C_s of these wells was ~ 35 nF. After *E. coli* K91 inoculation, on each C_s curve there was a critical segment called the detection time (DT). At DT, C_s stopped increasing and began to decrease quickly during the next 2–3 h, followed by a slow decrease to ~ 23 nF. For the wells with an initial *E. coli* K91 titration 5×10^7 , 5×10^4 and 5×10^1 CFU/mL, DT was at $t = 5.2$, 8.6 and 12.0 h, respectively. Notice that the oxidation of ferrocyanide to ferricyanide in aqueous solution has served as a model system in electrochemistry since $\text{Fe}(\text{CN})_6^{4-/-3-}$ reaction involves the transfer of a single electron and exhibits close to ideal quasi-reversible outer sphere kinetic behavior. Ferrocyanide has been known to interact with several oxidases including glucose oxidase and cholesterol oxidase (Jubete et al., 2009). The interaction of microbial cells with this redox couple is anticipated and warrants further investigation.

3.3. A simple method for bacterial doubling time measurements by ECIS spectra

The doubling time (τ) of bacterial multiplication is a very important index. Measuring τ by the traditional CFU counting method is tedious work. The three solid lines with dot symbols shown in Fig. 5 came from CFU counting of 2 days of work. In the first day at an interval of 40 min, bacterial samples were taken from three tubes with different initial titrations: 5.5×10^6 , 5.5×10^4 , and 5.5×10^2 CFU/mL. These samples were diluted and seeded on three LB agar plates, respectively. The LB agar plates were incubated overnight at 37 °C. On the second day, all the plates were taken out for counting of the colony forming units. As shown in the graph, the lag time of *E. coli* K91 was ~ 80 min. During the lag phase, the *E. coli* K91 titration was almost constant. After the lag time, the 10-based logarithm of *E. coli* K91 titrations displayed linear relationships with culture time (solid lines). From the slopes of these three straight lines obtained by linear regression, the estimated doubling time of *E. coli* K91 was 23.2, 22.2 and 21.8 min, respectively. When the *E. coli* K91 titration reached 1.47×10^9 CFU/mL, the exponential phase was terminated and the titration became constant (the stationary phase of *E. coli* K91). For example, the *E. coli* K91 came into the stationary phase at $t = 4.3$ h for the tube with the initial titration 5.5×10^6 CFU/mL.

In an ECIS experiment with two chips (A and B), each of the 16 wells was filled with 0.4 mL LB medium and 2.5 mM

Fig. 4. Application of ECIS for living bacteria detection with ferri/ferrocyanide as an indicator. Resistance R_s and capacitance C_s change with time at 4 kHz after *E. coli* K91 bacteria were injected into wells with 0.4 mL the LB medium. The symbol ①, ② and ③ showed ECIS spectra of the initial *E. coli* K91 titration in 8W10E wells was 5×10^7 , 5×10^4 and 5×10^1 CFU/mL, respectively. The only difference of experimental conditions between left and right graphs was that there was 2.5 mM ferri/ferrocyanide in the LB medium for the right graphs. The dash lines came from controls without bacteria.

ferri/ferrocyanide. The initial *E. coli* K91 titration in the well A1, A2, ..., A7 on chip A was 10^7 , 10^6 , ..., 10^1 , respectively. The well A8 was used as a negative control (no bacteria). The chip B was used as a repeat of chip A. The time was set to $t=0$ when bacteria were injected into these wells. The bacterial solution in each well at its detection time was taken out for titration measurements by CFU counting on a LB agar surface. In Fig. 5, the "x" symbols showed the titration at DT for 14 wells on the two ECIS chips, and the dash line came from the average (1.38×10^8 CFU/mL) of these 14 titrations at DT. Since titrations at DT of the 14 wells were very close to the average value, it is reasonable to assume that the titrations at DT were constant. Notice that the titration at DT was less than the stationary phase titration. At DT, *E. coli* K91 was still in its exponential phase. Based on the two facts at DT, we proposed a simple method to measure τ by ECIS spectra shown in Fig. 4D, and required no CFU counting. From two known initial titrations N_i and N_j , it was easy

to measure bacterial multiplication rate: the doubling time τ from the DT difference by the following equation:

$$DT_i - DT_j = -\frac{\tau}{\log(2)} \log \left(\frac{N_i}{N_j} \right) \quad (9)$$

As an example, from the capacitance curves ① and ③ in Fig. 4D, the detection time difference was 6.8 h, and their initial titration ratio was 10^{-6} . According to the above equation, the doubling time of *E. coli* K91 measured by ECIS was 20.5 min. Many kinds of bacteria were detected and their doubling times were measured by the method. Notice that 2.5 mM ferri/ferrocyanide affected the growth of some tested microorganisms including *Salmonella typhimurium* and *Salmonella enteritidis*. Therefore, the value estimated by ECIS was twice higher than the value obtained by CFU counting. *Staphylococcus epidermidis* and *Streptococcus pneumonia* could not be detected by the method, because the 2.5 mM ferri/ferrocyanide in LB medium was so toxic that they could not grow. The toxicity of ferri/ferrocyanide to these bacteria was confirmed by CFU counting of these bacteria cultured in LB medium with 2.5 mM ferri/ferrocyanide. A series of experiments was conducted to modify the gold surface. We found if electrodes of 8W1E or 8W10E were coated by L-cysteine SAM, all the above living bacterial strains could be directly detected in LB medium without ferri/ferrocyanide. The experimental data confirmed that modified gold electrodes of 8W1E or 8W10E were applicable for monitoring the above-mentioned living bacterial strains in the LB medium. The doubling times measured by ECIS agreed well with those obtained by CFU counting. We also tried D-cysteine, but we found D-cysteine was toxic to bacteria.

3.4. Monitoring bacteria resistance to antibiotics by ECIS

Bacteria with resistance to antibiotics, known as super bugs, are a severe threat to human beings and antibiotic therapy. Therefore, it is of importance to quickly screen such antibiotic resistant strains. Our study demonstrated that ECIS chips coated with L-cysteine SAM were capable of detecting the resistance of bacteria to antibiotics. The bacterial resistance was dependent on antibiotics concentration as well as the initial bacterial titration. Bacteria with higher

Fig. 5. The relationship between time and *E. coli* K91 titration ("•" symbols) measured by the traditional CFU counting method. The *E. coli* K91 samples initial titration was 5.5×10^6 , 5.5×10^4 , and 5.5×10^2 CFU/mL, respectively. From the slopes of the three solid lines, *E. coli* K91 doubling times could be obtained. The dash line represented the average of 14 CFU titrations ("x" symbols) of 14 K91 samples in 14 wells on two ECIS chips at their detecting times (DT).

Fig. 6. Application of ECIS for monitoring the resistance of *S. typhimurium* DT104A and DT104E to ampicillin in 8W1E wells coated with L-cysteine SAM. The dash lines were obtained from negative controls, and the thicker lines were obtained from positive controls (details in text). The ampicillin concentrations in LB medium were 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL, respectively. Both DT104A and DT104E were resistant to ampicillin, but the latter was more sensitive.

titration could tolerate higher antibiotics concentrations. To simplify ECIS measurement, we cultured two bacteria with the same initial titration in different ECIS mini wells to detect their responses to multiple concentrations of antibiotics. The ECIS spectra of *S. typhimurium* DT104A and DT104E cultured in ampicillin with multiply concentrations were shown in Fig. 6. At the beginning, DT104A and DT104E were diluted to 2×10^8 CFU/mL by mixing 100 µL the weekly stock with 900 µL LB medium. At $t = 0.7$ h after the two chips were connected into the ECIS instrument, the diluted DT104A solution (10 µL) was inoculated into each well on chip A, except for the well 8 which served as a negative control. The chip B was inoculated with DT104E, and all the operating conditions were similar to the chip A. The initial titration in each of the 14 wells on the two chips was 5.4×10^6 CFU/mL. The two chips were placed in a 37 °C incubator, and the ECIS instrument was operated at 4 kHz. The two dash lines in Fig. 6 came from negative controls (no bacteria growing in the LB medium); the two thicker lines came from positive controls (bacteria normally grow in the LB medium). Fig. 6A shows that the capacitance of all six wells with ampicillin decreased in the same way as the capacitance of well 7 without ampicillin (positive control) about 2 h after bacteria inoculation. The capacitance spectra indicated that 5.4×10^6 CFU/mL DT104A could grow normally even in the LB medium with ampicillin up to 100 µg/mL. The spectra shown in Fig. 6B also indicated that DT104E bacteria could survive in the LB medium with 100 µg/mL ampicillin. However, their multiplication rates were significantly sluggish if the ampicillin concentration was above 25 µg/mL. In the LB medium with ampicillin less than 25 µg/mL, 5.4×10^6 CFU/mL DT104E could grow normally. Both DT104A and DT104E were resistant to ampicillin, but the latter was more sensitive to ampicillin.

4. Conclusion

A theoretical model for ECIS technique has been presented to describe the ECIS impedance change with AC operating frequency. Some applications were demonstrated by modification of the gold surface with L-cysteine or the addition of ferri/ferrocyanide in the LB medium as a redox couple for detection of living bacteria. Although the two procedures were simple and straightforward, the latter was only applicable for bacterial strains that might tolerate ferri/ferrocyanide. Unlike traditional impedance methods which need special culture media and strict temperature control, our methods significantly enhanced the signal/noise ratio so bacteria could be cultured in general purpose culture media. Bacteria multiplication doubling times could be easily obtained from the detection times on ECIS spectra. With ECIS spectra coming from L-cysteine SAM coated chips, it was convenient and sensitive to monitor bacterial resistance to antibiotics.

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References

- Beriet, C., Pletcher, D., 1993. *J. Electroanal. Chem.* 361 (1–2), 93–101.
- Caruso, F., Furlong, D.N., Kingshott, P., 1997. *J. Colloid Interface Sci.* 186 (1), 129–140.
- Dézenclos, T., Ascon-Cabrera, M., Ascon, D., Lebeault, J.M., Paus, A., 1994. *Appl. Microbiol. Biotechnol.* 42, 232–238.
- Feyter, S.D., Schryver, F.C.D., 2003. *Chem. Soc. Rev.* 32, 139–150.
- Giaever, I., Keese, C.R., 1984. *Proc. Natl. Acad. Sci. U.S.A.* 81 (12), 3761–3764.
- Giaever, I., Keese, C.R., 1991. *Proc. Natl. Acad. Sci. U.S.A.* 88 (11), 7896–7900.
- Jubete, E., Loaiza, O.A., Ochoteco, E., Pomposo, J.A., Grande, H., Rodríguez, J., 2009. *J. Sensors* (Article ID 842575), 13 pages.
- K'owino, I.O., Sadik, O.A., 2005. *Electroanalysis* 17 (23), 2101–2113.
- Keese, C., Karra, N., Dillon, B., Goldberg, A., Giaever, I., 1998. *In Vitro Mol. Toxicol.* 11 (2), 183–192.
- Ko, K.S.C., Lo, C.-M., Ferrier, J., Hannam, P., Tamura, M., McBride, B.C., Ellen, R.P., 1998. *J. Microbiol. Methods* 34 (2), 125–132.
- Kowolenko, M., Keese, C.R., Lawrence, D.A., Giaever, I., 1990. *J. Immunol. Methods* 127, 71–77.
- Liedberg, B., Nylander, C., Lundström, I., 1983. *Sens. Actuators A: Phys.* 4, 299–304.
- Lo, C.M., Keese, C.R., Giaever, I., 1994. *Exp. Cell Res.* 213 (2), 391–397.
- Luong, J.H., Habibi Rezaei, M., Meghrou, J., Xiao, C., Male, K.B., Kamen, A., 2001. *Anal. Chem.* 73 (8), 1844–1848.
- Male, K.B., Lachance, B., Hrapovic, S., Sunahara, G., Luong, J.H.T., 2008a. *Anal. Chem.* 80 (14), 5487–5493.
- Male, K.B., Rao, Y.K., Tzeng, Y.-M., Montes, J., Karmen, A., Luong, J.H.T., 2008b. *Chem. Res. Toxicol.* 21 (11), 2127–2133.
- Male, K.B., Tzeng, Y.-M., Montes, J., Liu, B.-L., Liao, W.-C.K.A., Luong, J.H.T., 2009. *Analyst* 134 (7), 1447–1452.
- Mitra, P., Keese, C.R., Giaever, I., 1991. *Biotechniques* 11, 504–510.
- Pejic, B., Marco, R.D., 2006. *Electrochim. Acta* 51, 6217–6229.
- Silley, P., Forsythe, S., 1996. *J. Appl. Bacteriol.* 80, 233–243.
- Smith, T.J., Wang, H.S., Hogg, M.G., Henrikson, R.C., Keese, C.R., Giaever, I., 1994. *Proc. Natl. Acad. Sci. U.S.A.* 91 (11), 5094–5098.
- Stewart, G.N., 1899. *J. Exp. Med.* 4, 235–243.
- Warburg, E., 1899. *Ann. Phys. Chem.* 67, 493–499.
- Xiao, C., Luong, J.H.T., 2003. *Biotechnol. Prog.* 19 (3), 1000–1005.
- Xiao, C., Lachance, B., Sunahara, G., Luong, J.H., 2002a. *Anal. Chem.* 74 (22), 5748–5753.
- Xiao, C., Lachance, B., Sunahara, G., Luong, J.H., 2002b. *Anal. Chem.* 74 (6), 1333–1339.