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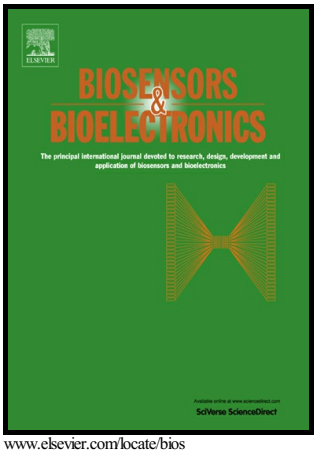

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Monitoring of bacteria biofilms forming process by in-situ impedimetric biosensor chip

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Abstract

A biosensor chip integrated interdigital microelectrodes was proposed and applied to monitor the formation process of *Salmonella* and *E. coli* biofilms in this paper. The biosensor chip was composed of a glass substrate with interdigital microelectrodes and PDMS layer with micro cavities. The electrochemical impedance spectroscopy (EIS) of *Salmonella* and *E. coli* biofilms was measured by the biosensor chip using alternating voltage of 100 mv in the frequency range from 1 Hz to 100 kHz for 48 h. It was illustrated that the changes of impedance spectroscopy of biofilms occurred with culture time. Furthermore, impedance spectroscopy of biofilms was fitted by an equivalent circuit model including the biofilms capacitance (C_b) and the biofilms resistance (R_b) parameters. The results indicated that the C_b presented a tendency to decrease first and then rise with culture time, while the R_b was in the opposite direction. These changing trends were consistent with the formation process of biofilms that bacteria adhered to electrodes surface, and then formed mature biofilms, finally escaped from biofilms. In addition, it was also demonstrated that the changing trends of C_b and R_b with culture time were quite different between *Salmonella* and *E. coli*. The results obtained by impedance detection were in accordance with the results of using crystal violet staining to analyze biofilms formation process, under the same conditions for bacterial culture. The biosensor chip provided a promising platform for further study of biofilms owing to its unique advantages of real time, continuity, and non-invasion for bacteria biofilms detection and in-situ monitoring.

Key words:

Biosensor chip; Electrochemical impedance spectroscopy; *Salmonella* biofilms; *E. coli* biofilms; Biofilms formation.

1. Instruction

Biofilms are defined as microorganisms communities composed of bacteria cells and self-produced extracellular polymeric substances, which lead to antibiotic resistance of bacteria and cause a great threat in the fields of medicine, food industries and so on (Coughlan et al. 2016; G et al. 2000; Jahid and Ha 2012). According to statistics, 65% of the iatrogenic infection is related to biofilms (Costerton et al. 1999). In addition, biofilms can bring various problems such as water pollution and food pollution (Satpathy et al. 2016; Wingender and Flemming 2011). In recent years, much more attention has been paid to the related research areas of bacteria biofilms, especially detection methods for biofilms (Azeredo et al. 2017). An efficient detection method for biofilms is of paramount significance for analyzing structural characteristics of biofilms, interpreting phenomenon of biofilms, and solving problems of biofilms (Driessche et al. 2014; Satpathy et al. 2016).

Biofilms formation is a dynamic process, which can be divided into several stages mainly including surface attachment of bacteria cells, biofilms maturation, and biofilms dispersion (Sauer 2003; Verstraeten et al. 2008). The structure and bacteria metabolism of biofilms are quite different at their different growth stages (Efstathios et al. 2015; Toyofuku et al. 2016). Close attention is paid to monitor bacteria survival conditions in biofilms and characterize the turning points of biofilms formation process (Zarabadi et al. 2017). The conventional detection methods for biofilms, such as crystal violet staining and fluorescent imaging methods using confocal laser scanning microscopy, require pre-treatment processes which destroy the structure of biofilms and are only applied for detecting and observing biofilms at a certain moment (Oubekka et al. 2012; Peeters et al. 2008; Pitts et al. 2003). Compared with conventional detection methods，EIS is a competitive one with many advantages (Daniels and Pourmand 2007; Retter and Lohse 2010). EIS is a non-destructive and real-time measurement tool suitable for analyzing the interfacial properties of electrode surfaces (Hashemi et al. 2017a). The electrochemical impedance is analyzed by equivalent circuit models contained various electrochemical parameters. Different electrochemical parameters were involved in different circuit models (Chang and Park 2010; Floyd et al. 2009). For example, the Randles equivalent circuit including several parameters: double layer capacitance C_{dl}, solution resistance R_s, Warburg impedance W, and charge transfer resistance R_{ct} was applied to the cocaine detection by changes of R_{ct} (Hashemi et al. 2017b). In recent years, EIS had been used to study the biofilms formed on various electrode surfaces (Ben-Yoav et al. 2011). Ward et al used screen printed carbon electrodes to measure *Pseudomonas aeruginosa* biofilms by EIS in a commercial culture cell (Ward et al. 2014). Kim et al monitored the impedance spectroscopy of *Pseudomonas aeruginosa* biofilms by gold electrodes in cylindrical electrolytic cell. It was found that bacteria adhesion and biofilms maturation brought the decrease of double-layer capacitance (Kim et al. 2011). Paredes et al investigated *Staphylococcus* biofilms by impedance detection with interdigital electrodes in Petri dishes, and further analyzed the impedance spectroscopy of biofilms using an equivalent circuit. It was shown that the biofilms capacitance, a parameter of equivalent circuit, decreased as extension of culture time (Paredes et al. 2014).

Accordingly, when EIS was used to detect and study biofilms, the capacitance and resistance associated with biofilms were the key parameters. Until now, there is a lack of high performance detection methods for characterization and differentiation of biofilms development stages. For the impedance detection of biofilms, it is essential to choose an appropriate electrode for improving the detection efficiency. Compared with traditional electrodes, interdigital microelectrodes array made up of a series of parallel micro band electrodes present promising advantages in terms of fast establishment of steady-state, low ohmic drop, rapid reaction kinetics, and improving signal-to-noise ratio (Varshney and Li 2009).

Here, a biosensor chip integrated with interdigital microelectrodes and relative detecting methodology based on EIS are proposed for monitoring biofilms formation process of *Salmonella* and *E. coli*. PDMS micro cavities on interdigital microelectrodes are designed to provide a place for biofilms growth. Two detection areas are arranged on the biosensor chip for detection biofilms of *Salmonella* and *E. coli*, respectively. The biofilms forming on the surface of interdigital electrodes are monitored in-situ by EIS. To research the electrochemical behavior of biofilms formation process, an equivalent circuit model containing C_b and R_b is established for fitting the impedance spectroscopy to obtain the related electrochemical parameters. Therefore, biofilms formation process of *Salmonella* and *E. coli* can be monitored in real-time, continuously, non-invasively by the biosensor chip and matching analysis method of biofilms formation process is established.

2. Materials and methods

2.1 Reagents and instruments

Luria-Berta broth was purchased from Beijing land bridge technology co., LTD (China). Anhydrous ethanol, sodium hydroxide, and hydrochloric acid were attained from Chongqing Chuan dong Chemical Co., Ltd (China). The phosphate buffer (pH=7.4) consisting of sodium chloride, potassium chloride, potassium hydrogen phosphate, and potassium dihydrogen phosphate, was prepared using distilled water and filtered through 0.22 μm membrane. Polydimethylsiloxane (PDMS) was purchased from Dongguan Yihui adhesive Co., Ltd (China). All solutions were prepared in distilled water and autoclaved at 121 °C for 15 min. *E. coli JM109* and *Salmonella* (ATCC 14028) were presented by Medical University of Chongqing.

Electrochemical workstation (VersaSTAT3, Princeton, USA) was used to measure impedance spectroscopy of biofilms. Bacteria treatment was performed on a clean bench (SWCJO, Suzhou, China). An inverted microscope (OLYMPUS IX71, Olympus, Japan) was used to obtain microscopic photographs of bacteria. Bacteria culture was injected into the biosensor chip by a micro injector. Microplate Reader (Multiskan FC, Shanghai, china) was used to detect the absorbance of biofilms.

2.2 Fabrication of the biosensor chip

The biosensor chip used in experiment was shown in scheme. 1. The bottom layer was a glass substrate with interdigital gold electrodes, which were prepared by MEMS standard process including mask fabrication, film deposition and micro graphic machining. The electrodes were cleaned by 0.1 mol/L hydrochloric acid, 0.1 mol/L sodium hydroxide, anhydrous ethanol and sterile distilled water in turn. And the top layer was the PDMS

with micro cavities prepared by molding method. The biosensor chip was successfully fabricated by bonding the PDMS layer to the glass substrate. A printed circuit board (PCB) matching the biosensor chip was designed. The interdigital microelectrodes were connected to the PCB by gold wire so that the biosensor chip could be mounted to electrochemical workstation to monitor biofilms by EIS.

2.3 Biofilms formation of *Salmonella* and *E. coli* in the biosensor chip

E. coli JM109 and *Salmonella* (ATCC 14028) were used for this study. Single colony of bacteria was selected from Luria-Berta agar, and then was transferred to Luria-Berta broth. Bacteria culture was incubated in a shaker for 12 h at 37℃ with gently shaking at 160 rpm to obtain bacteria of logarithmic phase. Then bacteria culture was diluted with sterile Luria-Berta broth to absorbance value at the wavelength of 600 nm (OD_{600}) of 0.05 in Luria-Berta broth. The biosensor chip was washed by sterile distilled water, anhydrous ethanol and sterile distilled water in sequence. Then, it was placed under an ultraviolet lamp to kill surface bacteria. Afterwards, bacteria culture of *Salmonella* and *E. coli* was respectively injected into the micro cavities of the biosensor chip. Finally, *Salmonella* and *E. coli* were cultured at temperature of 37℃. With the increase of culture time, the bacteria would adhere to the surface of interdigital microelectrodes, gradually formed biofilms. The control experiment, in which sterile Luria-Berta broth was added into micro cavity for EIS detection, was carried out under the same experimental conditions.

2.4 EIS detection for biofilms formation process by the biosensor chip

Electrochemical workstation was used to generate excitation signals for impedance detection. The electrode ports of the biosensor chip were connected to the electrochemical workstation. One contact pad of the interdigital microelectrodes was connected to working electrode and the other was connected to counter electrode. Impedance measurements were performed on biofilms at intervals with alternating voltage of 100 mV in the frequency range from 1 Hz to 100 kHz. For *Salmonella* and *E. coli* biofilms, the impedance monitoring lasted for 48h.

2.5 Crystal violet staining detection for biofilms formation process

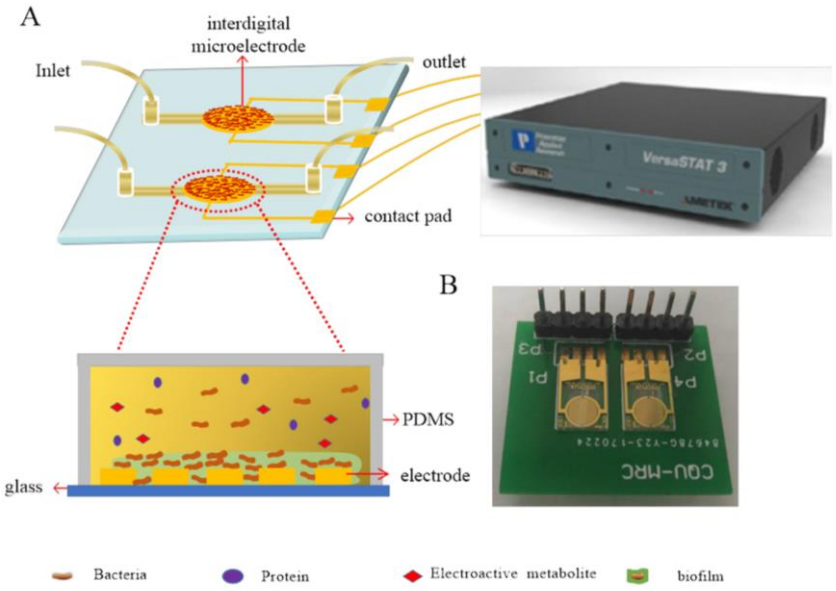
The Crystal violet staining experiment was carried out in 96-well plates. *Salmonella* (ATCC 14028) and *E. coli JM109* (OD_{600} of 0.05 in Luria-Berta broth) were used for biofilms formation process detection. Three parallel experiments were carried out. Firstly, *Salmonella* and *E. coli* culture were injected into 96-well plates with 200 μL per well and the control well was added to 200 μL sterile Luria-Berta broth. Then, they were placed in incubator to culture biofilms at temperature of 37℃. Secondly, the planktonic suspension was removed from wells and surface planktonic bacteria were cleaned with phosphate buffer (pH=7.4). After fixing biofilms by absolute ethanol, 200 μL of Crystal violet staining solution was added to each well and then dyed for 15 min. Finally, 200 μL of 33 % acetic acid was added to each well to release bound Crystal violet when the Crystal violet staining solution was removed. And the absorbance value was measured at 590 nm.

3. Results and discussion

3.1 Design and fabrication of the biosensor chip

In this experiment, the biosensor chip for detection biofilms formation process of *Salmonella* and *E. coli*

was designed, as shown in scheme. 1. Because of its good biocompatibility, transparency and permeability, PDMS was considered as the micro cavity material, which was beneficial to the growth of biofilms. For impedance monitoring of *Salmonella* and *E. coli* biofilms respectively, two same detection areas of interdigital microelectrodes were integrated into the biosensor chip. The interdigital microelectrode array consisted of 90 pairs of microelectrodes, with a circular diameter of 3.5 mm. The width and spacing of finger electrodes were both 10 μm. The diameter and depth of micro cavity was 3.5 mm and 500 μm respectively. The micro channel was connected with the micro cavity to ensure introduction of bacteria culture. Lead wires of interdigital microelectrodes were arranged on the same side of the glass substrate. Impedance monitoring of *Salmonella* and *E. coli* biofilms could be successfully achieved by connecting the biosensor chip to electrochemical workstation.



Scheme. 1. The biosensor chip for biofilms formation process monitoring. (A) Schematic layout of biofilms impedance detection system. (B) A photograph of the biosensor chip.

3.2 Optimization of operating parameters in EIS detection for biofilms

To meet the demand of bacterial growth and biofilms formation, the electrolyte was selected as Luria-Berta broth. As known, the excitation voltage and scanning speed used in impedance detection had an impact on growing status of biofilms. In this experiment, the impedance spectroscopy of biofilms was recorded at the excitation voltage 100 mV and in frequency range from 1 Hz to 100 kHz (Wang et al. 2017).

The contact area between biofilms and microelectrodes was significance for EIS detection. PDMS micro cavities with the diameters of 3.5 mm, 2 mm and 1 mm, were made and applied for detecting the impedance spectroscopy of *E. coli* biofilms. Bode plot of the impedance spectroscopy of *E. coli* biofilms was shown in fig. 1. It was illustrated that the smaller contact area was, the higher impedance value was at the same frequency. The diameter of PDMS micro cavities was chosen to be 3.5mm to ensure the sufficient contact area between bacteria and interdigital microelectrodes.

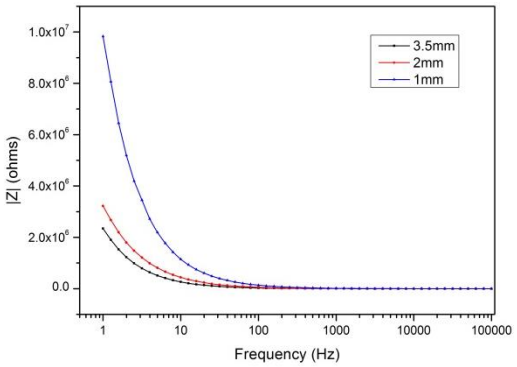


Fig. 1. Bode plot of the impedance spectroscopy of *E. coli* biofilms with different contact area

3.3 EIS monitoring of bacteria biofilms

EIS was a powerful method of analyzing the complex electrical resistance of electrochemical system and was sensitive to surface phenomena (Lisdat and Schäfer 2008). The impedance spectroscopy including Bode plot and Nyquist plot could be applied to analyze the development stages and state of bacteria biofilms, because the changes in the composition of bacteria biofilms, polysaccharides and proteins in extracellular polymeric substance lead to changes of impedance (Kim et al. 2012).

Impedance detection experiments were carried out by the way described at the 3.2 section. Fig. 2 presented the Bode plots of impedance spectroscopy of *Salmonella* biofilms (A), *E. coli* biofilms (B), and the control experiment (C) in frequency range of 1-100 kHz at different growth time. The impedance spectroscopy was enlarged to facilitate a clear view of changing trends over time as shown in the inserting pictures. As shown in fig. 2C, the impedance at different time was almost constant. It was indicated that the impedance remained almost unchanged when there was no biofilms formation on electrodes surface. It was seen from fig. 2A and fig. 2B that the changes of impedance were unobvious in the frequency range from 100 Hz to 100 kHz, while the changes of impedance were obvious in the frequency range from 1 Hz to 100 Hz. For EIS detection for biofilms, the changes of high frequency impedance were mainly caused by changes of electrolyte resistance. During the process of biofilms formation, the slight changes of electrolyte composition brought about a little changes of high frequency impedance. The changes of low frequency impedance were mainly caused by adhesion and formation of biofilms on electrode surface, which were closely related to growth process of bacteria biofilms.

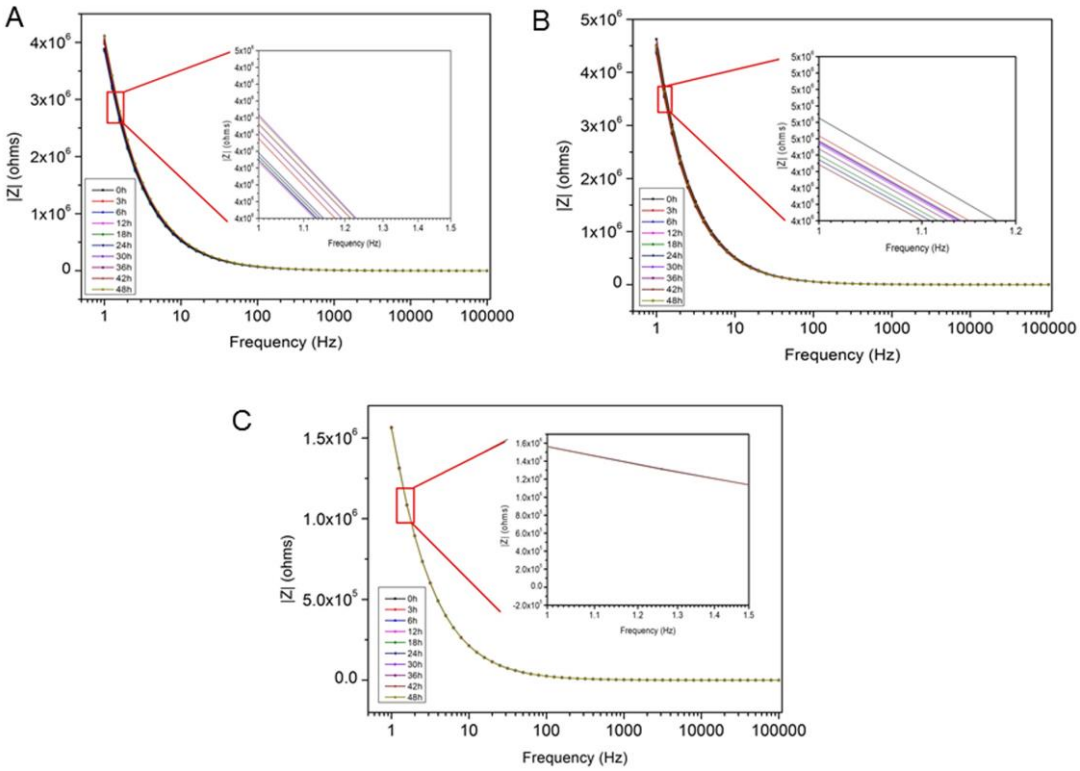


Fig.2. Bode plots of the impedance spectroscopy of *Salmonella* biofilms (A), *E. coli* biofilms (B), the control experiment (C) at different culture times.

3.4 Equivalent circuit model for EIS of biofilms

In order to characterize the relationship between electrochemical parameters and formation process of *Salmonella* and *E. coli* biofilms, an equivalent circuit model was established and applied to analyze impedance spectroscopy in depth. In general, the biofilms formation process included reversible adhesion stage, irreversible adhesion stage, early-mature stage, mature stage and dispersal stage shown in scheme. 2A. The impedance of biofilms monitoring was mainly non-faradaic impedance, because the electrolyte solution and gold electrodes were hardly oxidized or reduced. In the biosensor chip, bacteria would gradually attach and form biofilms on the interdigital microelectrodes surface. The growth of biofilms on the electrodes surface was the main factors that affected capacitance and resistance of electrochemical system. The solution resistance of electrochemical system was affected by many factors, such as electrically active metabolites produced by bacteria, nutrient consumption of culture medium and so on. The bacteria cells adhering to the electrode surface could be equivalent to a capacitor component and extracellular polymeric substances (EPS) of biofilms could be equivalent to a resistor component (Paredes et al. 2014). According to the developing process of biofilms shown in scheme.1A, the equivalent circuit model was selected to analyze the variation of impedance spectroscopy triggered by biofilms growth. As shown in scheme. 2B, the equivalent electrical circuit was consisted of five parameters. R_{sol} was the solution resistance; C_{dl} stood for the double layer capacitance of solution; R_b and C_b represented for the resistance and capacitance of the bacteria biofilms, respectively. And Z referred to the total impedance of electrochemical system. Equation (1), (2), (3), (4) showed the impedance of electrical parameters

represented in the equivalent electrical circuit. And Equation (5) indicated the calculation formula of the total impedance.

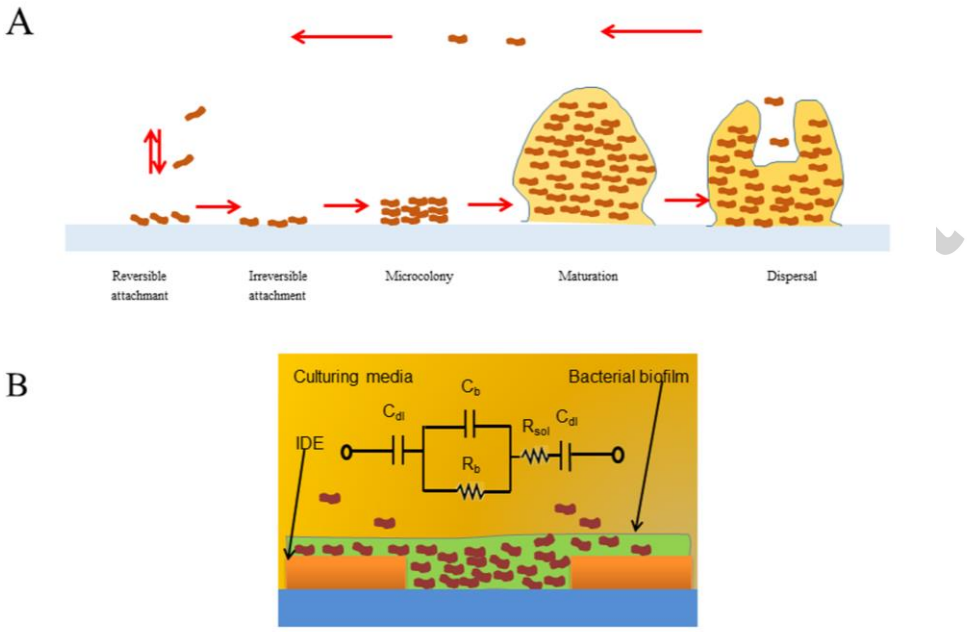
$$Z_{Rsol} = R_{sol} \quad (1)$$

$$Z_{Rb} = R_b \quad (2)$$

$$Z_{Cdl} = -j\ (1/\ \omega C_{dl}) \quad (3)$$

$$Z_{Cb} = -j\ (1/\ \omega C_b\ ) \quad (4)$$

$$Z = Z_{Rsol} + 2Z_{Cdl} + (Z_{Rb}Z_{Cb})\ /\ (Z_{Rb}+Z_{Cb}) \quad (5)$$



Scheme. 2. Impedance analysis for biofilms formation process. (A) Stages of biofilms formation process including reversible adhesion stage, irreversible adhesion stage, micro colony stage, mature stage and dispersal stage. (B) The equivalent circuit model for impedance analysis of *Salmonella* and *E. coli* biofilms.

The measured impedance spectroscopy of *Salmonella* and *E. coli* biofilms at different growth time intervals were curve fitted using the equivalent electrical circuit by Zsimpwin software. The almost constant value of R_{sol} and C_{dl} was obtained during biofilms formation process and this phenomenon indicated that the solution resistance and the double layer capacitance of solution were kept constant regardless of biofilms growth. While, the specific changes trends of C_b and R_b were acquired with culture time and they are closely related to the growth of biofilms. The fitting results of C_b and R_b were described in Table 1 and the changes trends of C_b and R_b with culture time was shown in Fig.3.

In the growth and maturation stages of biofilms, the number of bacteria cells adhering to electrodes surface increased gradually, and the EPS were also accumulated. And in the dispersal stage of biofilms, the biofilms would be degraded spontaneously. The adhesion of bacteria cells or biofilms on electrode surface could reduce the effective area of electrodes and resulted in the decrease of the C_b (Muñoz-Berbel et al. 2007). The production and aggregation of the EPS had influences on the R_b and could cause an increasing of the R_b. The

decreasing trend of C_b and the increasing trend of R_b could indicate that bacteria gradually developed into mature biofilms. And the increasing trend of C_b and the decreasing trend of R_b could indicate that the biofilms were degraded in dispersal stage. The biofilms maturation stage and biofilms dispersion stage could be discriminated by the changing trends of C_b and R_b.

For *Salmonella*, during 0-24 h, the value of C_b had a slight fluctuation in the range from 32.9±0.2 nF to 35.0±0.3 nF. While, the value of R_b dropped from 423.4±5.2 Ohms to 338.5±2.3 Ohms during the period of 0-6 h, and then continued to increase from 338.5±2.3 Ohms to 385.0±3.6 Ohms during the period of 6-24 h. Some electro active metabolic product secreted by bacteria cells might have influences on impedance and cause the decreasing of R_b in the range of 0-6 h. The fluctuations of C_b and R_b were probably owing to *Salmonella* biofilms in adhesion period, when bacteria could commit to biofilm lifestyle or leave electrode surface and return to the planktonic lifestyle. When culture time arrived at 30h, the value of C_b of *Salmonella* began to decrease sharply. And it dropped to the lowest value of 18.7±0.1 nF at 42 h. Meanwhile, the value of R_b continued to increase from 385.0±3.6 Ohms to 403.3±2.9 Ohms during the period of 24-42 h. In the process of biofilms maturing, bacteria could produce the EPS composed of polysaccharide, protein, DNA and so on. The increasing of EPS attached to the electrodes surface was the main reason for the increasing of the R_b. According to this, during the period of 24-42 h, *Salmonella* adhered successfully to electrode surface and gradually formed mature biofilms. After 42h, the value of C_b of *Salmonella* appeared little rebound, and the value of R_b of *Salmonella* show a trend of decline. As the nutrient was consumed, the bacteria could escape from biofilms and arrive into culture medium entering into dispersal period. During this period, the amount of biofilms and their EPS gradually decreased, which could lead to the increasing of the C_b and the decreasing of the R_b. Therefore, during the period of 42-48 h, *Salmonella* biofilms was in dispersal stage.

For *E. coli*, the value of C_b dropped from 238.7±3.0 nF to 207.1±1.8 nF and the value of R_b increased from 14.9±0.5 Ohms to 20.1±0.5 Ohms during the period of 0 -12 h. The reasons for the decrease of C_b and the increase of R_b were caused by *E.coli* biofilms growth. Therefore, the first 12 h was the transition period from adhesion to maturation of *E. coli* biofilms. During the period of 12 -24 h, the value of C_b increased from 207.1±1.8 nF to 221.0±2.0 nF, and the value of R_b decreased from 20.1±0.5 Ohms to 19.1±0.1 Ohms, owing to *E. coli* biofilms dispersal. During the period of 24-36 h, the value of C_b dropped back to 217.2±0.6 nF at 36 h, and the value of R_b increased to 20.3±0.1 Ohms at 36 h. At dispersal stage, bacteria could escape from mature biofilms, and then attached again to new interface. Dispersal stage was not only the final stage of biofilms lifecycle, but also the start of another biofilms lifecycle (Toyofuku et al. 2016). The decline in the C_b and the rise in the R_b once again were attributable to that *E. coli* was in next growth period of biofilms during 24 -36 h. After 36 h, the value of C_b gradually increased, and the value of R_b started to decline again. This was owing to *E. coli* biofilms being in next dispersal period.

In order to prove the reliability of impedance detection and analysis for *Salmonella* and *E. coli* biofilms formation process, biofilms formation process of *Salmonella* and *E. coli* was measured using crystal violet

staining in 96 well-plates under the same experimental conditions. The changes trends of absorbance value at the wavelength of 600 nm (OD_{600}) of biofilms over culture time were shown in fig. 3. For *Salmonella*, the OD_{600} almost remained constant during the period of 0-24 h, and continued to increase during 24-36 h. Meanwhile, the OD_{600} showed a declining trend during the period of 36-48 h. For *E. coli*, the OD_{600} increased dramatically during the period of 0-12 h, and dropped during the period of 12-30 h. And the OD_{600} started to go up again during the period of 30-39h, but declined again after 39h. The changes trends of the C_b and R_b over culture time were consistent with changes trends of the OD_{600} during biofilms formation process, which proved the reliability of the detection and analysis method based on the biosensor chip.

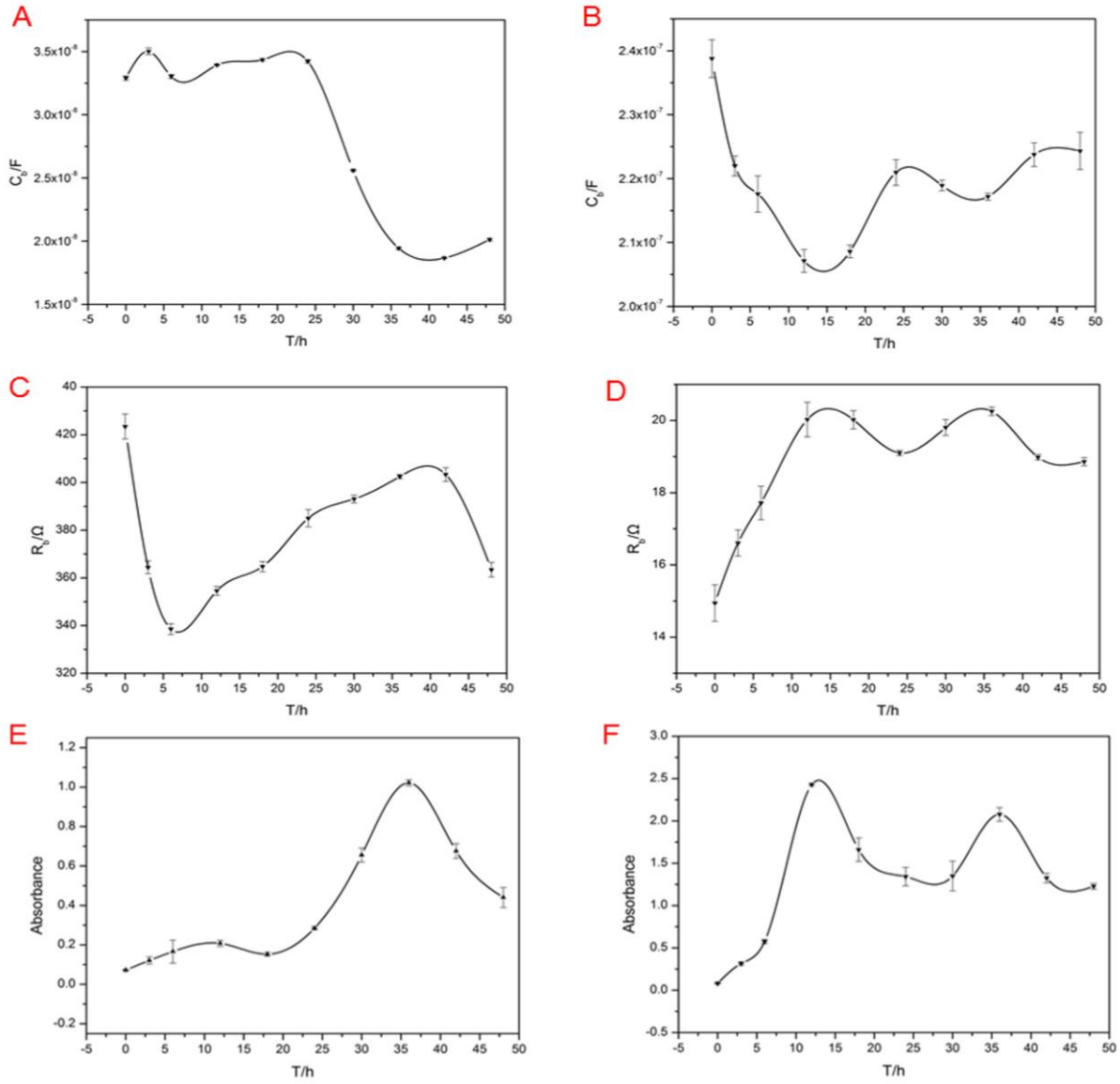


Fig. 3. Fitting results of the equivalent circuit model for EIS of *Salmonella* and *E. coli* biofilms as well as the results of Crystal violet staining experiments. (A) Trends of C_b during *Salmonella* biofilms formation process; (B) Trends of C_b during *E. coli* biofilms formation process; (C) Trends of R_b during *Salmonella* biofilms formation process; (D) Trends of R_b during *E. coli* biofilms formation process; (E) Trends of absorbance values at the wavelength of 600 nm of *Salmonella* biofilms over time; (F) Trends of absorbance values at the

wavelength of 600 nm of *E. coli* biofilms over time. Data represent mean values for n = 3 and the error bars represent standard deviation of the means.

Table 1 Fitting results of the C_b and R_b in equivalent circuit model at different growth times (n=3).

Time (h)	*Salmonella*		*E. coli*	
	C_b(nF)	R_b(Ω)	C_b(nF)	R_b(Ω)
0	32.9±0.2	423.4±5.2	238.7±3.0	14.9±0.5
3	35.0±0.3	364.4±2.6	222.0±1.6	16.6±0.4
6	33.0±0.2	338.5±2.3	217.6±2.8	17.7±0.5
12	33.9±0.1	354.5±1.9	207.1±1.8	20.1±0.5
18	34.3±0.1	364.7±2.1	208.6±1.0	20.0±0.3
24	34.2±0.1	385.0±3.6	221.0±2.0	19.1±0.1
30	25.6±0.1	393.1±1.6	219.0±0.9	19.8±0.2
36	19.4±0.1	402.5±0.8	217.2±0.6	20.3±0.1
42	18.7±0.1	403.3±2.9	223.7±1.9	20.0±0.1
48	20.1±0.1	363.4±3.1	224.3±2.9	18.9±0.1

3.5 The comparation of EIS detection and analysis for *Salmonella* and *E. coli* biofilms formation process

Compared with *Salmonella*, the C_b of *E. coli* began to decrease at the beginning of culture time, while the C_b of *Salmonella* showed a downward trend after an adjustment period for 24 h. While, both C_b of *E. coli* and *Salmonella* firstly decreased, and then appeared to increase over culture time, but the time turning points of the C_b remained difference between *E. coli* and *Salmonella*. These differences were due to *Salmonella* and *E. coli* owning different biofilms formation cycle. The biofilms formation process of *E. coli* was shorter, and *E. coli* were easier to attach on electrodes surface. The biofilms formation process of *Salmonella* was relatively long, and *Salmonella* successfully attached on electrodes surface requiring longer time. From the fitting data, the C_b of *E. coli* was larger than *Salmonella* at the same time, but R_b of *E. coli* was less than *Salmonella*, because of different structure of *E. coli* and *Salmonella* biofilms. In crystal violet staining experiments, the greater the absorbance value was, the more the mass of biofilms was (Peeters et al. 2008). Crystal violet staining results indicated that absorbance value of *E. coli* biofilms was greater than *Salmonella* at the same time, which proved that *E. coli* had a stronger ability to form biofilms than *Salmonella*.

4. Conclusion

A biosensor chip integrated interdigital microelectrodes was successfully prepared and applied for monitoring biofilms formation process of *Salmonella* and *E. coli*. An equivalent circuit consisting of the biofilms capacitance (C_b) and the biofilms resistance (R_b) was used to analyze EIS of biofilms. The growth and development of biofilms attached on the electrode surface was closely related to C_b and R_b, so biofilms formation process could be monitored in real-time by measuring the changes of C_b and R_b. It was indicated

that the C_b and R_b had specific changes trends with the growth of biofilms. When *Salmonella* and *E. coli* attached the electrode surface and then started to form mature biofilms, the C_b gradually decreased, and the R_b gradually increased. Subsequently, the C_b increased slightly, and the R_b decreased slightly, which was attributed to biofilms dispersal. Furthermore, the C_b and R_b had different changes trends over time between *Salmonella* and *E.coli* during biofilms formation process and the periodic characteristics of biofilms formation process were distinguished. The impedance detection and analysis method showed great advantages such as not destroying biofilms structure, non-requiring samples preparation and being able to characterize biofilms formation periods. Meanwhile, the detection method based the biosensor chip was confirmed by crystal violet staining. These results demonstrated that the biosensor chip could be applicable for monitoring of biofilms formation process.

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Highlights:

1. An impedance biosensor chip was designed to monitor biofilms forming process.
2. Periodic characteristics of biofilms were discriminated by changes of electrochemical parameters.
3. The method based on the biosensor chip had advantages of real time, continuity, and non-invasion.